FORMATION OF LYSINO-ALANINE AND LANTHIONINE IN VARIOUS FOOD PROTEINS, HEATED AT NEUTRAL OR ALKALINE pH

C. AYMARD, J. L. CUQ & J. C. CHEFTEL

Laboratoire de Biochimie et Technologie Alimentaires, Université des Sciences et Techniques, 34060 Montpellier, France

(Received: 22 December, 1976)

ABSTRACT

Lysino-alanine and lanthionine have been estimated by a rapid TLC procedure in several food proteins previously submitted to various heat treatments at different pH's. The kinetics and activation energy of the formation of lysino-alanine in heated casein have been assessed. Free lysino-alanine and lanthionine have been found to be partially labile in alkaline media.

INTRODUCTION

Lysino-alanine (LAL) and other inhabitual amino acids such as lanthionine (LAT) and ornithino-alanine are found in heat- and/or alkali-treated proteins. Their formation is assumed to proceed via a nucleophilic attack by the distal reactive group of lysine, cysteine or ornithine respectively, on a dehydro-alanine residue: the latter is formed by a β-elimination reaction from cysteine or from serine, *O*-phosphorylated or *O*-glycolysated serine (see Provansal *et al.*, 1975, for a review).

Lysino-alanine seems to be ubiquitous: it has been reported to be present, sometimes in great quantity, in several industrial or home-cooked foods, even when heat treatments were performed at moderate pH (Sternberg *et al.*, 1975*b*). LAL-containing proteins have been reported to be nephrotoxic to rats (De Groot *et al.*, 1975).

It is therefore of interest to obtain more information concerning the formation of LAL. Preliminary results on this subject are reported here.

Fd. Chem. (3) (1978)—
Printed in Great Britain

EXPERIMENTAL

Lysino-alanine dihydrochloride

This was obtained from Miles-Yeda, Rehovot-Israel.

Acid hydrolysis

Acid hydrolysis was carried out as previously described (Provansal *et al.*, 1975).

LAL and LAT determinations

These were performed by a thin layer chromatography method, adapted from Sternberg *et al.* (1975*a*); the composition of the solvent system was modified (*n*-butanol, methanol, formic acid, water: 10:10:4:4) in order to achieve analysis in a shorter time (about 4 h) with a single development on a 20 × 20 cm, 0·1 mm cellulose plate (from Merck AG). The differential determination of LAL and LAT (the R_f of which is very close to that of LAL) was made possible as follows. Two analyses were run in parallel, one with the 6N HCl hydrolysate, the other with the same hydrolysate treated with 0·5M H_2O_2 for 15 min at 50 °C (50 µl of 10M H_2O_2 added to 1 ml of hydrolysate). In these conditions and following revelation with a ninhydrin spray, the LAL and LAT spots are well discriminated: the R_f of LAT-sulphoxide quantitatively formed after H_2O_2 treatment is smaller than that of LAL (the R_f of which is unchanged). The mean R_f values measured at 20 °C are: LAL: 0·17; LAT: 0·19; LAT-sulphoxide: 0·12.

Kinetics of LAL formation

A 10 mg/ml, pH 10, casein solution was held at 70, 85 or 100 °C for various periods of time (20 to 400 min). The kinetic constants at these different temperatures were then measured as slopes in a plot of concentration variation versus time (apparent zero-order reaction).

Stability of LAL and LAT in alkaline media

A 2 mM LAL or LAT solution in 0·2N NaOH was kept for various periods of time (0 to 6 h) at 20 or 70 °C. TLC analysis was then performed.

RESULTS AND DISCUSSION

The TLC method was chosen for convenience and rapidity. Some analyses were also performed by ion exchange chromatography (NC 1 Technicon Autoanalyser, see Provansal *et al.*, 1975). The results of both analytical methods agreed by 20–30 %.

The analysis of several foods and food proteins shows that LAL is present, sometimes in large amounts, in most heat-treated proteins (Tables 1 and 2). It must be noted that LAT levels are lower than those of LAL. There is no correlation between the lysine over cysteine + cystine ratio on the one hand and the LAL over

TABLE 1
LYSINO-ALANINE AND LANTHIONINE[a] IN HEAT-TREATED PROTEINS

Protein food or protein	*Conditions of treatment in the laboratory*	*LAL + LAT (μg/g protein)*
Strasbourg sausage	No treatment	0
	Boiling water, 10 min	50
Evaporated milk	No treatment	700
Dried skim milk	—	<20
Canned corned-beef	—	<20
Whipping agents (from whey proteins spray-dried at high pH)		
Brand A	No treatment	30000 (20000)
Brand B	—	5000
Brand C	—	200 (230)
Bovine serum albumin[b]	120°C, 6 h, pH 6	8000
Casein[b]	75°C, 1 h, 0·2N NaOH	1250 (1300)
Sodium caseinate[b]		
Brand A	120°C, 6 h, pH 6	600
Brand B	—	200
Soya protein isolates		
Brand A	No treatment	1300
	200°C, 30 sec[c]	2000
Brand B	No treatment	20
	200°C, 30 sec[c]	650

[a] Determined by TLC (see section in the text headed 'Experimental'). Numbers in parentheses refer to determinations of lysino-alanine by ion exchange chromatography.
[b] Pure proteins were heat-treated as 0·5 or 1% w/v aqueous solutions (casein as a 7% w/v solution).
[c] Dry-heated in small sealed tubes in the presence of sucrose (20% w/w).

TABLE 2
LYSINO-ALANINE AND LANTHIONINE IN VEGETABLE PROTEIN PREPARATIONS

Vegetable protein	*LAL[a] (μg/g protein)*	*LAT[a] (μg/g protein)*
Defatted oilseed cakes		
Peanut	900	200
Soya	1500	300
Sunflower	900	300
Rapeseed	1500	100
Linseed	1400	150
Textured broad bean protein	350	150

[a] Determined separately by TLC (see section in the text headed 'Experimental').

LAT ratio (as calculated with the oilseed cakes) on the other. An alkaline pH of heat treatment enhances LAL formation but, as is well known, LAL may be found in products heat-treated at a moderate pH (Sternberg *et al.*, 1975*b*).

The pH value can be expected to influence both the formation of dehydro-alanine residues (β-elimination is a base-catalysed reaction) and the state of ionisation of the amino or sulphydryl group of lysyl or ornithyl or cysteinyl residues which are responsible for the nucleophilic attack on dehydro-alanine. Only unprotonated forms of these groups are reactive. However, even at a given acid pH, a high temperature may substantially increase the proportion of reactive unprotonated ε-

amino groups, as a consequence of the large positive ΔH for the ionisation equilibrium of these groups: + 11·5 kcal/mole (an almost hundred fold increase in the concentration of the unprotonated form may be expected for a rise in temperature of 100 °C).

After heating a casein solution at different temperatures for different lengths of time (see kinetics of LAL formation above), apparent overall velocity constants of LAL formation were calculated from the variation of the concentration of LAL as a function of time (in this experiment, lanthionine formed was considered as negligible and no differential determination of lysino-alanine and lanthionine was performed). The results are given in Table 3.

Introducing these values into the integrated form of Arrhenius' law gives an energy of activation of 18·5 to 21 kcal/mole. Attempts to determine energy of activation at lower pH were unsuccessful, because of the very low levels of LAL formed.

TABLE 3
OVERALL VELOCITY CONSTANTS OF LYSINO-ALANINE FORMATION

Temperature (°C)	*Velocity constant*[a] (μ *mole* . min^{-1} . l^{-1})
70	0·6
85	1·7
100	5·3

[a] Measured as a slope in a plot of concentration variation versus time (apparent zero-order reaction).

It is known that lysino-alanine slowly decomposes on heating at 110 °C in 6N HCl and it has been suggested to calculate lysino-alanine content by extrapolation to zero hydrolysis time (Sternberg *et al.*, 1975*a*). However, the question arises whether lysino-alanine and lanthionine formed in alkaline media are stable in this environment. Experiments previously performed in this laboratory showed a slight decrease in the LAL content of a sunflower protein isolate with time of treatment by 0·2N NaOH, at 80 °C (Provansal *et al.*, 1975). We studied stability of LAL and LAT as free amino acids, in solution in 0·2N NaOH, at 20° and 70 °C (see section above headed 'Experimental'). The formation of a new ninhydrin-positive compound (R_f = 0·5) from LAL as well as from LAT was observed on examination of TLC plates. These reactions are rapid and incomplete: a limit of reaction is reached after 1 h of treatment, the decrease in LAL and LAT being similar and close to 30%. Further experiments are needed to study this phenomenon which could be due to the formation of diastereoisomers. Lability of LAL and LAT in polypeptide chains should also be examined.

ACKNOWLEDGEMENTS

This study was supported in part by the 'Centre National de la Recherche

Scientifique', Paris (E.R.A. No. 614: Modifications Biochimiques et Nutritionnelles de Protéines Alimentaires).

REFERENCES

DE GROOT, A. P., SLUMP, P., VAN BEEK, L. & FERON, V. J. (1975). *Abstract—35th Ann. Meet. Inst. Food Technologists, USA.*

PROVANSAL, M. P., CUQ, J. L. & CHEFTEL, J. C. (1975). Chemical and nutritional modifications of sunflower proteins due to alkaline processing. Formation of amino acid cross-links and isomerization of lysine residues. *J. Agric. Food Chem.*, **23**, 938–43.

STERNBERG, M., KIM, C. Y. & PLUNKETT, R. A. (1975*a*). Lysino-alanine determination in proteins, *J. Food Sci.*, **40**, 1168–70.

STERNBERG, M., KIM, C. Y. & SCHWENDE, F. J. (1975*b*). Lysino-alanine: Presence in foods and food ingredients, *Science*, **190**, 992–4.

PRODUCTION OF VOLATILE COMPOUNDS BY MUSKMELON, *CUCUMIS MELO*†

K. YABUMOTO, M. YAMAGUCHI & W. G. JENNINGS

Departments of Vegetable Crops and Food Science and Technology, University of California, Davis, California 95616, *USA*

(Received: 13 January, 1977)

ABSTRACT

The volatile constituents of 'PMR 45', *'Top Mark', 'Honeydew' and 'Crenshaw' cultivars of Muskmelon were examined by trapping the essence on Porapak Q and analysing the eluted compounds on wall coated open-tubular glass capillary columns. The relative concentrations of forty-three compounds were determined. Also, by direct sampling of gas from the fruit cavity of 'PMR* 45' *and 'Top Mark' cantaloupe cultivars, the relative concentrations of fifteen compounds were measured daily over a period of eight days. Computer-constructed arrays of chromatograms revealed trends in the progressive changes of volatile compounds produced by the ripening fruits. The volatile constituents measured in this study could be fitted into groups, depending on the pattern exhibited by the production of that compound. One group showed a continuously accelerating rate of production (e.g. ethyl esters, acetaldehyde, ethanol) and another increased rapidly and then plateaued (e.g. acetate esters).*

INTRODUCTION

The respiration of melon fruits and the effect of ethylene on induced ripening has received considerable attention (Eskin *et al.*, 1971; Pratt & Goeschl, 1969; Pratt, 1971). In addition to these studies on the physiology of growing and ripening melons, the identification of the volatile compounds of ripe melons has also been investigated (Kemp *et al.*, 1971, 1972, 1973, 1974; Yabumoto, 1976; Yabumoto *et al.*, 1974). Commercially, melons are harvested green-ripe and permitted to ripen

† The work reported in this paper was supported in part by a research grant from the California Melon Advisory Board, and is from a thesis submitted by the senior author in partial satisfaction of the requirements for the Ph.D in Agricultural Chemistry.

Fd. Chem. (3) (1978)—
Printed in Great Britain

during shipment. Typically, the green-ripe fruit tastes relatively flat and lacks flavour; as it approaches the climacteric point, volatile constituents that are responsible for the full, ripe, fruity flavour are produced at a rapid rate. With continued ripening the fruit develops an undesirable flavour that appears to be associated with the over-production of certain volatiles. The results are presented on the identification of some of these volatile constituents in melon cultivars, the types of compound produced during ripening and the possible biosynthetic pathways.

EXPERIMENTAL

Materials

Fruits of PMR 45, Top Mark (cultivars of cantaloupe), Honeydew and Crenshaw—all cultivars of muskmelon—were harvested from experimental plots of the University of California, Davis.

Porapak concentrates

Six to ten (depending upon size) freshly harvested melons were placed in a 40 litre glass chamber which was swept with breathing quality compressed air further purified by passage through molecular sieves 5A and 13X, at a flow rate of 120 ml/min. The effluent air with its entrained volatiles was passed through a 10 cm length of 12 mm tubing containing 2 g of 80–100 mesh Porapak Q between glass wool plugs. The Porapak traps had received careful purging prior to use (Jennings *et al.*, 1974). After a 24 h trapping period, the traps were backflushed at 100 °C with purified nitrogen to glass trapping bulbs chilled with dry ice. Recovered samples varied from 5 to 500 μl. The trapping tube was then flame sealed and stored at −20 °C until used.

Sampling of volatiles during ripening

Three 'PMR 45' fruits at similar maturity (firm flesh, slightly yellowish–green rind) and three 'Top Mark' fruits were selected for daily analysis of the fruit cavity volatiles. A glass probe made of a disposable pipette was inserted into the melon flesh and sealed to the rind with fast-setting epoxy resin. The outside end of the probe was fitted with a rubber septum to allow repetitive sampling of the cavity gas. The probe-fitted melons were held at room temperature (26 °C), and a sample of cavity gas was taken for gas chromatographic analysis every 24 h for eight days, starting on the day after harvest. The melons were at eating ripeness on the fourth and fifth days of sampling. On the ninth day, the fruits were excessively over-ripe, mould developed on some so the experiment was terminated.

Gas chromatography

A Hewlett-Packard 5711A gas chromatograph with dual FID was adapted to wall-coated open-tubular (WCOT) glass capillary columns, 0·25 mm inside

diameter × 80 m coated with Carbowax 20M admixed with benzyltriphenylphosphonium chloride and Ionox (Jennings *et al.*, 1974) and highly linear glass inlet splitters (Jennings, 1975). The inlet was maintained at 250 °C and the split ratio was *ca.* 1:100. Linear velocity was adjusted to *ca.* 15 cm/sec as measured by methane injection. Detectors were supplied with 30 ml/min nitrogen make-up gas, 30 ml/min hydrogen and 240 ml/min air and maintained at 250 °C. Oven temperature was held at 80 ° for 16 min following injection, programmed at 1 °/min to 120 °C and held.

Identification of volatile compounds

Volatile essences prepared from melon cavity gas on Porapak Q were subjected to mass spectral analysis on a Finnigan GC–MS Model 3200, directly interfaced with a WCOT glass capillary column. Measurement of retention time on WCOT glass columns in two liquid phases, as well as IR spectra of certain fractions, confirmed the identifications.

Three-dimensional plots

To assist in differentiating random scatter from progressive trends, three-dimensional computer plots were applied to these data (Richard *et al.*, 1971). Based on the retention time data and normalised areas of all detected peaks, a computer-constructed chromatogram was produced for each chromatographic run. The simulated chromatograms representing the data from successive analyses on the same fruit were drawn in an offset stacked array by a Cal-Comp plotter (Fig. 1(a) and (b)).

RESULTS AND DISCUSSION

Composition of volatiles from ripe melons

The complexity of the volatile composition of cantaloupes required a degree of resolution achieved only with WCOT glass capillary columns. With liquid samples no problem is encountered using extremely low carrier gas flows of 0·6 to 0·7 ml/min which limited sample size and the amount of material available to the detector. However, when a very dilute gas sample is subjected to analysis with capillary columns, such low flow rates may become critical in the separations. Fortunately, the concentrations of the major volatile constituents in the melon cavity gas occurred at detectable levels for capillary analysis and the detection of minor constituents was sacrificed to improve resolution in these experiments.

Capillary columns also required the use of an inlet splitter to prevent the injected sample from occupying too long a section of the column because of the low flow rate of the carrier gas resulting in broad peaks. A 3 ml gas sample and a 1:100 split ratio with a 60 ml/min discharge from the splitter outlet was used in these analyses because the splitting merely decreased peak widths and had little effect on the

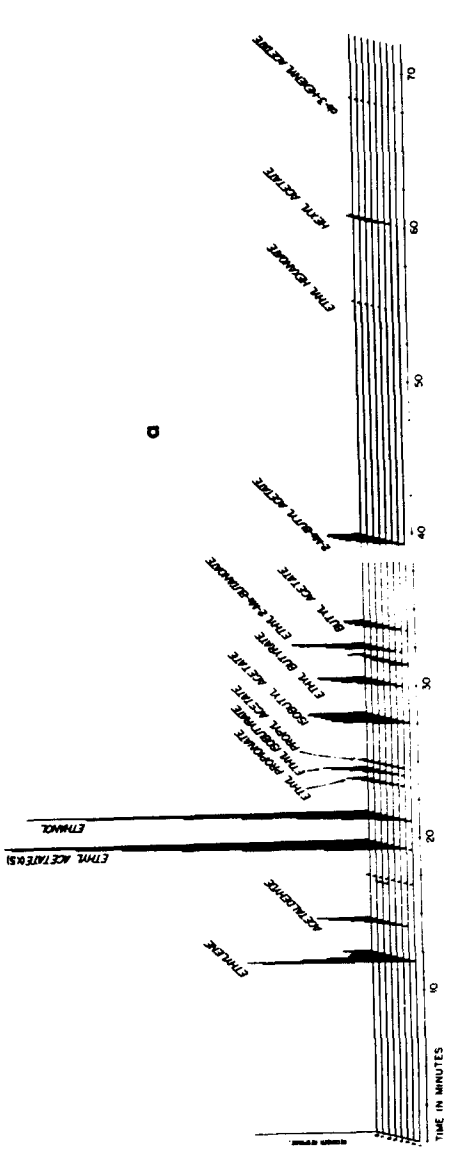


Fig. 1. (a) Typical 'PMR 45' melon; (b) typical 'Top Mark' melon. Computer-redrawn chromatograms of sequential analyses of fruit cavity gas. Integrator data on Carbowax 20M glass-capillary column runs were normalised on an artifact peak 1, immediately preceding ethyl acetate. The bottom level chromatogram, 1, represents analysis on the first day; the second level chromatogram, 2, on the second day; etc.

TABLE 1

COMPARISON OF THE VOLATILE COMPOSITION OF SEVERAL MELON CULTIVARS[a]

Peak number[b]	*Compound*	*Cantaloupe 'PMR-45'*	*Cantaloupe 'Top Mark'*	*Honeydew*	*Crenshaw*
		Peak Area			
3	Methyl acetate	0·06	0·11	1·67	0·32
4	Ethyl acetate	73·69	64·39	63·34	45·87
8	Propyl acetate	3·34	2·01	2·21	1·43
10	Isopropyl acetate	0·05	0·08	0·58	0·05
15	Butyl acetate	6·25	11·48	6·50	8·81
—	Isobutyl acetate	16·98	15·99	13·04	24·11
24	Pentyl acetate	0·22	0·37	0·36	0·25
20	2-Methylbutyl acetate	19·33	24·23	36·90	31·56
30	Hexyl acetate	1·81	6·52	8·16	4·75
31	*Cis*-3-hexenyl acetate	1·13	2·52	2·62	0·25
—	Methyl propionate	0·11	0·01	1·17	2·18
6	Ethyl propionate	5·38	2·54	2·16	0·60
—	Butyl propionate	0·04	0·14	0·06	0·01
17	Isobutyl propionate	0·38	0·16	0·17	0·01
—	2-Methylbutyl propionate	0·10	0·16	0·85	0·91
9	Methyl butyrate	0·58	0·32	2·02	4·18
12	Ethyl butyrate	14·67	15·01	4·32	0·51
—	Propyl butyrate	0·10	0·18	0·00	0·13
—	Butyl butyrate	0·03	0·18	0·04	0·20
23	Isobutyl butyrate	0·17	0·41	0·72	2·10
29	2-Methylbutyl butyrate	0·11	0·33	0·65	1·41
—	Methyl isobutyrate	0·14	0·07	0·99	1·70
7	Ethyl isobutyrate	4·14	1·85	0·99	0·12
—	Butyl isobutyrate	0·03	0·04	0·16	0·04
19	Isobutyl isobutyrate	0·14	0·21	0·87	1·98
18	Methyl pentanoate	0·01	0·00	0·22	0·69
21	Ethyl pentanoate	0·14	0·16	0·06	0·11
11	Methyl 2-methylbutanoate	3·09	1·77	4·37	9·39
13	Ethyl 2-methylbutanoate	19·57	10·21	7·04	0·36
22	Propyl 2-methylbutanoate	0·11	0·16	0·03	0·01
—	Butyl 2-methylbutanoate	0·02	0·15	0·01	0·03
25	Isobutyl 2-methylbutanoate	0·21	0·27	0·38	0·28
—	2-Methylbutyl 2-methylbutanoate	0·11	0·29	0·39	0·16
26	Methyl hexanoate	0·08	0·10	0·13	0·12
28	Ethyl hexanoate	1·30	1·91	1·82	1·53
—	Ethyl 3-hexanoate	0·10	0·18	0·06	0·00
—	Butanol	0·63	0·72	0·31	0·27
16	Isobutanol	1·74	0·49	0·13	0·25
—	Pentanol	0·05	0·08	0·11	0·05
27	2-Methylbutanol	3·76	0·82	0·47	0·32
—	Hexanol	0·57	0·79	0·39	0·06
—	*Cis*-3-hexenol	0·43	0·41	0·19	0·05
14	Dimethyl disulphide	0·03	0·02	—	—
1	Ethylene	+	+	+	+
2	Acetaldehyde	+	+	+	+
5	Ethanol	+	+	+	+

[a] Determined on Porapak Q concentrates of intact ripe melon volatiles; — not detected; + present but not quantified.

[b] Peak number of Fig. 1, volatiles from fruit cavity.

magnitude of the detector response. A reproducible artifact peak, which was more constant than a hydrocarbon vapour addition, was used as a standard for normalising peak areas throughout the series.

A typical programmed temperature chromatogram on a glass capillary column of gas sampled from the fruit cavity of a 'PMR 45' ripe fruit is shown in Fig. 2, and the identity of the compounds of the numbered peaks are listed in Table 1. Table 1 also shows the relative concentration of the volatiles obtained from gas concentrates on Porapak Q from intact ripe melon cultivars. The method of sample collection seems to be important in the analyses; ethylene, acetaldehyde and ethanol were prominent peaks in direct injections from the fruit cavity but these compounds were not

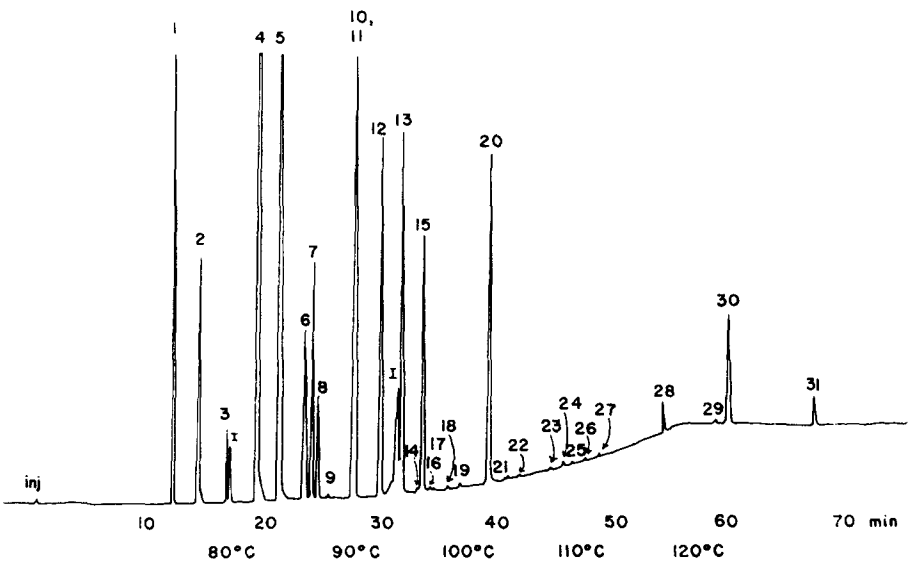


Fig. 2. Chromatogram, 'PMR 45' fruit cavity gas—direct injection on Carbowax 20M glass-capillary column. (See Table 1 for peak identification.)

detected in the Porapak Q concentrates obtained from gas sweeping of intact melon. Large quantities of ethyl, butyl, isobutyl and 2-methylbutyl acetate were common to all four cultivars. Ethyl butyrate exhibited high concentrations in the two cultivars of cantaloupes but considerably lower concentrations were present in the Honeydew and Crenshaw melons. Ethyl 2-methyl-butanoate showed this same pattern. Crenshaw melons had larger amounts of methyl esters than did the other melons and very low concentrations of ethyl propionate, butyrate, isobutyrate and 2-methylbutanoate, which were major volatile components of the other cultivars. Except for ethyl butyrate, the volatile ester composition of Honeydew melons was not widely different from that of cantaloupes, and only minor differences are apparent between the two cantaloupe cultivars.

Post-harvest production of volatiles by cantaloupes

Typical patterns of volatiles produced by two cultivars of cantaloupes, over the ripening period of eight days following harvest are depicted in Fig. 1. 'PMR-45' (Fig. 1(a)) is a more aromatic melon than 'Top Mark' (Fig. 1(b)) and the sequential diagrams show this to be the case. The production of ethylene, the ripening hormone (Pratt, 1971), was much more rapidly produced by 'PMR-45' than by 'Top Mark', which may account for the difference in the ripening patterns of the two cultivars (Yamaguchi *et al.*, 1974, unpublished data).

Patterns in volatile production by cantaloupes

Apparently, the regulation of volatile production in ripening melons is not independent for each compound but groups of compounds may be regulated simultaneously. Changes in the concentration of individual compounds seem to occur in definite patterns which can be grouped: acetaldehyde and ethanol make up one group, ethyl esters a second and acetate esters a third; ethyl acetate seems not to fit any of the above groups but is in a category by itself. The patterns of these groupings of 'PMR-45' and 'Top Mark' (fruits of Fig. 1(a) and (b)) are depicted in Fig. 3(a) and (b). Each curve is multiplied by an arbitrary factor so that all curves are of similar magnitude and the patterns are more easily comparable. The curves for ethanol and acetaldehyde showed a close relationship to that of the total ethyl esters. Ethyl acetate exhibited a curve with combined characteristics of the two distinguishable groups; this may relate to the fact that both the ethyl and the acetyl group are involved in the molecule.

Regulation of the volatile production

According to Conn & Stumpf (1973), biochemical reactions are regulated by several factors which can be summarised as follows: kinetic factors, which include concentrations of the enzyme, the substrate and coenzymes; physical conditions (i.e. temperature, pressure, pH, etc.) and structural factors which represent the organisation of cells and locality of the substances within the system; occasionally special positive or negative factors exist.

First, the substrate concentration may be the determining factor. It is generally believed that an ester is enzymically formed by combining an alcohol with an acyl group as acyl-CoA (Forss, 1972; White *et al.*, 1973). From this scheme, the ethyl esters share a common substrate (the ethyl moiety as ethanol) and the acetate esters share another (the acetyl group as acetyl-CoA). Both moieties contain two carbon units and have close relationships in metabolic networks; they are related through the immediate intermediate, pyruvic acid, even to requiring the same co-factors. Ethanol is produced by reduction of acetaldehyde which is the decarboxylated product of pyruvic acid.

$$\text{Pyruvic acid} \xrightarrow[(-CO_2)]{} \text{Acetaldehyde} \xrightarrow[\text{(reduction)}]{} \text{Ethanol} \xrightarrow[(+\text{Acyl-CoA})]{} \text{Ethyl esters}$$

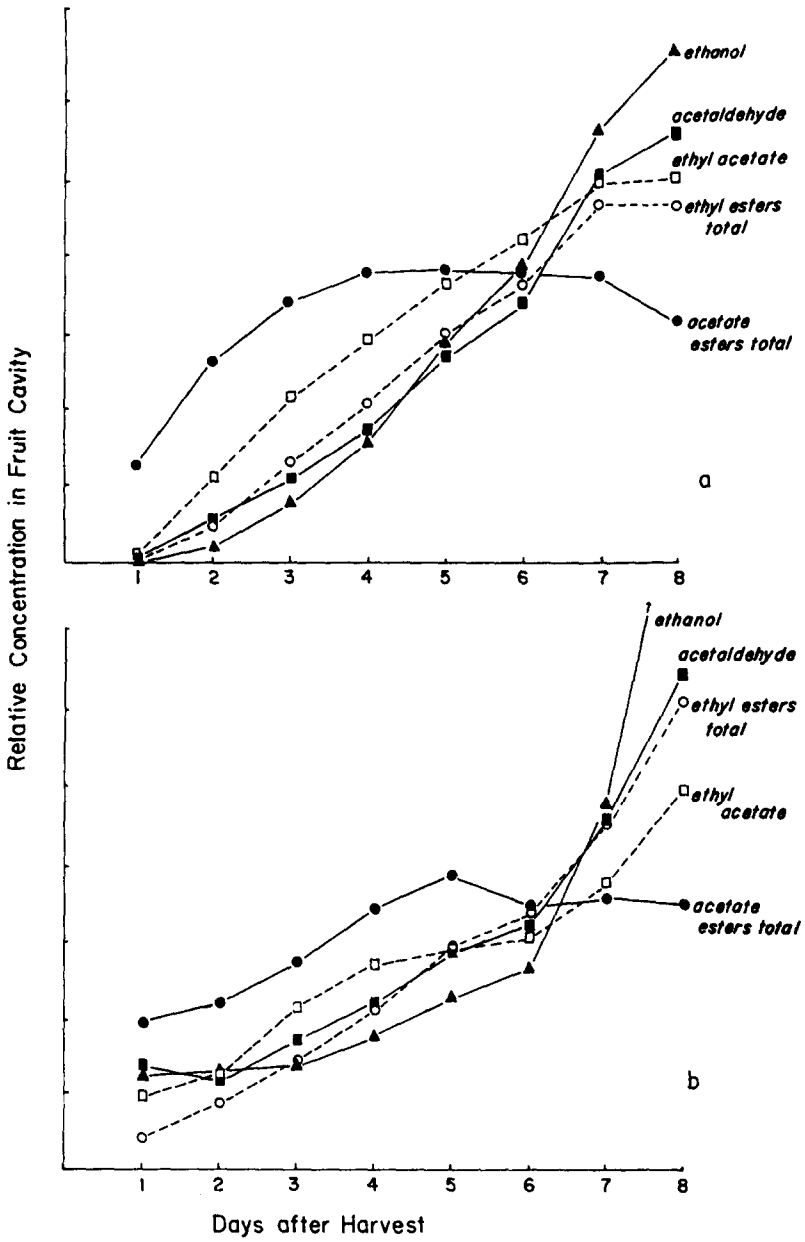


Fig. 3. (a) 'PMR 45' melon; (b) 'Top Mark' melon. Abundance of selected volatiles and groups of volatiles with ripening. Values of a given curve were multiplied by an appropriate factor to facilitate comparison.

The fact that acetaldehyde production exhibits the same pattern as that of the ethyl esters supports this pathway as an important regulatory factor.

Acetyl-CoA, which is thought of as the direct precursor of acetate esters, is generated by oxidative decarboxylation of pyruvic acid.

$$\text{Pyruvic acid} \xrightarrow[(-CO_2)]{} \xrightarrow[\text{(oxidation)}]{} \xrightarrow[(+\text{CoA-SH})]{} \text{Acetyl-CoA} \xrightarrow[(+\text{R—OH})]{} \text{Acetate esters}$$

If, indeed, these are the major sources of both the ethyl ester series and the acetate ester series, independent regulation of the two different pathways from pyruvic acid must be the factor which causes the significant difference in patterns exhibited by the ethyl ester group and the acetate ester group.

It is also possible that the enzyme or co-enzyme concentration may serve as the regulatory factor. Since one part of the esters is common within these groups, the ethyl ester production may be catalysed by a single enzyme; this could also be the case with the acetate ester group. This would explain the uniform progressive change within the groups and the unequal change between them.

In considering the above possibilities, it seems probable that the esters measured from the fruit cavity volatiles are produced from their precursors by enzymes of low specificity and that the production rates are largely due to the precursor concentration as increased mobilisation of the precursor(s) is achieved with increasing maturity.

REFERENCES

CONN, E. E. & STUMPF, P. K. (1973). *Outlines of biochemistry* (3rd ed.) New York, Wiley and Sons.

ESKIN, N. A. M., HENDERSON, H. M. & TOWNSEND, R. J. (1971). *Biochemistry of foods*, New York, London, Academic Press.

FORSS, D. A. (1972). Odor and flavor compounds from lipids. In: *Progress in the chemistry of fats and other lipids*, ed. R. T. Holman, Vol. 13, 181, New York, Pergamon Press.

JENNINGS, W. G., LEVIN, M. J. & WOHLEB, R. H. (1974). Isolation of volatile compounds for GLC analyses, *MBAA Tech. Quarterly*, **11**, 104–9.

JENNINGS, W. G. (1975). Glass inlet splitter for gas chromatography, *J. Chromatogr. Sci.*, **13**, 185–7.

KEMP, T. R., KNAVEL, D. E. & STOLTZ, L. P. (1971). Characterization of some volatile constituents of muskmelon fruit. *Phytochem.*, **10**, 1925–8.

KEMP, T. R., KNAVEL, D. E. & STOLTZ, L. P. (1972). Cis-6-Nonenal: A flavor component of muskmelon fruit, *Phytochem.*, **11**, 3321–2.

KEMP, T. R., KNAVEL, D. E. & STOLTZ, L. P. (1973). Volatile *Cucumis melo* components: Identification of additional compounds and effect of storage conditions, *Phytochem*, **12**, 2921–4.

KEMP, T. R., KNAVEL, D. E., STOLTZ, L. P. & LUNDIN, R. E. (1974). 3, 6-Nonadiene-1-ol from *Citrullus vulgarus* and *Cucumis melo*. *Phytochem.*, **13**, 1167–70.

KEMP, T. R., STOLTZ, L. P. & KNAVEL, D. E. (1972). Volatile components of muskmelon fruit. *J. Agr. Food Chem.*, **20**, 196–8.

PRATT, H. K. & GOESCHL, J. D. (1969). Physiological roles of ethylene in plants. *Ann. Rev. Plant Physiol.*, **20**, 541–84.

PRATT, H. K. (1971). Melons. In: *The biochemistry of fruits and their products*, ed. A. C. Hulme, Vol. 2, 207–32, New York, London, Academic Press.

RICHARD, H. M., RUSSELL, G. F. & JENNINGS, W. G. (1971). The volatile components of black pepper varieties. *J. Chromatogr. Sci.*, **9**, 560–6.

WHITE, A., HANDLER, P. & SMITH, E. L. (1973). *Principles of biochemistry* (5th ed.). New York, McGraw-Hill.

YABUMOTO, K., YAMAGUCHI, M., HUGHES, D. & JENNINGS, W. G. (1974). Changes in the volatile constituents of ripening cantaloupes. *Proceedings, IVth International Congress Food Science and Technology, Madrid*, 1a, 36–7.

YABUMOTO, K. (1976). Studies on Certain Volatile Constituents of Cantaloupe, *Cucumis melo*, Ph.D. Thesis, University of California, Davis.

STUDIES ON STORAGE OF MILLED RICE FOR A LONG PERIOD†

H. BOLLING, G. HAMPEL & A. W. EL BAYÂ

Federal Research Center for Cereal Processing, Detmold, Germany

(Received: 10 February, 1977)

ABSTRACT

Some studies of long term rice storage are presented. Swelling properties, consistency, gelatinisation and sensory properties are separately evaluated.

INTRODUCTION

Storage of cereals is a normal step between harvest and consumption. Grain must be stored at least from one harvest to the next. Buffer stocks are necessary as an insurance against possible crop failures or poor yields in the following year(s), to arrest fluctuations in price and in the event of a sudden outbreak of war—hence prolonged storage. Storage of freshly harvested rice for short periods under controlled conditions has important advantages: causing rice to age and improving culinary properties and general consumer acceptance. Ageing or post-harvest maturity is a natural process which occurs in the few weeks following harvest to complete the biochemical and physical changes in the kernel. Villareai *et al.* (1976) demonstrated that the hardness index, water absorption, the amylograph peak viscosity and free fatty acids of milled rice increased during storage for six months whilst salt-soluble protein decreased.

There is little, if any, available information concerning the physical and chemical changes occurring in rice during long periods of storage. Therefore it is important to fill this gap.

The present work describes changes in some physicochemical properties of milled rice stored for a long period. The effect of packing materials on the quality of rice during storage has also been examined.

† Publication No. 4327 of Federal Research Center for Cereal Processing.

Fd. Chem. (3) (1978)—
Printed in Great Britain

MATERIALS AND METHODS

Samples

Six rice samples—China (short-grain), Originario, Egyptian, Rinaldo Bersani, Super Patna and Blue Bonnet—were packed in 10 kg lots in paper and jute bags and stored at 20 °C for seven years.

Physicochemical analysis

The consistency of cooked rice was determined in the Haake's consistometer as described by Hampel (1965). The gelatinisation temperatures have been estimated on 100 g rice flour (under 150 μ) slurried in 400 ml distilled water in the Brabender viscograph. The swelling number of the rice samples was tested by the method of Halick & Keneaster (1956) on 1 g of the whole kernels. The increases in volume and cooking losses were determined according to the method of Borasio (1964). For the determination of acetic acid-soluble proteins a colorimetric method according to Jennings (1961) was used. The fatty acid numbers were determined according to the standard methods of the Association for Cereal Research (1971) in Detmold.

Cooking and organoleptic test

The cooking tests were conducted according to the method of Batcher *et al.* (1957, 1963) for 28 min in a covered jena glass jar and 5 min without the cover. The oven temperature was about 176 °C. Evaluation of appearance, cohesiveness, tenderness and taste was carried out by three experienced panellists who were asked to rate the cooked rices according to a hedonic rating scale. A score of 9 was considered as excellent and a score of 4 as acceptable for cohesiveness, tenderness and appearance. The taste of rice should be free from off flavours.

RESULTS AND DISCUSSION

As there are no significant differences between jute and paper packings, the data for jute bag storage only are presented.

Consistency of cooked rice

The consistency of cooked rice is significantly correlated with the total score of the sensoric test (Bolling *et al.*, 1974), the appearance and the separability of kernels after cooking. In general, the consistency of cooked rice markedly increased during storage (2–3 years) and decreased a little, but 1·5 to 2·5 times above the original values (Table 1), indicating that storage causes cooked rice to have a harder texture.

Gelatinisation temperatures

The gelatinisation temperatures seem to be an index of ease of cooking milled rice. Varieties with high gelatinisation temperatures, such as Patna 231, take a

longer time to cook than most long-grain varieties of intermediate gelatinisation temperature (Halick & Kelly, 1959). In general, the gelatinisation temperatures increased with all stored rice samples compared with the tests at the beginning of storage. The increase was greater during the first two years of storage than during the following years.

TABLE 1
EFFECT OF STORAGE ON THE CONSISTENCY (cP × 10^3) OF RICE

Samples stored at 20°C	*Storage period in years* 0	1	2	3	4	7
China (short-grain)	1980	3318	8050	4151	5488	5278
Originario	3700	4228	7448	9681	7910	7518
Egyptian	2650	3388	4900	8512	3542	5012
Rinaldo Bersani	1900	2030	7140	4480	3388	3682
Super Patna	11200	9660	16356	11977	11522	23464
Blue Bonnet	11600	11102	19782	9282	11102	16688

Swelling numbers

The swelling numbers show the hydration characteristics of rice grains and there are varietal differences in the tested rice samples. With very few exceptions, the swelling numbers for all rice samples examined decreased with progressive storage (Table 2). The form of packing seems to have no effect on the swelling numbers. This decrease may explain the decrease in cooking losses and the increased consistency of cooked rice during storage.

TABLE 2
EFFECT OF STORAGE ON THE SWELLING NUMBERS OF RICE

Samples stored at 20°C	*Swelling numbers* *Storage period in years* 0	1	2	3	4	7
China (short-grain)	20	19	11	11	6	9
Originario	26	25	13	10	8	7
Egyptian	15	18	8	8	7	8
Rinaldo Bersani	14	14	10	7	5	9
Super Patna	7	5	5	5	4	4
Blue Bonnet	7	4	5	5	5	4

Volume expansion and cooking losses

The volume expansion of most of the rice samples remained nearly unchanged except for Super Patna and Blue Bonnet in which the volume expansion decreased in the third and seventh years, regardless of the type of packing. The cooking losses of most rice samples decreased with increasing storage time but the effect of packing material was very small compared with the cooking losses (Table 3).

TABLE 3
EFFECT OF STORAGE ON COOKING LOSSES OF RICE

Samples stored at 20°C	*Cooking losses % Storage period in years*				
	0	1	2	3	7
China (short-grain)	13·4	11·8	12·7	11·4	10·9
Originario	13·2	12·2	11·8	11·1	11·2
Egyptian	12·5	11·3	11·4	10·6	10·6
Rinaldo Bersani	13·3	12·1	11·6	11·5	11·2
Super Patna	11·7	10·8	11·1	10·7	11·0
Blue Bonnet	11·8	11·0	10·4	11·1	10·5

Fatty acid numbers

Fat acidity is of special interest as it indexes the deterioration of milled rice during storage, having high correlations with kernel damage. Free fatty acids, measured as fatty acid numbers, increased in all rice samples of the two packings during storage up to the second year and, interestingly, decreased on further storage (Table 4). The increase in the fatty acid number of stored rice samples is due to the degradation of glycerides forming fatty acids which can complex with the amylose during long periods of storage. On the other hand, the free fatty acids undergo oxidation, giving carbonyl compounds which interact with other constituents forming petroleum ether insoluble compounds. Therefore, the decrease in the fatty acid numbers after the second year may be explained by the degradation of unsaturated fatty acids or by the formation of complexes with amylose, increasing the consistency values.

TABLE 4
EFFECT OF STORAGE ON THE FATTY ACID NUMBERS OF RICE

Samples stored at 20°C	*Fatty acid numbers Storage period in years*				
	0	1	2	3	7
China (short-grain)	36	58	65	45	37
Originario	25	43	51	39	21
Egyptian	68	80	96	76	53
Rinaldo Bersani	32	24	25	21	14
Super Patna	22	40	57	56	45
Blue Bonnet	23	29	42	41	29

Acetic acid-soluble proteins

There was a continuous decrease in the acetic acid-soluble proteins in all milled rice samples stored in the two types of packing (Table 5). Towards the end of the seventh year these values were almost half the original values. The decrease in the acetic acid-soluble proteins may be due to their interaction with sugars and other products formed from rice lipids. Changes in solubility of proteins by interaction with carbonyls have been reported by Barber (1972). The interaction of lipid

TABLE 5
EFFECT OF STORAGE ON THE ACETIC ACID SOLUBLE PROTEINS OF RICE

Samples stored at 20°C	*Acetic acid-soluble proteins %* *Storage period in years*				
	0	1	2	3	7
China (short-grain)	2·79	2·80	2·48	1·57	1·44
Originario	2·26	1·83	1·90	1·39	1·33
Egyptian	2·74	1·97	2·11	1·50	1·57
Rinaldo Bersani	4·06	3·78	3·37	3·16	2·03
Super Patna	2·33	1·59	1·33	1·26	1·33
Blue Bonnet	2·47	1·83	2·03	1·63	1·58

hydroperoxides, formed during storage with proteins, lowers the solubility, as suggested by Premavalli *et al.* (1973). Also, Pomeranz *et al.* (1968) have reported decreased amounts of globulins and albumins in storage-damaged flours. The increased intensity of the yellow colour of milled rice stored for one year at 35 °C in comparison with storage at 2 °C (Pelshenke *et al.*, 1967) may be attributed to the non-enzymic browning reaction of proteins with reducing sugars which are concentrated in the surface layer of milled rice. Similar results have been reported by Barber (1972).

Cooking and organoleptic tests

Sensory evaluation tests showed that the total score of the sensoric test remained unchanged during the storage period. However, an improvement in the cooking quality of the Egyptian sample was observed after the first year of storage and remained until the end of the storage period. The odour of the uncooked rice samples was normal up to the first year. However, during the seven years of the storage period, all the rice samples were acceptable in odour and taste after cooking.

SUMMARY

The effects of prolonged storage on the physicochemical characteristics and cooking quality of milled rice were investigated and the relative merits of paper and jute as a packing material studied. It was found that the consistency of cooked rice and the gelatinisation temperature of rice flour increased during storage, whilst the swelling number of the rice kernels and the cooking losses decreased. The increased fatty acid numbers of the stored rice samples up to the second year can be attributed to the formation of free fatty acids by lipolysis. After the second year the fatty acid numbers decreased with increasing storage time. A marked gradual decrease of acetic acid-soluble proteins has been observed. The organoleptic tests of cooked rice showed that the total score of the sensoric test did not change markedly during the storage period.

REFERENCES

ASSOCIATION FOR CEREAL RESEARCH (1971). *Standard-Methoden für Getreide, Mehl und Brot der Arbeitsgemeinschaft Getreideforschung*, 5th edn., Verlag Moritz Schäfer, Detmold.

BARBER, S. (1972). Milled rice and changes during aging, in *Rice: Chemistry and technology* (D. F. Housten, ed.), pp. 215–63. American Cereal Chemists, St Paul, Minn.

BATCHER, O. M., DEARY, P. A. & DAWSON, E. H. (1957). Cooking quality of twenty-six varieties of milled white rice. *Cereal Chem.*, **24**, 277–85.

BATCHER, O. M., STALEY, M. G. & DEARY, P. A. (1963). Palatability characteristics of foreign and domestic rice cooked by different methods. Parts I and II. *Rice Journal*, **66**(9), 19–24 and (10), 13–16.

BOLLING, H., HAMPEL, G., EL BAYÂ, A. W. & ISIK, N. (1974). Evaluation of some physicochemical characteristics of rice varieties grown in Turkey as related to their cooking quality. *Il Riso*, **23**, 399–403.

BORASIO, L. (1964). Aspect actuel de l'analyse et de l'appréciacion des riz. *Report of the ICC-Congress, Wien.*

HALICK, J. V. & KENEASTER, K. K. (1956). The use of a starch-iodine-blue test as a quality indicator of white milled rice. *Cereal Chem.*, **33**, 315–19.

HALICK, J. V. & KELLY, V. J. (1959). Gelatinization and pasting characteristics of rice varieties as related to cooking behavior. *Cereal Chem.*, **36**, 91–8.

HAMPEL, G. (1965). Untersuchung über die Qualität von Weißreis. *Getreide und Mehl*, **15**, 132–6.

JENNINGS, A. C. (1961). Determination of the nitrogen content of cereal grain by colorimetric methods. *Cereal Chem.*, **38**, 467–79.

PELSHENKE, P. F. & HAMPEL, G. (1967). Studies of the effect of extreme storage conditions on white rice. *Milling*, **149**, 192–6.

POMERANZ, Y., DAFTARY, R. D., SHOGREN, M. D., HOSENEY, R. C. & FINNEY, K. F. (1968). Changes in biochemical and bread-making properties of storage-damaged flour. *J. Agr. Food Chem.*, **16**, 92–6.

PREMAVALLI, K. S., LEELA, R. K., ARYA, S. S., PARIHAR, D. B. & NATH, H. (1973). Studies on packing and storage of wheat flour (Atta) under tropical conditions. *J. Fd. Sci. Technol. (India)*, **10**, 27–30.

Standard-Methoden für Getreide, Mehl und Brot (1971). 5. *Auflage Verlag Moritz Schäfer, Detmold.*

VILLAREAL, R. M., RESURRECCION, A. P., SUZUKI, L. B. & JULIANO, B. O. (1976). Changes in physicochemical properties of rice during storage. *Die Stärke*, **28**, 88–94.

THE RELATIONSHIP OF ADDED NITRATE TO PIGMENT FORMATION IN NATIVE PORK-SAUSAGE (*LONGANIZA*)

E. S. LUIS, A. O. LUSTRE and C. H. LACEBAL

Food Research Department, Industrial Research Center, National Institute of Science and Technology, Pedro Gil St., cor. Taft Ave., Manila, Philippines

(Received: 3 March 1977)

ABSTRACT

The nitrosomyoglobin, residual nitrate and nitrite concentrations of the native pork-sausage (longaniza) *were analysed within a ten-day storage period. Several levels of nitrate* (50, 100, 300, 500 *and* 2000, 3500 *and* 5000 *ppm*) *were employed. These were examined to determine the minimum amount of nitrate that could be added to the curing mixture for the making of* longaniza *whilst still obtaining acceptable organoleptic properties, especially colour.*

Pigment formation in longaniza *due to the added nitrate occurs from the onset and reaches its maximum on the third or fourth day. The desirable pink to red colour in the cured meat was obtained even with amounts of nitrate as low as* 50 *ppm. All treated* longaniza *showed high and similar colour scores compared with the control. However, higher colour stability was obtained with the use of greater than* 500 *ppm nitrate. The amount of residual nitrate and nitrite is directly proportional to the initial quantity of nitrate added. The use of* 50 *to* 500 *ppm nitrate poses no danger to public health while the use of* 2000 *to* 5000 *ppm nitrate enhances the possibility of carcinogenic nitrosamine formation and incidence of methemoglobinemia.*

INTRODUCTION

Determining the proper levels of use of food additives in local recipes for processed products will go a long way towards educating processors and therefore minimising potential hazards to public health which the indiscriminate use of additives can bring about.

The native pork-sausage or *longaniza* is one of the many kinds of cured meat (e.g. ham, bacon, tocino, etc.) that has gained popularity in the Filipino diet. Although each cured meat product has its specific component and proportion of the curing

Fd. Chem. (3) (1978)—
Printed in Great Britain

mixture employed, common to them all is *salitre* (potassium nitrate) which is used as a basic ingredient. Close examination of published formulations for the making of *longaniza* shows that there has been an indiscriminate use of potassium nitrate. Mendoza (1961) recommended the use of 0·5 % KNO_3 based on the weight of meat and De Leon (1966), one teaspoonful of KNO_3/kg meat. In both cases, the quantity of added KNO_3 amounts to about 4000 to 5000 ppm nitrate. The permissible limit of nitrate in cured meats is 500 ppm, as prescribed by the Food and Drug Administration of the United States, and as adapted by the Food and Drug Administration of the Philippines.

Studies conducted on the use of excessive nitrates and nitrites, either as their potassium or sodium salts in meat curing, showed that they pose a potential health hazard. The possibility of forming carcinogenic nitrosamines in nitrate/nitrite treated meat is already well established (Lijinsky & Epstein, 1970; IFT, 1972; Fiddler *et al.*, 1972; Newton *et al.*, 1972; Sebranek & Cassens, 1973; Wolff & Wasserman, 1972; Dooley *et al.*, 1973; Fan & Tannenbaum, 1973; Patterson & Mottram, 1974 and Dethmers *et al.*, 1975). The incidence of methemoglobinemia, a type of food poisoning due to the consumption of meat products where excessive amounts of nitrates/nitrites are present, has also been reported (Orgeron *et al.*, 1957 and Fasset, 1966).

In spite of the potential hazards that accompany the use of nitrates/nitrites in meat curing, their elimination cannot be recommended. The use of nitrates/nitrites is technologically necessary in maintaining flavour, colour and texture characteristics associated with and expected of certain meat products (Fine, 1972; Knowles *et al.*, 1974; Kemp *et al.*, 1974; Brown *et al.*, 1974 and Hadden *et al.*, 1975). Besides, nitrates/nitrites exert an inhibitory effect on the growth of *Clostridium botulinum* (Perigo & Roberts, 1968; Wasserman & Huhtanen, 1972 and Christiansen, 1975), *Clostridium perfringens* (Riha & Solberg, 1973, 1975), *Clostridium sporogenes* (Perigo *et al.*, 1967), *Staphylococcus aureus* (Buchanan & Solberg, 1972) and other putrefactive organisms (Bulman & Ayres, 1952).

Under Philippine conditions, the indiscriminate addition of *salitre* during meat curing accrues from several factors. The seemingly innocuous physical appearance of *salitre* (which resembles ordinary salt), traditional user's ignorance on the possibility of nitrosamine formation and anxiety over the likelihood of colour fading in the cured products, all combine to aggravate the situation. Very recently, Madlansacay *et al.* (1975), recommended the use of 0·05 % KNO_3 based on the weight of meat for the making of native pork-sausage. This yields 400–500 ppm of nitrate which is within the permissible level. No mention was made of the organoleptic properties of the resulting product.

This paper thus presents a completed portion of a project whose overall objective is to determine the effect of food additives in local recipes for processed foods. The project is carried out with the purpose of being able to recommend proper levels of use of various food additives in local formulations for processed foods. Specifically, this study was undertaken to determine the minimum amount of nitrate that could

be added to the curing mixture for the making of *longaniza* whilst still maintaining favourable organoleptic qualities, especially colour. The experiment was conducted at the Food Research Department of the Industrial Research Center, NIST/NSDB.

MATERIALS AND METHODS

Preparation of native pork-sausage

The native pork-sausage used in this experiment was prepared using the recipe of De Leon (1966). The quantities and proportions of the ingredients employed were as specified except for the amount of *salitre* (potassium nitrate) added. The amounts of nitrate added were: 0, 50, 100, 300, 500, 2000, 3500 and 5000 ppm. The pork used in the preparation was bought from Paco Market, Manila.

Chemical analysis

(a) The residual nitrate and nitrite were determined according to the procedure of Jacobs (1965).

(b) The pigment (nitrosomyoglobin) formed in the products was quantitatively analysed by the method of Hornsey (1956).

Sensory evaluation

The colour was evaluated by a panel of judges from the Food Research Department, IRC, NIST staff members using the 9-1 hedonic rating scale.

RESULTS AND DISCUSSION

Pigment formation

The progress of the amount of pigment formed coming from the nitrate added was determined daily over a ten-day storage period at room temperature. The quantity of nitrate added to the curing mixture was divided into two groups, one within (50, 100, 300 and 500 ppm NO_3^- added) and the other above (2000, 3500 and 5000 ppm NO_3^- added) the permissible level (500 ppm NO_3^-). The *longaniza* samples were hung inside a wire screen enclosure to permit free air circulation. All the operations involved in the determination of the nitrosomyoglobin concentration in the cured meat product were conducted under subdued light conditions. Only freshly opened *longaniza* (sausage) pieces were used. The results are shown in Figs. 1 and 2.

The changes in nitrosomyoglobin concentration expressed in ppm haematin with time for the *longaniza* samples treated with 50, 100, 300 and 500 ppm nitrate are shown in Fig. 1. It can be seen from the curves that all the treatments indicated had very similar trends. The quantity of nitrosomyoglobin extractable by 80 % aqueous-acetone mixture increased gradually from the first to the third day, and abruptly from the third to the fourth day in which maximum pigment formation was reached. On the other hand, the quantity of pigment extractable from the fourth to the fifth

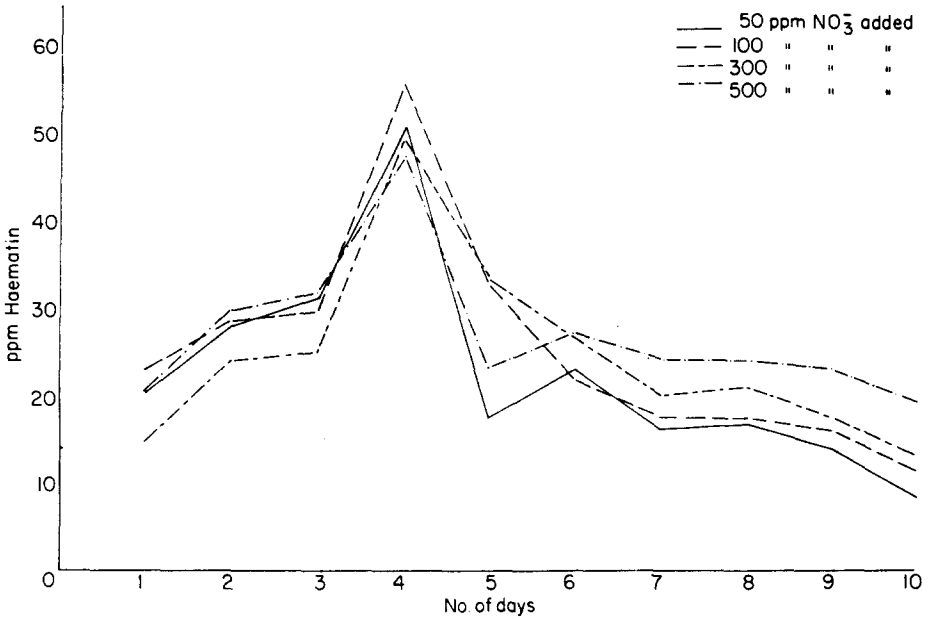


Fig. 1. Changes in nitrosomyoglobin concentration expressed in ppm haematin with time of the *longaniza* treated with 50, 100, 300 and 500 ppm nitrate.

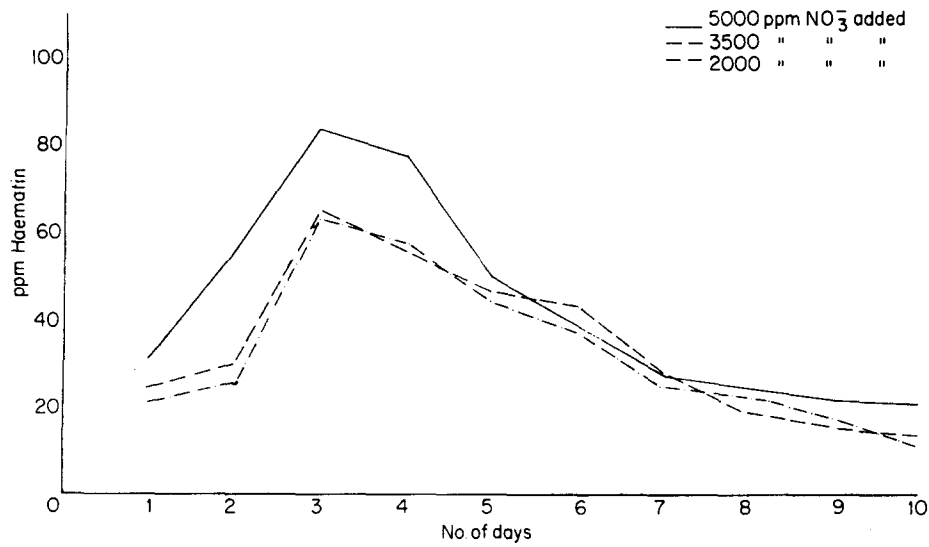


Fig. 2. Changes in nitrosomyoglobin concentration expressed in ppm haematin with time of *longaniza* treated with 2000, 3500 and 5000 ppm nitrate.

day declined abruptly, a net gradual decrease from the fifth day to the tenth day then ensuing.

The behaviour depicted by the curves suggests that pigment formation occurred from the time the preparation of the *longaniza* was completed until its fourth day of holding. This lapse of time is reasonable since the nitrate added must first be converted into nitrite then to nitric oxide, the reactive species that combines with the myoglobin present in the meat to form the pink–red nitrosomyoglobin pigment. The conversion of nitrate to nitrite has been known to be effected by the action of some species of bacteria (IFT, 1972) present in the meat. Thus, the rate of pigment formation can be said to be dependent upon the rate of the transformation:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \xrightarrow{\text{myoglobin}} \text{NO-myoglobin}$$

The progressive decline in the amount of nitrosomyoglobin extracted by the 80% aqueous-acetone solution after the pigment formation has reached its peak apparently suggested that the nitrosomyoglobin was transformed into some other

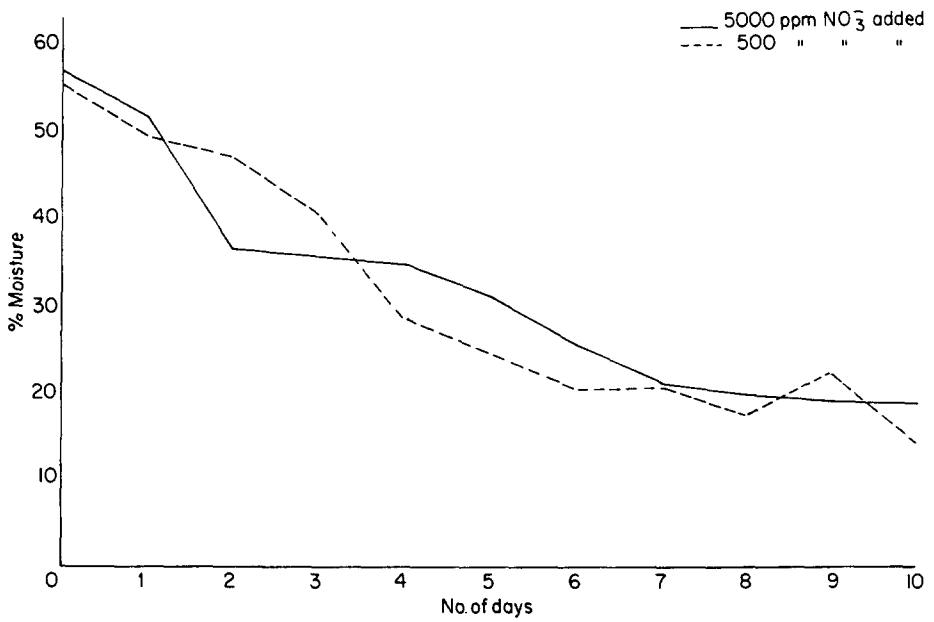


Fig. 3. Changes in moisture content with time of the *longaniza* treated with 500 and 5000 ppm nitrite.

form which is insoluble in the extractant. Perhaps attendant conformational change and/or denaturation occurred in the protein moiety of the pigment which rendered it insoluble. This could probably occur since continuous dehydration of the *longaniza* samples (Fig. 3) occurred with time of holding. The formation of

nitrosylhemochrome (a denatured species of the pigment which also has a pink to red colour) is very unlikely since this pigment is also very soluble in polar solvents.

Based on the curves in Fig. 1 and from the result of the colour evaluation (Table 1), it could be seen that the colour in the meat had already formed considerably even after only the first day. However, the measured changes in nitrosomyoglobin concentration did not reflect in colour scores. Table 1 shows that the treated samples already had high hedonic colour ratings on the first day of analysis while the pigment concentration was still at a low level. Apparently, the myoglobin content determined the amount of pigment formed since the maximum level of nitrosomyoglobin produced is very similar in all treatments. This indicated that the different amounts of nitrate added to each curing mixture may have been sufficient for colour formation, suggesting that, with respect to colour development, 50 ppm nitrate suffices to give the desirable colour in *longaniza*.

TABLE 1

THE AVERAGE COLOUR SCORES OF *longaniza* (CONTROL AND NITRATE TREATED AT 50, 100, 300 AND 500 ppm LEVEL). THE SCORING WAS BASED ON THE 9-1 HEDONIC RATING SCALE

Days of holding	*Average control*	*Colour score ppm NO_3^- added*			
		50	100	300	500
1	4·4	6·8	6·6	7·4	7·2
2	4·4	6·8	6·9	7·1	6·8
3	4·0	6·7	6·2	7·1	6·7
4	4·1	6·4	6·9	7·0	7·1
7	4·2	6·1	6·4	6·3	6·5

Figure 2 shows the changes with time in the nitrosomyoglobin concentration expressed in ppm haematin for the *longaniza* samples treated with 2000, 3500 and 5000 ppm nitrate. The curves in Fig. 2 show trends comparable to those shown in Fig. 1. The measurable quantities of the pigment formed increased progressively until a maximum was reached and then continuously declined on holding. Pigment formation apparently occurred at a faster rate, as indicated by the relatively higher amount of nitrosomyoglobin formed after the first day, and the highest colour development was obtained on the third day. The faster rate observed in colour development with the use of higher nitrate concentration could be due to the greater saturation of the available myoglobin in the meat by the nitrous oxide coming from the added nitrate. Again, the maximum quantities of nitrosomyoglobin formed are similar among treatments. This reiterates that the quantity of pigment formed is dependent on the concentration of myoglobin present in the meat. At the levels of nitrate added in the curing mixture, they are more than sufficient to convert all the myoglobin into nitrosomyoglobin.

The difference in the quantity of pigment formed between the treatments within and those above the permissible level was not remarkably distinguishable. The F-

TABLE 2
THE F VALUES OBTAINED BY THE ANALYSIS OF VARIANCE OF THE COLOUR SCORES OF *longaniza* TREATED WITH 50, 500 AND 5000 ppm NITRATE

Days of holding	*F-value*		
	Computed	*Tabulated* (5%)	(1%)
1	0·49	3·55	6·01
4	2·21		
10	7·37		

values (Table 2) computed from the result of the colour evaluation of *longaniza* treated with 50, 500 and 5000 ppm nitrate revealed no significant differences in colour between treatment on the first and fourth day of storage. It was only on the tenth day that significant differences in colour were obtained. Perhaps greater concentration of nitrate provided conditions for the greater stability of the pigment formed. However, the colour stability on storage of the *longaniza* treated with 5000 ppm nitrate does not give a marked advantage since *longaniza*, when stored in the manner described in this paper, is seldom kept beyond the fifth or sixth day.

Residual nitrates and nitrites

The residual nitrates and nitrites were determined at one day intervals over the ten day holding period. The residual nitrates were analysed at the start of and on the second, fourth, sixth, eighth and tenth days of storage. On the other hand, the residual nitrites were obtained on the first, third, fifth, seventh and ninth days. The results are shown in Figs. 4 and 5.

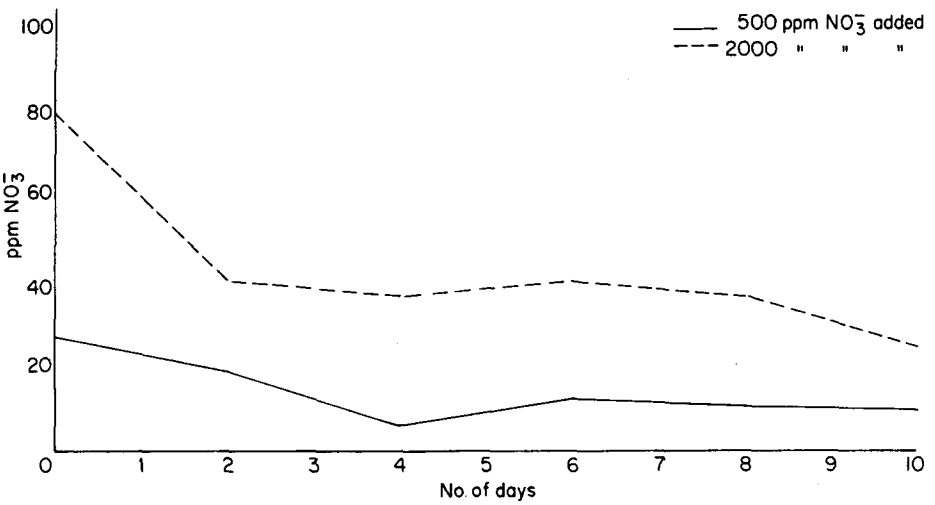


Fig. 4. Changes in residual nitrate concentration with time of the *longaniza* treated with 500 and 2000 ppm nitrate.

Figure 4 shows representative data on the changes in residual nitrate concentration with time for the *longaniza* treated with from 50 to 500 and from 2000 to 5000 ppm nitrate. The lower curve represents the 500 ppm nitrate treated *longaniza* and the upper one, the 2000 ppm nitrate treated sample.

On average, as represented by the curve for the 500 ppm nitrate treated sample, there was first a gradual conversion of the added nitrate (into NO_2^- and NO) which then increased rapidly until it reached its minimum residual value. This characteristic is indicated in the parts of the curve covering from zero to the second day and from the second to the fourth days, respectively. The remaining nitrate may be further converted into NO_2^- then to NO or remain as residual nitrate. The curve also shows that an increased amount of nitrate added to the curing mixture adds up only to the amount of residual quantities of NO_3^- without significantly effecting enhanced pigment formation (see also Fig. 1). This is quite important in relation to minimising the amount of residual nitrate which could serve as a reservoir of reactants involved in the formation of carcinogenic nitrosamines.

The upper curve in Fig. 4 revealed similar net changes between treatments of from 50 to 500 and from 2000 to 5000 ppm nitrate.

For those treated with from 2000 to 5000 ppm nitrate, the inflection point (as represented by the curve for the 2000 ppm nitrate treated sample) signifies the rapid

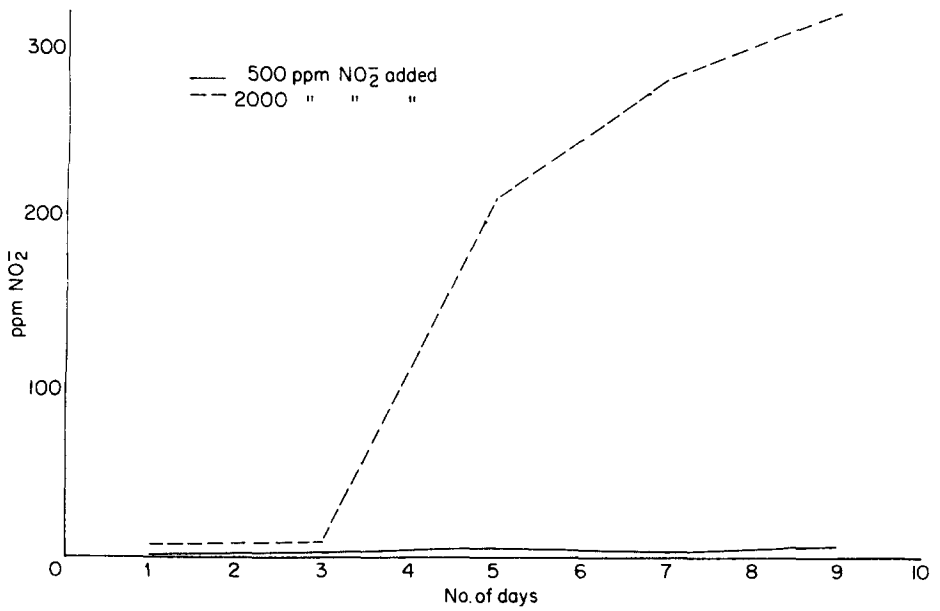


Fig. 5. Changes in residual nitrite concentration with time of the *longaniza* treated with 500 and 2000 ppm nitrate.

transformation of NO_3^- into subsequent forms which occurred earlier. Apparently, the kinetics of the chemical transformation in the curing process are enhanced by larger quantities of nitrate present in the curing mixture. This advantage is completely offset by the increased quantity of starting materials that may lead to nitrosamine formation.

Figure 5 shows representative data on the changes in residual nitrite concentration with time of the *longaniza* treated with from 50 to 500 and with from 2000 to 5000 ppm nitrate. The lower curve is for the 500 ppm treated sample and the upper one for the 2000 ppm treated sample. In all cases, a net increase in residual nitrite was obtained. The maximum inflection point in the lower curve again coincided with the time at which pigment development reaches its peak. On the other hand, no such maximum inflection point is present in the upper curve. The difference in the behaviour of the curves can be attributed to nitrate and nitrite saturation of the cured meat product. Those treated beyond the permissible nitrate level form excess amounts of residual nitrite and present a very dangerous situation. The tolerable limit of residual nitrite in any cured meat is 200 ppm. The possibility of nitrosamine formation and the outbreak of methemoglobinemia is very probable in 2000, 3500 and 5000 ppm nitrate treated *longaniza*. It can also be seen from Fig. 5 that after the *longaniza* has formed its maximum colour and possibly all the myoglobin has already been reacted, most of the remaining nitrate is converted into residual nitrite, probably including the gaseous products such as NO and N_2O which may be lost by diffusion.

TABLE 3
NITROGEN-BALANCE STUDY IN *longaniza* BY KJELDAHL ANALYSIS

Weight of sample	*ppm* NO_3^- *added*	*ppm nitrogen*	
		Water extract	*Meat residue*
50	600	100	1100

Fate of the NO_3^- *added*

Summing up, the total amounts of nitrate converted into nitrosomyoglobin and residual nitrates and nitrites for a particular treatment and holding time would indicate that the majority of the added nitrogen could not be accounted for in these compounds. A nitrogen balance study conducted by Kjeldahl analysis of the water extract used for nitrate determination and the meat residue showed that some of the nitrates added become bound to the complex cured meat system or converted into unknown nitrogenous compounds. The result of such an experiment is shown in Table 3. The 100 ppm N in the water extract represents that of the water soluble in the aqueous medium. The non-nitrate compounds may include nitrites, free amino acids and nitrogenous compounds formed from nitrate addition. The 1200 ppm N

in the meat residue represents the organic nitrogen in the meat, bound NO_3^- and NO_2^-, NO-myoglobin produced and unknown nitrogenous compounds formed during processing. Thus, the discrepancy between the actual amounts of nitrate added and the sum total of those converted to nitrosomyoglobin (and perhaps including nitrosylhemochrome), residual nitrates and nitrites may be accounted for as bound to proteins (Bard & Towsend, 1970 and Sebranek *et al.*, 1973), lost in the form of gaseous products such as N_2O and NO (Woolford *et al.*, 1972) and converted into unknown nitrogenous compounds.

Retrospect

The amount of nitrate to be added to the curing mixture must take account of the desired colour and other organoleptic properties, as well as control of microbial growth. Thus, although the addition of 50–5000 ppm of nitrate seems sufficient for colour development, there is a need to conduct microbial analysis on the products to ascertain the effect of the added nitrate on the microflora. Bulman & Ayres (1952) indicated that 400 to 800 ppm nitrite is required to have a preservative effect. For colour development, 10 ppm (Mohler, 1965) to 100 ppm (Takagi *et al.*, 1971) nitrite is needed for the development of the desired colour in meat. The 200 ppm nitrite and 500 ppm nitrate as limits in cured meat agree fairly well with the cited findings. In this study, the use of 2000 to 5000 ppm nitrate is definitely excessive. However, no definitive level can be pinpointed as fairly reasonable on the *longaniza* treated within the permissible level because of the absence of data on the microflora of the resulting products. This, therefore, should be the object of the next phase of the study.

SUMMARY AND CONCLUSIONS

The nitrosomyoglobin, residual nitrate and nitrite concentration of the native pork-sausage (*longaniza*) were analysed within a ten-day storage period. The *longaniza* was prepared according to the recipe of De Leon (1966) with varying levels of nitrate added (50, 100, 300, 500 and 2000, 3500 and 5000 ppm). The nitrosomyoglobin concentration was determined daily from zero to the tenth day; the residual nitrates were determined at the start of storage and on the second, fourth, fifth, eighth and tenth days of storage; and the residual nitrites on the first, third, fifth, seventh and ninth days. Sensory evaluation with respect to the colour of the *longaniza* was also undertaken.

The changes in nitrosomyoglobin concentration expressed in ppm haematin with time indicated that pigment formation in the meat due to the addition of nitrates occurs from the start and reaches its maximum on the third to fourth days. The desirable pink to red colour in the cured meat can be obtained with as low as 50 ppm nitrate. This was demonstrated by the high and similar colour scores given to the

treated *longaniza* compared with the untreated *longaniza* even after only one day of storage. There is no significant colour difference between treatments, although the *longaniza* treated above the permissible level of nitrate (500 ppm) showed more colour stability. The rate of colour development is faster with the use of higher concentrations of nitrate in the curing mixture.

The *longaniza* undergoes continuous dehydration on storage. This is accompanied by the transformation of the nitrosomyoglobin into a form which is insoluble in 80% aqueous-acetone solution. The protein moiety of the pigment might have undergone conformational change and/or denaturation.

The amount of residual nitrates and nitrites increases with increased quantity of nitrate added to the curing mixture. The use of 50–500 ppm nitrate produces almost negligible quantities of residual nitrate and nitrite. Thus, the possibility of the production of carcinogenic nitrosamines and the incidence of methemoglobinemia is minimal or even perhaps absent. On the other hand, the use of 2000–5000 ppm nitrate definitely poses public health hazards as the residual nitrite accumulates to levels much higher than the permissible level of 200 ppm.

REFERENCES

BARD, V. & TOWSEND, W. E. (1971). Meat curing. In: *The science of meat and meat products*. 452–70, San Francisco, W. H. Freeman and Co.

BROWN, C. L., HEDRICK, H. B. & BAILEY, M. E. (1974). Characteristics of cured ham as influenced by levels of sodium nitrite and sodium ascorbate. *J. Food Sci.*, **39**, 977–9.

BUCHANAN, R. L. & SOLBERG, M. (1972). Interaction of nitrite, oxygen and pH on growth of *Staphylococcus aureus*. *J. Food Sci.*, **37**, 81.

BULMAN, C. & AYRES, J. C. (1952). Preservative action of various concentrations of curing salts in comminuted pork, *Food Technol.*, **6**, 255–9.

CHRISTIANSEN, L. M. (1975). Effect of sodium nitrite and nitrate on *Clostridium botulinum* growth and toxin production in a summer style sausage. *J. Food Sci.*, **40**, 488–90.

DE LEON, S. Y. (1966). Curing of fish and meat. In: *Preservation of Philippine foods*. 60–61, Quezon City, Philippines, Alemar Phoenix Publishers.

DETHMERS, A. E. *et al.* (1975). Effect of added sodium nitrite and sodium nitrate in sensory quality and nitrosamine formation in thwringer sausage. *J. Food Sci.*, **40**, 491–5.

DOOLEY, C. J., WASSERMAN, E. A. & OSMAN, S. (1973). A contaminant in N-nitrosodimethylamine confirmation by high resolution mass spectrometry, *J. Food Sci.*, **39**, 1096.

FAN, T. Y. & TANNENBAUM, S. R. (1973). Natural inhibitors of nitrosation reactions. The concept of available nitrite, *J. Food Sci.*, **38**, 1067–9.

FASSET, D. W. (1966). Nitrates and nitrites. Toxicants occurring naturally in foods. NAS/NRC, Pub. 1354, p. 250. (Cited by IFT (1972). *J. Food Sci.*, **37**, 4.)

FIDDLER, W. *et al.* (1972). The effect of sodium nitrite concentration in N-nitrosodimethyl formation of frankfurters, *J. Food Sci.*, **37**, 688–70.

FINE, S. D. (1972). *Food additives* (21 CFR Part 121). *Federal Register*, **37**, 213. (Cited by Dethmers, A. E., *loc. cit.*)

HADDEN, J. P. *et al.* (1975). Influence of sodium nitrite in the chemical and organoleptic properties of comminuted pork, *J. Food Sci.*, **40**, 626–30.

HORNSEY, H. C. (1956). The colour of cooked cured pork: I-Estimation of the nitric oxide-haem pigments, *J. Sci. Fd. and Agric.*, **7**, 534–40.

JACOBS, M. B. (1965). Nitrates/nitrites in flesh foods. In: *Chemical analysis of foods and food products*, 760–6, New York, D. Van Nostrand Co., Inc.

KEMP, J. D., POX, J. D. & MOODY, W. G. (1974). Cured ham properties as affected by nitrate and nitrite and fresh pork quality. *J. Food Sci.*, **39**, 972–6.

KNOWLES, M. E., GILBERT, J. & MCWEENEY, D. J. (1974). Stability of red food colours in the presence of nitrite in canned pork luncheon meat. *J. Sci. Fd. Agric.*, **25**, 1239–48.

LIJINSKY, W. & EPSTEIN, S. S. (1970). Nitrosamines as environmental carcinogens. *Nature*, **225**, 21.

MADLANSACAY, P. L. *et al.* (1975). Methods of meat preparation. In *Handbook on animal products utilization*. Bureau of Animal Industry, Department of Agriculture, Philippines, p. 48.

MENDOZA, J. N. (1966). Meat and meat products. In: *Philippine food processing and manufacture*. Manila, Philippines, Phil. Educ. Co., 300–1.

MOHLER, K. (1965). Reaction products of nitrite in meat production. Paper presented to 11th European Meeting, Meat Research Workers, Belgrade, Yugoslavia.

NEWTON, B. E., SAXBY, M. J. & WALTERS, C. L. (1972). Nitrosation of food amines under simulated stomach condition, *Biochem. Journal*, **130**, 82.

ORGERON, J. D. *et al.* (1957). Methemoglobinemia from eating meat with high nitrite content. (Cited by W. W. Fiddler (1972).) Functions of nitrates and nitrites in cured meats. In: *Quality and its preservation in processed foods*, 9. U.S.D.A., Agricultural Research Service.

PATTERSON, R. L. S. & MOTTRAM, D. S. (1974). The occurrence of volatile amines in uncured and cured pork meat and their possible role in nitrosamine formation in bacon. *J. Sci. Fd. Agric.*, **25**, 1419–25.

PERIGO, J. A. *et al.* (1967). Observations on the inhibition of vegetative cells of *Clostridium sporogenes* by nitrite which has been autoclaved in a laboratory medium, discussed in the context of sub-lethally processed cured meats. *J. Food Technol.*, **2**, 377–80.

PERIGO, J. A. & ROBERTS, T. A. (1968). Inhibition of *Clostridia* by nitrite. *J. Food Technol.*, **3**, 91–4.

RIHA, W. E. & SOLBERG, M. (1973). Instability of $NaNO_2$ in chemically defined microbiological medium. *J. Food Sci.*, **38**, 1–3.

RIHA, W. E. & SOLBERG, M. (1975). *Clostridium perfringens* inhibition by sodium nitrite as a function of pH, inoculum size and heat. *J. Food Sci.*, **40**, 439–42.

SEBRANEK, J. G. *et al.* (1973). N^{15} tracer studies of nitrite added to a comminuted meat product. *J. Food Sci.*, **38**, 1220–3.

SEBRANEK, J. G. & CASSENS, R. G. (1973). Nitrosamines: A review. *J. Milk Food Technol.*, **36**, 76–91.

WASSERMAN, A. E. & HUHTANEN, C. N. (1972). Nitrosamines and the inhibition of *Clostridia* in medium treated with sodium nitrite. *J. Food Sci.*, **37**, 785–6.

WOLFF, I. A. & WASSERMAN, A. E. (1972). Nitrates, nitrites, and nitrosamines. *Science*, **177**, 15–19.

WOOLFORD, G., CASSELDAN, W. C. & WALTERS, C. L. (1972). Gaseous products of the interaction of sodium nitrite with porcine skeletal muscle. *Biochem. Journal*, **130**, 82P–83P.

IFT EXPERT PANEL (1972). Nitrites, nitrates, and nitrosamines in food: A dilemma. A scientific status summary by the Institute of Food Technologists Panel of Experts. *J. Food Sci.*, **37**, 4.

TAKAGI, S. *et al.* (1971). Studies on the curing of meat. II. Effects of nitrate during curing. *J. Food Sci. Tech. (Tokyo)*, **18**, 1–7.

STUDIES ON ENZYME-MODIFIED PROTEINS AS FOAMING AGENTS: EFFECT OF STRUCTURE ON FOAM STABILITY

T. HORIUCHI & D. FUKUSHIMA

Noda Institute for Scientific Research, Noda-shi, Chiba-ken, Japan

&

H. SUGIMOTO & T. HATTORI

Central Research Laboratories, Kikkoman Shoyu Co., Noda-shi, Chiba-ken, Japan

(Received: 8 March, 1977)

ABSTRACT

A correlation between foam stabilities and molecular structures was estimated for five kinds of protein hydrolysed with pepsin. The protein hydrolysates which exhibited large surface absorption showed large foam stability within the region in which the Gibbs' rule was applicable. At the same time, the content of the external hydrophobic region demonstrated a good correlation with foam stability. Intra-molecular disulphide bonds seem to participate strongly in the formation and stabilisation of the hydrophobic region on the surface of molecules. However, the secondary structures and the content of the total hydrophobic amino acids in the samples bore no correlation with their foam stabilities. The influence of disulphide bonds on foam stabilities is discussed.

INTRODUCTION

There are many kinds of food which contain foaming agents. Among these egg albumin and cream are natural types which have been used from ancient times. They have several merits; particularly, the foam of egg white is fixed by heat coagulation, but good foam formation is not normal at high temperatures.

In general, foaming agents originating from proteins are prepared by their partial hydrolysis, through which the foaming power is increased. However, the foam stability, one of the most important properties of foaming agents, is decreased by this treatment. The decrease of the foam stability might be ascribed to the change of molecular structure during hydrolysis. This paper therefore deals with the relationship between foam stabilities of hydrolysed proteins and their structures.

Fd. Chem. (3) (1978)—

MATERIALS AND METHODS

Materials

Five kinds of protein were used as the materials. They were gelatin (Nitta Gelatin Co.), egg albumin (Koso Chemical Co.), casein (Hammerstein), wheat gluten (Tokyo Kasei Kogyo Co.) and soybean protein. Soybean protein was prepared by isoelectric precipitation of the water-extract from soybean flakes defatted with hexane at low temperature.

Modification of proteins with enzyme

Each of the five per cent protein solutions preliminarily adjusted to pH 1·5 was partially hydrolysed with 0·1 % pepsin (Mikuni Kagaku Sangyo Co. 1:5000) at 50 °C for 20 h and then the pH of the resultant solution was brought to 4·5 by the addition of NaOH. After the precipitant produced was removed, the pH of the solution was adjusted to 6·5 and heated at 90 °C for 10 min. After removing the resultant precipitation, the solution was stored in a refrigerator.

Adjustment of protein concentration

The protein concentration was measured by the Biuret method. The basic sample solution was prepared by dilution of the above solution.

Foam stabilities (St)

Fifteen millilitres of the sample solution was measured into a 50 ml cylinder with a stopper and shaken horizontally for 30 sec (about 100 cycles a minute, 15 cm amplitude of shaking). The shaking was repeated three times successively. The volume of foam was measured and it was ascertained that there was no change of the foam volume at each shaking step. The resultant solution was allowed to stand until the foam volume had decreased to one half its initial level and the foam disappearing velocity (mm/min) was determined from the time at which half the foam volume disappeared. Next, the time in which 100 mm of foam layer disappeared was calculated from this foam disappearing velocity. The buffer solutions used were 0·1M CH_3COOH–0·1M CH_3COONa at pH 4·5, 0·1M KH_2PO_4–0·1M Na_2HPO_4 at pH 6·5 and 0·1M NH_4Cl–0·1M NH_4OH at pH 9·0.

Surface tension

A Du Nouy surface tension meter (Shimazu Seisakujo) was used for measuring surface tension. The measurements were carried out after the sample solutions were placed in glass dishes with aluminium foil covers at 25 °C for 12 h.

Ultraviolet difference spectra

After heating the sample solutions at 60 °C for 30 min in the presence and absence of 8M urea and 0·01M 2-mercapto-ethanol, the difference spectra were measured at 285 nm with a Hitachi type 323 automatic spectrophotometer. The results were

expressed as the ratio of the optical densities of the difference spectra at 285 nm to the optical densities at 280 nm in the sample solutions containing 1·0 mg/ml.

Measurements of hydrophobic region in molecular surface

The estimation of the hydrophobic region in the surface of the molecule was carried out by measuring the fluorescence resulting from binding of 1-anilinonaphthalene-8-sulphonate (ANS) to the hydrophobic region (Kirtley & Koshland, 1972) using a Hitachi MPF-II spectrofluorophotometer. The values are obtained as relative fluorescence at the protein concentration of 5 mg/ml. The excitation and the emission were at 386 nm and 465 nm, respectively. The buffer solutions used were the same as in foaming stabilities.

Intrinsic viscosities

Intrinsic viscosity was measured with an Ostwald viscometer at 25 °C using the following three kinds of solution; 0·1M CH_3COOH–0·1M CH_3COONa at pH 4·5, 0·1M glycine–0·1M NaOH at pH 10·0 with 8M urea and 0·05M 2-mercapto-ethanol.

Determination of disulphides

Disulphides in samples were determined by the method of De Marco *et al.* (1966) using N-ethylmaleinimide. The values were expressed as g cystine per 100 g protein. The results thus obtained were also supported by the amino acid analyses using an Hitachi KLA-5 automatic amino acid analyser after hydrolysing the samples with 6N HCl for 20 h at 105 °C.

RESULTS AND DISCUSSION

(1) *Foam stabilities and molecular structures of protein hydrolysates*

Many investigators have reported on the foam stability (*St*) from various viewpoints. For instance, there have been several discussions of viscosity, surface viscosity, surface denaturation of protein, desorption energy, the Marrangoni effect, DLVO theory and so on (Bikerman, 1973). In general, foam stabilities relate to the extent of the absorption of a molecule to the surface. Since the extent of the absorption of a molecule to the surface can be recognised to run parallel with its hydrophobicity, it is very important to examine the effect of the hydrophobicities of foaming molecules. However, the extent and the order of the foam stabilities (*St*) of the five kinds of hydrolysed proteins were not always followed by the contents of the compositive hydrophobic amino acids, as shown in Table 1.

From this, the idea arises that the existence of a hydrophobic region in a molecule may probably relate to this phenomenon.

First, the extent of the internal hydrophobic region of the samples was estimated from the ratio of the optical densities of the difference spectra at 285 nm ($\Delta OD_{285\,nm}$) to optical densities at 280 nm ($OD_{280\,nm}$) on the assumption that tyrosine and tryptophane residues distribute uniformly in the molecules. $OD_{280\,nm}$

TABLE 1
PHYSICAL AND CHEMICAL PROPERTIES OF THE PROTEIN HYDROLYSATES

Hydrolysates	*Gelatin*	*Soybean protein*	*Casein*	*Gluten*	*Albumin*
Foam stability (min/100 mm)	4·4	17·4	7·8	340·9	100·0
$\Delta OD_{285\,nm}/OD_{280\,nm}$ (Internal hydrophobic region)	0	0·0571	0·0856	0·0365	0·0692
Content of hydrophobic amino acid (%)[a]	24·1	33·1	49·2	34·9	37·8
Content of cystine (g/100 g protein)	0·0	1·7	0·4	2·0	3·2

[a] Valine, leucine, isoleucine, methionine, phenylalanine, proline, tyrosine and tryptophane were accounted as hydrophobic amino acids.

indicates the total quantities of tyrosine and tryptophane residues, and $\Delta OD_{285\,nm}$ indicates the quantities buried in the hydrophobic regions of the protein molecules. As shown in Table 1, a parallel relation between the internal hydrophobic region and the foam stability could not always be found, as in the case of the compositive hydrophobic amino acids.

Secondly, the hydrophobic region on the surface of the samples was estimated with ANS as a hydrophobic probe. The results illustrated in Fig. 1 showed that the foam stabilities of the samples possessed a good correlation with the content of external hydrophobic region at three separate pH values.

Accordingly, the foam stability of the protein hydrolysates seemed to have a close relation to their external hydrophobic region, not to their internal hydrophobic region. This was supported by the fact that there was a linear relationship, except in casein, between the logarithm of foam stability ($\log St$) and the extent of surface absorption, which can be calculated from the Gibbs' rule, eqn. (1), as shown in Fig. 2:

$$a = -c/RT \cdot d\gamma/dc \tag{1}$$

In eqn. (1), a, R and T are quantity of absorbed solute, gas constant ($8{\cdot}314 \times 10^7$ ergs/mole/degree) and absolute temperature, respectively. This equation is applicable for a solution of comparatively low concentration so that a linear relationship may be formed between surface tension (γ) and solute concentration (c).

Thus, a molecule having a large hydrophobic region on the surface is more absorbable on a surface than that having a small one, because of its strong tendency to avoid its surrounding water. Further, the former needs more energy than the latter, to pull the absorbed molecule back into the water from the surface. As a result, the molecules are more concentrated on the surface and the foam is more stabilised.

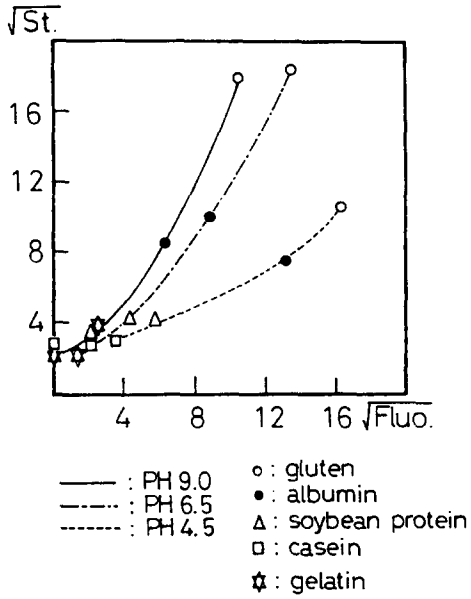


Fig. 1. Correlation between foam stability (*St*) and content of the hydrophobic region on the molecular surface (relative fluorescence = Fluo.).

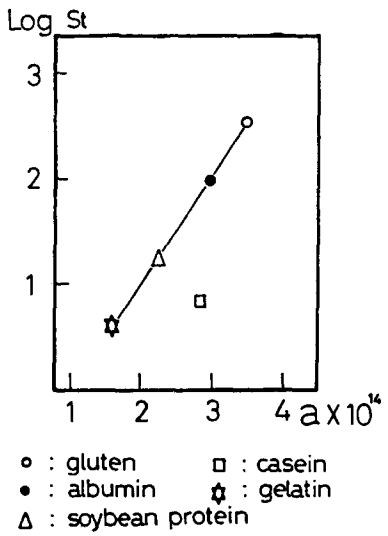


Fig. 2. The relationship between quantity of molecule absorbed to surface (*a*) and foaming stability (*St*). (The concentration of protein hydrolysates was 0·1 mg/ml for surface tension and 0·33 mg/ml for foam stability in 0·1M phosphate buffer, pH 6·5.)

(2) *Effect of disulphide linkages*

As shown in Fig. 3, a good correlation was found between the square root of relative ANS fluorescence (the hydrophobic region on the molecular surface) and the content of cystine in the protein hydrolysates (except that of gluten). In general, the hydrophobic region of molecules in aqueous solution is to be found not on the molecular surface, but in the centre of the molecules. The parallel relation between the hydrophobic region of a molecular surface and the disulphide content indicates that the disulphide bonds serve as an energy barrier for the rearrangement of the peptide chains.

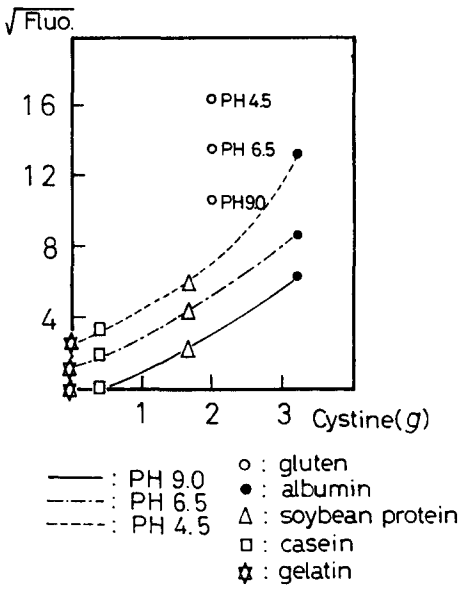


Fig. 3. Correlation between content of hydrophobic region on the molecular surface (Fluo.) and disulphide bond (Cystine). Symbols are the same as in Fig. 1.

No change of foam stabilities was observed on the addition of 2-mercaptoethanol, as shown in Table 2, indicating that the sulphide bonds which existed on the molecular surface had no effect on their foam stabilities, because 2-mercaptoethanol would not penetrate into the centre of the molecules, but would disrupt only the disulphide bonds located at the surface.

In spite of a lower cystine content in gluten than in albumin, the former shows an extremely high relative ANS fluorescence. This distinct difference between foam stabilities of the two samples could not be explained by the secondary structure data obtained from optical rotatory dispersion and infrared spectra.

TABLE 2
EFFECT OF 2-MERCAPTOETHANOL ON FOAM STABILITY

Hydrolysates	*Gelatin*	*Soybean protein*	*Casein*	*Gluten*	*Albumin*
Control	4·4	17·4	7·8	340·9	100·0
+0·05M ME[a]	3·6	22·1	5·3	449·4	125·4

[a] Foam stability with 2-mercaptoethanol (ME) was measured by exchanging the air in the measuring cylinder with nitrogen gas.

The intrinsic viscosities of these proteins were measured, in order to obtain an indication of the effective hydrodynamic volume of the molecules. In general, the protein molecules at pH 10 in the presence of 8M urea are most completely unfolded in the loosest state of polypeptide chains and possess the largest values of effective hydrodynamic volumes. The ratio of intrinsic viscosities of these two hydrolysates at pH 4·5 and pH 10 without the presence of urea to those at pH 10 in the presence of 8M urea is shown in Table 3. These values at pH 4·5 are smallest, because of the least electrostatic repulsion, while the values at pH 10 are larger, because of the large electrostatic repulsion between the negative charges of the peptide chains. As shown in Table 3, the effective hydrodynamic volume of albumin hydrolysate changed markedly between pH 4·5 and 10. In gluten hydrolysate, however, the change of the

TABLE 3
VARIATION OF INTRINSIC VISCOSITY AGAINST THE CHANGE OF pH VALUES BETWEEN GLUTEN AND ALBUMIN HYDROLYSATES

Hydrolysates	*Gluten*	*Albumin*
$(\eta)_{pH10,8M\ urea}$ (dl/g)	0·0123	0·0055
$(\eta)_{pH4\cdot5}/(\eta)_{pH10,8M\ urea}$	55	27
$(\eta)_{pH10}/(\eta)_{pH10,8M\ urea}$	59	80

values was very small. This indicates that the polypeptide chains of the gluten molecules are fixed firmly against the electrostatic repulsions which act mutually among the peptide chains at pH 10. Probably the disulphide linkage plays an important role in this fixation of the chains. The disulphide bonds in gluten molecules might bind the chains at long distances. As a result, the protein molecule is, on the whole, unexpanded and the hydrophobic region at the molecular surface is also maintained, leading to a stable foam. On the other hand, in the case of albumin hydrolysate, disulphide linkages might exist between adjacent cysteine residues in a chain or between the cysteines in the peptide chains at a close distance. Consequently, the peptide chains would not be fixed by sulphide bonds as a whole in spite of the higher contents of cystine.

ACKNOWLEDGEMENT

The authors wish to express their sincere thanks to Dr I. Koshiyama of this Institute for valuable discussions and advice.

REFERENCES

BIKERMAN, J. J. (1973). *Foams*, Springer-Verlag, Berlin, Heidelberg, New York.

DE MARCO, C., GRAZIANI, M. T. & MOSTI, R. (1966). Spectrophotometric determination of thiols and disulfides with N-ethylmaleinimide. *Analytical Biochemistry*, **15**, 40–4.

KIRTLEY, M. E. & KOSHLAND, D. E. JR. (1972). Environmentally sensitive groups attached to protein. In *Methods in enzymology*, **XXVI**. (Hirs, C. H. W. & Timasheff, S. N. (eds.)), 578. Academic Press, New York.

CONTENT OF SOME ORGANIC ACIDS IN CLOUDBERRY (*RUBUS CHAMAEMORUS* L.)

PERNILLE BAARDSETH & HELLMUT RUSSWURM JR

Norwegian Food Research Institute, Postboks 50, N-1432 Aas-NLH, Norway

(Received: 11 March, 1977)

ABSTRACT

Cloudberry (Rubus chamaemorus *L.*) *is very resistant to deterioration by micro-organisms and has a good keeping quality. In the present study the natural content of some organic acids, often used as food preservatives, has been determined by gas chromatography. Benzoic acid was found in the greatest amount (about 50 mg/100 g cloudberry). Sorbic acid, salicylic acid, p-hydroxy-, methyl-p-hydroxy- and propyl-p-hydroxy-benzoic acid were found in smaller amounts (less than 1 mg/100 g cloudberry).*

The amount of benzoic acid changes very little with the degree of ripeness or place of growth.

INTRODUCTION

Benzoic acid, sorbic acid and p-hydroxybenzoic acid esters are permitted in limited amounts as preservatives in the food industries. It is important to know the natural content of these acids in the raw materials in order to be able to keep the amounts within the legal limits in the actual products and to avoid specific taste sensations.

It has long been known that cranberry (*Vaccinium vitisideae*) contains considerable amounts of benzoic acid (Fellers & Esselen, 1965). p-Hydroxybenzoic and salicylic acid are found in strawberry and raspberry, but in small concentrations (Herrmann, 1973). Other edible wild berries like blueberry (*Vaccinium myrtillus*) have been shown to contain benzoic acid together with salicylic acid (Hulme, 1971), but the concentrations have not been reported.

Chemical analyses of cloudberry have been carried out by several workers (Turpeinen & Roine, 1960, Kuusi, 1970; Simola, 1945; Erkama, 1946 and Nordnes & Werenskjold, 1952) but none of these includes the determination of benzoic,

Fd. Chem. (3) (1978)—
Printed in Great Britain

sorbic or similar acids. The good keeping quality of the berries indicates the presence of substances which display preservative action.

During the preparation of this paper, Hankanen & Pyysalo (1976) published a paper in which the amount of benzoic acid (33 mg/100 g) in cloudberry is mentioned, but not the methods used.

The aim of this investigation was to determine the presence and levels of benzoic, sorbic and *p*-hydroxybenzoic acid, as well as esters of the latter, in cloudberry. Total acidity total solids, pH and ascorbic acid content were also determined.

MATERIALS AND METHODS

Materials

Four cloudberry samples from the central area of Norway and one sample from Finland, were gathered in 1973. In 1974 six samples from the central and northern parts of Norway, specified as to maturity, locality and collection time, were purchased.

The berries were deep-frozen after arrival at the Institute in 1973. The 1974 samples were deep-frozen immediately upon picking. The samples were stored at −20 °C until analysed. The berries were thawed at room temperature and homogenised before analysis.

Methods

The organic acids were analysed by gas chromatography after extraction and silylation, using a method described by Gosselé (1971). To obtain reproducible quantitative results, the method was modified slightly. Butyl-*p*-hydroxybenzoic acid was used as an internal standard instead of methyl gallate and chloroform was used as solvent instead of pyridine during the silylation procedure.

The silylated acids were chromatographed in 0·6 µl sample volumes on two columns, under the following conditions:

Column I: Four metre stainless steel, id 2 mm packed with 6% Dexil 300 (Supelco) on Chromosorb WAW DMCS 80/100 mesh.

Column II: Three metre stainless steel, id 2 mm packed with 5% SE 30 (Supelco) on Chromosorb WAW 80/100 mesh.

A Perkin-Elmer 900 gas chromatograph equipped with a flame ionisation detector was used. GC parameters: Oven, column I: 120° for 4 min, then 8 °C/min to 250 °C; column II: 120° for 4 min, then 4 °C/min to 210 °C. Injector and detector set at 290 °C and 300 °C, respectively. Carrier gas (nitrogen) flow rate 21 ml/min.

The retention times of the peaks were compared with the retention times of authentic compounds chromatographed under the same conditions. The organic acids were identified by identical retention times with authentic samples on the two

columns. The recoveries for each acid in the samples tested were in the range 96 to 100%.

Total solids were determined by drying 10 g of the samples in a vacuum oven for 3 h at 70 °C (Horwitz, 1970, p. 369). pH was measured by a Radiometer pH meter type with a combination electrode.

Total acidity was determined by potentiometric titration with sodium hydroxide after dilution with water (Horwitz, 1970, p. 377). Ascorbic acid was determined by Tilman's method with 2,6-dichlorophenol-indophenol (Horwitz, 1970, p. 777).

RESULTS AND DISCUSSION

The content of benzoic acid varied from 43 to 65 mg/100 g cloudberry (Table 1).

Apart from benzoic acid, small amounts of the following acids appeared in the gas chromatograms (milligramme/100 g cloudberry): Sorbic acid 0·1–0·3; salicylic acid 0·2–0·9; methyl-*p*-hydroxybenzoic acid 0·1–0·4, *p*-hydroxybenzoic acid 0·3–0·9.

TABLE 1
THE AMOUNTS OF SOME ORGANIC ACIDS IN CLOUDBERRY

Location		*Milligramme/100 g cloudberry*					
		Benzoic acid	*Sorbic acid*	*Salicyclic acid*	*Methyl-p-hydroxy-benzoic acid*	*p-Hydroxy-benzoic acid*	*Propyl-p-hydroxy-benzoic acid*
1973							
CENTRAL NORWAY							
Sørli		51	0·2	0·5	0·3	0·3	0·2
Norli		43	0·2	0·3	0·1	0·5	0·1
Steinkjaer		65	0·3	0·9	0·4	0·9	0·6
Steinkjaer		53	0·2	0·4	0·2	0·3	0·1
FINLAND		58	—	0·2	0·1	0·3	—
1974							
NORTHERN NORWAY							
Prestmyra	Unripe	55	0·1	0·3	0·4	0·4	0·1
Prestmyra	Ripe	61	0·1	0·5	0·2	0·4	0·4
CENTRAL NORWAY							
Rognlihøgda	Unripe	42	0·2	0·3	0·3	0·3	0·5
Rognlihøgda	Ripe	51	0·1	0·3	0·1	0·4	0·3
Faergenheia	Unripe	48	0·2	0·6	0·1	0·6	0·3
Faergenheia	Ripe	51	0·2	0·4	0·1	0·4	0·2

The difference in content of benzoic acid between unripe and ripe berries from the same location (1974 samples) was 3–9 mg/100 g cloudberry, which does not indicate a significant variation with the stage of ripening.

The variations between samples from different locations are also slight.

TABLE 2
TOTAL SOLIDS, pH, TOTAL ACIDITY AND ASCORBIC ACID IN CLOUDBERRY

Location		*Total solids* (%)	*pH*	*Total acidity as citric acid* (%)	*Ascorbic acid* (*mg*/100 *g*)
1973					
CENTRAL NORWAY					
Sørli		14·9	3·4	1·1	109
Norli		14·9	3·6	0·8	37
Steinkjaer		16·1	3·4	1·2	69
Steinkjaer		15·0	3·5	1·0	102
FINLAND		14·6	3·5	0·9	80
1974					
NORTHERN NORWAY					
Prestmyra	Unripe	14·7	3·4	1·2	76
Prestmyra	Ripe	14·5	3·4	1·2	90
CENTRAL NORWAY					
Rognlihøgda	Unripe	13·6	3·6	0·9	21
Rognlihøgda	Ripe	14·0	3·3	1·1	52
Faergenheia	Unripe	13·6	3·5	0·8	14
Faergenheia	Ripe	15·2	3·4	1·1	51

The total solids (13·6–16·1 %) and pH (3·3–3·6) in the samples of cloudberries were found to be similar to the values reported by Kuusi (1970). The ascorbic acid content in the 1974 samples seemed to increase with the degree of ripeness, which confirms the findings of Nordnes & Werenskjold (1952). Total acidity increases with ripeness, which has also been reported for other berries (Hulme, 1971) (Table 2).

REFERENCES

ERKAMA, J. (1946). Kolorimetrisestä määritykesestä 2,6-diklorfenoli-indofenolilla. *Suomen Kemistilekti*, **19**, 21–5.

FELLERS, C. R. & ESSELEN, W. B. (1965). *Cranberries and cranberry products*. Univ. of Mass. Agr. Exp. Stat. Bulletin 481.

GOSSELÉ, J. A. W. (1971). Gas chromatographic determination of preservatives in foods. *J. Chromatogr.* **63**, 429–32.

HANKANEN, E. & PYYSALO, T. (1976). *Z. Lebensm. Unters-Forsch*, **160**, 393–400.

HERRMANN, K. (1973). Review article: Über das Vorkommen der hydroxy-benzosäure-Verbindungen im Pflanzenreich und in pflanzlichen Lebensmitteln, *Z. Lebensm. Unters-Forsch*, **153**, 170–6.

HORWITZ, W. (1970). *Official methods of the Association of Official Analytical Chemists* (11th ed.).

HULME, A. C. (1971). The biochemistry of fruits and their products, **2**, 388–90. London, Academic Press.

KUUSI, T. (1970). Über die chemische Zusammensetzung und Kennzahlen einigen finnischen einheimischen Beeren, *Flüssigs. Obst.*, **37**, 188–200 and 253–63.

NORDNES, T. & WERENSKJOLD, B. Q. (1952). The variation of the ascorbic acid content in raw and preserved cloudberries, *Rubus chamaemrus* L., *Food Res.*, **17**, 117–22.

SIMOLA, P. E. (1945). Suomalaisten ruoka-aineiden vitami-inipitoris understa, *Suomen Kemistilekti*, **18**, 4–10.

TURPEINEN, O. & ROINE, P. (1960). Ruska-ainetanlukko 3. painos. Helsinki. Keuruu.

METAL–POLYSACCHARIDE COMPLEXES—PART I†

J. A. RENDLEMAN

Northern Regional Research Center, Agricultural Research Service, US Department of Agriculture, Peoria, Illinois, USA, 61604

(Received: 1 May, 1977)

ABSTRACT

Many factors, including linear charge density, effective nuclear charge of the cation, basicity of the donor group, chelation, intermolecular binding of two or more donor groups by a single cation and steric fit, contribute—or possibly contribute—to the formation and stability of metal ion–polysaccharide complexes. In order to form a proper foundation for studying and interpreting metal ion–polysaccharide interactions, the author introduces into his treatise on polysaccharide complexes much current information concerning the interaction of metal ions with simple unidonor and simple multidonor ligands. For convenience of discussion, the subject of polysaccharide complexes is divided into two categories: interactions of neutral polysaccharides, such as cellulose and starch, and interactions of anionic polysaccharides, such as alginate, pectate, mucopolysaccharides and carrageenan. On the one hand, neutral polysaccharides form weak complexes with cations in neutral or non-alkaline media. Only in alkaline solution is there any great affinity between cation and donor. On the other hand, anionic polysaccharides have a strong affinity for metal counterions, even at low concentrations. Attempts are made to provide reasonable explanations for the formation of these complexes and for the often large differences in affinity between metals for a particular polysaccharide or between polysaccharides for a particular metal. In some instances, rheological behaviour of polysaccharide systems can be related to structural features of macromolecular complexes.

† Part II of this paper will appear in the next issue of *Food Chemistry*.
The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

Fd. Chem. (3) (1978)—
Printed in Great Britain

I. INTRODUCTION

Primordial life on earth probably had its origin in the oceans and, still today, an aqueous environment is essential for all living systems. The distribution of anions and cations in both aquatic and terrestrial organisms reflects the composition of the seas. Sodium, potassium, calcium, magnesium and chloride ions constitute the major ionic constituents of lakes, streams and oceans. These same ions preponderate in the ionic environment of living cells. In addition to large amounts of these metals, trace amounts of at least nine others are essential for some form of life: V, Cr, Mn, Fe, Co, Cu, Zn, Mo and Sn (Frieden, 1972).

In sea water, concentrations of the essential trace metals are extremely small (≤ 10 parts per billion) compared with concentrations of the major metallic constituents (concentrations in weight %: Na, 1·05; K, 0·038; Mg, 0·14 and Ca, 0·040) (Goldberg, 1963). In freshwater lakes and streams, Na, K, Ca and Mg also predominate, relative to amounts of trace elements (Garrels & Mackenzie, 1967; Kunze & Smith, 1966; Raspletina *et al.*, 1967).

Factors that determine the availability or unavailability of various metallic elements in the earth's waters are not well understood. In river waters, sorption of certain metal ions by organic colloids is possibly a determinant (Bergh *et al.*, 1956). Heavy metals in brooks and streams generally arise from ground waters that have penetrated greater depths before emptying onto the surface. Actually, such metals have been known to disappear within several hundred metres from their point of emergence from the ground (Bergh *et al.*, 1956). In the waters of certain dystrophic lakes of interior Alaska, more than 50 % of the trace elements Co, Mn and Zn are in highly dispersed colloidal form (Barsdate, 1970). Possibly metal ions are associated with organic colloids, polyaromatic ligands containing many carboxyl and hydroxyl groups that serve as binding sites for metal ions. Although complexation of this type may not necessarily occur in marine environments, colloidal particles of Mn, Cu and Zn have been found in ocean water (Slowey *et al.*, 1967). However, little is known of the origin, character, stability or biological and geochemical significance of these metal-containing entities.

In soils, both inorganic and organic substances participate in controlling the availability of metal ions in living organisms. The special stability of Ni and Cr cations in the octahedral sites of soil silicates explains the low abundance of these two metals in living systems (Williams, 1967). Soil polysaccharides (Mortensen, 1960), in addition to humic and fulvic acids (Mortensen, 1963; Stewart, 1963), are capable of forming water-soluble complexes with copper. A polysaccharide extracted from clay loam with hot water and composed of rhamnose, fucose, xylose, arabinose, mannose, glucose, galactose and small quantities of uronic acid residues formed complexes with Fe(III) and Al(III). The interactions also release hydrogen ions (Saini, 1966).

In certain salt solutions, biological fluids and tissue culture media, multimolecular crystalline 'complexes' of high calcium content form (Činátl, 1969). Such complexes are called calcareous structures. When grown in biological fluids, these structures contain such organic substances as lipids, polysaccharides and proteins. Certain similarities between calcareous structures and living organisms led Činátl (1969) to propose that the structures were significant for the origin of life. He also proposed a possible relationship between development of these high calcium complexes and the deposition of calcium, phosphorus, lipids and polysaccharides in various organisms.

The importance of metal ions in protein chemistry is quite extensive and well documented (Friedman, 1974; Neurath, 1970). Nearly one-third of all known enzymes involve a metal ion as an essential participant. To some enzymes the cation is firmly bound in a fixed stoichiometric ratio. Yet to other enzymes, the cation may be only loosely bonded. Certain metalloproteins function in a non-catalytic manner by transporting oxygen and various metals.

In polysaccharide chemistry the function of the metal ion in biological processes has not yet been well defined. Polysaccharides that either occur in nature as metal complexes or are believed to behave in some fashion as complexing agents in the living organism fall into the category of polyelectrolytes. These are hydrocolloids that contain acid groups, such as sulphate or carboxylate. Mucopolysaccharides, pectic substances and certain algal polysaccharides, such as alginate and carrageenan, are naturally-occurring polyelectrolytes already widely studied.

Mucopolysaccharides (acidic glycosaminoglycans) (Jeanloz, 1970) are components of the extracellular amorphous ground substance that surrounds collagen and elastin fibres and the cells of connective tissue in animals. Their solutions are highly viscous and they form gels readily. Polysaccharides may affect physiological and pathological processes, i.e. induction of calcification, control of the movement of metabolites, ions and water, healing of wounds, lubrication of joints and absorbing shock.

Various sulphated polysaccharides—such as dextran sulphate (Oncley *et al.*, 1957), sulphated amylopectin (Bernfeld *et al.*, 1957), heparin (Miller, 1960) and carrageenan (Turner & Magnusson, 1962)—are known to precipitate proteins by forming polysaccharide–protein complexes that can be dissociated into their components with high concentrations of salt. Although the mechanism of this complex formation is not known, metal ions are possibly essential to it.

Alginic acid (McNeely & Pettitt, 1973) is a structural component of seaweed and occurs naturally as an insoluble complex of K, Na, Ca and Mg. Carrageenan (Percival, 1970), also a structural component of seaweed, presumably exists in the natural state as a salt complex. Both polysaccharides are possibly involved in the ion-exchange mechanism responsible for selective absorption of $K^{\oplus}$ and $Na^{\oplus}$ from sea water by algae.

Pectic substances (Aspinall, 1970), that group of complex plant polysaccharides in which D-galacturonic acid is the principal constituent, occur in primary cell walls and intercellular layers in land plants. In cell walls, the substances exist partially in salt form (usually Ca); however, in intercellular layers, only the acid form occurs. Polysaccharides in which a portion of the acid residues is esterified as methyl esters are termed pectinic acids or pectins. Pectinic acids possess considerable gelling power and are widely used in making jellies.

Substances belonging to a class of compound called lectins are gaining considerable attention because of their ability to agglutinate (aggregate) red blood cells (Mosher & Price, 1975) and to interact with various carbohydrates, including branched-chain polysaccharides and glycoproteins (Agrawal & Goldstein, 1967). Concanavalin A is an example of a lectin now commercially available. These compounds are high molecular weight polymers of both plant and animal origin. Almost all are glycoproteins and generally they contain a metal ion, Ca^{2+} or Mn^{2+}, held in the folds of the protein chain. Removal of these ions by dialysis destroys the agglutinating activity of the lectin but return of the ions restores the activity. Changing the metal-ion content of Concanavalin A causes a change in the ability of the compound to bind carbohydrates (Karlstam, 1973). Binding ability increases with increasing metal content. Although the exact function of the cation in binding these carbohydrates is not understood, the importance of the cation warrants mention of these protein–carbohydrate complexes.

Only in the past few years have extensive efforts been initiated to elucidate interactions between polysaccharides and cations. The many studies of complex formation between cations and small simple carbohydrates now under way will contribute to an understanding of macromolecular behaviour. Because of the greater number of metal-binding sites on macromolecules, the chemistry involved in complexation of polysaccharides is understandably more difficult to study than that of carbohydrate monomers. My purpose is to draw attention to those characteristics of metal cations and ligands of which some knowledge is essential for an understanding of complexation of polysaccharides. Where possible, rheological behaviour will be related to the structural features of macromolecular complexes.

In Sections II and III below, I shall describe the general behaviour of metal ions and ligands, with emphasis on small ligands containing few donor groups. The behaviour of macromolecular ligands will be discussed in Section I of Part II of this paper.

II. COMPLEXATION AND NATURE OF THE METAL ION

A complex has been generally defined as a species formed by association of two or more simpler species, each of which is capable of independent existence (Rossotti & Rossotti, 1961). Bonding arises from the attraction that the metal (acceptor) ion has

for one or more lone pairs of electrons on the donor groups of a ligand. In complexes where ligands are uncharged, attraction is between the cation and the dipole (static plus induced) of the donor group. The bond may have some covalent character. When the ligand contains charged groups (e.g. carboxylate, sulphate or anionic oxygen), the bond is probably largely ionic (electrostatic) with varying amounts of covalent character.

Another common definition of a metal complex is that species formed when solvent molecules bound to a metal ion are replaced by other polar molecules or by anions. In aqueous solution, the number of water molecules replaced would depend upon the number of donor atoms involved. Actually, this definition is not completely descriptive because the solvated cation itself is a complex and the cation in a complex may, in some instances, be separated from the ligand donor atom by solvent molecules. To avoid confusion, solvated cations will not be referred to as complexes.

A complete understanding of complexation phenomena is acquired only by knowledge of the discrete molecular nature of the solvent and its dynamic interaction with ions (Evans & Matesich, 1973). In water, tetrahedral orientation of the hydrogen bonds results in the formation of a three-dimensional solvent network. In monohydric alcohols, solvent structuring is limited to linear chains of associated molecules of alcohol. Unsubstituted amides are capable of forming a three-dimensional network, but not so easily as water. Both cations and anions are effectively solvated in each of these three types of hydrogen-bonded solvent. Somewhat anomalously, cation–solvent association is weak in polyfluorinated alcohols, such as CF_3CH_2OH, although anion–solvent association is strong. Apparently, fluorine atoms reduce the basicity of hydroxyl oxygen and thereby the strength of cation–solvent bonding. In dipolar aprotic solvents, such as ketones, nitriles and nitro compounds, only cations are strongly solvated.

In aqueous solution, metal ions are highly solvated. Bound water molecules are so oriented that the negative (oxygen) end of the water dipole is directed towards the metal cation. Arrangement and number of water molecules about the cation may vary according to the particular metal involved and are known to depend upon such conditions as temperature, concentration and ionic strength (Chaberek & Martell, 1959). The strength of a metal–solvent bond is greater the shorter the cationic radius. Li^+ and Mg^+ are examples of small ions to which molecules in the primary hydration sphere (the shell of solvent molecules making direct contact with the metal cation) are bound tightly. The water molecules attached to Li^+ and Mg^{2+} are displaced with greater difficulty than those co-ordinated to larger ions in the same periodic group (Rendleman, 1966*a*). Kistenmacher *et al.* (1974) have estimated by quantum-mechanical computation that co-ordination numbers for hydrated forms of Li^+, Na^+ and K^+ are, respectively, 4, 5–6 and 5–7; cation-oxygen distances are, respectively, 1·9–2·0 Å, 2·3–2·4 Å and 2·8–2·9 Å. A large proportion of hydration energy in the primary shell of Li^+ may be due to hydrogen bonding among bound water molecules (Talekar, 1975). Hydrogen bonding possibly contributes also to the

hydration energy of Na^+, but to a much smaller extent. However, in primary hydration shells of K^+, Rb^+ and Cs^+, O–O distances between neighbouring water molecules are too great to permit H-bond formation. Study of model systems has indicated strongly that there is no covalency in metal–water bonds of hydrated alkali metal ions (Talekar, 1975). Orientation of water molecules with respect to the metal ion is such that lone pairs of electrons on the ligand oxygen do not face the alkali metal ion. Therefore, there can be no overlap of lone pairs with vacant alkali metal ion orbitals. In other words, delocalisation of electrons from the ligand water molecule to the alkali metal ion, which would impart covalency to the metal–water bond, is not consistent with the geometric parameters of the metal hydrate structure. However, with organic ligands containing oxygen donor atoms, such as nonactin and valinomycin, donor oxygens are so oriented towards the alkali metal ion (as shown by x-ray diffraction studies (Kilbourne *et al.*, 1967; Shemyakin *et al.*, 1969)) that delocalisation should occur and covalency of the metal–ligand bond is expected.

Infrared spectroscopy has provided evidence that transition metal ions interact covalently with water molecules (Zundel, 1969). Bonding would involve an interaction of lone electron pairs with incompletely occupied *d* orbitals of the cations.

Complexation between a cation and an anion can be regarded simply as formation of an ion pair. Maximum distance of separation between the two ions corresponds to that distance at which coulombic attraction is balanced by forces tending to produce random thermal motion of the ions. Binding a metal cation to either a charged or an uncharged ligand is best described as an association process involving the equilibria represented by eqns. (1) to (3) where, for illustrative purposes, water is the solvent medium and both cation and ligand are univalent ions. Symbol *n* represents the number of water molecules in the primary solvation sphere of the cation. For the sake of simplicity, any water of hydration surrounding the ligand donor group will be ignored.

$$M(H_2O)_n^+ + L^- \rightleftharpoons \underset{\text{I}}{M(H_2O)_n^+ \cdots L^-} \tag{1}$$

$$M(H_2O)_n^+ \cdots L^- \overset{\text{slow}}{\rightleftharpoons} \underset{\text{II}}{M(H_2O)_{n-1}^+ \cdots L^-} + H_2O \tag{2}$$

$$M(H_2O)_{n-1}^+ \cdots L^- \overset{\text{fast}}{\rightleftharpoons} \underset{\text{III}}{LM(H_2O)_{n-1}} \tag{3}$$

Species I is called an outer-sphere complex (or encounter complex) whose formation precedes rupture of the metal–water bond (eqn. (2)). Rupture of the metal–water bond is the slow, rate-determining step in the formation of an inner-sphere complex (species III) in which true cation–ligand contact occurs. The stronger the cation–

solvent bond, the smaller should be the tendency for a given ligand to replace a solvent molecule and form an inner-sphere complex. The mechanism outlined above applies not only to reactions of unidentate ligands but also to chelation where a multidentate ligand is involved in forming multiple co-ordinate bonds to a single metal ion (Rorabacher & Moss, 1970).

A. *Stability as a function of cationic radius*

Stability of a complex is greatly influenced by the ease with which the donor group is polarised and by the ability of the cation to polarise the donor group. Stability increases with increasing polarisability of the donor and with increasing ability of the cation to polarise. Mutual polarisation of interacting components also contributes to strengthening the complex bond by augmenting the coulombic energy of attraction.

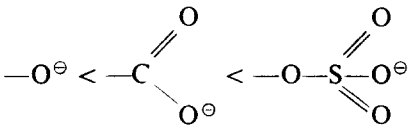
Cations of shorter radius and higher ionic charge have a lower ability to be polarised but a greater ability to cause polarisation. However, it is not ionic valence that determines bonding strength, but the effective nuclear charge (i.e. the effective charge at the surface of the cation). The greater the effective charge, the greater the external coulombic field. Effective nuclear charge increases with decreasing degree of screening of the nuclear field by inner-orbital electrons (Duncan, 1959). Ions of alkali metals, alkaline-earth metals, and rare-earth metals have non-bonding electron cores that effectively shield the cation nucleus from donor groups.

TABLE 1
NON-BONDING CORE AND CLASS OF ACCEPTOR METAL ION

Non-bonding core	*Metal ion*	*Class*
$1s^2$	Light metals, Li^+, Be^{++}, B^{3+}	A
ns^2np^6	Pre-transition metals, e.g. Na^+, Ca^{++}, Sc^{3+}	A
$(n-1)d^{10}$	Post-transition metals, e.g. Cu^+, Zn^{++}, Ga^{3+}	B
$(n-1)d^{10}ns^2$	Inert pair metals, e.g. Tl^+, Pb^{++}	B
$(n-1)d^1 \rightarrow (n-1)d^9$	Transition metals	A+B
$(n-1)(f^1-f^{13})ns^2np^6$	Lanthanide metals ($n=4$) and actinide metals ($n=5$)	A

Acceptor metal ions can be divided conveniently into two classes (A and B) according to the effectiveness of inner-core shielding (Craig & Nyholm, 1964) (Table 1) because the type of non-bonding core relates to the metal class. Class A has greater shielding than Class B and tends to co-ordinate better with less polarisable ligands. The degree of covalency in bonding of Class A cations is probably low compared with that of Class B cations because the external field of the former is lower. Stability of Class A cations increases with increasing cationic charge. Class B, with its lower degree of inner-core shielding, has a greater affinity for the more polarisable ligands; the most stable complexes are those formed between a cation of high, effective nuclear charge and a donor of low electronegativity. Transition metal ions have an intermediate character, changing gradually from Class A to Class B with increasing atomic number.

n the absence of chelation effects, the relative ability of different oxyanions to m complexes with a given cation is determined largely by basicity of the donor ygen (i.e. availability of the electron). The greater the basicity, the greater the bility of the complex. Basicity of the donor oxygen decreases with increase in rge delocalisation. Delocalisation of charge increases in the order:



e sulphate group can be expressed as a hybrid of three canonical structures, ereas there are only two canonical structures for the carboxylate ion.

Certain monocarboxylate complexes of Zn(II), Cd(II), Pb(II) and Cu(II) serve to strate the effects of basicity (Filipović *et al.*, 1968). With each of these cations, er of increasing stability parallels order of increasing basicity of the ligand:

$$\text{Formate} < \text{Acetate} < \text{Propionate}$$

bility of acetate and propionate complexes increases in the order:

$$\text{Zn} < \text{Cd} < \text{Cu} < \text{Pb}$$

der of stability for the formate complex (Zn < Cd < Pb < Cu) is only slightly erent. The relative positions of Zn, Cd and Pb are in accord with the increasing lity of the cation to polarise the anion. Stabilisation effects, other than arisation, are probably involved in complexations with Cu(II) because of the remely high stability of copper complexes. Each metal ion in the sequence above ble to complex with more than one monocarboxylate ligand. However, bond ngth decreases abruptly when more than three ligands are bonded.

Where inner chelation is possible, as in metal lactate complexes, stability is often siderably greater than that which would be expected solely on the basis of anion icity. The subject of inner chelation will be discussed in Section II.B below.

nvestigations of complexation between trivalent rare-earth cations and rganic anions (Rard & Spedding, 1975; Nelson & Irish, 1971; Hale & Spedding, 2) have shown that nitrate ions form stronger complexes with the rare-earths n do chloride or perchlorate ions. Presumably, chloride and perchlorate ions n only outer-sphere complexes, whereas nitrate ions form mixtures of inner- and er-sphere complexes. In concentrated nitrate solution, the predominant complex robably the inner-sphere type with binding occurring through oxygens of the ate ions. Sulphate complexes might also be mostly innersphere. stallographic studies on hydrated rare-earth (III) chlorides (Bel'skii & chkov, 1965; Graeber *et al.*, 1966; Marezio *et al.*, 1961) reveal that only two of three chloride ions are bound to the cation by inner-sphere coordination. These chloride ions are probably displaced by water molecules when the crystalline s are dissolved in water (Spedding *et al.*, 1977).

With increasing atomic number of a lanthanide ion, degree of inner-sphere complexation with inorganic anion diminishes. This decrease is probably a result of lanthanide contraction (the radius of a lanthanide ion decreases with increasing atomic number). With decreasing radius of a lanthanide ion, the number of bound water molecules and the strength of cation–water bonds probably increase, making replacement of a water molecule by an anion more difficult and thus causing ion-pair stability to drop.

With polar non-ionic ligands, both permanent dipole moment (or electronegativity of the donor atom) and polarisability govern ligand basicity. In reactions of the inert-gas type of ion, such as the alkali metal and alkaline–earth metal ions, the permanent dipole moment of the ligand is of greater importance than polarisability. This condition is exemplified by the behaviour of metal ammines. Alkali and alkaline-earth metal ammines decompose when dissolved in water because H_2O has a greater dipole moment than NH_3. Ammonia molecules are replaced by water molecules in the primary co-ordination sphere of the cation. In contrast to the behaviour of the inert-gas type of ion, complexation of transition metal ions is dominated by polarisation—that is, the ability of the cation to induce a dipole. Most transition metal ions form complexes with ammonia that are stable in water: explained best by greater polarisability of the NH_3 molecule relative to polarisability of H_2O.

B. *Contribution of chelation to complex stability*

Sometimes strength (ΔH) of the metal–ligand bond in a metal chelate may be approximately the same as that in a corresponding non-chelate complex and yet, because of a difference in the entropy change during complex formation (fewer degrees of freedom are lost during chelation than are lost in the formation of a non-chelate), stability of the chelate is greater than that of the non-chelate. Stability constants (K) are related to free-energy change (ΔG), entropy change (ΔS) and enthalpy change (ΔH) as follows:

$$\Delta G = \Delta H - T\Delta S = -RT\ln K \qquad (4)$$

Because some terms that contribute to the overall entropy change have been described by Duncan (1959), they are not dealt with here.

Of two similar multidentate ligands, that which forms the greater number of chelate rings with a given metal ion will form the more stable complex. Five- and six-membered rings are the most stable. Generally, rings containing more than six members have low stability compared with six-membered rings. Five-membered rings are usually more stable than six-membered rings.

When a given multidentate ligand interacts with a series of metal ions in the same oxidation state, order of decreasing chelate stability does not often depend upon the nature of the chelating molecule. For divalent cations, Mellor & Maley (1948) found the following order of decreasing reactivity:

$$Pb > Cu > Ni > Co > Zn > Cd > Fe > Mn > Mg$$

compounds, can be explained on the basis of cation hydration and cation radius. When N or S atoms are substituted for O atoms in the polyethers, affinity of the polyether for alkali metal ions is reduced; stability constants decrease with decreasing electronegativity of the donor atom: O > NR > NH > S. As the partial negative character of the heteroatom drops, electrostatic attraction diminishes between donor atom and cation.

Mention should be made that Ag^+ is bound more strongly to N- or S-containing polyethers (Frensdorff, 1971). Silver ion, which has a much higher effective nuclear charge than an alkali metal ion, would tend to bind a less electronegative, or more polarisable, donor group.

Alkaline–earth metal ions react with acyclic alkanedicarboxylates (such as malonate and succinate) to form chelates of 1:1 combining ratio. The order of stability is $Ca^{2+} > Sr^{2+} > Ba^{2+}$; the position of Mg^{2+} is irregular, often being equal to, or greater than, that of Ca^{2+} (Cannan & Kibrick, 1938). In 1:1 complexes (non-chelates) of acyclic alkanemonocarboxylate ions (such as acetate, propionate and butyrate), the order is $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+}$ (Cannan & Kibrick, 1938). These orders of stability indicate that alkaline–earth metal complexes of alkanecarboxylates are predominantly inner-sphere complexes, regardless of whether the complexes are chelate or non-chelate. Dicarboxylate complexes will be discussed further in Section III.C (below) and in Part II, Section I.B.1, the order of affinity of many carboxylate-containing *polymers* for alkaline-earth metal ions will be shown to be the reverse of that presented here for simple alkanecarboxylates.

C. *Stability constants*

Where a simple monodentate ligand (L) is involved in complexation with a metal ion (M), stability of the metal complex in solution is concerned with an equilibrium:

$$M + nL \rightleftharpoons ML_n \tag{5}$$

The overall equilibrium constant, also known as a formation or stability constant, is best expressed in terms of activities of the various species (eqn. (6)). Unfortunately, many constants reported in the literature were calculated on the basis of concentrations rather than activities. Use of concentrations leads to errors in values for free energies, enthalpies and entropies.

$$\beta_n = \frac{[ML_n]}{[M][L]^n} \tag{6}$$

Where $n > 1$, the reaction usually occurs stepwise. To illustrate, where $n = 3$:

$$M + L \rightleftharpoons ML \qquad K_1 = \frac{[ML]}{[M][L]} \tag{7}$$

$$ML + L \rightleftharpoons ML_2 \qquad K_2 = \frac{[ML_2]}{[ML][L]} \tag{8}$$

$$ML_2 + L \rightleftharpoons ML_3 \qquad K_3 = \frac{[ML_3]}{[ML_2][L]} \tag{9}$$

$$\beta_3 = K_1 . K_2 . K_3 \tag{10}$$

These expressions are also applicable to chelate formation between a polyvalent cation and a simple multidentate ligand containing two or more ionic donor groups. Each donor group (L) co-ordinates stepwise to the metal ion. With high molecular weight polyelectrolytes, such as alginate and carrageenan, one monomer unit or a group of two or more monomer units may be treated as a single ligand to determine formation constants. One method for determining overall constants involves a competition between a cation-exchange resin and the polysaccharide (salt form) in question (Schubert, 1952 and 1956; Triffitt, 1968). The formation constant is given by:

$$K_f = \frac{(K_d^\circ / K_d)}{A^n} \tag{11}$$

where K_d° and K_d are the coefficients of distribution of metal between resin and solution in the absence and presence of polysaccharide, respectively, A is the molar concentration of polysaccharide ligand (each ligand consisting of one or more of the polymer residues) and n is the number of moles of ligand per metal ion in the complex. The distribution coefficient is defined as:

$$K_d = \frac{\% \text{ of metal in resin}}{\% \text{ of metal in soln.}} \times \frac{\text{vol. of soln.}}{\text{wt. of resin}} \tag{12}$$

Another method for determining overall formation constants for calcium complexes involves murexide as a competing ligand (Raaflaub, 1951; Buddecke & Drzenieck, 1962). Both free (uncomplexed) murexide and complexed murexide (i.e. calcium murexide) can be determined spectrophotometrically in an aqueous mixture of polysaccharide and calcium murexide. The amount of calcium bound to the polysaccharide is obtained by difference. Equation (6) may then be used to calculate a constant. Because murexide as an indicator is dependent upon pH, the use of pH-independent tetramethylmurexide has often been preferred in determinations of alkaline-earth metal ions (Raaflaub, 1962; Kohn & Furda, 1967*a* and *b*; Kohn, 1969).

Formation constants for alkali metal–polysaccharide complexes are obtained easily if the concentration of free metal ion can be measured potentiometrically by means of an ion-selective electrode. The electrode measures the cation activity which, for most practical purposes, may be assumed to equal the concentration of cations not bonded to the anionic donor groups of the polysaccharide. However, with polyvalent cations, the measured activity coefficient for a simple salt can be considerably less than 1·0, even when the salt is reported to be completely, or almost completely, dissociated into ions. An example of such a salt is $CaCl_2$ which is generally thought to be highly dissociated over a wide range of concentration and

yet has a low activity coefficient, even in very dilute solution. There has been obtained, nevertheless, some evidence that the species $CaCl^+$ does exist to an appreciable extent, even at concentrations of calcium chloride as low as 0·025M (Nakayama, 1971). Osmometry, polarography, dialysis equilibrium measurements and electrical conductance and transference measurements are also effective in determining the fraction of free cations and the fraction of cations bound by a polysaccharide.

III. NATURE OF THE LIGAND

A. *Polar ligands in neutral or non-alkaline media*

In dilute aqueous solutions, the degree to which polyvalent metal cations interact with neutral sugars and alditols would be virtually non-existent were chelation not a factor contributing to the stability of the complex. Monohydric alcohols are essentially unable to displace water of hydration from around a metal ion in aqueous solution. However, detectable or measurable displacement of these molecules of hydration can occur, if the ligand contains two or more hydroxyl groups in favourable steric arrangement for chelation (Angyal, 1973). Through the use of nuclear magnetic resonance (NMR), Angyal (1972) established that in

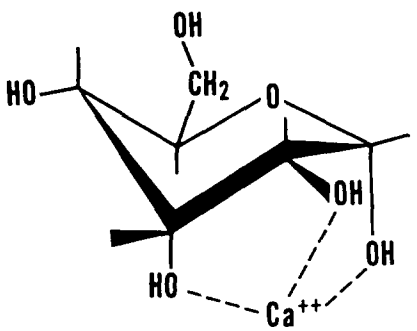


Fig. 1. α-D-allopyranose-Ca^{2+} complex.

hydroxylic solvents, a variety of metal cations (provided by such salts as NaCl, $MgCl_2$, $CaCl_2$, $Ca(NO_3)_2$, $Sr(NO_3)_2$, $BaCl_2$, $Pb(OAc)_2$, $SnCl_2$, $SnCl_4$, $Y(NO_3)_3$ and $LaCl_3$) form 1:1 complexes with cylitols and sugars containing an *axial–equatorial–axial* sequence of three hydroxyl groups in a six-membered ring or a *cis,cis* sequence in a five-membered ring. Orientation of three hydroxyls is most suitable when oxygen atoms form an equilateral triangle whose sides are about 2·8 Å in length. The oxygen atoms probably enter the primary co-ordination sphere of the cation with concurrent displacement of three water molecules. Examples of these ring types are shown in Figs. 1 and 2. Lanthanum had the greatest reactivity of the elements studied.

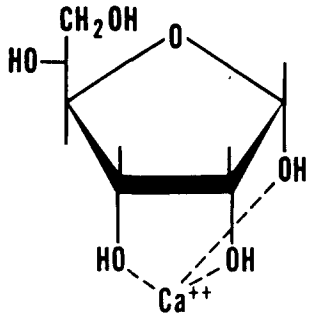


Fig. 2. α-D-allofuranose-Ca^{2+} complex.

TABLE 2
APPARENT STABILITY CONSTANTS FOR 1:1 COMPLEXES OF D-ALLOSE WITH VARIOUS METAL IONS[a]

Salt	*Molarity*		β_1	
	Salt	*Allose*	*Pyranose form*	*Furanose form*
NaCl	1·6	0·7	0·12	—
$MgCl_2$	0·6	0·6	~0·19	—
$CaCl_2$	0·74	0·75	5·2	1·6
$Ca(NO_3)_2$	0·69	0·66	6·4	5·4
$Sr(NO_3)_2$	0·79	0·66	5·9	2·7
$BaCl_2$	0·73	0·6	2·9	1·2
$Pb(OAc)_2$	0·55	0·55	~2·4	—
$SnCl_2$	1·1	0·3	0·3	—
$SnCl_4$	1·29	0·3	0·3	—
$Y(NO_3)_3$	0·35	0·35	1·9	0·7
$LaCl_3$	0·90	0·59	10·4	8·7

[a] Angyal (1972).

Apparent formation constants for various metal complexes of D-allopyranose are given in Table 2. Obviously, the anion exerts an appreciable influence on stability constants. In some instances the influence of an anion might arise from incomplete dissociation of the salt, particularly where salt concentrations are high. A significant proportion of some polyvalent cations possibly exists as a species whose charge is not equal to the oxidation state of the metal. Since different species of the same metal ion can react differently towards a polyhydroxy compound, salt concentration (and therefore salt activity) should greatly influence the apparent stability constant. Indeed, Angyal (1972) did observe that the magnitude of apparent stability constants changes with changes in salt concentration. Rendleman (1966*b*) observed that magnesium acetate causes no electrophoretic migration of sugars in methanolic media, whereas magnesium chloride does cause a measurable movement. This difference in the ability of different salts (possessing a common cation) to promote migration can be attributed to a difference in the degree of ionic

dissociation of the salt or to some property of the salt that causes effective concentration of cation to be less than actual concentration. Kohn *et al.* (1968) showed that the activity coefficient for Ca^{2+} in aqueous 0·0015M $CaCl_2$ solution is only 0·75, indicating that even in dilute solution the behaviour of Ca^{2+} is not that of a completely free ion; the effective concentration of Ca^{2+} is therefore considerably less than the actual concentration.

Angyal (1972) also examined the behaviour of other metal ions in addition to those in Table 2. Cadmium(II) ion, like Sn^{2+} and Sn^{4+}, formed weak complexes. The extent of complexation of K^+ and Zn^{2+} was too small to measure and no detectable complex formation occurred in solutions of LiCl, CsCl, RbCl, $BeSO_4$, $TlNO_3$, $Al_2(SO_4)_3$, $AgNO_3$ and $Hg(OAc)_2$. In each periodic group, those metal ions that form complexes most easily seem to have a radius of about 1·0 Å.

Electrophoretic experiments (Angyal, 1972) with α-D-allofuranose (Fig. 2) in aqueous solution have shown that the substitution of a methoxyl for a hydroxyl group at C-1 does not prevent complexation. However, the corresponding β anomer, which does not have a *cis,cis* sequence of three oxygen atoms and does not migrate electrophoretically, probably undergoes little or no complexation. That a methoxyl group might function as a donor has also been suggested by NMR studies of methyl α-D-gulopyranoside in aqueous solutions of lanthanide salts (Grasdalen *et al.*, 1975). Eu^{3+} and Pr^{3+} form 1:1 complexes with the glycoside and are bound near the *ax–eq–ax* sequence of oxygens at C-1, C-2 and C-3.

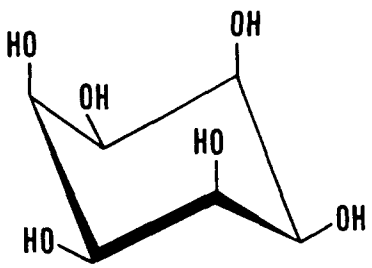


Fig. 3. *cis*-Inositol.

A triad of three axial hydroxyl groups offers a potential site for strong complexing. *cis*-Inositol (Fig. 3) contains one such group, in addition to three *ax–eq–ax* sequences. It is not surprising, therefore, that this polyol forms extremely stable complexes (Angyal, 1973).

X-ray crystallographic analysis of β-D-mannofuranose . $CaCl_2$. H_2O has revealed (Craig *et al.*, 1972) that Ca^{2+} in this complex is co-ordinated to O–1, O–2 and O–3 of one sugar moiety and to O–5 and O–6 of another. The site of the metal ion in the crystal suggests that such a sequence of three hydroxyls at C-1, C-2 and C-3 of furanoses would also favour complexation in solution.

A few years ago, electrophoretic and NMR studies of the interaction of lanthanide ions (Eu^{3+} and Pr^{3+}) with straight-chain polyols were described (Angyal *et al.*, 1974; Kieboom *et al.*, 1975). Strong complexing occurs at the site of three consecutive oxygen atoms that are in a *gauche–gauche* configuration (Fig. 4). Because such a group contains two oxygen atoms in an energetically unfavourable orientation, the predominant conformation of uncomplexed alditol will not have this configuration. The extent of complexation is determined by the energy required to form a *gauche–gauche* arrangement by rotation about carbon–carbon bonds. The similarity between structures in Fig. 4 and Fig. 1 is obvious. Although acyclic

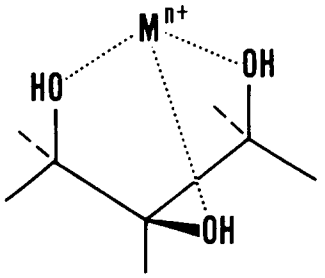


Fig. 4. Complexation of a metal ion with a straight-chain polyol.

polyols only appear rarely in naturally-occurring polysaccharides, groupings of three (or more) hydroxyl groups, which might impart exceptional stability to a macromolecular complex, are important.

The electronic structures of various glycerol chelates of different non-transition metals have been calculated by means of a molecular orbital method (Savransky & Pilipenko, 1971). A six-membered ring is more stable than a five-membered one when glycerol is treated as a bidentate ligand. Furthermore, a chelate in which glycerol functions as a tridentate ligand is more stable than a chelate involving glycerol as a bidentate ligand.

The subtle influence of cation radius on the stability of complexes of straight-chain polyols was revealed by studies of interactions of lanthanide ions with 1,2-ethanediol, 1,2-dihydroxypropane and glycerol (Manku & Chadha, 1972). Stability constants for these complexes increase with increasing atomic number from lanthanum(III) to lutetium(III). In other words, ions of smaller radius (and therefore stronger coulombic field) favour stronger co-ordination.

Such enolates as maltolate and kojate (Beélik, 1956) form stable chelates. Maltol reacts with Cu(II) according to equilibria (13) and (14) (Chiacchierini *et al.*, 1973):

$$M + HL \rightleftharpoons ML + H^+ \tag{13}$$

$$ML + HL \rightleftharpoons ML_2 + H^+ \tag{14}$$

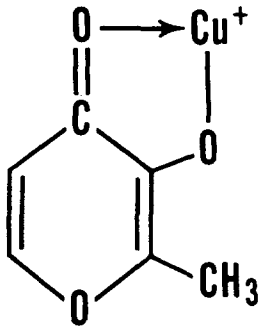


Fig. 5. Monomaltolato complex of Cu(II) (Chiacchierini *et al.*, 1973).

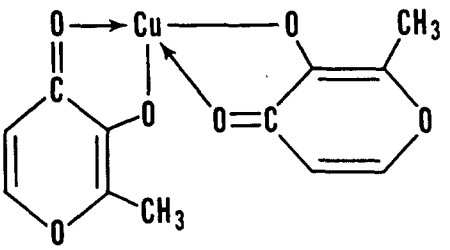
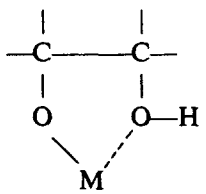


Fig. 6. Dimaltolato complex of Cu(II) (Chiacchierini *et al.*, 1973).

Equilibrium (13) prevails at high acidity (pH 3–4), whereas equilibrium (14) only occurs at somewhat lower acidity (pH 4·7–5·5). Under both acidic conditions only mononuclear species (Figs. 5 and 6) are formed (Smith & Pilbrow, 1974). (*Note:* Copper and many other metals of the transition series are known to form polynuclear complexes in which each molecular species contains two or more metal ions.)

B. *Polar ligands in alkaline media*

In alkaline solution, a polyhydroxy compound may lose one or more hydroxylic protons to give a metal–alcoholate complex that is more stable than that formed by simple cation–dipole interaction. In all likelihood, metal alcoholates are stabilised by inner chelation of the metal ion by neighbouring hydroxyl groups (Rendleman, 1966*a*):



Mannitol is known to form strong complexes with Fe(II), Fe(III), Mn(II), Mn(III) and Mn(IV) in highly alkaline solutions (1–3M OH^-) (Doležal & Langmyhr, 1972). Co(II) and Co(III) form 1:1 complexes with mannitol in alkaline media (Doležal *et al.*, 1973). With Cu(II) and with mannitol in large excess, a 1:1 complex forms; however, with ligand in only small excess, or with reactants in equimolar proportion, a 2:1 copper–mannitol complex predominates. Reaction of Ni(II) with mannitol is weak and its stoichiometry is uncertain (Doležal *et al.*, 1973).

From steric considerations and from knowledge concerning the reaction of borate ion with mannitol and glycosides (borate ion reacts with adjacent *cis*-hydroxyl groups in galactosides and mannosides (Malcolm *et al.*, 1964)), mannitol is capable of behaving as a bidentate ligand over two contiguous hydroxyl groups. It should also behave as a tridentate ligand through groups of hydroxyls at positions 1, 2, 4 or 2, 3, 5 or 3, 4, 6. However, the positions of attachment and the number of hydroxyls involved in chelation are not known.

In the absence of solvent, a polyhydroxy compound can react with the oxide or hydroxide of a polyvalent metal to yield alcoholate-type products. The structure of the product depends upon temperature, as was found for the reaction of CaO with glycerol in a phase study by Fujii & Kondo (1968), who reported the following phase equilibria:

$$\underset{\text{Calcium diglyceroxide . 4 Glycerol}}{Ca(C_3H_7O_3)_2 \cdot (C_3H_8O_3)_4} \overset{18^\circ}{\rightleftharpoons} \underset{\text{Tricalcium hexaglyceroxide . 2 Glycerol}}{Ca_3(C_3H_7O_3)_6 \cdot (C_3H_8O_3)_2}$$

$$Ca_3(C_3H_7O_3)_6 \cdot (C_3H_8O_3)_2 \overset{44^\circ}{\rightleftharpoons} \underset{\text{Calcium diglyceroxide}}{Ca(C_3H_7O_3)_2}$$

$$Ca(C_3H_7O_3)_2 \overset{75^\circ}{\rightleftharpoons} \underset{\text{Calcium monoglyceroxide}}{CaC_3H_6O_3}$$

C. *Ligands with multiple anionic groups*

The presence of multiple oxyacid groups in a ligand confers especially great stability upon a metal complex when two or more of those groups are able to chelate with a single polyvalent cation. This condition is well illustrated by the familiar reactions of EDTA with various metal cations (Garvan, 1964). Oxalate ion forms strong bonds with such divalent cations as Ca^{2+}, Sr^{2+} and Ba^{2+} because of the five-membered chelate ring. The corresponding malonate complex, with a six-membered ring, is somewhat lower in stability. Furthermore, stability drops off considerably with succinate ion and alkanedicarboxylates of greater length (Table 3). Table 3 shows the influence of steric factors (compare malonate with methylmalonate and dimethylmalonate). Because equilibria may be affected by ionic strength of the reaction mixture, comparisons of stability constants should be made, if possible, only where studies have been performed at the same ionic strength (μ). Sulphate, thiosulphate and selenate ions were included in Table 3 to point out that certain inorganic divalent anions are also able to chelate with polyvalent metal cations.

TABLE 3

STABILITY CONSTANTS[a] OF 1:1 CHELATES OF DIVALENT METAL IONS WITH DICARBOXYLATES AND DIVALENT INORGANIC OXYANIONS

Ligand	μ	t (°C)	$\log K^{M}_{MA}$						*References*
			Zn(II)	*Cu(II)*	*Mg(II)*	*Ca(II)*	*Sr(II)*	*Ba(II)*	
Oxalate	1·0	25	6·16						Moriya & Sekine (1974)
Malonate	1·0	25	3·80						Moriya & Sekine (1974)
	0·1	25		5·04	2·11	1·51	1·30	1·22	Ostacoli *et al.* (1968)
	~0·035	?		5·80	2·86	2·35	—	1·85	Jones & Stock (1962)
Methylmalonate	0·1	25	2·55	4·89	1·73	1·65	1·43	1·42	Ostacoli *et al.* (1968)
Dimethylmalonate	0·1	25	1·90	4·57	1·55	1·52	1·33	1·35	Ostacoli *et al.* (1968)
Succinate	~0·035	?	3·48						Jones & Stock (1962)
	1·0	25	2·00						Moriya & Sekine (1974)
Glutarate	~0·035	?	3·00						Jones & Stock (1962)
	1·0	25	1·74						Moriya & Sekine (1974)
Adipate	~0·035	?	3·02						Jones & Stock (1962)
	1·0	25	1·78						Moriya & Sekine (1974)
Suiphate	1·0	25	1·48						Moriya & Sekine (1974)
Thiosulphate	1·0	25	2·28						Moriya & Sekine (1974)
Selenate	1·0	25	1·38						Moriya & Sekine (1974)

[a] These are overall stability constants for the reaction $M^{2+} + A^{2-} \rightleftharpoons MA$.

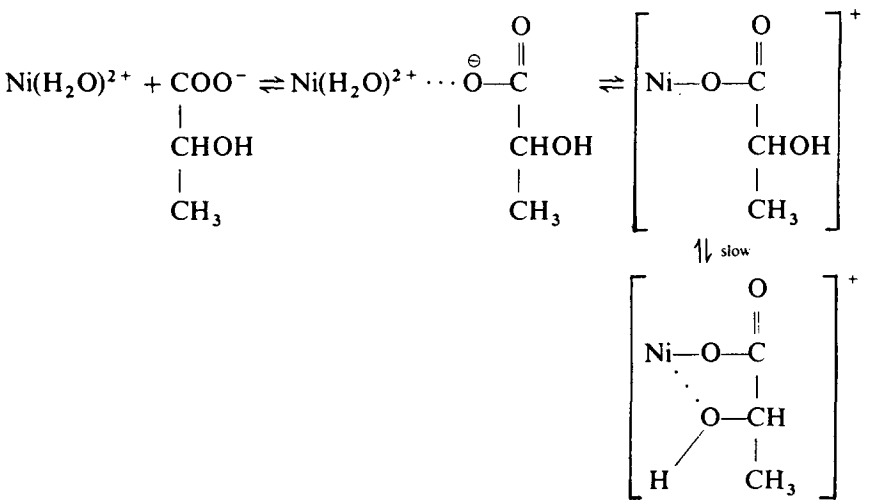
The order of stability of the malonate complexes of the first-transition metal group is the same as the Irving–Williams order of stabilities: Zn < Cu > Ni > Co > Fe > Mn (Jones & Stock, 1962).

Malonate and oxalate complexes in which the ratio of dicarboxylate ion to polyvalent cation is greater than 1:1 are known. Examples of this for Co(III) and Cr(III) are $Co(C_3H_2O_4)_3^{3-}$ (Russell & Douglas, 1973) and $Cr(C_2O_4)_3^{3-}$ (Butler & Snow, 1971). Interestingly, Th(IV) forms chelates (Tomat *et al.*, 1972) with one and two malonate ligands, but it can chelate with only one succinate. Glutarate ion does not chelate with Th(IV); however, the monohydrogen glutarate ion (HL^-) behaves as a monodentate donor and forms the complexes $Th(HL)^{3+}$ and $Th(HL)_2^{2+}$.

D. *Hydroxy acid ligands*

Hydroxyl or amino groups close to an anionic donor group in an organic ligand might also function as donors so as to permit the formation of an inner chelate. For example, propionate ion serves as a monodentate ligand for Zn(II), whereas 2-aminopropionate (Brezina, 1968) and 2-hydroxypropionate (Filipović *et al.*, 1973) behave as bidentates; aminomalonate serves as a tridentate ligand (Brezina, 1968).

In the formation of an inner-sphere complex involving a monodentate ligand, the rate-determining step is the dissociation of a water molecule from the inner co-ordination sphere (primary hydration sphere) of the metal ion. In the formation of an inner chelate, the rate may in some instances be determined by the ring-forming step that follows bonding of an anionic donor atom to the cation. This was indicated by studies of interaction between Ni(II) and lactate (2-hydroxypropionate) ion (Harada *et al.*, 1973), the probable mechanism for which is:



For convenience, only one water molecule is shown in the inner co-ordination sphere of the nickel ion. The slow, rate-determining step is the one where the hydroxyl group becomes bonded to nickel.

Recent investigations of various hydroxycarboxylates (Filipović *et al.*, 1973; Nushi *et al.*, 1973) have contributed greatly towards an understanding of the effect of ligand basicity and hydroxyl-group position in complex stability, in particular with Zn(II) (Tables 4 and 5). The pK_a (acid-ionisation constant) values in Table 5 characterise ligand basicity. In many instances, Zn(II) is capable of binding as many as four ligands, including unsubstituted *n*-butyrate. However, the most stable of the alkanecarboxylate complexes are those with a hydroxyl group in the α position,

TABLE 4
INFLUENCE OF HYDROXYL GROUPS ON THE CUMULATIVE STABILITY CONSTANTS OF Zn(II)-ALKANECARBOXYLATE COMPLEXES AT 25 °C AND $\mu = 2$[a]

Ligand	β_1	β_2	β_3	β_4
3-Hydroxypropionate	7·2	13	27	—
2-Hydroxybutyrate	52	1059	6974	17415
3-Hydroxybutyrate	9·8	51	31	313
4-Hydroxybutyrate	9·2	36	34	239
Butyrate	9·4	45	54	373

[a] Filipović *et al.* (1973).

TABLE 5
INFLUENCE OF LIGAND BASICITY ON THE STABILITY OF Zn(II)-ALKANECARBOXYLATE COMPLEXES AT 25 °C AND $\mu = 2$

Ligand	β_1	pK_a	*Reference*
Acetate	7·0	4·74	Thun *et al.* (1967)
2-Hydroxyacetate	52	3·77	Thun *et al.* (1967)
Propionate	9·9	4·89	Thun *et al.* (1967)
2-Hydroxypropionate	40·7	3·82	Piljac *et al.* (1973)
3-Hydroxypropionate	7·2	4·56	Filipović *et al.* (1973)
Butyrate	9·4	4·86	Filipović *et al.* (1973)
2-Hydroxybutyrate	52	3·80	Filipović *et al.* (1973)
3-Hydroxybutyrate	9·8	4·53	Filipović *et al.* (1973)
4-Hydroxybutyrate	9·2	4·85	Filipović *et al.* (1973)

regardless of whether basicity of α-hydroxyl ligands is considerably lower than that of either unsubstituted alkanecarboxylate or the 3- and 4-substituted ligands. The similarity in complexing ability between 3-hydroxybutyrate, 4-hydroxybutyrate and butyrate shows that the presence of a hydroxyl group at positions other than C-2 does not influence complex stability in a straight-chain hydroxy acid. *n*-Alkanecarboxylates and 3- and 4-hydroxyalkanecarboxylates function only as monodentate ligands; binding occurs only through the carboxylate group. The high stability of 2-hydroxyalkanecarboxylate complexes presumably is caused by inner chelation involving the 2-OH group.

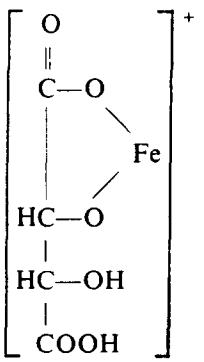
In contrast to the behaviour of Zn(II), Cd(II) (Filipović *et al.*, 1973) shows no preference for 2-hydroxybutyrate. Because no difference in stability is discernible between cadmium complexes of substituted and unsubstituted monocarboxylates, stabilisation through inner chelation must be greatly influenced by the size of the

cation. Zinc (atomic number 30) and Cd (atomic number 48) are both in periodic group IIb.

The order of stability for lactate and 3-hydroxybutyrate complexes of Co(II), Ni(II) and Cu(II) is Cu $\gg$ Ni > Co (Savić *et al.*, 1972), which obeys the Irving–Williams order of complex stability. Complexes of 1-hydroxycyclopentanecarboxylate (Powell & Rowlands, 1967) also fall into this sequence. In addition, similar studies with *cis*-2-aminocyclohexane-1-carboxylate (Nikolasev *et al.*, 1969) also show that stability increases with increasing atomic number and also follows the Irving–Williams order. Calcium(II) forms only one lactate complex, $Ca(lactate)^+$, whereas copper(II) forms two, $Cu(lactate)^+$ and the uncharged $Cu(lactate)_2$ (Ghosh & Nair, 1970). It is not surprising that the stability of the Ca(II) complex ($\log\beta_1 = 1{\cdot}55$ at 25° and $\mu = 0{\cdot}1$) is considerably lower than that of the Cu(II) complex ($\log\beta_1 = 2{\cdot}36$; $\log\beta_2 = 3{\cdot}90$), considering the low effective nuclear charge of the calcium ion.

At low pH (3·6–5·8) and in the presence of Cd(II) ion, tartaric acid gives up two protons to form a neutral complex, Cd(tartrate) (Tripathy *et al.*, 1972). At a slightly higher pH (6·0–8·5), a third proton is released to form another complex—$Cd(tartrate)^-$—which, in turn, can release a fourth proton at pH 9·0–9·5 to form still another complex—$Cd(tartrate)^{2-}$. The stability constant for the formation of Cd(tartrate) from Cd^{2+} and $(tartrate)^{2-}$ is quite high, 73·2 at 33° and $\mu = 0{\cdot}1$. Similar reactions of tartaric acid or tartrate ion occur with Co(II) (Tripathy & Patnaik, 1970) and Mn(II) (Tripathy & Patnaik, 1967). In alkaline solution, tartaric acid is known to liberate four protons in the presence of Sb(III) (Iyer *et al.*, 1972), Al(III) (Pavlinova, 1947) and Zr(IV) (Ermakov *et al.*, 1967). However, in no instance is there complete certainty about the structure of any of these tartrate complexes.

In acid solution (pH 1), Fe(III) reacts with tartaric acid, liberating two protons and forming a 1:1 complex. Niac & Andrei (1967) offer evidence that at least one carboxylate group is involved in the structure.



Lactic acid at pH 1–2 releases a proton to form an Fe(III) complex that possibly has the chelate structure a (Fig. 7). At pH 3 a second proton is released with the production of a 1:1 complex that, presumably, has structure b. At pH 4–7 a 2:1 lactate-Fe(III) complex is formed that is negatively charged and which probably has structure c.

Many of these transition-metal complexes of hydroxy acids possibly exist as dimers. Electron paramagnetic resonance (EPR) measurements on Cu(II) citrate chelates at pH 7–11 indicate dipole–dipole coupling of Cu(II) ions arising from dimer formation (Dunhill *et al.*, 1966). Dimer formation is further discussed in Section III.E below.

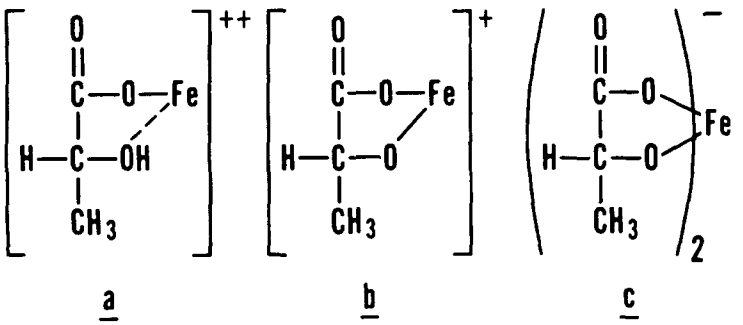


Fig. 7. Proposed structures for Fe(III)-lactate (Niac & Andrei, 1967) in solutions of different pH: a at pH 1–2; b at pH 3 and c at pH 4–7.

Gluconate ion forms soluble chelates with a variety of different metals, among which are the transition metals, rare-earth metals and alkaline-earth metals (Sawyer, 1964). Stability of alkaline–earth metal complexes varies with pH; it is low in weakly basic media, but increases greatly as the solution becomes highly alkaline (pH ~ 12–13). Reportedly, gluconate ion is four times as effective as EDTA in sequestering polyvalent metals from hard water (Colaric *et al.*, 1957). Stoichiometry of the formation of gluconate complexes may vary greatly depending upon the specific metal, the molar ratio of metal to gluconate in the reaction mixture and the pH of the solution. The stoichiometry of reaction with Co(II) is 1:1 (Bermejo Martinez & Branas Miguez, 1971). Examination of the influence of pH on ultraviolet (UV) absorbance of the Co(II) complex suggests that at least one hydroxyl group, if not more, releases a proton. NMR studies of Pb(II)-gluconate complexation indicate that Pb(II) is co-ordinated to anionic oxygens at the α, β and γ positions, in addition to being bound to the carboxylate group (Sawyer & Brannan, 1966). From careful pH measurements and optical rotation studies, Sipes (1969) found that in the pH region of 1–11, two calcium gluconate complexes

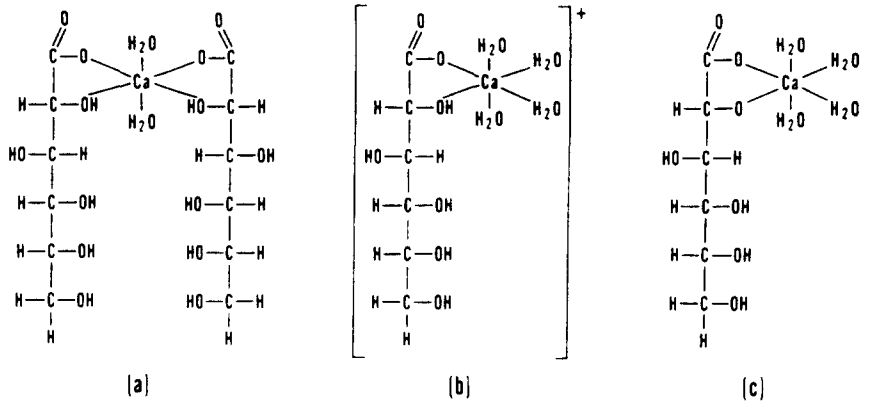


Fig. 8. Suggested structures for calcium gluconate complexes (Sipes, 1969) in solutions of different pH: (a) and (b) at pH 1–11; (c) at pH 11–12.

can exist (Fig. 8(a) and (b)). In this region no hydroxylic protons are released. At pH 11–12, another species, possibly that shown in Fig. 8(c), forms with concurrent proton removal. At higher alkalinity, there is evidence of further proton removal to form a 2:1 Ca(II)-gluconate species.

Crystal structure analysis of calcium D-arabinonate . $5H_2O$ (Furberg & Helland, 1962) (Fig. 9), calcium D-*xylo*-hex-5-ulosonate . $4H_2O$ (Balchin & Carlisle, 1965)

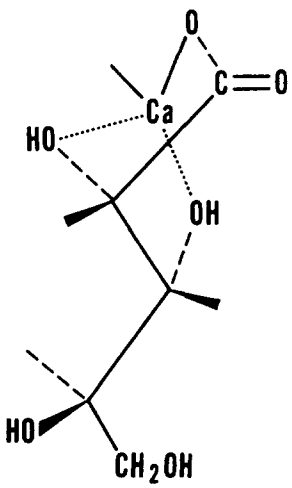


Fig. 9. Structure of calcium D-arabonate (Furberg & Helland, 1962).

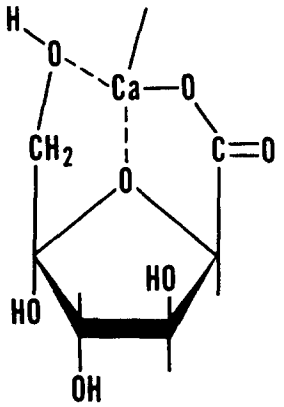


Fig. 10. Structure of calcium D-*xylo*-hex-5-ulosonate (Balchin & Carlisle, 1965).

(Fig. 10), α-D-glucuronate . CaBr . $3H_2O$ (DeLucas *et al.*, 1975), calcium sodium D-galacturonate hexahydrate (Gould *et al.*, 1975) and strontium 4-*O*-(4-deoxy-β-L-*threo*-hex-4-enosyl)-α-D-galacturonate . 4 . $5H_2O$ (Gould *et al.*, 1976) has revealed that inner chelation in the solid state may involve not only hydroxyl groups but also ring oxygens (as in Fig. 10). In the first two of these compounds, a triad of three oxygen atoms from each carbohydrate ligand is involved in chelation with Ca^{2+}. In the other three complexes there are bidentate arrangements around the alkaline-earth metal cations. It must be stressed that although tridentate complexes may occur in the crystalline state, chelation of hydroxy acids in solution does not necessarily require a triad of oxygen atoms. Lactate complexes exemplify a situation where only a bidentate complex is possible. However, a hydroxy acid anion capable of functioning as a tridentate ligand will form a stronger chelate than a bidentate anion of similar structure.

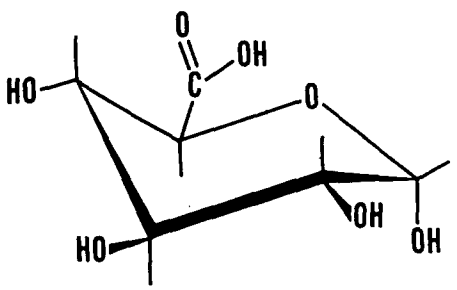


Fig. 11. α-D-glucuronic acid.

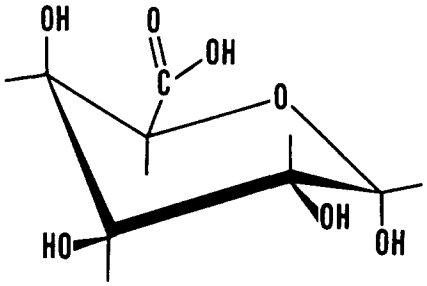


Fig. 12. α-D-galacturonic acid.

Gould & Rankin (1970) determined stability constants for 1:1 complexes of calcium ion with the anion of D-glucuronic acid (Fig. 11) and of D-galacturonic acid (Fig. 12). For the α anomers, the respective values reported for D-glucuronate and D-galacturonate are 37 and 101 and for the respective β anomers, 28 and 61. The influence of the anomeric hydroxyl is probably of only secondary importance since the stability constants for 1:1 calcium complexes of methyl α-D-glucuronosidate and methyl β-D-galacturonosidate (40 and 56, respectively) do not differ greatly from constants for the corresponding uronate complexes. The greater strength of the galacturonate complex, relative to that of the glucuronate complex, can be related to the hydroxyl group on C-4 which is axial in galacturonate and equatorial in glucuronate. Involvement of an axial oxygen at C-4 of D-galacturonate is strongly suggested by NMR studies in which Eu^{3+} was the paramagnetic probe for the Ca^{2+} site (Anthonsen *et al.*, 1973). Crystal structure analysis (Gould *et al.*, 1975) has shown that in crystalline calcium D-galacturonate, contrary to what is thought to occur in aqueous solution, the axial oxygen at C-4 is not bonded to Ca^{2+}. In the solid, the cation is bonded to the carboxylate, to the ring oxygen and to a water molecule.

It seems to be a general rule that, in the crystalline state, uronate ions will co-ordinate to Ca^{2+} through a carboxylate oxygen and an oxygen function on the α-carbon atom, with these two oxygens in an eclipsed or near-eclipsed conformation (Gould *et al.*, 1975). In solution, however, where packing considerations are not important, the system might possibly deviate from the eclipsed conformation to adopt an entropy favoured arrangement involving a triad of oxygens from each carbohydrate residue.

Stability constants reported by Gould & Rankin (1970) for calcium D-glucuronate and calcium D-galacturonate at anomeric equilibrium, 32 (±2) and 70 (±4), respectively, are considerably higher than those reported by Buddecke & Drzenieck (1962), 5·3 and 5·0. Perhaps differences in ionic strength (μ) of the media are responsible for the differences between the two sets of data. Only in the studies of Buddecke & Drzenieck was μ reported. Neither group of investigators specified

conditions of temperature. In Table 6, stability constants are compared for 1:1 calcium complexes of the two uronates and various other monocarboxylate ions in neutral solution at similar ionic strengths. Even without a hydroxyl substituent in the ligand, carboxylate groups tend to complex moderately well with polyvalent cations. For example, in a solution that is 0·1M with respect to both Ca^{2+} and OAc^{-}, the extent of ion pairing to form $Ca(OAc)^{+}$ would be approximately 22%; however, at 0·01M concentration the extent of complexation would be only 3%.

TABLE 6
STABILITY CONSTANTS OF 1:1 CALCIUM COMPLEXES OF VARIOUS SUBSTITUTED AND UNSUBSTITUTED MONOCARBOXYLATE IONS IN NEUTRAL SOLUTION (pH 7) AND AT μ = 0·15–0·20

Substituent	*Anion*	μ	°C	β_1	*Reference*
None	Acetate	0·16	25	3·4	Cannan & Kibrick (1938)
	Propionate	0·2	25	3·2	Cannan & Kibrick (1938)
	Butyrate	0·16	25	3·2	Cannan & Kibrick (1938)
	Hydrogen malonate	0·16	25	3·0	Cannan & Kibrick (1938)
	Hydrogen succinate	0·16	25	3·3	Cannan & Kibrick (1938)
α-Hydroxy	Lactate	0·2	?	11·8	Cannan & Kibrick (1938)
	Glycerate	0·2	?	15·1	Cannan & Kibrick (1938)
	2-Hydroxyacetate	0·2	?	12·9	Cannan & Kibrick (1938)
	D-Gluconate	0·15	?	16·2	Cannan & Kibrick (1938)
β-Hydroxy	3-Hydroxybutyrate	0·2	?	4·0	Cannan & Kibrick (1938)
	D-Glucuronate	0·15	?	5·3	Buddecke & Drzenieck (1962)
	D-Galacturonate	0·15	?	5·0	Buddecke & Drzenieck (1962)

In many oligo- and polysaccharides, the oxygens at C-1 and C-4 are involved in glycosidic linkages. Because the acetal oxygen O–5 in D-galacturonate and D-glucuronate ions can co-ordinate to Ca^{2+}, it is conceivable that acetal oxygens O–1, O–4 and O–5 in some of the oligo- and polysaccharides might also function as donors, at least in the solid state.

From existing knowledge of the ability of Ca^{2+} to serve as a bridge between two or more simple carbohydrate residues, it is reasonable to assume that Ca^{2+} is also able to link polysaccharide chains together and that the mechanism for the interaction is stereospecific.

E. *Polynuclear transition-metal ion complexes of hydroxy acids*

The occurrence of metal-ion dimer (binuclear) units in many transition-metal complexes is well documented (Smith & Pilbrow, 1974). Polymeric species containing more than two metal ions are also known. X-ray diffraction studies, magnetic susceptibility measurements and EPR studies have contributed to elucidating this phenomenon. EPR measurements provide information on metal ion-metal ion separation which, together with information derived from molecular-model studies, permits decisions to be made concerning the actual structure of the complex in solution. For example, the short Cu^{2+}–Cu^{2+} separation of 3·1 Å in aqueous solutions of citrate and Cu^{2+} ions led to the proposed structure (Boas *et*

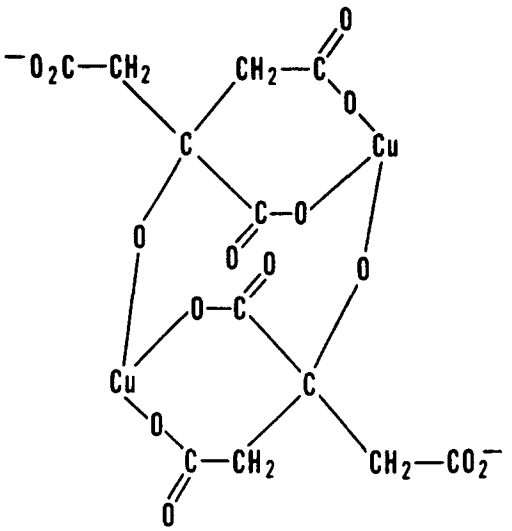


Fig. 13. Proposed structure for a Cu(II) citrate dimer in aqueous solution (Boas *et al.*, 1969).

al., 1969) for the Cu(II) species shown in Fig. 13. The hydroxyl group in a hydroxycarboxylate complex is not always involved in complexation in neutral media, as found by similar studies of malate ion and Cu^{2+} (Boas *et al.*, 1969).

EPR studies have been made of Cu(II) chelates formed by reactions with saccharic acid (D-glucaric acid), lactobionic acid, gluconic acid, and mucic acid (galactaric acid) (Toy *et al.*, 1971). Evidence was provided for the formation of dimeric, as well as monomeric, species, the relative proportions of each being dependent upon pH conditions of the solution. Dimer formation results from ionisation of the hydroxyl groups at high alkalinity (pH 11–13). The copper–copper distance of about 3·4–4·0 Å suggests a bridging arrangement involving a terminal carboxylate group and an ionised hydroxyl group on a β-carbon atom.

The EPR low-field signals, which provide the evidence for the presence of dimeric species, are observed only when basicity of the solution is raised to a certain level.

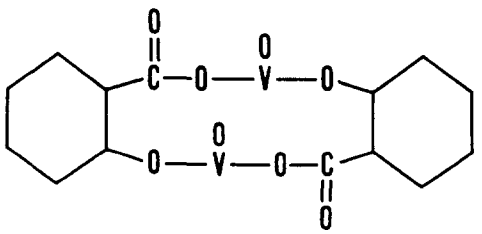


Fig. 14. Suggested structure for the dimeric form of vanadyl 1-hydroxycyclohexanecarboxylate complex (Smith *et al.*, 1971).

Because this level of basicity is much higher than that necessary to ionise both carboxyl groups of saccharic or mucic acid, more than just the presence of two carboxylate groups is needed for dimerisation; dimerisation apparently occurs only at a pH high enough to ionise hydroxyl groups appreciably.

EPR spectra of dimethylformamide solutions containing 1-hydroxycyclohexanecarboxylic acid, vanadyl ions and triethylamine (which functions as a base) indicate the formation of a dimer in which ionised hydroxyl group and carboxylate group are both involved (Smith *et al.*, 1971). The copper–copper distance of about 3·6 Å is consistent with the structure shown in Fig. 14.

REFERENCES

AGRAWAL, B. B. L. & GOLDSTEIN, I. J. (1967). Protein–carbohydrate interaction. VI. Isolation of concanavalin A by specific adsorption on cross-linked dextran gels. *Biochim. Biophys. Acta*, **147**, 262–71.

ANGYAL, S. J. (1972). Complexes of carbohydrates with metal cations. I. Determination of the extent of complexing by NMR spectroscopy. *Aust. J. Chem.*, **25**, 1957–66.

ANGYAL, S. J. (1973). Complex formation between sugars and metal ions. *Pure Appl. Chem.*, **35**, 131–46.

ANGYAL, S. J., GREEVES, D. & MILLS, J. A. (1974). Complexes of carbohydrates with metal cations. III. Conformations of alditols in aqueous solution. *Aust. J. Chem.*, **27**, 1447–56.

ANTHONSEN, T., LARSEN, B. & SMIDSRØD, O. (1973). NMR studies of the interaction of metal ions with poly(1,4-hexuronates). II. The binding of europium ions to sodium methyl α-D-galactopyranosiduronate. *Acta Chem. Scand.*, **27**, 2671–3.

ASPINALL, G. O. (1970). Pectins, plant gums, and other plant polysaccharides. In *The carbohydrates* (Pigman, W. & Horton, D. (eds)). **IIB**, 515–36, New York, Academic Press.

BALCHIN, A. A. & CARLISLE, C. H. (1965). The crystal structure of calcium 5-keto-D-gluconate (calcium D-*xylo*-5-hexulosonate). *Acta Cryst.*, **19**, 103–11.

BARSDATE, R. J. (1970). Transition metal binding by large molecules in high latitude waters. *Symp. Org. Matter Nat. Waters* (1968), *University of Alaska, College, Alaska*, 485–93.

BEĔLIK, A. (1956). Kojic acid. *Adv. Carbohydr. Chem.*, **11**, 145–83.

BEL'SKII, N. K. & STRUCHKOV, YU. T. (1965). The crystal structure and optical properties of europium(III) chloride hexahydrate. *Kristallografiya*, **10**, 21–8.

BERGH, A., GEDEON, A. & STEGENA, L. (1956). A geochemical research method based on the determination of heavy metals in river waters. *Geofiz. Kozl.*, **5**, 31–8.

BERMEJO MARTINEZ, F. & BRAÑAS MIGUEZ, G. (1971). Spectrophotometric determination of cobalt with gluconic acid. *Z. Anal. Chem.*, **253**, 353–4.

BERNFELD, P., DONAHUE, V. M. & BERKOWITZ, M. E. (1957). Interaction of human serum β-lipoglobulin with polyanions. *J. Biol. Chem.*, **226**, 51–64.

BOAS, J. F., DUNHILL, R. H., PILBROW, J. R., SRIVASTAVA, R. C. & SMITH, T. D. (1969). Electron spin resonance studies of copper(II) hydroxy-carboxylic acid chelates in aqueous and non-aqueous solutions. *J. Chem. Soc.* **A**, 94–108.

BREZINA, F. (1968). Correlation between the equilibrium constants and the structure and constitution of complexes. *Acta Univ. Palacki. Olomuc., Fac. Rerum Nat.*, 277–83.

BUDDECKE, E. & DRZENIECK, R. (1962). Stability constants of the Ca complexes of acid mucopolysaccharides. *Z. Physiol. Chem.*, **327**, 49–64.

BUTLER, K. R. & SNOW, M. R. (1971). Piper's model for optical activity: Absolute configurations of the complex ions $(+)_{589}[Cr(malonate)_3]^{3-}$, $(+)_{546}[Co(malonate)_2(ethylenediamine)]^-$, and $(+)_{589}[Cr(oxalate)_3]^{3-}$. *J. Chem. Soc.*, **D**, 550–1.

CANNAN, R. K. & KIBRICK, A. (1938). Complex formation between carboxylic acids and divalent metal cations. *J. Am. Chem. Soc.*, **60**, 2314–20.

CHABEREK, S. & MARTELL, A. E. (1959). *Organic sequestering agents*, **2**, New York, John Wiley and Sons, Inc.

CHIACCHIERINI, E., DE ANGELIS, G. & BALESTRIERI, F. (1973). The reaction between copper(II) and maltol in 0·5M aqueous sodium perchlorate medium. *Gazz. Chim. Ital.*, **103**, 387–91.

ČINÁTL, J. (1969). Inorganic-organic multimolecular complexes of salt solutions, culture media and biological fluids and their possible significance for the origin of life. *J. Theor. Biol.*, **23**, 1–10.

COLARIC, R. B., BALLUM, A. T., SNIKERIS, P. & KARABINOS, J. V. (1957). Evaluation of sequestrants in bottle washing. *Soap Chem. Spec.*, **33**(9), 47–9.

CRAIG, D. C., STEPHENSON, N. C. & STEVENS, J. D. (1972). An X-ray crystallographic study of β-D-mannofuranose . $CaCl_2 . 4H_2O$. *Carbohydr. Res.*, **22**, 494–5.

CRAIG, D. P. & NYHOLM, R. S. (1964). The nature of the metal-ligand bond. In *Chelating agents and metal chelates* (Dwyor, F. P. & Mellor, D. P. (eds.)). 51–93, New York, Academic Press.

DELUCAS, L., BUGG, C. E., TERZIS, A. & RIVEST, R. (1975). Calcium binding to D-glucuronate residues: Crystal structure of a hydrated calcium bromide salt of D-glucuronic acid. *Carbohydr. Res.*, **41**, 19–29.

DIETRICH, B., LEHN, J. M. & SAUVAGE, J. P. (1969). The cryptates. *Tetrahedron Lett.*, 2889–92.

DOLEŽAL, J., KLAUSEN, K. S. & LANGMYHR, F. J. (1973). Studies in the complex formation of metal ions with sugars. Part I. The complex formation of cobalt(II), cobalt(III), copper(II), and nickel(II) with mannitol. *Anal. Chim. Acta*, **63**, 71–7.

DOLEŽAL, J. & LANGMYHR, F. J. (1972). Some possibilities of redox titrations with iron(II) sulfate in an alkaline mannitol medium. *Anal. Chim. Acta*, **61**, 73–81.

DUNCAN, J. F. (1959). Complex formation by inorganic ions. *Aust. J. Chem.*, **12**, 356–81.

DUNHILL, R. H., PILBROW, J. R. & SMITH, T. D. (1966). Electron spin resonance of copper(II) citrate chelates. *J. Chem. Phys.*, **45**, 1474–81.

ERMAKOV, A. N., MAROV, I. N. & KAZANSKII, L. P. (1967). Infrared spectra of complex compounds of zirconium with hydroxycarboxylic acids. *Zh. Neorg. Khim.*, **12**, 2725–8; *Chem. Abstr.*, **68** (1968) 44411.

EVANS, D. F. & MATESICH, M. A. (1973). Ionic association in hydrogen-bonding solvents. *J. Solution Chem.*, **2**, 193–216.

FILIPOVIĆ, I., PILJAC, I., MEDVED, A., SAVIĆ, S., BUJAC, A., BACH-DRAGUTINOVIĆ, B. & MAYER, B. (1968). On the polarographic determination of stability constants of formato, acetato, propionato, butyrato, and lactato complexes of copper, zinc, cadmium, and lead. *Croat. Chem. Acta*, **40**, 131–42.

FILIPOVIĆ, I., PILJAC, I., BACH-DRAGUTINOVIĆ, B., KRUHAK, I. & GRABARIĆ, B. (1973). Stability constants of zinc complexes with 3-hydroxypropionate and 2-, 3-, and 4-hydroxybutyrates. *Croat. Chem. Acta*, **45**, 447–52.

FRENSDORFF, H. K. (1971). Stability constants of cyclic polyether complexes with univalent cations. *J. Am. Chem. Soc.*, **93**, 600–6.

FRIEDEN, E. (1972). Chemical elements of life. *Sci. Am.*, **227**(1), 52–7; 59–60.

FRIEDMAN, M. (Ed.) (1974). *Adv. Exp. Med., Biol.*, **48**.

FUJII, K. & KONDO, W. (1968). Calcium glyceroxides formed in the system of calcium oxide-glycerol. *Z. Anorg. Allg. Chem.*, **359**, 296–304.

FURBERG, S. & HELLAND, S. (1962). The crystal structure of the calcium and strontium salts of arabonic acid. *Acta Chem. Scand.*, **16**, 2372–83.

GARRELS, R. M. & MACKENZIE, F. T. (1967). Origin of the chemical compositions of some springs and lakes. *Adv. Chem. Ser.*, **67**, 222–42.

GARVAN, F. L. (1964). Metal chelates of ethylenediaminetetraacetic acid and related substances. In *Chelating agents and metal chelates* (Dwyer, F. P. & Mellor, D. P. (eds.)) 283–333. New York, Academic Press.

GHOSH, R. & NAIR, V. S. K. (1970). Studies of metal complexes in aqueous solution. I. Calcium and copper lactates. *J. Inorg. Nucl. Chem.*, **32**, 3025–32.

GOLDBERG, E. D. (1963). The oceans as a chemical system. In *The sea* (Hill, M. N. ed.) **2**, 3–25, New York, John Wiley.

GOULD, R. O. & RANKIN, A. F. (1970). Calcium complexes of uronic acid monomers. *J. Chem. Soc.*, **D**, 489–90.

GOULD, S. E. B., GOULD, R. O., REES, D. A. & SCOTT, W. E. (1975). Interactions of cations with sugar anions. Part I. Crystal structures of calcium sodium galacturonate hexahydrate and strontium sodium galacturonate hexahydrate. *J. Chem. Soc., Perkin Trans.*, **2**, 237–42.

GOULD, S. E. B., GOULD, R. O., REES, D. A. & WIGHT, A. W. (1976). Interactions of cations with sugar anions. Part II. Crystal structure of strontium 4-*O*-(4-deoxy-β-L-*threo*-hex-4-enosyl)-α-D-galacturonate . 4 . 5 water. *J. Chem. Soc., Perkin Trans.*, **2**, 392–8.

GRAEBER, E. J., CONRAD, G. H. & DULIERE, S. F. (1966). Crystallographic data for solvated rare earth chlorides. *Acta Crystallogr.*, **21**, 1012–13.

GRASDALEN, H., ANTHONSEN, T., LARSEN, B. & SMIDSRØD, O. (1975). NMR studies of the interaction of metal ions with poly(1,4-hexuronates). III. Proton magnetic resonance study of the binding of lanthanides to methyl α-D-gulopyranoside in aqueous solution. *Acta Chem. Scand.*, **B29**, 17–21.

HALE, C. F. & SPEDDING, F. H. (1972). Effect of high pressure on the formation of aqueous $EuSO_4^+$ at 25°. *J. Phys. Chem.*, **76**, 2925–9.

HARADA, S., TANABE, H. & YASUNAGA, T. (1973). Kinetic studies of the nickel lactate complex formation in solution by the pressure-jump method. *Bull. Chem. Soc., Jpn.*, **46**, 3125–7.

IRVING, H. & WILLIAMS, R. J. P. (1953). The stability of transition-metal complexes. *J. Chem. Soc.*, 3192–210.

IYER, R., KRISHNA, DESHPANDE, S. G. & RAO, G. S. (1972). Complexes of tartaric acid. I. Antimony(III)-tartaric acid system. *J. Inorg. Nucl. Chem.*, **34**, 3351–6.

JEANLOZ, R. W. (1970). Mucopolysaccharides of higher animals. In *The carbohydrates* (Pigman, W. & Horton, D. (eds.)), **IIB**, 589–625, New York, Academic Press.

JONES, R. H. & STOCK, D. I. (1962). The dissociation constants in water of some bivalent metal alkanedicarboxylates. *J. Chem. Soc.*, 306–11.

KARLSTAM, B. (1973). Evidence of the requirement of a full complement of manganese and calcium ions for optimal binding of carbohydrates to electrophoretically homogeneous concanavalin A. *Biochim. Biophys. Acta*, **329**, 295–304.

KIEBOOM, A. P. G., SPOORMAKER, T., SINNEMA, A., VAN DER TORN, J. M. & VAN BEKKUM, H. (1975). Proton NMR study of the complex formation of alditols with multivalent cations in aqueous solution using praseodymium(II) nitrate as shift reagent. *Recl. Trav. Chim. Pays-Bas*, **94**, 53–9.

KILBOURNE, B. T., DUNITZ, J. D., PIODA, L. A. R. & SIMON, W. (1967). Structure of the K^+ complex with nonactin, a macrotetrolide antibiotic possessing highly specific K^+ transport properties. *J. Mol. Biol.*, **30**, 559–63.

KISTENMACHER, H., POPKIE, H. & CLEMENTI, E. (1974). Study of the structure of molecular complexes. VIII. Small clusters of water molecules surrounding Li^+, Na^+, K^+, F^-, and Cl^- ions. *J. Chem. Phys.*, **61**, 799–815.

KOHN, R. (1969). Spectrophotometric determination of magnesium, calcium, strontium, and barium in pairs by use of tetramethylmurexide. *Chem. Zvesti.*, **23**, 721–35.

KOHN, R. & FURDA, I. (1967*a*). Calcium ion activity in solutions of calcium pectinate. *Collect. Czech. Chem. Commun.*, **32**, 1925–37.

KOHN, R. & FURDA, I. (1967*b*). Interaction of calcium and potassium ions with carboxyl groups of pectin. *Collect. Czech. Chem. Commun.*, **32**, 4470–84.

KOHN, R., FURDA, I., HAUG, A. & SMIDSRØD, O. (1968). Binding of calcium and potassium ions to some polyuronides and monouronates. *Acta Chem. Scand.*, **22**, 3098–102.

KUNZE, H. L. & SMITH, J. T. (1966). Base-flow studies, Upper Guadalupe River Basin, Texas: Quantity and quality, March 1965. *Tex. Water Dev. Board Rep.*, **29**.

MCNEELY, W. H. & PETTITT, D. J. (1973). Algin. In *Industrial gums* (Whistler, R. W. (ed.)) 49–81, New York, Academic Press.

MALCOLM, E. W., GREEN, J. W. & SWENSON, H. A. (1964). A study of the borate-carbohydrate complex. *J. Chem. Soc.*, 4669–76.

MANKU, G. S. & CHADHA, R. C. (1972). Lanthanon(III) complexes with ethanediol, propane-1,2-diol and glycerol in aqueous medium. *J. Inorg. Nucl. Chem.*, **34**, 357–9.

MAREZIO, M., PLETTINGER, H. A. & ZACHARIASEN, W. H. (1961). The crystal structure of gadolinium trichloride hexahydrate. *Acta Crystallogr.*, **14**, 234–6.

MELLOR, D. P. & MALEY, L. (1948). Stability constants of internal complexes. *Nature*, **159**, 370.

MILLER, D. (1960). Heparin precipitability of the macroglobulin in a patient with Waldenstrom's macroglobulinemia. *Blood*, **16**, 1313–17.

MILLS, J. A. (1961/62). Association of polyhydroxy compounds with cations in solution. *Biochem. Biophys. Res. Commun.*, **6**, 418–21.

MORIYA, H. & SEKINE, T. (1974). A solvent-extraction study of zinc(II) complexes with several divalent anions of carboxylic and inorganic acids. *Bull. Chem. Soc., Jpn.*, **47**, 747–8.

MORTENSEN, J. L. (1960). Physicochemical properties of a soil polysaccharide. *Trans. Int. Congr. Soil Sci., 7th*, **2**, 98–104. University of Wisconsin, Madison, Wisconsin.

MORTENSEN, J. L. (1963). Complexing of metals by soil organic matter. *Soil Sci. Soc. Am., Proc.*, **27**, 179–86.

MOSHER, M. W. & PRICE, H. C. (1975). Plant-derived lectins. *Chemistry*, **48**(7), 6–9.

NAKAYAMA, F. S. (1971). Calcium complexing and the enhanced solubility of gypsum in concentrated sodium-salt solutions. *Soil Sci. Soc. Am., Proc.*, **35**, 881–3.

NELSON, D. L. & IRISH, D. E. (1971). Interactions in lanthanide systems. I. A raman and infrared study of aqueous gadolinium nitrate. *J. Chem. Phys.*, **54**, 4479–89.

NEURATH, H. (Ed.) (1970). *The proteins*, V, New York, Academic Press.

NIAC, G. & ANDREI, Z. (1967). Complexes of trivalent metals with organic hydroxy acids. XXIV. Spectrophotometric investigation of ferritartrate. *Rev. Roum. Chim.*, **12**, 801–11.

NIKOLASEV, V., GÖNDÖS, G. & BERNÁTH, G. (1969). Equilibrium correlations of complexes of Co(II), Cu(II), Hg(II), Cd(II), and Mn(II) with *cis*-2-aminocyclohexanecarboxylic acid. *Acta Phys. Chem.*, **15**, 147–50.

NUSHI, S., PILJAC, I., GRABARIĆ, B. & FILIPOVIĆ, I. (1973). Polarographic determination of stability constants of cadmium and lead complexes in buffer solution of 3-hydroxypropionic acid and 2-, 3-, and 4-hydroxybutyric acids. *Croat. Chem. Acta*, **45**, 453–6.

ONCLEY, J. L., WALTON, K. W. & CORNWELL, D. G. (1957). A rapid method for the bulk isolation of β-lipoproteins from human plasma. *J. Am. Chem. Soc.*, **79**, 4666–71.

OSTACOLI, G., VANNI, A. & ROLETTO, E. (1968). Complex formation between alkyl-substituted malonic acids and bivalent metal ions in aqueous solutions. *Ric. Sci.*, **38**, 318–21.

PAVLINOVA, A. V. (1947). Tartrate aluminum compounds. *J. Gen. Chem. USSR*, **17**, 3–10.

PERCIVAL, E. (1970). Algal polysaccharides. In *The carbohydrates* (Pigman, W. & Horton, D. (eds.)), **IIB**, 537–68, New York, Academic Press.

PILJAC, I., GRABARIĆ, B. & FILIPOVIĆ, I. (1973). Improved technique for determination of stability constants by polarographic method. *J. Electroanal. Chem. Interfacial Electrochem.*, **42**, 433–42.

PIODA, L. A. R., WACHTER, H. A., DOHNER, R. E. & SIMON, W. (1967). Complexes of nonactin and monactin with sodium, potassium, and ammonium ions. *Helv. Chim. Acta*, **50**, 1373–6.

POWELL, J. E. & ROWLANDS, D. L. G. (1967). The stabilities of some transition metal 1-hydroxycyclopentanecarboxylic acid complexes. *J. Inorg. Nucl. Chem.*, **29**, 1729–33.

RAAFLAUB, J. (1951). A photometric method for determining ionized calcium. *Hoppe Seyler's Z. Physiol. Chem.*, **288**, 228–36.

RAAFLAUB, J. (1962). The photometric determination of ionized calcium in biological fluids, particularly in urine. *Hoppe Seyler's Z. Physiol. Chem.*, **328**, 198–203.

RARD, J. A. & SPEDDING, F. H. (1975). Electrical conductances of some aqueous rare earth electrolyte solutions at 25°. III. The rare earth nitrates. *J. Phys. Chem.*, **79**, 257–62.

RASPLETINA, G. F., UL'YANOVA, D. Z. & SHERMAN, E. E. (1967). Hydrochemistry of Lake Ladoga. *Tr. Lab. Ozeroved., Akad. Nauk SSSR*, **23**, 60–122.

RENDLEMAN, J. A., JR. (1966*a*). Complexes of alkali metals and alkaline-earth metals with carbohydrates. *Adv. Carbohydr. Chem.*, **21**, 209–71.

RENDLEMAN, J. A., JR. (1966*b*). Alkali metal complexes of carbohydrates. I. Interaction of alkali metal salts with carbohydrates in alcoholic media. *J. Org. Chem.*, **31**, 1839–45.

RORABACHER, D. B. & MOSS, D. B. (1970). Steric effects in chelation kinetics. II. Role of alcoholic oxygen as a donor atom and the internal conjugate base effect in cobalt(II)-poly(amino alcohol) reactions. *Inorg. Chem.*, **9**, 1314–8.

ROSSOTTI, F. J. C. & ROSSOTTI, H. S. (1961). *The determination of stability constants*, New York, McGraw Hill.

RUSSELL, R. L. C. & DOUGLAS, B. E. (1973). Resolution of the tris(malonato)cobaltate(III) ion. *Inorg. Nucl. Chem. Lett.*, **9**, 1251–4.

SAINI, G. R. (1966). Sequestering of iron and aluminum by soil polysaccharides. *Curr. Sci.*, **35**, 259–61.

SAVIC, J., SAVIC, M. & FILIPOVIC, I. (1972). Spectrophotometric determination of stability constants of lactato and β-hydroxypropionato complexes of cobalt(II), nickel(II), and copper(II). *Croat. Chem. Acta*, **44**, 305–15.

SAVRANSKY, L. I. & PILIPENKO, A. T. (1971). Structure of glycerol chelates with non-transition metals. *Dopov. Akad. Nauk Ukr. RSR, Ser. B*, **33**, 436–8.

SAWYER, D. T. (1964). Metal-gluconate complexes. *Chem. Rev.*, **64**, 633–43.

SAWYER, D. T. & BRANNAN, J. R. (1966). Proton nuclear magnetic resonance studies of metal gluconate complexes. I. Lead(II) and bismuth(III). *Inorg. Chem.*, **5**, 65–70.

SCHUBERT, J. (1952). Ion exchange studies of complex ions as a function of temperature, ionic strength, and presence of formaldehyde. *J. Phys. Chem.*, **56**, 113–8.

SCHUBERT, J. (1956). Measurement of complex ion stability by use of ion-exchange resins. *Methods Biochem. Anal.*, **3**, 247–63.

SCHWEIGER, R. G. (1962). Acetylation of alginic acid. II. Reaction of algin acetates with calcium and other divalent ions. *J. Org. Chem.*, **27**, 1789–91.

SHEMYAKIN, M. M., OVCHINNIKOV, YU. A., IVANOV, V. T., ANTONOV, V. K., VINOGRADOVA, E. I., SHKROB, A. M., MALENKOV, G. G., EVSTRATOV, A. V., LAINE, I. A., MELNIK, E. I. & RYABOVA, I. D. (1969). Cyclodepsipeptides as chemical tools for studying ionic transport through membranes. *J. Membr. Biol.*, **1**, 402–30.

SIPES, G. E. (1969). *Structure and stability constants of boron, calcium, and magnesium gluconate chelates from optical rotation and computer analysis.* Ph.D. Thesis, University of Illinois, Urbana, Illinois.

SLOWEY, J. F., JEFFREY, L. M. & HOOD, D. W. (1967). Evidence for organic complexed copper in sea water. *Nature*, **214**, 377–8.

SMITH, T. D., LUND, T., PILBROW, J. R. & PRICE, J. H. (1971). An electron spin resonance study of the vanadyl ion separation in dimeric vanadyl 1-hydroxycyclohexanecarboxylate. *J. Chem. Soc.*, **A**, 2936–9.

SMITH, T. D. & PILBROW, J. R. (1974). The determination of structural properties of dimeric transition metal ion complexes from EPR spectra. *Coord. Chem. Rev.*, **13**, 173–278.

SPEDDING, F. H., DEKOCK, C. W., PEPPLE, G. W. & HABENSCHUSS, A. (1977). Heats of dilution of some aqueous rare earth electrolyte solutions at 25°C. 3. Rare earth chlorides. *J. Chem. Eng. Data*, **22**, 58–70.

STEWART, I. (1963). Chelation in the absorption and translocation of mineral elements. *Annu. Rev. Plant Physiol.*, **14**, 295–310.

TALEKAR, S. V. (1975). The nature of metal-ligand bonding in the primary hydration shell and in ionophores with alkali metal ions. *Biochim. Biophys. Acta*, **375**, 157–64.

THUN, H., GUNS, W. & VERBEEK, F. (1967). The stability constants of some bivalent metal complexes of α-hydroxyisobutyrate and lactate. *Anal. Chim. Acta*, **37**, 332–8.

TOMAT, G., MAGON, L., PORTANOVA, R. & CASSOL, A. (1972). Complexes of thorium(IV) with dicarboxylate ligands in aqueous solution. *Z. Anorg. Allg. Chem.*, **393**, 184–92.

TOY, A. D., SMITH, T. D. & PILBROW, J. R. (1971). An electron spin resonance study of dimer formation by the copper chelates of polyhydroxycarboxylic acids. *J. Chem. Soc.*, **A**, 2925–9.

TRIFFITT, J. T. (1968). Binding of calcium and strontium by alginates. *Nature*, **217**, 457–8.

TRIPATHY, K. K. & PATNAIK, R. K. (1967). Tartrate complex of manganese(II). *Indian J. Chem.*, **5**, 511–14.

TRIPATHY, K. K. & PATNAIK, R. K. (1970). Studies on tartrate complex of cobalt(II). *J. Indian Chem. Soc.*, **47**, 331–40.

TRIPATHY, K. K., PATNAIK, R. K. & PANI, S. (1972). Tartrate complex of cadmium(II) *J. Indian Chem. Soc.*, **49**, 345–50.

TURNER, K. J. & MAGNUSSON, B. J. (1962). Carrageenin as a protein precipitant. *Nature*, **194**, 451–2.

WILLIAMS, R. J. P. (1967). Heavy metals in biological systems. *Endeavour*, **26**, 96–100.

WIPF, H. K., PIODA, L. A. R., ŠTEPHANAC, Z. & SIMON, W. (1968). Complexes of enniatins and other antibiotics with alkali metal ions. *Helv. Chim. Acta*, **51**, 377–81.

ZUNDEL, G. (1969). Hydration structure and intermolecular interaction in polyelectrolytes. *Angew. Chem.* (Int. Ed.), **8**, 499–509.

BOOK REVIEW

World Review of Nutrition and Dietetics. Volume 27. Some Aspects of Human Nutrition. Edited by G. H. Bourne, Atlanta, Georgia. S. Karger: Basel, Munich, Paris, London, New York, Sydney. 1977. x + 180 pp., 17 Figures, 81 Tables. Price: £25·00 (approx.).

This volume in the series *World Review of Nutrition and Dietetics* is divided into five sections written by experts in the respective fields.

The first two sections deal with nutritional problems and their remedies amongst young children in India. Section 1 describes the nutritional problems of pre-school children with case histories and how nutritional imbalance at this stage can affect the rest of the child's life. At a stage of vast redevelopment in India, where these young people will be needed in the future, these nutritional problems are very important. Following on this theme, Section 2 describes how malnutrition may be overcome by feeding a balanced diet based on locally grown produce which apparently contains all necessary nutrients. Consumer acceptability of the new dishes is poor, however, the people preferring established dishes.

Sections 3, 4 and 5 deal with worldwide aspects of nutrition. The importance of dietary fibre is discussed and its role in the prevention of some alimentary diseases is described. New definitions of fibre are proposed but whether plantix (plant + matrix) or complantix (complex plantix) will ever replace the word 'fibre' is debatable. Useful analyses of some common foods are also provided.

The adaptation of the endocrine pancreatic secretions to the hydrolysis products of dietary components (fats, proteins and carbohydrates) is discussed in Section 4. The role of hormones in this process is discussed and it is suggested that the quantity of fatty acids, amino acids and glucose in the intestinal pools may be the start of the sequence which controls the hydrolysing enzymes.

The final section deals with pangamic acid. Its identification, distribution and metabolism in the body and its biochemical actions are described. The author

Fd. Chem. (3) (1978)—

concludes that whilst the compound has obvious actions its physiological importance remains in doubt and that it warrants further investigation.

At approximately £25·00 the book is not cheap but by today's standards is a useful library supplement for the references in the text as much as for the text itself.

M. W. KEARSLEY

OXIDATION OF METHIONINE RESIDUES OF FOOD PROTEINS AND NUTRITIONAL AVAILABILITY OF PROTEIN-BOUND METHIONINE SULPHOXIDE†

JEAN-LOUIS CUQ,‡ PIERRE BESANCON,†† LILIANE CHARTIER†† & CLAUDE CHEFTEL‡

‡ *Laboratoire de Biochimie et Technologie Alimentaires.*
†† *Laboratoire de Physiologie de la Nutrition, Université des Sciences et Techniques,* 34060 *Montpellier, France*

(Received: 22 December, 1976)

ABSTRACT

The extent of oxidation of methionine residues has been determined in food proteins submitted to various oxidative treatments.

The nutritional value of oxidised casein, in which 98% *of the methionine residues were converted to the corresponding sulphoxide, was assessed both by proteolysis* in vitro *and on rats.*

The nutritional availability of residues of methionine sulphoxide appears to be only slightly inferior to that of methionyl residues.

INTRODUCTION

Sulphur amino acids, which are the limiting factors of most diets, may be destroyed or made nutritionally unavailable by the treatments, especially the oxidative ones, to which protein foods are submitted (Bender, 1970; Bender, 1972; Mauron, 1973).

Such oxidative treatments include the use of hydrogen peroxide for the sterilisation of milk, whey and milk containers (Fox & Kosikowski, 1967; Naguib & Hussein, 1972; Reed, 1966), and for bleaching or detoxifying protein concentrates (Anderson *et al.*, 1975; Rasekh *et al.*, 1972). Oxidising agents such as benzoyl peroxide are used to bleach and improve flour. Heat, traces of metals, photon and γ-irradiation are also known to catalyse the oxidation of various food components by oxygen. Lipid

† This study was presented in part at the IVth International Congress of Food Science and Technology, Madrid, Spain, 23 September, 1974.

Fd. Chem. (3) (1978)—
Printed in Great Britain

peroxides and orthoquinones, which may be present in various food systems, are also strong oxidising agents.

The two main derivatives of methionine formed by oxidation are methionine sulphoxide and methionine sulphone; the formation of the latter requires relatively severe oxidative conditions while the formation of methionine sulphoxide readily occurs under mild oxidative conditions (Cuq *et al.*, 1973).

The nutritional consequences of the oxidation of free or protein-bound methionine have been studied by several investigators (Anderson *et al.*, 1976; Anderson *et al.*, 1975; Ellinger & Palmer, 1969; Miller & Samuel, 1968; Miller & Samuel, 1970; Miller *et al.*, 1970; Njaa, 1962; Rasekh *et al.*, 1972; Slump & Schreuder, 1973). It appears from these studies that free methionine sulphoxide may partly replace methionine in the diet. On the other hand, free methionine sulphone cannot replace methionine and may even be toxic (Anderson *et al.*, 1976; Anderson *et al.*, 1975; Miller & Samuel, 1968; Miller & Samuel, 1970).

There is some discrepancy in the results concerning the nutritional availability of protein-bound methionine sulphoxide. Ellinger & Palmer (1969) found oxidised casein to display a lower net protein utilisation (NPU) for rats than normal casein. Such an oxidised casein appears to contain methionine sulphoxide but not methionine sulphone (Cuq *et al.*, 1973). According to Slump & Schreuder (1973) the reduction in the NPU of casein oxidised by hydrogen peroxide together with perchloric acid results from the presence of residues of methionine sulphone and not from that of methionine sulphoxide. According to Anderson *et al.* (1975) the decrease in nutritional value of rapeseed flours detoxified by treatment with 0·9M hydrogen peroxide is due to the formation of residues of methionine sulphone, methionine sulphoxide and oxidised tryptophan derivatives. The decrease in nutritional value could not be completely reversed by methionine supplementation.

In the present work, using model systems and chemical methods, the effects of various treatments on the oxidation of methionine residues in food proteins were first determined. Secondly, the nutritional availability of methionine sulphoxide residues in the absence of toxic sulphone residues was investigated both *in vitro* after enzymatic hydrolysis and *in vivo*.

MATERIALS AND METHODS

Reagents

All chemicals were from Merck, unless otherwise stated.

Amino acid analysis

Amino acid analysis of acid, alkaline or enzymatic hydrolysates of food proteins and free amino acid analysis of rat plasma and muscle were carried out on a Technicon NCl autoanalyser. Norleucine was used as an internal standard. Chromatography using a single column of Chromobeads A resin (diameter 0·6 cm;

length 140 cm) maintained at 60 °C and an elution gradient as first described by Piez & Morris (1960) and Miller *et al.* (1965) and later modified (Cuq *et al.*, 1973) allowed the separation and determination of the two isomers of L-methionine sulphoxide, methionine sulphone and other amino acids.

A more rapid analysis was obtained using a single column of Chromobeads C_2 resin (diameter 0·6 cm; length 75 cm) maintained at 60 °C (Robin & Robin, 1971); amino acid analysis was completed in 6 h instead of 21. However, the separation of methionine sulphoxide and methionine sulphone was not possible using this second technique.

Determination of methionine sulphone and methionine sulphoxide residues

It is known that methionine sulphoxide partially reverts to methionine during acid hydrolysis (Ray & Koshland, 1960). Therefore the methionine, methionine sulphoxide and methionine sulphone contents of protein oxidised by hydrogen peroxide or other treatments were determined after either alkaline hydrolysis (Blackburn, 1968; Neumann, 1967) or acid hydrolysis with and without preliminary carboxymethylation-performic acid oxidation (Neumann, 1967). Results obtained by both methods were similar except at low levels of oxidation, when the alkaline hydrolysis method is more accurate (Cuq *et al.*, 1973).

Treatment of casein, milk and sunflower protein isolate with hydrogen peroxide

(a) *Treatment of casein at pH* 8*:* Casein solutions, 5 % w/v, were stirred for 30 min at 50 °C in the presence of hydrogen peroxide 0 to 0·12M. Twenty milligrammes of catalase (Merck 5183) per gramme of casein were then added in order to remove excess hydrogen peroxide. The elimination of hydrogen peroxide was checked by iodometry.

(b) *Treatment of casein at pH* 7·1*:* Casein solutions, 6 and 8 % w/v, maintained at 50 °C were stirred for 30 min and treated with hydrogen peroxide of final molarities 0·2M and 0·27M respectively.

Catalase (catalase/casein = 1 % w/w) was added to remove excess hydrogen peroxide.

Similarly treated casein solutions were used in the experimental diets given to the rats.

(c) *Treatment of milk:* Pasteurised milk was treated by 0·018M hydrogen peroxide at 50 °C for 30 min. Excess hydrogen peroxide was removed with catalase (20 mg/g casein).

(d) *Treatment of sunflower protein isolate at pH* 9·5*:* A 2·5 % (w/v) solution of sunflower protein isolate (12·6 % N) was bleached at pH 9·5 and 50 °C for 1 h by hydrogen peroxide of molarity between 0·05M and 0·3M. After precipitation at pH 4·8, centrifugation at 12,000 × *g* and 4 °C for 15 min, the isolate was freeze-dried.

Treatment of casein by sodium sulphite in the presence of Mn^{2+}

A 3 % w/v casein solution (adjusted to pH 7 with NaOH) was incubated for 3 h at

20 °C in the presence of Mn^{2+} and SO_3^{2-} ions, in air and under oxygen. Molarity ratios were respectively as follows: methionine residues/sodium sulphite/manganese sulphate: $1/4/3{\cdot}5 \cdot 10^{-3}$ and 1/10/0·5. Analysis of methionine sulphoxide residues was performed after alkaline hydrolysis.

Treatment of casein by caffeic acid

A 5% casein solution was incubated for 3 h at pH 11·3 and 50 °C or at pH 7·0 and 0 °C in the presence of caffeic acid (caffeic acid/casein = 1% w/w) and of mushroom polyphenoloxidase (Sigma T.7755, Tyrosinase grade III) (polyphenoloxidase/caffeic acid = 1% w/w). After precipitation at pH 4·8 and centrifugation at 12,000 × *g*, 4 °C for 15 min, the casein was freeze-dried. Analysis of methionine sulphoxide residues was performed after alkaline hydrolysis.

Storage of casein in the presence of autoxidising lipids

A model system consisting of 5 g casein, 0·5 ml methyl linoleate (Sigma L 1876) and 13 ml distilled water was emulsified under nitrogen. The mixture was freeze-dried, then placed in desiccators in which a controlled relative humidity was maintained by either phosphoric anhydride or aqueous saturated salt solutions (0% RH by anhydrous P_2O_5, 31% RH by $MgCl_2$ at 40 °C and 68% RH by $SrCl_2$ at 40 °C). Storage was carried out either in air or under oxygen for four months at 40 °C.

Fat extraction was carried out at room temperature using 250 ml of a chloroform–methanol mixture (2/1 = v/v) to 5 g casein, with a magnetic stirrer. After filtration through paper (Whatman No. 2) the casein was oven-dried for 20 min at 40 °C. The aggregates thus obtained were ground in a mortar to a fine and homogeneous powder.

Analyses of methionine sulphoxide residues were carried out after both alkaline hydrolysis and acid hydrolysis with or without carboxymethylation-performic acid oxidation.

In vitro *proteolytic digestion of oxidised casein*

The solutions of casein and oxidised casein were subjected to hydrolysis by different proteases, namely:

> Pepsin (Calbiochem 51639) followed by pancreatin (Calbiochem, 5067).
> Proteases from *Streptomyces griseus* (Pronase, Calbiochem 53702; or proteases Sigma P.5130).

In the case of hydrolysis by pepsin and pancreatin, the experimental conditions adopted were those described by Mauron (1973) for the evaluation of the 'pepsin and pancreatin protein digestion index'. Four hundred milligrammes of casein were incubated with 6 mg pepsin in 60 ml 0·1N hydrochloric acid at 37 °C for 3 h. The pH was adjusted to 8, first with 2N sodium hydroxide (about 2·5 ml), then with 0·1N sodium hydroxide. Sixteen milligrammes of pancreatin were added and the mixture

was incubated for 24 h at 37 °C. The pH was held constant and equal to 1·2 during the first phase of hydrolysis, and equal to 8 during the second, by the addition of 0·1 N sodium hydroxide by means of a pH stat (Radiometer Titrator TT2, Autoburette ABU 11).

In the case of the hydrolysis by proteases from *Streptomyces griseus*, two hydrolytic systems were used.

In the first of these systems (system No. 1), a solution of casein was used whose concentration was similar to that of the test pepsin–pancreatin mixture. Four hundred milligrammes of casein were incubated at 50 °C for 5 h with 4 mg of proteases from *Streptomyces griseus* (Calbiochem or Sigma). The total volume of the reactants was 60 ml. The pH was maintained at pH 8 during the course of the hydrolysis.

In the second system (system No. 2), 50 ml of a 5 % (w/v) casein solution were hydrolysed at 50 °C and at pH 8 for 5 h. Proteases from *Streptomyces griseus* were then added at the rate of 10 mg per gramme of casein.

Free amino acids were determined on the autoanalyser after sulphosalicylic acid treatment according to Hamilton (1962). This treatment did not produce any notable protein precipitation. Peptides only minimally interfered with the baseline.

Biological assays

In vivo assays were carried out on rats of the Sprague-Dawley strain, specific pathogen-free. The animals, weaned and raised to one month of age before the start of the experiment, were placed in individual metabolic cages in a chamber with temperature control at 25 °C. The cages allowed individual control of the food intake and quantitative collection of urine and faeces separately.

Four experiments were carried out. The food, in semi-liquid form, containing 30 % solids, was fed *ad libitum* in the first three experiments and in pair-feeding in the last experiment in order to equalise food intake between the different groups. The composition of the diets, in which casein was the sole protein source at levels of 9, 11 and 13 % of solids, is shown in Table 1. They differ by the presence of casein, oxidised or not, with and without methionine supplementation at the level of 2·5 g of L-methionine per 100 g casein, corresponding to the methionine content of casein, with and without tryptophan supplementation at the level of 1 g of L-tryptophan per 100 g casein. Five diets were used:

(i) Diet C: Purified casein (Merck Hammarsten).

(ii) Diet CO: Casein oxidised in the presence of hydrogen peroxide at pH 7·1, in which 98 % of the methionine residues were oxidised to the sulphoxide form, but which contained no sulphone residues (see 'Results and Discussion' below).

(iii) Diet CM: Casein supplemented with 2·5 g L-methionine per 100 g casein.

(iv) Diet COM: Oxidised casein, supplemented with 2·5 g L-methionine per 100 g casein.

(v) Diet COMT: Oxidised casein, supplemented with 2·5 g L-methionine and 1 g L-tryptophan per 100 g casein.

TABLE 1
COMPOSITION OF THE DIETS
(g per 100 g of dry diet)

Experiment	1	2	3 *and* 4
Protein level (N × 6·25)	13	11	9
Casein	14	12	10
Glucose monohydrate	4	4	4
Wheat starch	60	62	64
Fats (animal and vegetable)	7	7	7
Cellulose	4	4	4
Minerals[a]	10	10	10
Vitamins[b]	1	1	1

[a] Composition of salt mixture, g per kg: $CaHPO_3$: 430; KCl: 100; NaCl: 100; $MgCl_2$: 50; $MgSO_4$: 50; Fe_2O_3: 30; $FeSO_4$, $7H_2O$: 50; $MnSO_4$, H_2O: 24·5; $CuSO_4$, $5H_2O$: 5; $ZnSO_4$, $7H_2O$: 20; $CoSO_4$, $7H_2O$: 0·04; KI (stabilised): 0·08. Cellulose to 1 kg. Content of minerals per kg dry diet: Ca: 10 g; P: 7·75 g; K: 6 g; Na: 4 g; Mg: 1 g; Fe: 0·2 g; Mn: 80 mg; Zn: 45 mg; Cu: 12·5 mg; Co: 0·09 mg; I: 0·49 mg.

[b] Content of vitamins per kg dry diet: retinyl acetate: 19,800 IU; cholecalciferol: 6000 IU; D-L-α-tocopherol acetate: 170 mg; menadione: 40 mg; choline (HCl): 1360 mg; nicotinic acid: 100 mg; thiamine (HCl): 20 mg; riboflavine: 15 mg; pyridoxine (HCl): 10 mg; L-ascorbic acid: 800 mg; Ca-pantothenate: 70 mg; myoinositol: 150 mg; folic acid: 5 mg; biotin: 0·3 mg; *p*-aminobenzoic acid: 50 mg.

In order to avoid all risk of oxidation or browning, the rations were prepared shortly before distribution to the animals, by mixing without heating a 6 or 8% aqueous casein solution and a dry mix containing the other ingredients of the diet. The dry mix was purchased from U.A.R. (91360 Villemoisson Sur Orge) and stored at −18 °C.

In addition, in some experiments, one group of rats received a protein-free but otherwise well balanced diet.

After an adaptation period of about a week, during which the rats received diet C, the animals were divided into groups of six or eight rats each, and fed the experimental and control diets.

The experimental period varied in length between 11 and 28 days depending on the experiment. The rats were weighed every two days and their daily intakes of total solids and protein were recorded individually. The growth rate in g/day and the protein efficiency ratio (PER = gain in weight in g/day per g of protein ingested in g/day), were calculated.

The net protein ratio (NPR) was also calculated.

During the last eight days of the experimental period, nitrogen balances were carried out: faeces were individually collected and freeze-dried; urine was acidified with 1% hydrochloric acid. From this were calculated the protein digestibility coefficient (N adsorbed % N ingested), the biological value (BV; N retained % N

absorbed) and the net protein utilisation (NPU), which is the product of biological value and digestibility. The corrections for endogenous faecal and urinary nitrogen are calculated from the results obtained on animals fed a protein-free diet during the same period. In one experiment, the NPU was determined after 11 days by the analysis of the eviscerated carcass of the rats and represented the gain in body nitrogen (minus that of the group receiving a protein-free diet) divided by the weight of the protein ingested.

At the end of the experimental period the rats were killed using ether; blood was immediately drawn from the abdominal aorta, collected in heparinised tubes and centrifuged. The plasma was then frozen; a piece of muscle tissue was also removed and frozen immediately. The carcasses were then eviscerated, ground and freeze-dried.

The nitrogen content of carcasses, faeces and urine was determined by the Kjeldahl method (AOAC, 1975). The free plasma amino acids were determined after deproteination with sulphosalicylic acid according to Gerritsen *et al.* (1965); the free muscle amino acids were analysed according to Pawlak & Pion (1966). Picric acid was eliminated by means of an ion-exchange resin (Amberlite IRA-410, 20–50 mesh). Norleucine was used as internal standard. Carcass fat was determined by Soxhlet extraction with a chloroform/methanol solvent (2/1, v/v).

Statistical comparisons were made using the Student–Fisher *t* test (Schwartz, 1963).

RESULTS AND DISCUSSION

Oxidation of methionine residues by hydrogen peroxide

Under the conditions described above, treatment of casein by hydrogen peroxide at pH 8 oxidises methionine residues to the corresponding sulphoxide residues. The ratio of oxidation is a function of hydrogen peroxide molarity. At the relatively mild molarity of 0·12M, the percentage of oxidation was above 90 (Cuq *et al.*, 1973). In the casein treated with 0·2 or 0·27M hydrogen peroxide at pH 7·1, 98% of the initial methionine residues were oxidised to the corresponding sulphoxide residues.

None of the samples was found to contain methionine sulphone residues. When milk was treated with 0·018M hydrogen peroxide, a concentration sometimes used for sterilising milk or whey, more than 50% of methionine residues were oxidised to methionine sulphoxide residues (Cuq *et al.*, 1973). The oxidation to sulphoxide amounted to 93% of the initial methionyls when the casein was treated with hydrogen peroxide in a hydrochloric acid–methanol medium (Toennis & Kolb, 1939). Such a treatment did not result in the formation of residues of methionine sulphone.

Recently, Slump & Schreuder (1973) have noticed the formation of methionine sulphoxide and methionine sulphone when casein was oxidised by hydrogen

peroxide in a perchloric acid medium. It is likely that oxidation as far as the sulphone stage results from the presence of perchloric acid in the reaction medium.

Treatments of sunflower protein isolates by 0·05 to 0·3M hydrogen peroxide were carried out in our laboratory and found to give rise to the total oxidation of the initial methionine residues to sulphone or sulphoxide derivatives. For a hydrogen peroxide molarity of 0·3M, oxidation to the sulphone stage reached 11%. The appearance of methionine sulphone residues in this latter system could result from the presence of oxidation catalysts (possibly traces of metals) in the sunflower protein isolate.

Oxidation of methionine residues by other treatments

The treatment of casein by sodium sulphite in the presence of Mn^{2+} ions also gave rise to the oxidation of methionine residues. With methionine residues/sodium sulphite/manganese sulphate ratios of $1/4/3{\cdot}5 . 10^{-3}$ and 1/10/0·5, the percentages of methionine oxidation into its sulphoxide were 66 and 99%, respectively (incubation in an atmosphere of oxygen), or 39 and 45%, respectively (incubation in air). These results are similar to those reported by Yang (1970) and Inoue & Hayatsu (1971) with free methionine.

Incubation of casein with a polyphenol (caffeic acid) undergoing oxidation (in a neutral or alkaline medium, in the presence of polyphenoloxidase) brought about a mild oxidation of methionine residues. In this way, 20% and 7% of initial methionine residues were oxidised to sulphoxide at pH 11·3 and pH 7, respectively.

Storage of casein in the presence of methyl linoleate also caused the oxidation of methionine residues into methionine sulphoxide (Table 2). No residues of methionine sulphone were formed. Oxidation was maximal for the sample stored under oxygen, in which 58% of the initial methionine residues were oxidised to the sulphoxide. This oxidation was probably due to the presence of lipid peroxides formed during storage. Similar results were reported by Tannenbaum *et al.* (1969).

TABLE 2

FORMATION OF METHIONINE SULPHOXIDE DURING STORAGE OF CASEIN IN THE PRESENCE OF METHYL LINOLEATE (40°C)

Length of storage (days)	*Relative humidity (%)*	*Atmosphere*	*Methionine sulphoxide residues (mmoles per 100 g casein)*	
			Direct method[a]	*Indirect method*[b]
103	68	oxygen	10·8	10·4
99	0	air	5·7	6·6
120	31	air	5·0	5·1
120	68	air	5·4	5·9
Control casein stored for 99 days in air at 40°C, without methyl linoleate			0	1·9

[a] Alkaline hydrolysis.

[b] *S*-carboxymethylation, performic oxidation and acid hydrolysis.

Oxidation of methionine residues was only slightly influenced by the relative humidity of the atmosphere in which the samples were stored (Table 2). Studies with model systems of this type have nevertheless shown that the rate of unsaturated lipid oxidation is decreased within a relative humidity range between 20 and 50 % (Labuza *et al.*, 1970).

The casein samples stored in the presence of methyl linoleate show a yellow coloration. This coloration is particularly pronounced for the sample with the highest content in methionine sulphoxide (sample stored under oxygen). It is likely that browning reactions take place between casein and lipid oxidation derivatives (Tannenbaum *et al.*, 1969; Yanagita *et al.*, 1973).

The data reported above show that a relatively moderate oxidative treatment (such as hydrogen peroxide 0·2M, pH 7·1) brings about the oxidation of almost all the methionine residues of casein to methionine sulphoxide. The contact between casein and lipids undergoing oxidation also gives rise to the oxidation of methionine residues. Polyphenols undergoing oxidation and sodium sulphite in the presence of Mn^{2+} and oxygen are also capable of oxidising methionine residues to their corresponding sulphoxide. None of these treatments leads to the formation of methionine sulphone residues, contrary to the oxidation treatment with 0·11M hydrogen peroxide at pH 2·5 in the presence of perchloric acid as described by Slump & Schreuder (1973). Since the treatment of casein in an acid medium (Toennis & Kolb, 1939) leads only to methionine sulphoxide residues, it is not the low pH of the reaction medium used by Slump & Schreuder (1973) which may alone be regarded as responsible for the oxidation to methionine sulphone. The formation of the latter is probably due to the presence either of perchloric acid or, in the case of the sunflower protein isolate, of impurities which catalyse the oxidation.

In vitro *proteolytic digestion of oxidised casein*

It is known that free methionine sulphoxide, but not free methionine sulphone, can partially replace methionine in rat diets (Miller & Samuel, 1970; Miller *et al.*, 1970; Njaa, 1962). Methionine sulphoxide utilisation increases with the age of the animal (Miller *et al.*, 1970). In order to assess the nutritional availability of methionine sulphoxide residues, we first compared the *in vitro* digestibility of non-treated casein with that of oxidised casein.

Enzyme hydrolyses by pepsin and pancreatin on the one hand, and by proteases from *Streptomyces griseus* on the other (system No. 1, see section above headed 'Materials and Methods'), were carried out on the samples of casein and oxidised casein (hydrogen peroxide molarity: 0·27; ratio of oxidation of methionine residues to sulphoxide: 98 %).

The release of amino acids shown in Table 3 was similar using either of the three procedures for proteolysis. This preliminary observation confirmed that proteases from *Streptomyces griseus* have a high proteolytic activity. In order to obtain an equivalent release of amino acids by means of pepsin and pancreatin, it was

TABLE 3
AMINO ACID RELEASE FROM CASEIN (C) OR FROM HYDROGEN PEROXIDE-TREATED CASEIN (CO) BY PEPSIN AND PANCREATIN OR BY PROTEASES FROM *Streptomyces griseus*

Amino acid	*Amino acid release (g/100 g casein)*[a]					
	Hydrolysis by pepsin and pancreatin			*Hydrolysis by proteases from* Streptomyces griseus (*System No.* 1)		
	C	*CO*	*Pepsin–pancreatin autolysis control*	*C*[b]	*CO*[b]	*CO*[c]
Methionine sulphoxide	0	0·2	0	0	0·3	0·4
Serine	0·3	0·4	0·1	0·8	0·5	0·7
Glutamic acid	0·2	0·8	0·1	0·2	0·5	0·4
Proline[d]	—	—	—	0·2	0·2	0·3
Glycine	0·1	0·1	traces	0·2	0·1	0·2
Alanine	0·5	0·5	0·1	0·8	0·5	0·7
Valine	0·8	0·7	0·1	1·6	1·4	1·7
1/2 cystine	0	0	0	0	0	0
Methionine	0·9	0	traces	1·2	0	0
Isoleucine	0·6	0·6	0·1	1·6	1·2	1·6
Leucine	4·6	4·7	0·3	4·5	4·4	4·5
Tyrosine	2·5	2·8	0·2	2·9	2·8	3·0
Phenylalanine	2·7	2·5	0·2	2·3	2·2	2·5
Lysine	4·6	5·0	0·2	3·3	2·5	1·8
Histidine	0·4	0·5	traces	0·7	0·7	0·9
Arginine	2·7	2·7	0·1	1·2	1·0	0·9

[a] Hydrolysates were deproteinated by means of sulphosalicylic acid. The analyses were carried out on a Technicon Autoanalyser NCl (column 0·6 cm in diameter and 75 cm long, Chromobeads C_2 resin, 60 °C, 6 h).
[b] Pronase purchased from Calbiochem.
[c] Proteases purchased from Sigma.
[d] Proline was released but could not be measured owing to interference with other amino acids (the same situation holds for aspartic acid and threonine).

necessary to use levels of these enzymes 5·5 times greater than that of *Streptomyces griseus* proteases and to carry on the hydrolysis five times longer.

The oxidation of casein by hydrogen peroxide does not cause significant changes in the overall digestibility. The release of methionine, however, was negligible in the case of the oxidised casein sample.

On the other hand, if a value of 100 is assigned to the quantity of methionine released from the sample of non-oxidised casein, the amount of methionine sulphoxide released from the oxidised casein barely reaches 20 for the three proteolysis procedures. In all cases, it may be considered that the proteases active on methionyl peptide linkages do not hydrolyse or hydrolyse more slowly the same linkages once this methionyl residue is oxidised to the sulphoxide.

Previous Pronase proteolysis tests (system No. 2, see section above headed 'Materials and Methods') applied to various casein samples previously treated with hydrogen peroxide of different molarities between 0 and 0·12M showed that the release of methionine is inversely proportional to the content of methionine sulphoxide residues (Cuq *et al.*, 1973). The total absence of release of methionine

sulphoxide reported previously differs from the results of Table 3. Former and present Pronase proteolysis tests differ only in the casein concentration of the reaction medium, which is 7·5 times lower in the recent experiments given in Table 3. At higher protein concentration, inhibition of the proteases by the products of hydrolysis could provoke a reduction in the rate of hydrolysis of peptide linkages in which a methionine sulphoxide residue is present. The release of methionine sulphoxide may thus have been too low to be measurable.

Samples of casein oxidised in the presence of methyl linoleate were also submitted to proteolysis with proteases from *Streptomyces griseus* (system No. 2, see section above headed 'Materials and Methods'). The release of methionine is inversely related, but not inversely proportional, to the content of methionine sulphoxide residues (Table 4).

TABLE 4

EFFECTS OF THE OXIDATION OF METHIONINE RESIDUES IN CASEIN STORED IN THE PRESENCE OF METHYL LINOLEATE (ML) ON THE ENZYMATIC RELEASE[a] OF METHIONINE

Samples	*Oxidation of methionine residues*[b] (%)	*Decrease in methionine released*[c] (%)
Casein-ML-oxygen RH 68%	58	82
Casein-ML-air RH 0%	34	45
Casein-ML-air RH 31%	27	27
Casein-ML-air RH 68%	30	36

[a] Proteolysis with proteases from *Streptomyces griseus* (System No. 2).
[b] 100% corresponds to the initial number of methionine residues (= 2·75 g/100 g casein).
[c] 0% corresponds to the quantity of methionine released from the casein control (1·1 g/100 g casein).

On the whole the various *in vitro* proteolysis tests, applied to samples of casein and oxidised casein, give comparable results: the overall digestibility of casein is not modified by oxidation. In all cases, the oxidation of methionine residues to their sulphoxide derivatives induces a significant decrease in their release. As mentioned above, it is likely that this phenomenon is related to a reduction in the rate of hydrolysis of peptide linkages in which methionine sulphoxide residues are involved. Other modifications of the side chains of amino acid residues of proteins are known to affect *in vitro* digestibility: thus Bennett *et al.* (1972) have shown that methionine sulphone residues are only partially released, even when the proteins are submitted to the action of proteases which lead to almost total hydrolysis. Kussendrager *et al.* (1972) also found that lysosomal enzymes do not hydrolyse peptide linkages involving cysteic acid residues. In general, 'acidic' amino acids (see Table 3) (Mauron, 1973) are relatively poorly released by *in vitro* enzymatic hydrolysis. As

methionine sulphoxide is an 'acidic' amino acid, this may explain why its release was lower than that of methionine. Moreover, *in vitro* enzymatic hydrolysis is not analogous to *in vivo* digestion, either in reaction rate or in the final level of the amino acids released; a low release rate of an essential amino acid *in vivo* does not necessarily imply a lowering in the nutritional value of the protein.

Biological determination of the nutritional value of oxidised casein

In order to find out if *in vitro* release of methionine sulphoxide gives a true picture of the availability of methionine sulphoxide residues *in vivo*, the nutritional value of oxidised casein was studied in rats. The results are summarised in Tables 5, 6, 7 and 8.

TABLE 5

BIOLOGICAL DETERMINATION OF THE NUTRITIONAL VALUE OF OXIDISED CASEIN. EXPERIMENT No. 1

Diets[a]	*C*	*CO*	*CM*	*COM*	*COMT*
N intake (mg/day)	317 ± 5[b]	326 ± 6	325 ± 4	299 ± 7	307 ± 10
Growth (g/day)	4·9 ± 0·3	4·7 ± 0·3	5·2 ± 0·2	5·1 ± 0·9	5·3 ± 0·3
PER	2·2 ± 0·3	2·0 ± 0·2	2·3 ± 0·1	2·3 ± 0·1	2·4 ± 0·1
Apparent digestibility, N absorbed % N ingested	87·5 ± 0·7	86·9 ± 0·4[c]	ND	89·0 ± 0·3[c]	ND
Retention N retained % N ingested	62·6 ± 2·7[d]	49·1 ± 4·9[de]	ND	69·1 ± 0·5[e]	ND

[a] Rats fed *ad libitum*; initial weight: 83 g; protein level in diet: 13%; PER are calculated on 25 days; N balances (digestibility and retention) are determined on 8 days.
[b] Means ± SEM; means with the same superscripts [c d e] are significantly different at the level $p \leq 0{\cdot}01$; other means are not significantly different.
ND: Not determined.

TABLE 6

BIOLOGICAL DETERMINATION OF THE NUTRITIONAL VALUE OF OXIDISED CASEIN. EXPERIMENT NO. 2

Diets[a]	*C*	*CO*	*COM*	*Significance level* between *C* and *CO*	between *CO* and *COM*
N intake (mg/day)	295 ± 8[b]	241 ± 7	305 ± 8	$p<0{\cdot}001$	$p<0{\cdot}001$
Growth (g/day)	6·1 ± 0·2	4·4 ± 0·2	7·6 ± 0·2	$p<0{\cdot}001$	$p<0{\cdot}001$
PER	3·2 ± 0·1	2·8 ± 0·1	3·8 ± 0·1	$p<0{\cdot}01$	$p<0{\cdot}01$
True digestibility N absorbed % N ingested	94·5 ± 1·6	91·4 ± 0·8	95·4 ± 0·8	NS	$p<0{\cdot}05$
Biological value	60·3 ± 3·9	54·4 ± 1·9	78·3 ± 0·7	NS	$p<0{\cdot}001$
NPU	58·7 ± 4·6	49·7 ± 1·9	74·8 ± 0·9	NS	$p<0{\cdot}001$

[a] Rats fed *ad libitum*; initial weight: 83 g; protein level in diet: 11%. PER are calculated on 4 weeks; N balances (digestibility, biological value and NPU) are determined on 8 days.
[b] Means ± SEM.
NS: Not significantly different.

TABLE 7
BIOLOGICAL DETERMINATION OF THE NUTRITIONAL VALUE OF OXIDISED CASEIN. EXPERIMENT NO. 3

Diets[a]	*C*	*CO*	*COM*	*Significance level*		
				between C and CO	*between CO and COM*	*between C and COM*
N intake (mg/day)	148±7[b]	113±10	164±15	$p \leq 0.05$	$p < 0.05$	NS
Growth (g/day)	3·9±0·2	2·7±0·3	5·6±0·7	$p < 0.05$	$p < 0.001$	$p < 0.05$
PER	4·2±0·1	3·8±0·2	5·5±0·3	NS	$p < 0.01$	$p < 0.01$
NPU	67·2±2·2	69·8±4·4	83·6±3·9	NS	$p < 0.01$	$p < 0.01$

[a] Rats fed *ad libitum*; initial weight: 64 g; protein level in diet: 9%. PER and NPU (carcass method) are calculated on 11 days.
[b] Means ± SEM.
NS: Not significantly different.

TABLE 8
BIOLOGICAL DETERMINATION OF THE NUTRITIONAL VALUE OF OXIDISED CASEIN. EXPERIMENT NO. 4

Diets[a]	*C*	*CO*	*CM*	*COM*	*Significance level*	
					between C and CO	*between CM and COM*
N intake (mg/day)	116±4[b]	109±6	117±3	120±2	NS	NS
Growth (g/day)	1·9±0·1	1·6±0·2	2·6±0·1	2·5±0·1	NS	NS
PER	2·5±0·1	2·3±0·1	3·4±0·2	3·3±0·1	NS	NS
True digestibility	91·1±1·2	90·2±1·2	88·2±1·1	90·9±0·6	NS	NS
Biological value	80·6±3·6	76·5±2·3	88·1±0·7	85·6±0·4	NS	$p < 0.05$
NPU	74·0±3·4	69·0±1·9	77·7±1·1	77·7±0·7	NS	NS

[a] Pair-fed rats; initial weight: 70 g; protein level in diet: 9%. PER and N balances (digestibility, BV and NPU) are determined on 11 days.
[b] Means ± SEM.
NS: Not significantly different.

In *ad libitum* feeding experiments (Tables 5, 6 and 7), the presence of oxidised casein in the diet appears to reduce the spontaneous food intake, except in the first experiment (at a protein level of 13%).

The digestibility of nitrogen (Tables 5 and 6), determined at two levels of protein (11 and 13%), was not found to be affected significantly by the oxidation of casein.

PER and NPU were, in most cases, 10–20% reduced by the ingestion of oxidised casein; however, the differences are not always significant (according to the Student–Fisher *t* test (Schwartz, 1963) at the 5% significance level). It also appears difficult to establish logical correlations between, on the one hand, PER or NPU reductions and, on the other, the decrease in food intake, the protein level in the diet, or changes in body composition. The differences in PER between normal and oxidised casein were found to be less marked when the food intakes were equal (Tables 5 and 8).

Data from animals receiving oxidised casein plus free methionine show that methionine supplementation not only compensates for, but largely exceeds, the

supposed methionine deficit due to the formation of methionine sulphoxide. Data from animals receiving oxidised casein plus both free methionine and trytophan (Table 5) show that tryptophan is not a limiting nutritional factor in oxidised casein; this does not rule out the oxidation of tryptophan residues by hydrogen peroxide, but such an oxidation, if it occurred, affected only a few residues or did not significantly reduce the nutritional availability of tryptophan. These results also indicate that hydrogen peroxide did not significantly affect the nutritional availability of other essential amino acids and that methionine remains the limiting nutritional factor of oxidised casein as it is that of non-treated casein.

It appears from these experiments that a large proportion of the residues of methionine sulphoxide is released *in vivo* from casein, absorbed, reduced and used for protein synthesis; it is, however, not possible to conclude that methionine sulphoxide residues are completely available for the rat.

These observations are confirmed by the *analyses of plasmatic and muscular free amino acids* which were performed at the end of two feeding experiments (11 and 13% protein level). The results are shown in Table 9.

It is known from the studies of various investigators that the level of free methionine in animal tissues can reflect the availability of methionine in the diet (Pawlak & Pion, 1966).

In the present experiments, free methionine sulphoxide increased considerably when rats were fed oxidised casein, with or without methionine supplementation. This confirms that a proportion of the methionine sulphoxide in casein can be released and absorbed. The fact that, in these animals, free methionine sulphoxide accumulated to a higher level than free methionine in animals fed normal casein indicates that free methionine sulphoxide is not used as efficiently as methionine by the rat; this could be due to an incomplete or slow reduction to methionine.

The moderate rise in free methionine found in rats fed oxidised casein is difficult to explain but is in agreement with the results of some experiments concerning free methionine sulphoxide-fed rats (Anderson *et al.*, 1976; Miller *et al.*, 1970).

The level of free plasmatic and muscular serine appears to be inversely proportional to the methionine content of the diet. These variations should be considered with respect to the mechanisms of induction of serine dehydratase (Mauron *et al.*, 1973); these authors suggest that the regulatory amino acid role played by methionine is partially decreased when the methionine supply consists of methionine sulphoxide.

It can be concluded from these experiments that, at least for the young rat, the nutritional availability of methionine residues is only slightly reduced by their oxidation to methionine sulphoxide. These conclusions stand midway between those of other investigators who reported either more marked or negligible nutritional effects of methionine oxidation to methionine sulphoxide (Anderson *et al.*, 1975; Ellinger & Palmer, 1969; Miller & Samuel, 1970; Miller *et al.*, 1970; Njaa, 1962; Slump & Schreuder, 1973).

Although it is probably not possible to explain the low methionine availability of

TABLE 9

FREE AMINO ACIDS IN PLASMA AND MUSCLE OF RATS FED OXIDISED CASEIN[a]

Amino acids	*Animal series*[b]	*Protein level:* 11% (28 *days*) *Free amino acids in plasma*[c] (*μmoles/100 ml plasma*)			*Free amino acids in muscle*[c] (*μmoles/100 g muscle*)			*Protein level:* 13% (25 *days*) *Free amino acids in plasma*[c] (*μmoles/100 ml plasma*)				*Free amino acids in muscle*[c] (*μmoles/100 g muscle*)			
		C	*CO*	*COM*	*C*	*CO*	*COM*	*C*	*CO*	*COM*	*CM*	*C*	*CO*	*COM*	*CM*
Methionine sulphoxide		2	20	20	7	87	170	2	23	21	3·5	12	245	245	15
Aspartic acid		13	2	3	13	19	7	2	3	2	3	14	20	32	29
Threonine + glutamine		120	130	94	530	580	440	105	120	78	74	775	900	600	440
Serine		51	60	37	200	290	120	51	62	33	36	335	490	280	170
Glutamic acid		13	13	16	120	115	100	22	21	23	26	185	185	250	180
Proline[d]		34	28	26	81	73	64	32	27	27	26	120	110	110	80
Glycine		34	31	29	500	440	370	39	48	28	31	630	685	570	340
Alanine		64	60	87	360	270	400	62	68	52	53	520	505	560	400
Valine		19	16	19	22	36	26	18	14	14	14	28	32	32	23
1/2 Cystine		0	0	0	0	0	0	0	0	0	0	0	0	0	0
Methionine		4	6	7	5	13	12	5	7	9	7	2	14	17	3
Isoleucine		8	8	6	10	18	11	8	6	8	8	13	16	17	14
Leucine		13	10	14	19	33	21	12	8	11	12	19	26	24	21
Tyrosine		8	4	8	15	12	17	11	7	9	8	18	10	11	11
Phenylalanine		5	4	6	8	18	12	6	5	5	6	12	14	12	11
Ornithine		16	6	9	7	16	9	—	—	—	—	—	—	—	—
Lysine		53	51	58	90	91	140	52	44	41	48	120	79	72	88
Histidine[e]		11	10	8	105	72	89	7	9	6	7	140	145	145	105
Arginine		3	11	12	16	25	32	8	13	12	13	40	65	61	41

[a] Analyses were carried out on a Technicon NCl autoanalyser, column diameter 0·6 cm, length 75 cm, Chromobeads C_2 resin, 60 °C, 6 h.
[b] See experiments Nos. 1 and 2 in Tables 5 and 6.
[c] Analysis of one sample containing pooled plasma or muscle from six rats.
[d] Possible interference of citrulline.
[e] Possible interference of 1-methyl histidine.

some processed foods by methionine oxidation, the risk of methionine sulphone formation should not be overlooked when considering oxidative technological processing.

SUMMARY

Casein, milk or a sunflower protein isolate were submitted to various oxidative conditions, namely: treatment with hydrogen peroxide, incubation with sodium sulphite in the presence of oxygen and metal ions and contact with lipids or with polyphenols undergoing oxidation reactions.

Amino acid analyses indicated that even mild oxidative conditions caused an extensive oxidation of the methionine residues of food proteins into methionine sulphoxide residues.

The nutritional value of oxidised casein, in which 98 % of the methionine residues were converted to the corresponding sulphoxide, was determined.

Enzymatic hydrolyses carried out *in vitro* indicated that the overall digestibility of the protein was not modified; the concomitant release of methionine sulphoxide was, however, very low—sometimes negligible.

In vivo measurements of the true protein digestibility and of the nutritional value of oxidised casein in one-month-old rats led to the conclusion that a large proportion of methionine sulphoxide residues is released from casein, absorbed, converted to methionine and used for protein synthesis. Ten to fifteen per cent, sometimes non-significant, reductions in the protein efficiency ratio and the net protein utilisation of casein due to oxidation suggest that methionine sulphoxide residues are slightly less efficiently utilised than those of methionine. The accumulation of free methionine sulphoxide in the muscle and plasma at a level higher than that of methionine in rats fed normal casein, together with modifications in free amino acid patterns, appear to indicate that the reduction of methionine sulphoxide to methionine is a relatively slow or incomplete reaction, at least in young rats.

ACKNOWLEDGEMENT

The participation of J. C. Beaussier in parts of this work and the technical help of A. Cuadrado are gratefully acknowledged.

This study was supported in part by the Délégation Générale à la Recherche Scientifique et Technique (contract No. 71.7.2763), the Institut National de la Santé et de la Recherche Médicale, the Fondation pour la Recherche Médicale Française and the Centre National de la Recherche Scientifique (E.R.A. No. 614).

REFERENCES

ANDERSON, G. H., ASHLEY, D. V. M. & JONES, J. D. (1976). Utilization of L-methionine sulfoxide, L-methionine sulfone and cysteic acid by the weanling rat, *J. Nutrition*, **106**, 1108–14.

ANDERSON, G. H., LI, G. S. K., JONES, J. D. & BENDER, F. (1975). Effect of hydrogen peroxide treatment on the nutritional quality of rapeseed flour fed to weanling rats, *J. Nutrition*, **105**, 317–25.

AOAC (1975). *Official methods of analysis of the Association of Official Analytical Chemists: Improved Kjeldahl method* (Horwitz, W. (ed.)) 12th ed. 15–16, New York.

BENDER, A. E. (1970). Factors affecting the nutritive value of protein foods. In *Evaluation of novel protein products* (Bender, A. E., Kihlberg, R., Lofqvist, B. & Munck, L. (eds.)), 319–30, Pergamon Press, Oxford.

BENDER, A. E. (1972) Processing damage to protein foods. In *Protein Advisory Group Bulletin* 13, **2**, 10–19, United Nations, New York.

BENNETT, H. P., ELLIOT, D. F., EVANS, B. E., LOWRY, P. J. & MCMARTIN, C. (1972). Complete amino acid analysis of peptides and proteins after hydrolysis by a mixture of Sepharose-bound peptidases, *Biochem. J.*, **129**, 695–701.

BLACKBURN, S. (1968). Alkaline hydrolysis. In *Amino acid determination*, 22–4, M. Dekker Inc., New York.

CUQ, J. L., PROVANSAL, M., GUILLEUX, F. & CHEFTEL, C. (1973). Oxidation of methionine residues of casein by hydrogen peroxide. Effects on *in vitro* digestibility, *J. Food Sci.*, **38**, 11–13.

ELLINGER, G. M. & PALMER, R. (1969). The biological availability of methionine sulphoxide, *Proc. Nutr. Soc.*, **28**, 42A.

FOX, P. F. & KOSIKOWSKI, F. V. (1967). Some effects of hydrogen peroxide on casein and its implications in cheese making, *J. Dairy Sci.*, **50**, 1183–8.

GERRITSEN, T., REHBERG, M. L. & WAISMAN, H. A. (1965). On the determination of free amino acids in serum. *Anal. Biochem.*, **11**, 460–6.

HAMILTON, P. B. (1962). Ion exchange chromatography of amino acids. Micro-determination of free amino acids in serum, *Annals N.Y. Acad. Sci.*, **102**, 55–62.

INOUE, M. & HAYATSU, H. (1971). Interactions between bisulfite and amino acids. Formation of methionine sulfoxide from methionine in the presence of oxygen, *Chem. Pharm. Bull.*, **19**, 1286–8.

KUSSENDRAGER, K. D., DE JONG, Y., BOUMA, J. M. W. & GRUBER, M. (1972). The digestion of the B chain of oxidised insulin by extracts of rat liver lysosomes, *Biochim. Biophys. Acta*, **279**, 75–86.

LABUZA, T. P., TANNENBAUM, S. R. & KAREL, M. (1970). Water content and stability of low-moisture and intermediate-moisture foods, *Food Technology*, **24**, 543–50.

MAURON, J. (1973). The analysis of food proteins: Amino acid composition and nutritive value. In *Proteins in human nutrition* (Porter, J. W. G. & Rolls, B. A. (eds.)), 139–54, Academic Press, London and New York.

MAURON, J., MOTTU, F. & SPOHR, G. (1973). Reciprocal induction and repression of serine dehydratase and phosphoglycerate dehydrogenase by proteins and dietary-essential amino acids in rat liver, *Eur. J. Biochem.*, **32**, 331–42.

MILLER, E. L., HARTLEY, A. W. & THOMAS, D. C. (1965). Availability of sulphur amino acids in protein foods. 4. Effect of heat treatment upon the total amino acid content of cod muscle. *Brit. J. Nutr.*, **19**, 565–73.

MILLER, D. S. & SAMUEL, P. (1968). Methionine sparing compounds, *Proc. Nutr. Soc.*, **27**, 21A.

MILLER, D. S. & SAMUEL, P. D. (1970). Effects of the addition of sulphur compounds to the diet on utilisation of protein in young growing rats, *J. Sci. Food Agric.*, **21**, 616–18.

MILLER, S. A., TANNENBAUM, S. R. & SEITZ, A. W. (1970). Utilization of L-methionine sulfoxide by the rat, *J. Nutr.*, **100**, 909–15.

NAGUIB, K. & HUSSEIN, L. (1972). The effect of hydrogen peroxide treatments on the bacteriological quality and nutritive value of milk, *Milkwissenschaft*, **27**, 758–62.

NEUMANN, N. P. (1967). Analysis for methionine sulfoxides. In *Methods in enzymology* (Colowick, S. P. & Kaplan, N. O. (eds.)), **XI**, 487–90, Academic Press, New York.

NJAA, L. R. (1962). Utilization of methionine sulphoxide and methionine sulphone by the young rat, *Br. J. Nutr.*, **16**, 571–7.

PAWLAK, M. & PION, R. (1966). Les amino acides libres du sang et du muscle comme méthode d'évaluation des protéines. In *Proceedings of the Seventh International Congress of Nutrition*, **4**, Verlag Friedr. Vierweg & Sohn Gmbh, Braunschweig, West Germany.

PIEZ, K. A. & MORRIS, L. (1960). A modified procedure for the automatic analysis of amino acids, *Anal. Biochem.*, **1**, 187–201.

RASEKH, J., STILLINGS, B. R. & SIDWELL, V. (1972). Effect of hydrogen peroxide on the color composition and nutritive quality of fish protein concentrate, *J. Food Sci.*, **37**, 423–5.

RAY, W. J. & KOSHLAND, D. E. (1960). *Comparative structural studies of phosphoglucomutase and chymotrypsin.* Brookhaven Symposium of Biology, **13**, 135–50.

REED, G. (1966). Hydrogen peroxide-catalase treatment. In: *Enzymes in food processing*, 274–6, Academic Press, New York.

ROBIN, P. & ROBIN, D. (1971). Stabilité du tryptophane en milieu sodique et son recouvrement selon différentes conditions d'hydrolyses. Application à une farine de céréales, *Bulletin de la Société Scientifique d'Hygiène Alimentaire de l'Association Française des Techniciens de l'Alimentation Animale et de l'Association Française de Zootechnie*, **57**, 173–84.

SCHWARTZ, D. (1963). *Méthodes statistiques à l'usage des médecins et des biologistes*, 151–62. Flammarion, Paris.

SLUMP, P. & SCHREUDER, H. A. W. (1973). Oxidation of methionine and cystine in foods treated with hydrogen peroxide, *J. Sci. Food Agric.*, **24**, 657–61.

TANNENBAUM, S. R., BARTH, H. & LE ROUX, J. P. (1969). Loss of methionine in casein during storage with autoxidizing methyl linoleate, *J. Agr. Food Chem.*, **17**, 1353–4.

TOENNIS, G. & KOLB, J. J. (1939). Methionine studies. II. DL methionine sulfoxide, *J. Biol. Chem.*, **128**, 399–403.

YANAGITA, T., SUGANO, M., CHO, S. & WADA, M. (1973). Changes in available lysine and *in vitro* digestibility of casein accompanied with oxidation of ethyl linoleate. (Effect of oxidized fats on nutritive value of proteins Part I.) *J. Agr. Chem. Soc.*, **47**, 73–8.

YANG, S. F. (1970). Sulfoxide formation from methionine or its sulfite analogs during aerobic oxidation of sulfite. *Biochemistry*, **9**, 5008–14.

VARIATION ON STORAGE OF ASCORBIC ACID LEVELS IN PREPARED INFANT FEEDS

DAVID J. CAMERON

School of Nutritional Science, Robert Gordon's Institute of Technology, Queens Road, Aberdeen, AB9 2PG, Scotland

(Received: 14 March, 1977)

ABSTRACT

(a) Infant milks, supplemented with ascorbic acid, were investigated to evaluate the effectiveness of the supplementation procedure.

(b) Storage of feeds, after preparation, caused gradual loss of ascorbic acid.

(c) The procedure of re-heating the feeds after storage increased the loss of ascorbic acid.

(d) There appeared to be a correlation between form of vitamin supplement and stability after preparation, ascorbic acid being less susceptible to oxidation than sodium ascorbate.

INTRODUCTION

Infant milks are supplemented with ascorbic acid. The manufacturers' declared levels of ascorbic acid are such as to provide adequate amounts of the vitamin to infants fed on these milks. It was found in each brand investigated that the actual levels of ascorbic acid were approximately one-and-a-half to twice the declared levels. Thus, assuming no oxidation of the vitamin, more than adequate quantities should be present in the infant feeds.

It is the practice in some hospitals—and a recommendation of some manufacturers—to prepare feeds for a 24 h period and store them in a refrigerator until required. The levels of ascorbic acid may alter during this storage period and are possibly lowered by oxidation to dehydroascorbic acid and/or diketogulonic acid. If this is so then the ascorbic acid available over a 24 h period may be less than the daily recommended intake.

The current studies were confined to four proprietary brands of infant milks using feeds recommended for month-old infants. At this age it is possible that no vitamin supplements are being administered and the infant's total diet consists only of its bottle feeds.

Fd. Chem. (3) (1978)—
Printed in Great Britain

MATERIALS AND METHODS

Reagents	*Manufacturer*
Metaphosphoric acid	B.D.H.
Dichlorophenol indophenol tablets	B.D.H.
L-ascorbic acid	Analar
Ostermilk Two	Glaxo Ltd
Cow and Gate Babymilk 2	Cow and Gate Ltd for National Health Service
National Dried Milk	
S.M.A.	J. Wyeth & Brothers Ltd

Procedures

Ascorbic acid was measured spectrophotometrically using dichlorophenol indophenol (Cameron, 1975).

Feeds were prepared using the weights recommended for month-old infants. These feeds were prepared using water at 90 °C as recommended.

RESULTS

Experiment 1

Feeds were prepared and held at 4 °C in a refrigerator, samples for ascorbic acid analysis being removed at regular intervals. The amount of ascorbic acid present in a 120 ml feed was calculated and this was expressed as a percentage of the dry powder level.

A month-old infant would receive six feeds at four-hourly intervals during a 24 h period.

Experiment 2

Feeds were prepared and stored at 4 °C as in Experiment 1. In this experiment the samples were held at 50 °C for 5 min prior to ascorbic acid estimation. This was to simulate re-heating of the feed after refrigerated storage.

DISCUSSION

With all four brands of infant milks examined there is a trend towards decreasing ascorbic acid levels with respect to time of storage after preparation. By normalising the results, as shown by Figs. 1 and 2, it is possible to compare each brand with the others investigated. In Fig. 1 it is seen that the Ostermilk, S.M.A. and Cow and Gate feeds react almost identically with regard to ascorbic acid oxidation during storage.

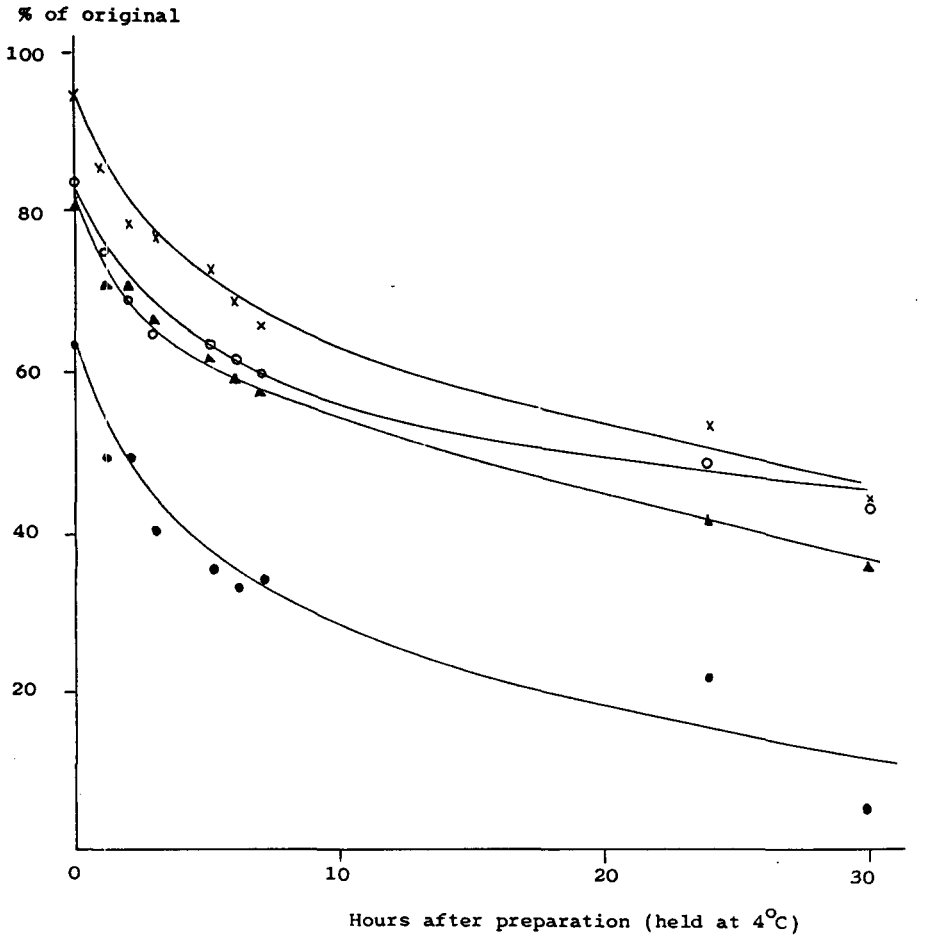


Fig. 1. Percentage of ascorbic acid remaining versus time. The estimated dry powder ascorbic acid level used as 100% value. ×—× Ostermilk; ○—○ Cow and Gate; ▲—▲ S.M.A. and ●—● National Dried Milk (mean of fourteen experiments).

The National Dried Milk, however, reacts differently from the other three with more rapid oxidation of ascorbic acid. This is seen not only with the more rapid drop in ascorbic acid levels with time but also in the initial level on preparation, 64%. The Ostermilk, S.M.A. and Cow and Gate products have levels of 93%, 82% and 84% immediately after preparation, these reductions in ascorbic acid levels being due to initial oxidation on addition of water at 90 °C and the very short time interval while the feed is being mixed before sampling occurs. It is possible that this relationship can be explained in terms of the form in which the vitamin C is added to the

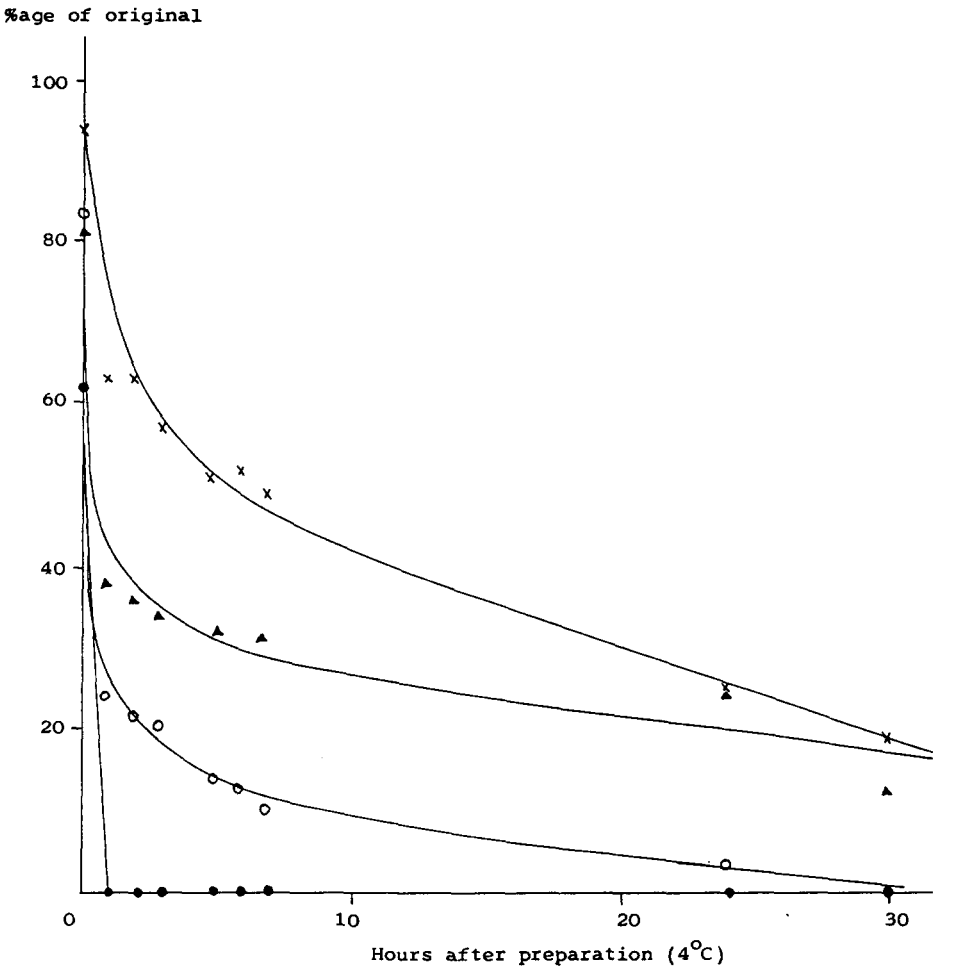


Fig. 2. Percentage of ascorbic acid remaining versus time. The estimated dry powder ascorbic acid level used as 100% value and zero time sample not held at 50 °C for 5 min before analysis. × — × Ostermilk; ○—○ Cow and Gate; ▲—▲ S.M.A. and ●—● National Dried Milk (mean of seven experiments).

individual products. The National Dried Milk has vitamin C added as sodium ascorbate whereas the other three products have the vitamin addition in the form of ascorbic acid. Possibly sodium ascorbate, in solution, is more susceptible to oxidation than ascorbic acid.

Experiment 2 has shown that in all four products re-heating of the sample increases the rate of ascorbic acid oxidation. The degree of increase varies, depending on the individual product concerned. Ostermilk and S.M.A. react

identically as before with a slight increase in oxidation rate; compare Figs. 1 and 2. The Cow and Gate product in this instance reacts more like the National Dried Milk. In an attempt to rationalise this fact, if the total ascorbic acid received by an infant over a 24 h period from these feeds is considered, an interesting relationship is found.

Taking the ratios of the levels shown in Table 1 to those shown in Table 2, the Ostermilk and S.M.A. have figures of 0·72 and 0·65, respectively. The other two products, however, both have the ratio 0·34. It seems obvious that Ostermilk and S.M.A. behave in a similar manner on heating with regard to ascorbic acid oxidation, but differently from Cow and Gate and National Dried Milk. The latter pair also react identically. This may be explicable by the actual amounts of ascorbic acid present in the product. Table 3 shows this relationship and although the manufacturers' declared levels uphold this theory, the actual levels found do not show such a clear cut relationship.

TABLE 1

AMOUNT OF ASCORBIC ACID (mg) DERIVED FROM SIX FOUR-HOURLY FEEDS USING LEVELS OF ASCORBIC ACID DERIVED FROM EACH FEED AS SHOWN IN FIG. 3

Brand	*Feed number*						*Total*[a]
	1	2	3	4	5	6	
Ostermilk	9·5	5·6	4·4	3·7	3·4	2·9	29·5
Cow and Gate	6·7	1·3	0·7	0·5	0·4	0·3	9·9
S.M.A.	7·9	3·2	2·8	2·4	2·2	2·0	20·5
National Dried Milk	3·6	0·0	0·0	0·0	0·0	0·0	3·6

[a] Recommended daily intake 15 mg (DHSS, 1969).

TABLE 2

AMOUNT OF ASCORBIC ACID (mg) DERIVED FROM SIX FOUR-HOURLY FEEDS USING LEVELS OF ASCORBIC ACID DERIVED FROM EACH FEED AS SHOWN IN FIG. 4

Brand	*Feed number*						*Total*[a]
	1	2	3	4	5	6	
Ostermilk	9·2	7·5	6·5	6·1	5·8	5·6	40·7
Cow and Gate	6·5	5·2	4·7	4·4	4·2	4·1	29·1
S.M.A.	7·9	6·0	5·1	4·6	4·2	3·9	31·7
National Dried Milk	3·6	2·2	1·6	1·3	1·0	0·9	10·6

[a] Recommended daily intake 15 mg (DHSS, 1969).

As stated above, it is the practice in some regions for infants of this age to receive no vitamin supplements. This being so, two of the products investigated—Ostermilk and S.M.A.—supply more ascorbic acid than the recommended daily intake over a 24 h period (Table 1). The other two products have lower total levels. In the case of National Dried Milk this is possibly an unfair assessment as the manufacturers do

TABLE 3
ASCORBIC LEVELS OF THE DRY FORMULA

Product	*Declared ascorbic acid levels (mg/100 g)*	*Estimated ascorbic acid levels (mg/100 g)*
Ostermilk	35·2	72
Cow and Gate	28·0	56
S.M.A.	45·0	66
National Dried Milk	26·4	52

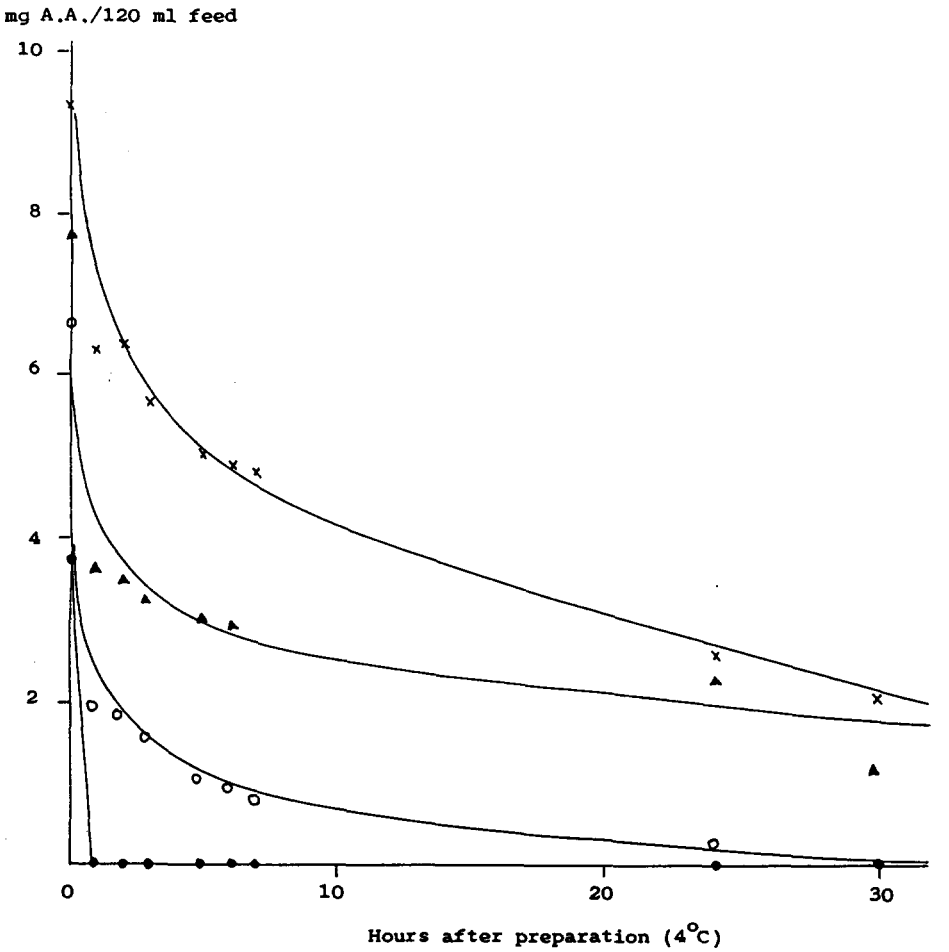


Fig. 3. Ascorbic acid levels in a 120 ml feed versus time. Feeds subjected to heat treatment before analysis. ×—× Ostermilk; ○—○ Cow and Gate; ▲—▲ S.M.A. and ●—● National Dried Milk (mean of seven experiments).

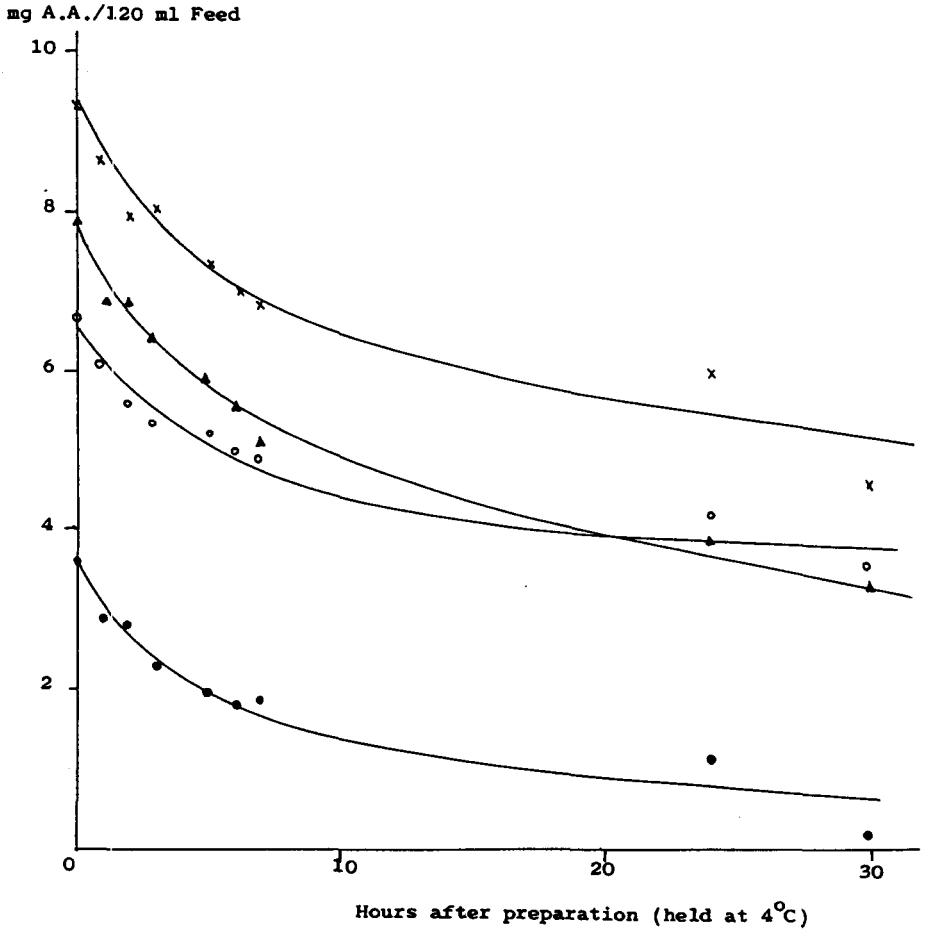


Fig. 4. Ascorbic acid levels in a 120 ml feed versus time. ×—× Ostermilk; ○—○ Cow and Gate; ▲—▲ S.M.A. and ●—● National Dried Milk (mean of fourteen experiments).

not recommend the preparation of feeds in advance. If, however, the initial level at preparation is taken for National Dried Milk and multiplied by 6 then this total level is still lower, at 20·4 mg ascorbic acid, than that of other brands over a 24 h period. At this level any delay in feeding will bring the ascorbic acid level below the recommended daily intake. It is therefore possible that before administration of vitamin supplements some infants will be receiving less than the recommended daily intake of ascorbic acid.

There is some confusion as to whether vitamin C, if administered in the form of

dehydroascorbic acid, is as useful to the body as ascorbic acid. In this paper only the reduced form, ascorbic acid, has been measured, basically because the DHSS (1969) recommended intake quotes vitamin C in the form of ascorbic acid. In the work of McCance & Widdowson (1960) there is confusion as to the form of vitamin C measured. Some of the figures are for ascorbic acid alone and others for ascorbic acid plus dehydroascorbic acid. Until this confusion is cleared up it would seem logical to quote and measure ascorbic acid levels when referring to vitamin C. It is intended to extend this work by looking at the relationship between ascorbic acid and dehydroascorbic acid levels on storage.

REFERENCES

CAMERON, D. J. (1975). Rapid spectroscopic method for the estimation of ascorbic acid in infant milks. *Proc. Nut. Soc.*, **34**, 7A.

DHSS (1969). *Recommended Intakes of Nutrients for the United Kingdom.* Reports on Public Health and Medical Subjects No. 120.

MCCANCE, R. A. & WIDDOWSON, E. M. (1960). *The Composition of Foods.* Medical Research Council, Special Report Series No. 297.

RAPID METHOD FOR THE DETERMINATION OF MEAT IN FOOD PRODUCTS

G. R. SKURRAY & V. A. LYSAGHT

School of Food Sciences, Hawkesbury Agricultural College, Richmond, New South Wales 2753, *Australia*

(Received: 25 March, 1977)

ABSTRACT

A method for the determination of meat in food products is described based on the determination of protein-bound 3*-methylhistidine. The method involved high pressure liquid chromatography and reduced the elution time of previous methods from* 7 *h to* 10 *min. The accuracy of the method was increased by using electronic integration. The method was used to determine the meat content of nine meat pies and three hamburger patties.*

INTRODUCTION

It has been established that the amino acid 3-methylhistidine occurs in the myofibrillar tissue of animals (Johnson *et al.*, 1967). Hibbert & Lawrie (1972) suggested the use of 3-methylhistidine as a possible indicator of meat protein content because it was heat stable and was thought to be present at similar levels in animal tissue. A significant correlation was found between the 3-methylhistidine content and the percentage of meat in mixtures of beef and soya protein that had been sterilised in cans. Further studies by Rangeley & Lawrie (1976) showed that there was a similar concentration of 3-methylhistidine in lamb and beef muscle but the concentrations in pork were higher and varied markedly between samples. This variation was found to be due to a dipeptide containing 3-methylhistidine in pork and could be removed by washing the sample with distilled water.

The method of Rangeley & Lawrie (1976) involved experimental errors of the order of 10% involving calculation of chromatographic peak areas. Furthermore, the elution time was of the order of 7–10 h. High pressure liquid chromatography offers the advantages of speed, low running costs and accurate electronic integration of peak areas.

Fd. Chem. (3) (1978)—
Printed in Great Britain

The aim of the present study was to use high pressure liquid chromatography to determine the 3-methylhistidine content of meat and meat products.

MATERIALS AND METHODS

Source of materials

All meat samples were prepared from *longissimus dorsi* muscle of freshly killed beef or lamb. Meat products were purchased fresh or frozen from local supermarkets and L-3-methylhistidine was obtained from Sigma Chemicals.

Chemical analysis

The meat products were prepared according to the methods used by Rangeley & Lawrie (1976). The nitrogen content of the samples was determined by the method described by Pearson (1962). Only the filling portion of meat pies was analysed. Samples were hydrolysed by placing 10 g in 450 ml of 6N HCl and refluxing for 22 h. The hydrolysate was filtered through sintered glass and evaporated to dryness twice using a rotary evaporator. The hydrolysate was made up to 25 ml with 5% $NaHCO_3$. After 10 min, freshly prepared 1-fluoro-2,4-dinitrobenzene (FDNB) solution (1 ml of FDNB dissolved in 20 ml ethanol) was added. The mixture was stirred and allowed to stand for 18 h and then the ethanol was evaporated on a steam bath. The remaining mixture was extracted twice with 20 ml of diethyl ether to remove DNP derivatives of non-basic amino acids. A 10 μl aliquot of the mixture was applied to a spherisorb silica (5 μm) column (8 × 250 mm) and eluted with degassed, distilled water. A flow rate of 0·5 ml/min was obtained using a Spectra Physics 3500 B high pressure liquid chromatograph with a 250 nm ultra-violet detector. Peak areas were determined by an electronic integrator (Linear Corporation). Standard solutions of DNP derivatives of 3-methylhistidine (20–100 mg/litre) were used.

The meat content of the products was calculated from their 3-methylhistidine content relative to skeletal muscle.

RESULTS

The DNP derivative of 3-methylhistidine was separated from the other DNP amino acids by high pressure chromatography (Fig. 1). The 3-methylhistidine content of beef and lamb skeletal muscle was 5·4 and 5·2 (±0·1) mg/gN respectively compared with the value of 6·0 (±0·7) mg/gN obtained by Rangeley & Lawrie (1976).

The protein and 3-methylhistidine content of the commercial samples of meat pies and hamburger patties varied markedly (see Table 1). There was no significant correlation between the protein content of the products and their meat content (*r*

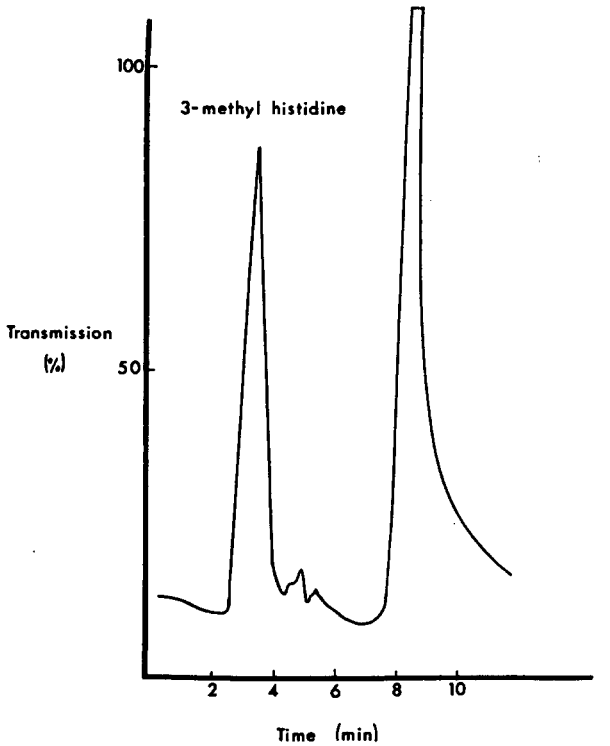


Fig. 1. Chromatogram of acid hydrolysate of bovine skeletal muscle showing the separation of DNP 3-methylhistidine.

$= 0{\cdot}26, p > 0{\cdot}05$) as calculated from their 3-methylhistidine content. The meat pie, sample 4, contained the highest amount of protein but the lowest calculated amount of meat.

DISCUSSION

The 3-methylhistidine contents of *longissimus dorsi* muscle of beef and lamb obtained by the method described are similar to those obtained by Rangeley & Lawrie (1976). However, high pressure liquid chromatography with electronic integration of peak areas resulted in a chromatographic time of 10 min with an experimental error of 1·9% compared with the method of Rangeley & Lawrie (1976) which had a chromatographic time of 7 h with an experimental error of 11·7%.

The new method described involves the preparation of a DNP derivative of 3-methylhistidine. This has the advantage of increasing the sensitivity of the method

TABLE 1
PROTEIN, 3-METHYLHISTIDINE AND MEAT CONTENT (WET WEIGHT BASIS) OF MEAT PRODUCTS

Sample	*Protein* (*g* %)	3-*Methylhistidine* (*g*/*gN*)	*Meat* (*g*/100 *g*)
Longissimus dorsi			
Beef	25·7	5·4	100
Lamb	23·4	5·2	100
Meat Pie			
1	8·2	4·1	76
2	4·8	2·3	43
3	5·3	4·5	83
4	11·5	1·9	35
5	6·2	3·4	63
6	6·7	5·2	96
7	5·7	4·5	83
8	6·1	3·4	63
9	6·5	3·8	70
Hamburgers			
1	2·5	1·3	24
2	2·3	1·1	20
3	2·8	1·6	30
Standard error of mean	0·08	0·06	0·83

due to the high extinction coefficient of the DNP derivative and the solubility of the DNP 3-methylhistidine in acid solution allows the separation of other DNP amino acids which would interfere with the chromatographic separation.

The lack of correlation between the protein content of the meat products and their meat content calculated from their 3-methylhistidine content suggests that significant amounts of non-skeletal muscle protein were used in their preparation. The hydroxyproline content of the samples would indicate whether the protein was connective tissue or vegetable protein (Skurray & Herbert, 1974) and this would have a significant bearing on the nutritional value of the products (Skurray & Osborne, 1976).

REFERENCES

HIBBERT, I. & LAWRIE, R. A. (1972). Technical note: The identification of meat in food products. *J. Fd. Technol.*, **7**, 333–5.

JOHNSON, P., HARRIS, C. I. & PERRY, S. V. (1967). 3-Methylhistidine in actin and other muscle proteins. *Biochem. J.*, **105**, 361–9.

PEARSON, D. (1962). *The chemical analysis of foods*, 5th edn. London, Churchill.

RANGELEY, W. R. D. & LAWRIE, R. A. (1976). Methylamino acids as indices in meat products. *J. Fd. Technol.*, **11**, 143–59.

SKURRAY, G. R. (1976). The nutritive value of non-skeletal meat tissue. *Proc. Aust. Nut. Soc. Conf.*, Sydney.

SKURRAY, G. R. & HERBERT, L. S. (1974). Batch dry rendering: Influence of raw materials and processing conditions on meat meal quality. *J. Sci. Fd. Agric.*, **25**, 1071–9.

SKURRAY, G. R. & OSBORNE, C. (1976). Nutritional value of soya protein and milk co-precipitates in sausage products. *J. Sci. Fd. Agric.*, **27**, 175–80.

ISOLATION AND PARTIAL PURIFICATION OF A PROTEOLYTIC ENZYME PREPARATION FROM *CLOSTRIDIUM PERFRINGENS*

LYNN T. HAPCHUK & A. M. PEARSON

Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan 48824, *USA*

(Received: 22 April, 1977)

ABSTRACT

Although two strains of Clostridium perfringens (*ATCC* 12915 *and* 13124) *exhibited excellent growth on amino acid and peptone media, only one* (*ATCC* 13124) *produced measurable proteolytic enzyme activity. Thus, subsequent purification steps concentrated on isolation of a proteolytic enzyme preparation produced by this strain. Purification and concentration were carried out by precipitating the crude enzyme fraction from the culture filtrate with* $ZnCl_2$, *extracting with saturated disodium phosphate and reprecipitating by* 60% *saturation with* $(NH_4)_2SO_4$. *The precipitate was then redissolved in borate buffered saline solution and further purified by successively passing it through a Bio-Gel P-*100 *column, a DEAE-cellulose column and a Bio-Gel P-*200 *column. The final step resulted in a* 159*-fold purification with* 12% *recovery and a final specific activity of* 79 *azocoll units/milligramme protein. Although each successive purification step eliminated some of the impurities, the final fraction still showed considerable heterogeneity upon disc-gel electrophoresis.*

INTRODUCTION

Clostridium perfringens is a common contaminant of meat and other muscle foods. In addition to its role as a causative agent of enteritis, *C. perfringens* is known to cause extensive disruption of muscle tissue, such as occurs in gas gangrene (MacFarlane & MacLennan, 1945; Strunk *et al.*, 1967). Hasegawa *et al.* (1970) demonstrated that *C. perfringens* caused extensive proteolysis of muscle which suggests that the organism produces enzyme(s) capable of degrading muscle proteins. Further support for the viewpoint that meat spoilage organisms cause enzymatic breakdown of muscle was obtained by Tarrant *et al.* (1973) working with *Pseudomonas fragi*. Porzio & Pearson (1975) later isolated and purified a single

Fd. Chem. (3) (1978)—
Printed in Great Britain

proteolytic enzyme from culture filtrates of *P. fragi* and characterised the enzyme. More recently, Hapchuk *et al.* (1976) demonstrated that *C. perfringens* degraded both sarcoplasmic and myofibrillar proteins.

The present investigation was undertaken to isolate, partially purify and characterise proteolytic enzyme(s) produced by *C. perfringens*.

METHODS

Culturing the organism

Stock cultures of *C. perfringens* (ATCC 12915 and 13124) were obtained from a frozen culture collection maintained in the Meat Laboratory at Michigan State University. After thawing, the cultures were inoculated into Difco thioglycollate broth, incubated anaerobically at 37 °C and loop transferred daily up to a maximum of 30 days, at which time a new frozen stock culture was used. The thioglycollate broth containing actively growing cells served as the source of inoculum throughout this study.

Culturing for enzyme production was carried out anaerobically using either a peptone medium supplemented with Difco Bacto beef or a synthetic amino acid mixture as outlined by Murata *et al.* (1968). The organisms grew well on both media, which appeared to be equally satisfactory. Two litres of peptone broth in a flask were inoculated with 1 ml of culture (ATCC 13124) and incubated for 16 to 18 h at 37 °C.

Measurement of enzyme production

The length of time required for enzyme production was determined using both peptone and amino acid media. Cultures (ATCC 12915 and 13124) were inoculated into peptone broth and incubated at 37 °C. Samples were removed at 1–2 h intervals in order to monitor growth and activity. Growth was estimated by turbidity measurements using a Beckman DU-2 spectrophotometer equipped with a Gilford optical density converter. The bacterial cells were removed by centrifugation at 2000 $\times$ *g* for 15 min using an International PR-6 refrigerated centrifuge.

Enzyme activity in the supernatant was measured by a modification of the azocoll method of Kameyama & Akama (1970) using an incubation time of 1 h. Activity was arbitrarily expressed as the amount of enzyme required to raise absorbance at 520 nm of the azocoll suspension by 0·1 unit in 15 min. A sample inactivated by boiling for 10 min served as the blank.

Purification scheme

Figure 1 outlines the steps used in enzyme purification. A 1 % solution of $ZnCl_2$ was used to precipitate the enzyme(s) from the culture filtrate and the inactive supernatant was discarded. The precipitate was extracted with cold saturated Na_2HPO_4 and after centrifugation the remaining inactive precipitate was

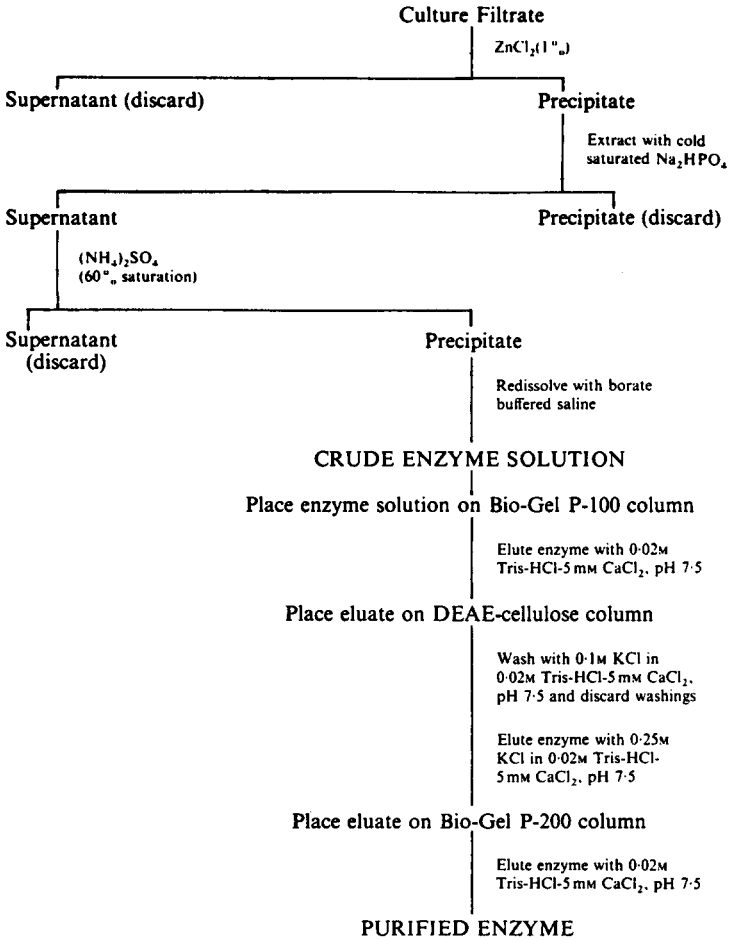


Fig. 1. Purification scheme used for preparation of enzyme fraction.

discarded. The supernatant was then salted out of solution by stepwise addition of solid $(NH_4)_2SO_4$ to 60% saturation. The precipitate was redissolved in borate buffered saline solution (0·05M $Na_2B_4O_7 . 10H_2O$, 0·02M H_3BO_4, 0·15M NaCl) to give the crude enzyme solution, which was placed on a Bio-Gel P-100 column and eluted with 0·02M Tris-HCl-5 mM $CaCl_2$ buffer at pH 7·5. The pooled fraction was then put on a DEAE-cellulose column and washed with 0·1M KCl in 0·02M Tris-HCl-5 mM $CaCl_2$ at pH 7·5 and the washings were discarded. The enzyme preparation was eluted with 0·25M KCl in 0·02M Tris-HCl-5 mM $CaCl_2$ at pH 7·5. The pooled fraction was loaded on a Bio-Gel P-200 column and again eluted with 0·02M Tris-HCl-5 mM $CaCl_2$ to give the final enzyme preparation. Further purification with CM-cellulose

chromatography was attempted with no increase in specific activity. Therefore, this step was omitted from the final purification scheme. Each step in the purification procedure was monitored for purity by disc-gel electrophoresis and for enzyme activity by the azocoll procedure.

Disc-gel electrophoresis

Disc-gel electrophoresis of the enzyme preparation was carried out using the method described by Davis (1964) to show the purity following each purification step. The gels contained 7% acrylamide. The position and intensity of the protein bands were recorded using a modified Kontes Chromaflex K-495000 densitometer.

Protein concentration

The protein concentration was determined following each step in purification using the method of Lowry *et al.* (1951).

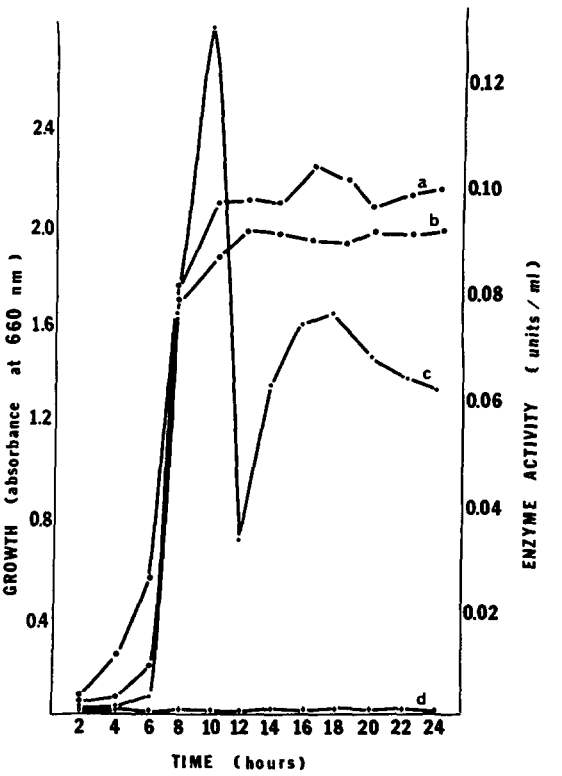


Fig. 2. Absorbance and enzyme production by *C. perfringens* ATCC 13124 and 12915 in amino acid medium: (a) absorbance of *C. perfringens* ATCC 13124; (b) absorbance of *C. perfringens* ATCC 12915; (c) enzyme activity from *C. perfringens* ATCC 13124; (d) enzyme activity from *C. perfringens* ATCC 12915.

RESULTS AND DISCUSSION

Growth and enzyme production by *C. perfringens* strains ATCC 13124 and ATCC 12915 grown in an amino acid medium are presented in Fig. 2. Although both strains grew well and their growth curves were similar, ATCC 12915 showed little enzyme activity. On the other hand, ATCC 13124 showed marked proteolytic enzyme activity which peaked after 8–9 h of incubation. All subsequent purification studies were limited to *C. perfringens* ATCC 13124.

Figure 3 shows absorbance, which is roughly indicative of growth, and enzyme production for *C. perfringens* ATCC 13124 in a peptone medium. Little enzyme production occurred until after 4 h of incubation but by 7 h both absorbance and enzyme production had reached a maximum.

Figure 4 shows the effect of enzyme concentration and time of incubation upon the azocoll reaction. It is apparent that the reaction rate becomes non-linear at longer incubation times. Since the reaction was essentially linear for 15 min, it was used as the standard incubation period. However, the increase in absorbance was not linear as the concentration of enzyme increased. In fact, at absorbance values above 0·6, the reproducibility of the assay declined. Consequently, the level of enzyme used in the assay was adjusted so as to give absorbance values below 0·6.

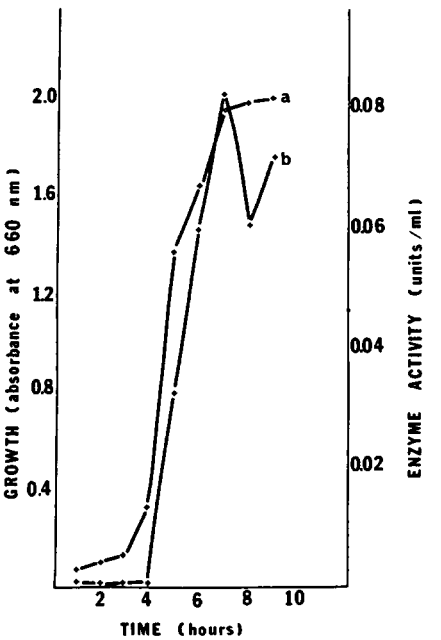


Fig. 3. Absorbance and enzyme production by *C. perfringens* ATCC 13124 in peptone medium: (a) absorbance; (b) enzyme activity.

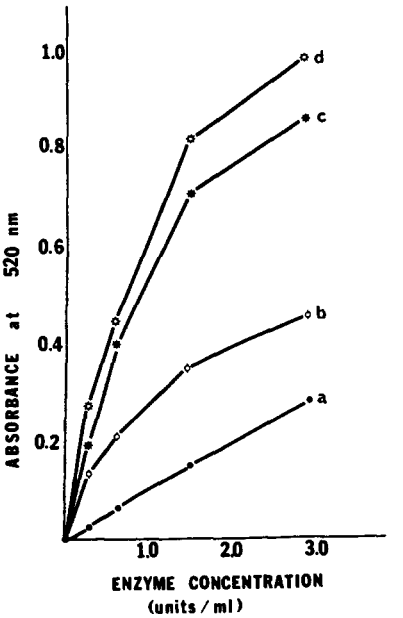


Fig. 4. Effects of enzyme concentration and reaction time upon the azocoll reaction: (a) 15 min incubation; (b) 30 min incubation; (c) 45 min incubation; (d) 60 min incubation.

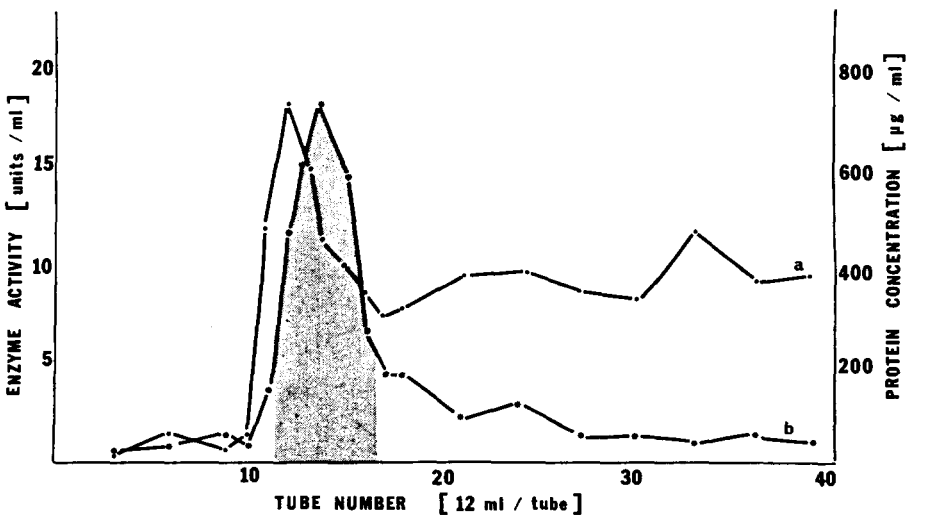


Fig. 5. Elution pattern showing protein concentration and enzyme activity upon Bio-Gel P-100 fractionation: (a) protein concentration; (b) enzyme activity; shaded area — pooled fractions 12–16 used in subsequent steps.

A typical elution pattern from the Bio-Gel P-100 column is shown in Fig. 5. The fractions with the highest activity (tubes 12–16) were pooled as indicated by the shaded area. The pooled fraction, which was used in all subsequent steps, was purified 3·66-fold with 118% recovery of the activity and increased in specific activity from 6·57 to 24·1 azocoll units/mg protein. The major portion of the enzyme activity was eluted just behind the major protein peak, but prior to the diffuse low molecular weight proteins. This indicated that the enzyme(s) fell in the molecular weight range of 80,000 to 113,000 daltons which is in agreement with the values reported for kappa toxin by Kameyama & Akama (1971) and for collagenase by Levdikova (1966), respectively.

A typical DEAE-cellulose elution pattern using a stepwise increase in salt concentration is shown in Fig. 6. The fractions showing the highest activity (tubes 66–69) were pooled and used in all subsequent purification steps. Purification was 1·84-fold with a recovery of 83·5% of activity and an increase of specific activity from 24·1 to 44·3 azocoll units/mg protein.

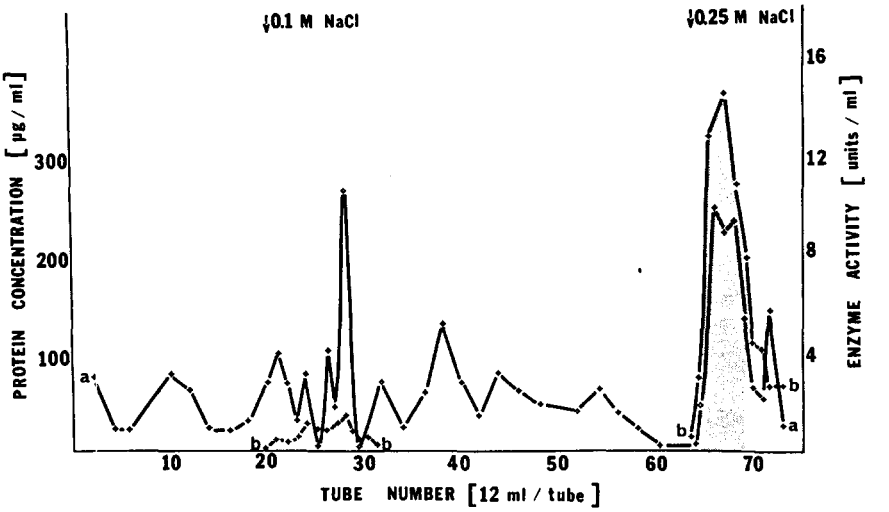


Fig. 6. Elution pattern showing protein concentration and enzyme activity upon stepwise DEAE-cellulose fractionation; (a) protein concentration; (b) enzyme activity; ↓ change in concentration of eluent pooled; shaded area — fractions 66–69 used in subsequent steps.

Figure 7 shows the gradient salt elution pattern for the enzyme preparation from the DEAE-cellulose column. The majority of the enzymatic activity was eluted between 0·15M and 0·23M NaCl, which corresponds closely to the major protein peak. The preparation absorbed on the column had a specific activity of 62 azocoll units/mg protein whereas the eluate fractions 17–23 increased to 114 azocoll units/mg protein which showed a 1·8-fold increase in purification.

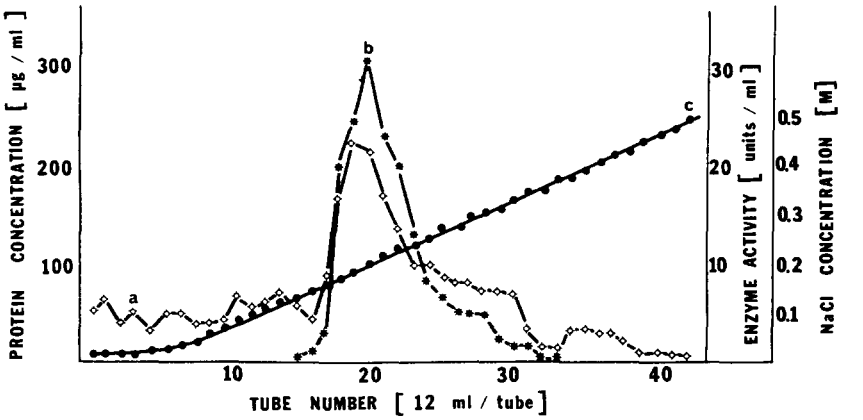


Fig. 7. Elution pattern on DEAE-cellulose gradient fraction showing protein concentration and enzyme activity: (a) protein concentration; (b) enzyme activity; (c) NaCl concentration.

A typical elution pattern from Bio-Gel P-200 column fractionation is shown in Fig. 8. While some proteolytic enzyme activity was eluted in the initial protein peak, the major active fraction coincided with the second protein peak. The preparation placed on the column had a specific activity of 59·7 azocoll units/mg protein whereas the eluate increased to 224 units/mg protein, giving a 3·7-fold purification and 34·8 % recovery.

Table 1 summarises the results of each step in the enzyme purification scheme. It clearly demonstrates that each step increased the specific activity and improved the purification. On the other hand, yield of total protein and the recovery of activity declined with each successive purification step, which is in agreement with earlier work on purification of an enzyme(s) from *P. fragi* (Tarrant *et al.*, 1973; Porzio & Pearson, 1975).

Disc-gel electrophoresis separated the culture filtrate into eight peaks with 74 % of the protein being localised in the four overlapping peaks with R_m values from 0·33 to 0·46. Upon electrophoresis of the resolubilised $ZnCl_2$ precipitate, there were a total of six peaks with 60 % of protein being in the peaks at R_m 0·38 and 0·53. The resolubilised $(NH_4)_2SO_4$ precipitate gave seven peaks upon electrophoresis, with two of the peaks (R_m 0·37 and 0·48) containing 68 % of the protein. The eluate from the Bio-Gel P-100 column separated into ten peaks with the two major ones (R_m 0·39 and 0·43) containing 52 % of the protein. The eluate from the DEAE-cellulose column separated into six peaks upon electrophoresis, with the two peaks at R_m 0·37 and 0·47 containing 60 % of the protein and the peak at R_m 0·34 comprising 20 %. The final enzyme preparation gave five peaks upon disc-gel electrophoresis which suggests heterogeneity was still present. However, the three major peaks (R_m 0·38, 0·46 and 0·33) contained 79 % of the protein.

TABLE 1

PURIFICATION SCHEME FOR A PROTEASE PRODUCED BY *Clostridium perfringens* ATCC 13124

Procedure	*Final volume (ml)*	*Activity*[a] *(units/ml)*	*Total activity (units)*	*Protein (mg/ml)*	*Specific activity (units/mg protein)*	*Yield (% protein)*	*Enzyme recovery (% activity)*	*Purification (fold)*
1. Culture filtrate	1815	2·42	4390	4·86	0·498	100	100	0
2. Zn Cl_2 precipitation	412	4·4	1812	1·32	3·33	6·2	41·3	6·7
3. Ammonium sulphate precipitation	59	21·9	1292	3·30	6·57	2·2	29·4	13·2
4. Bio-Gel P-100 fractionation	141	10·82	1526	0·45	24·1	0·7	34·8	48·4
5. DEAE-cellulose fractionation	135	9·75	1316	0·22	44·3	0·3	30·0	89·0
6. Bio-Gel P-200 fractionation	129	3·95	510	0·05	79·0	0·07	11·6	159

[a] One unit of enzyme activity is defined as that amount of enzyme required to raise the absorbance by 0·1 in 15 min.

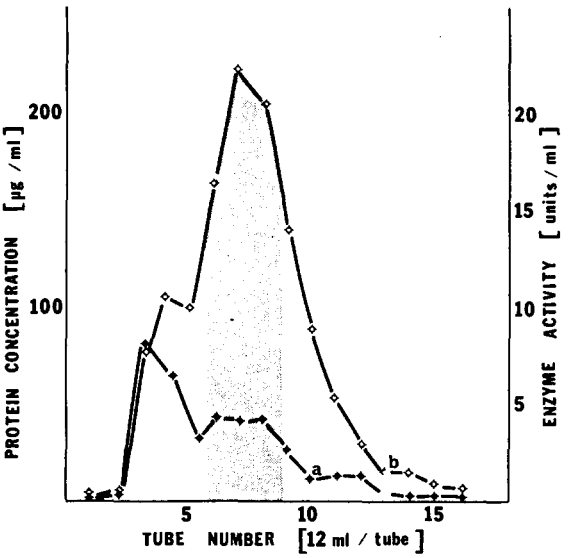


Fig. 8. Elution pattern on Bio-Gel P-200 fractionation showing protein concentration and enzyme activity: (a) protein concentration; (b) enzyme activity, shaded area — pooled fractions 6–8 used in subsequent studies with muscle.

The results reported herein show that one strain of *C. perfringens* (ATCC 13124) exhibited excellent growth and produced considerable proteolytic activity in culture media enriched with either peptone or amino acids as described by Murata *et al.* (1968). An enzymatically active fraction was isolated and purified by the procedure outlined herein. The final preparation was purified 159-fold with 12% recovery and had a final specific activity of 79 azocoll units/mg protein. However, disc-gel electrophoresis revealed that the isolated enzyme fraction still showed some heterogeneity. The heterogeneity of the enzyme, as well as its rapid production, helps to explain the extensive tissue breakdown caused by *C. perfringens*.

ACKNOWLEDGEMENTS

Michigan Agricultural Experiment Station Journal Article No. 7911. This investigation was supported in part by US Public Health Service Research Grant No. FD-00097 from the Food and Drug Administration.

REFERENCES

DAVIS, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.*, **121**, 404–27.

HAPCHUK, L. T., PEARSON, A. M. & PRICE, J. F. (1976). Action of *Clostridium perfringens* organisms on porcine muscle. *J. Food Sci.*, **41**, 1042–6.

HASEGAWA, T., PEARSON, A. M., PRICE, J. F. & LECHOWICH, R. V. (1970). Action of bacterial growth on sarcoplasmic and urea soluble proteins from muscle. I. Effects of *Clostridium perfringens*, *Salmonella enteritidis*, *Achromobacter liquifaciens*, *Streptococcus faecalis* and *Kurthia zopfii*, *Appl. Microbiol.*, **20**, 117–22.

KAMEYAMA, S. & AKAMA, K. (1970). Titration of kappa toxin of *Clostridium perfringens* and kappa antitoxin by a modified azocoll method. *Jap. J. Med. Sci. Biol.*, **23**, 31–46.

LEVDIKOVA, G. A. (1966). Vydelenie i nekotorye svoistva kollagenzy *Clostridiut perfringens*. (Isolation and properties of *Clostridium perfringens* collagenase.) *Biokhimiya*, **31**, 821. Cited by M. V. ISPOLATOVSKAYA (1971). Type A *Clostridium perfringens* toxin. In *Microbial toxins IIA*, p. 109. (Kadis, S., Mountie, T. C. & Ajl, S. J. (eds.)), New York, N.Y. Academic Press.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–75.

MACFARLANE, R. G. & MACLENNAN, J. D. (1945). The toxaemia of gas gangrene. *Lancet*, **249**, 328–31.

MURATA, R., SODA, S., YAMAMOTO, A. & ITO, A. (1968). Further investigation on the influence of inorganic cations on growth and toxin production by *Clostridium perfringens* PB6K. *Jap. J. Med. Sci. Biol.*, **9**, 81–91.

PORZIO, M. A. & PEARSON, A. M. (1975). Isolation of an extracellular neutral proteinase from *Pseudomonas fragi*. *Biochim. Biophys. Acta*, **384**, 235–41.

STRUNK, S. W., SMITH, C. W. & BLUMBERG, J. M. (1967). Ultrastructural studies on the lesion produced in skeletal muscle fibers by crude type A *Clostridium perfringens* toxin and its purified α fraction. *Amer. J. Pathol.*, **50**, 89–107.

TARRANT, P. J. V., JENKINS, N., PEARSON, A. M. & DUTSON, T. R. (1973). Proteolytic enzyme preparation from *Pseudomonas fragi*: Its action on pig muscle. *Appl. Microbiol.*, **25**, 996–1005.

METAL–POLYSACCHARIDE COMPLEXES—PART II†

J. A. RENDLEMAN

Northern Regional Research Center, Agricultural Research Service, US Department of Agriculture, Peoria, Illinois, USA 61604

(Received: 1 May, 1977)

I. COMPLEXATION OF METAL IONS WITH POLYSACCHARIDES

A. *Neutral polysaccharides*

(1) *In neutral or non-alkaline media:* In aqueous media neutral polysaccharides have little affinity for cations of low acidity (i.e. cations with little ability to polarise a donor atom) such as alkali metal and alkaline-earth metal ions. Isolation of these complexes from aqueous solution necessitates adding ethanol, which not only insolubilises the adduct but also stabilises the bonding between cation and carbohydrate. Senti & Witnauer (1952) isolated several alkali metal salt complexes of amylose by using aqueous alcoholic media. These were 2:1 adducts of amylose with KOAc, KBr, KI and K formate and 1:1 adducts of amylose with KOAc and K propionate. (The ligand in these adducts is the D-glucose residue.) Of these complexes, KBr–amylose has been given the most intensive study. X-ray diffraction data (Senti & Witnauer, 1952; Jackobs *et al.*, 1968) show both cation and anion in an environment of oxygen atoms and hydroxyl groups. The O–2 atoms from adjacent chains are in contact with K^+, while O–3 and O–4 are located very near. Examination of models (Jackobs *et al.*, 1968) has indicated that the amylose chain is a left-handed helix with four D-glucose units per turn.

The low degree of carbohydrate–salt interaction in water is appropriately illustrated by the virtual inability of locust-bean gum (maximum concentration, 0·03M on a monosaccharide-residue basis) to affect the conductivities of numerous ions (H^+, Na^+, K^+, Ag^+, Ba^{2+}, OAc^-, Cl^-, NO_3^-, SO_4^{2-} and HSO_4^-) (Barry & Halsey, 1963). High viscosity and low solubility limit the polymer concentration to 0·5% by weight. Locust-bean gum contains about 88% of D-galacto-D-

† Part I of this paper appeared in Vol. 3, No. 1, pp. 47–79.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

Fd. Chem. (3) (1978)—
Printed in Great Britain

mannoglycan, 4% of pentan, 6% of protein, 1% of cellulose and 1% of mineral elements (Griffiths, 1949 and 1952).

Generally speaking, there has been little research in the area of complexation between metal cations and neutral polysaccharides. The little attention that has been devoted to this area has been focused largely upon starch and, to a much lesser degree, cellulose. Some study has been given to the relative effects of different cations and anions in 'salting-out' phenomena but no measurements have been made on the extent to which complexation contributes to insolubilisation of polysaccharides. That complexation contributes a substantial amount can be inferred because, when dextran is salted out of an aqueous ethanolic solution by means of $CaCl_2$, a considerable amount of salt (as much as 10% by weight) remains with the polymer (Sloneker, 1975) and subsequent dialytic treatment is necessary in order to obtain a salt-free polysaccharide.

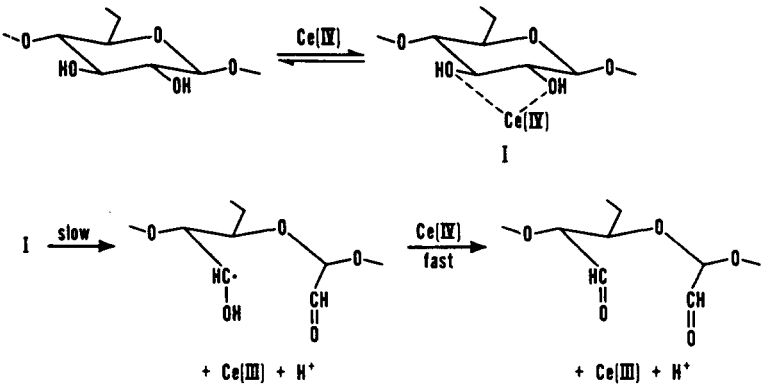
When samples of Douglas fir are impregnated with salts of Cr, Co, Cu, Fe and Zn, the metal cation becomes bound within the cell wall and at least a proportion of the bound ions is present in the form of a metal–cellulose complex, as shown by electron diffraction analysis (Belford *et al.*, 1957 and 1958; Belford & Preston, 1960). Cellulose from cotton is known to form complexes with Cu, Ag and Au (Cronshaw *et al.*, 1958). However, the interaction of Cu(II) with cellulose has been investigated the most extensively (Belford *et al.*, 1958). Adding a sample of cellulose to a dilute copper salt solution makes the pH fall, an effect which suggests that complexation is accompanied by release of hydroxylic protons to form metal–oxygen bonds. In the absence of a swelling agent, such as sodium hydroxide, sorption of the ions occurs only on the surface of the microfibrils and is in accordance with Langmuir's adsorption isotherm. Dilute acid instantly decomposes the complex, a not surprising reaction since transition-metal complexes of this type are sensitive to pH.

Because identical electron diffraction patterns (Belford *et al.*, 1958) are obtained from celluloses treated with a variety of metal salts, the spacings of the unit cell must relate to the outer layer of cellulose molecules. Because spacings of the unit cell of the complex bear no obvious relationship to spacings of the unit cell for pure cellulose, the surface structure of a microfibril must be different from the inner structure.

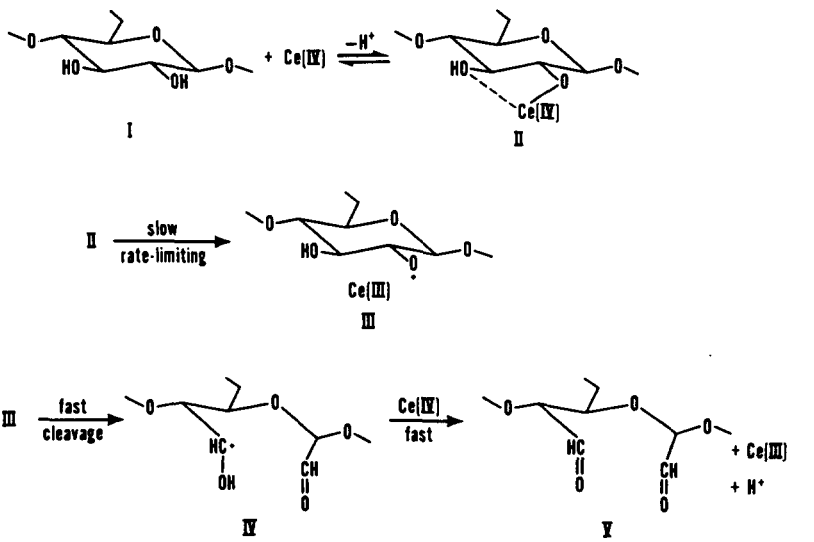
In strongly alkaline solution, there is a large uptake of copper(II) by cellulose, with sorption occurring apparently within the fibrils, as well as on their surface (Roschier & Hyvärinen, 1953). Here also, the copper uptake is in accordance with Langmuir's isotherm.

Cerium(IV) ion can react with hydroxyl groups on neutral polysaccharides in acidic solution (1M H^+) to produce complexes that slowly dissociate by single electron transfers. The reaction mechanism for the reaction of cellulose with Ce(IV) was advanced by Pottenger & Johnson (1970) as in Scheme I.

More likely, Ce(IV) reacts initially to form an alkoxide type of chelate as I suggest in Scheme II. This scheme agrees with the fact that the degree of complex formation is known to decrease with increasing acid concentration (Gugliemelli *et al.*, 1972).



Scheme I. Mechanism for the oxidation of cellulose by Ce(IV) as proposed by Pottenger & Johnson (1970).



Scheme II. Alternate mechanism for the oxidation of cellulose by Ce(IV).

Furthermore, tetravalent cerium ion has a strong tendency towards covalent bond formation (Sidgwick, 1950). In the ionic state, Ce(IV), as well as Fe(III) and Co(III), is highly unstable because of the magnitude of its charge. This instability can be reduced by forming covalent bonds with oxygens of alcoholic hydroxyl groups, which formation promotes the release of hydroxyl protons. In weakly acidic solution, many highly charged cations, including Ce^{4+}, tend to undergo hydrolysis, which has a stabilising effect on unstable ions. Even at 1M H^+, some ceric ions

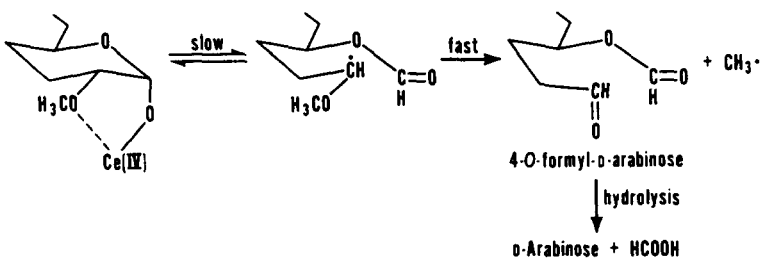
undergo the following hydrolytic reaction to produce ionic species less reactive than Ce^{4+} in oxidising alcohols (Rangaswamy & Santappa, 1969):

$$Ce^{4+} + H_2O \rightleftharpoons CeOH^{3+} + H^+$$

$$2CeOH^{3+} \rightleftharpoons (Ce\text{–}O\text{–}Ce)^{6+} + H_2O$$

Oxidation of vicinal diols by Ce(IV) is most easily explained by assuming the formation of alkoxy free radicals in solution as in Scheme II. Meyer & Roček (1972) postulated alkoxide intermediates in the oxidation of monohydric alcohols.

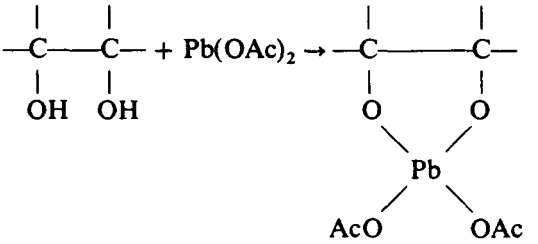
Evidence for chelation in the mechanism of diol oxidation has been provided by a study of the behaviour of D-glucose and 2-deoxy-D-glucose (Pottenger & Johnson, 1970). With no hydroxyl group at C-2, 2-deoxy-D-glucose is unable to form a chelate with a Ce^{4+} ion attached to O–1. This inability would explain why the oxidation rate of D-glucose, which can form a chelate intermediate, is thirty times that of the 2-deoxy compound. Significantly, 2-*O*-methyl-D-glucose is quite reactive (five times more reactive than D-glucose) (Pottenger & Johnson, 1970). This high reactivity suggests to me that the methoxyl group is able to participate in forming a chelate that undergoes cleavage (Scheme III). Both D-glucose and 2-deoxy-D-glucose yield D-arabinose.



Scheme III. Possible mechanism for the oxidation of 2-*O*-methyl-D-glucose by Ce(IV).

In D-glucose, oxidation by Ce(IV) involves hydroxyls at C-1 and C-2 whereas, in cellulose and starch, reaction occurs at C-2 and C-3. The reaction with starch has been investigated by Gugliemelli *et al.* (1972) because of current interest in Ce(IV)-initiated graft copolymerisation of vinyl monomers on starch.

There is a certain similarity in the proposed mechanism for Ce(IV) oxidation of vicinal diols and that proposed for Pb(IV) oxidation (Perlin, 1959) of vicinal diols. Both involve cyclic intermediates. In acetic acid, lead tetraacetate reacts with a *cis*-1,2-diol such as α-D-glucofuranose (Perlin, 1964), presumably in the following manner:



Lead tetraacetate oxidations of polyhydroxy compounds must be carried out in non-aqueous solvents to achieve good yields. In aqueous acetic acid, cellulose, amylose and amylopectin are only partially oxidised and the rate of reaction is slow (Abdel-Akher *et al.*, 1963). However, in methylsulphoxide (containing acetic acid), neutral polysaccharides are easily oxidised and the products are isolable in excellent yield by precipitation with alcohol (Zitco & Bishop, 1966). One of the main factors contributing to high reactivity in methylsulphoxide is high carbohydrate solubility. Where solubility is low and where the reaction mixture is consequently heterogeneous, consumption of $Pb(OAc)_4$ is poor.

(2) *In alkaline media:* The release of hydroxylic protons by neutral carbohydrates contributes greatly to complex stability. Unlike many highly charged transition-metal cations and certain lanthanide ions, the alkali metal, the alkaline-earth metal and trivalent lanthanide ions are unable to effect this release. In the presence of a base, however, polyhydroxy compounds undergo ionisation (Rendleman, 1973), which facilitates the formation of metal–alcoholate complexes.

(a) Cellulose in alkaline media: Studies with microcrystalline, water-insoluble, native-cotton cellulose (Haworth *et al.*, 1969; Rowland *et al.*, 1969, 1971, 1974) have indicated that the hydroxyls at C-2, C-3 and C-6 are selectively available rather than equally available for reaction. The reactions occur only on the surfaces of the elementary fibril. This selective availability has been attributed to the crystalline order and to the intramolecular and intermolecular hydrogen bonds that tie up almost all the C-3 hydroxyls and some C-6 hydroxyls, making them unavailable for reaction under only moderately alkaline conditions (i.e. 2M hydroxide or less).

Natural cellulose has a twofold helical conformation. Under the action of sodium hydroxide, it undergoes a series of changes that produce a crystalline material commonly called sodium cellulose II, which contains both Na^+ and OH^- in its lattice. X-ray fibre diffraction patterns (Whitaker *et al.*, 1974) indicate that the cellulose chains are threefold left-handed helices and are so arranged that six chains surround a hole containing sodium ions, hydroxide ions and water molecules. The proposed structure has the C-2 and C-3 hydroxyls pointing towards the central hole.

Because the stoicheiometry of reactions of alkali metal hydroxide with cellulose has been reviewed elsewhere (Rendleman, 1966*a*), there is no need to repeat here. It is

sufficient to say that stoicheiometry is variable, that 'stable' adducts (perhaps the products are mixtures of adduct and alcoholate) are formed whose combining ratios vary according to alkali concentration. For NaOH complexes, the reported ratios (D-glucose residue:metal ion) are 1:1, 2:1, 3:1, 3:2 and 4:3; for KOH, 1:1, 2:1, 3:2 and 4:3 and for RbOH and CsOH, 3:1.

Deserving at least brief comment is the Normann compound (Normann, 1906), often called sodium cupri-cellulose. It is water-insoluble and can be prepared by immersing cotton in concentrated NaOH solution containing dissolved cupric hydroxide. The structure of the compound is not known; however, careful analyses (Davidson & Spedding, 1958) have shown that the ratio of D-glucose residue to copper remains nearly 2:1 over a wide range of alkali concentrations (5–10N NaOH). The reactive Cu(II) species is probably the stable cuprate ion $Cu(OH)_4^{2-}$. However, the behaviour of such a negatively charged species towards polyhydroxy compounds has never received sufficient study to permit a determination of the mode of reaction. Perhaps the mechanism resembles that involved in the reaction of carbohydrates with oxyanions, such as germanate, tellurate, arsenate and borate. In numerous studies (Antikainen & Huttunen, 1973; Antikainen, 1974) these oxyanions were found to combine only with polyhydroxy compounds containing at least two vicinal diol groups. The interaction involves the removal of a hydroxylic proton from each polyhydroxy ligand to give anionic chelates of 1:1 or 1:2 metal–ligand ratio.

The reaction between cellulose and Cu(II) in strongly basic media appears to be rapid and reversible; such bases as KOH and concentrated ammonium hydroxide may be used in place of NaOH. The complexes prepared with different bases all generate characteristic EPR spectra (Hinojosa *et al.*, 1974). Reeves (1951) has discussed the interactions of Cu(II) in strong ammonium hydroxide solution with polyols to form cuprammonium complexes. He suggested that complexation occurs most readily with vicinal hydroxyl groups that are true *cis* with respect to each other (i.e. the dihedral angle formed by the two C–O bonds equals 0°). Complexation also occurs where the dihedral angle equals 60°, but it would be weaker than that for a true *cis* diol; there would be no complexation where the angles are 120° or 180°. The dihedral angles formed by the hydroxyls at C-2 and C-3 in the glucose residues of cellulose are 60°.

Aqueous alkaline solutions of an iron–sodium tartrate complex are known to dissolve cellulose (Jayme, 1971; Achwal & Chaugule, 1975). In this reaction, tartrate ion and ionised polysaccharide possibly function as co-ligands in such a way that the cellulose acquires the quality of high solubility. About this I can only conjecture, because no structure determinations have ever been attempted.

Perhaps closely related to the iron–sodium tartrate–cellulose 'complex' are some of the iron–dextran complexes (Murphy & Whistler, 1973), which have been studied intensively because of their potential use in the treatment of iron-deficiency

anaemia. They are soluble, non-ionic, non-dialysable and stable over a fairly wide pH range (4–11). The method of preparation may vary somewhat but, in essence, involves mixing dextran, ferric salt and alkali (e.g. Na_2CO_3) in the proper proportions. Some formulas include sodium citrate, which may well be bound to the same ferric ions to which dextran is bound.

(b) Amylose in alkaline media: Senti & Witnauer (1948) treated amylose with either LiOH, KOH or CsOH in 25% aqueous ethanol to obtain products having a 1:3 ratio of hydroxide to D-glucose residue. In the preparative medium, since these adducts are stable only over a limited range of hydroxide concentration, the stoicheiometry is variable. Champetier & Yovanovitch (1951) treated corn starch with aqueous sodium hydroxide of various concentrations and obtained adducts having 1:2, 1:1 and 2:1 ratios.

Banks *et al.* (1971) made what may be an important observation. They found that when KCl is added portionwise to an aqueous alkaline solution of amylose (pH 12), the viscosity number of the solution decreases with increasing concentration of salt (Fig. 1). A basic solution is necessary for the KCl to have this effect; salt alone does not cause this marked change. Iodine and 1-butanol have the ability to cause a viscosity decrease in neutral solution (Banks & Greenwood, 1971), and both are known to form helical complexes with amylose (Rundle, 1947; Rundle & Baldwin, 1943; Rundle & Edwards, 1943; Rundle & French, 1943*a* and 1943*b*). Addition of 1-butanol to an alkaline solution of amylose also causes a decrease in viscosity (Fig. 1).

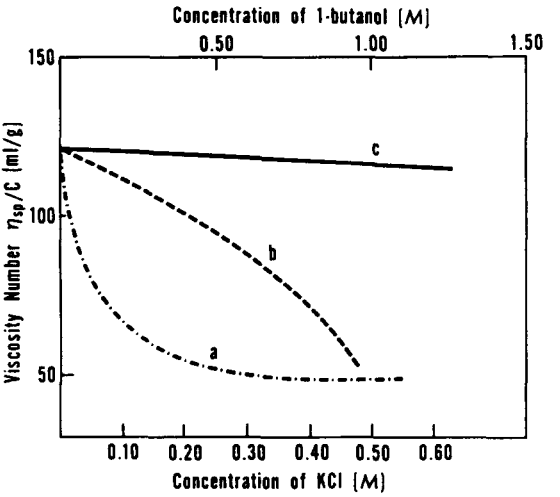
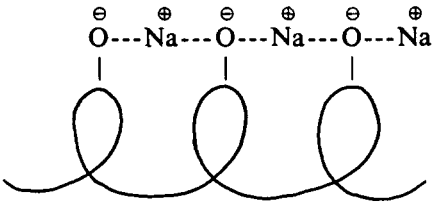


Fig. 1. Effect on viscosity number of amylose (in 0·01M KOH; pH 12) of adding (a) 3M KCl in 0·01M KOH, (b) 1-butanol and (c) 0·01M KOH (Banks & Greenwood, 1972). Experiment (c) is a control run in which the volumes of KOH solution added to the amylose solution are the same as the volumes of KOH–KCl solution added in experiment (a).

Banks & Greenwood (1972) argue convincingly that in a salt-free alkaline solution, the conformation of amylose should be predominantly random coil, not helical, and that in alkaline KCl solution, the conformation is helical. Ionisation of the polysaccharide, caused by the presence of base, would tend to 'expand' the chain because of repulsion between the anionic oxygens. Such repulsion would lead to increased viscosity, which has been observed by various investigators (Rao & Foster, 1963; Erlander & Purvinas, 1968). However, when increasing amounts of K^+ are added to an alkaline amylose solution, the viscosity number drops not to that value expected for a neutral solution of amylose but to an appreciably lower value. Part of the decrease must be due to 'neutralisation' of the negative charges through increased ion pairing. According to Banks *et al.* (1971), an additional contribution to the viscosity drop is probably made by some kind of complexation that imparts a helical character to the amylose chain. These workers suggest that within the helical complex there is an ionised hydroxyl group on each turn of the helix and that the ionised groups lie adjacent to each other and are co-ordinated to Na^+ ions in the fashion:



However, this proposed structure suffers from the unlikely assumption that each Na^+ can be bonded to more than one anionic oxygen. In my opinion, it is more likely that each Na^+ is co-ordinated to one anionic oxygen and at least two neighbouring hydroxyl groups.

In connection with the ideas expressed by Banks *et al.* (1971), I have presented in Table 1 some unpublished data concerning the effect of alkali metal cation on degree of interaction between high-amylose starch and hydroxide ion in aqueous solution.

TABLE 1

EFFECTS OF HYDROXIDE CONCENTRATION AND KIND OF CATION ON COMBINING RATIO FOR INTERACTIONS AT 25 °C BETWEEN HIGH-AMYLOSE STARCH AND ALKALI METAL HYDROXIDE[a]

Run	*Salt added*	*Salt concentration* (M)	*KOH concentration* (M)	*Millitres of reaction mixture*	D-*Glucose Residues/Cation*
1	None	—	0·011	61	11·2
2	LiCl	0·3	0·011	61	11·7
3	NaCl	0·3	0·011	61	6·94
4	KCl	0·3	0·011	61	6·40
5	None	—	0·06	11	5·8
6	KCl	0·3	0·06	11	4·9
7	KCl	0·3	0·25	11	4·5

[a] Each solution contains 0·09 g of anhydrous high-amylose starch (71% apparent amylose). For precipitation, 3 ml of ethanol was added for each millilitre of aqueous solution.

Metal hydroxide complexes of high-amylose starch were precipitated from aqueous solution by the addition of ethanol; the precipitates were washed with ethanol, dried at 25 °C under vacuum and then analysed for metal content by acid titration. (Since such complexes are highly unstable in aqueous methanol, washing with methanol would have led to erroneous conclusions.) Tests for Cl^- were negative ($< 1\ Cl^-$ per 400 glucose residues).

Although it is probable that the isolated complexes were mixtures of alcoholate and metal-hydroxide adduct, the products listed in Table 1 have been treated as if they were totally alcoholate. It is apparent from runs 1 to 4 that the addition of Li^+ has no significant effect on combining ratio, whereas the addition of Na^+ or K^+ has a considerable effect. Potassium ion has a slightly greater stabilising effect on the complex than does Na^+. In other words, complex stability increases with increasing cationic radius: $Li^+ \ll Na^+ < K^+$. This sequence is rather surprising because it is also the theoretically expected order of increasing ease with which water molecules are displaced from the primary co-ordination sphere of the cation. Yet the high basicity of ionised hydroxyl groups should lead to inner-sphere complexes between metal ion and anionic oxygen and the order of increasing stability of an inner-sphere complex would be $K^+ < Na^+ < Li^+$—exactly the reverse of what is obtained experimentally for alcoholates of high-amylose starch.

To explain the starch–alcoholate sequence of metal ions, there must be in operation a factor of overriding importance. Inner chelation might be that factor. The ability of the metal cation of an undissociated metal alcoholate to form a bond with a neighbouring hydroxyl group should increase in the order $Li^+ < Na^+ < K^+$, the order of increasing ease with which water molecules can be displaced from the primary hydration shell of the cation.

It is tempting to treat the combining ratios for the isolated complexes (Table 1) as though they are the actual ratios prevailing in aqueous solution before addition of ethanol. However, there is really no justification for making such an assumption since the presence of ethanol in the reaction media probably has a stabilising effect on the complexes. Nevertheless, a K:glucose ratio of 1:6 (run 4) was obtained at the same concentration of hydroxide and KCl that gave minimum viscosity in the study of Banks & Greenwood (1972) (Fig. 1). A considerably higher concentration of KOH (0·25M) does not greatly change the combining ratio. If it is assumed that the amylose chain is a sixfold helix, the Table 1 data indicate approximately one ionised hydroxyl group per helical turn. In solution the complex would be in a constant state of rapid formation and dissociation. Each glucose unit would, at some time, be participating in complexation as an oxyanion but, at any one instant, only one glucose unit in about six would be ionic. Such alcoholate formation should stabilise a helix, provided there is inter-residue chelation. In Fig. 2 is drawn a proposed chelate structure for a segment of amylose alcoholate. In this structure, I bonded the potassium ion to an anionic oxygen at C-1, to a neighbouring hydroxyl at C-2 and to a C-3 hydroxyl on an adjacent glucose unit.

In support of the proposition that inter-residue binding is involved are some data

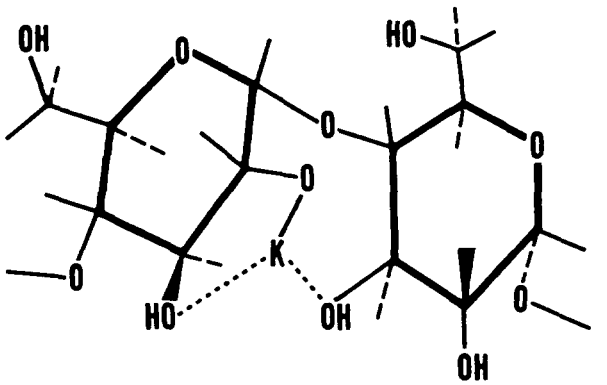


Fig. 2. Proposed inner-chelate structure of potassium alcoholate of amylose.

from studies of sugars in alcoholic media (Rendleman, 1966*c*). In many instances when a metal alcoholate is precipitated from ethanolic media containing an excess of sugar, the alcoholate binds a neutral (non-ionised) molecule of sugar. The ability of a given metal alcoholate to associate with a neutral carbohydrate ligand increases with increasing cationic radius: $Li^+ \ll Na^+ < (K^+, Cs^+)$. This is the order of increasing ease of displacing water from the hydration shell of the cation and also the order of increasing diameter and surface area of the cation.

Surface area should be important for accommodating multiple donor groups. The larger the area, the greater the capability. Cation diameter should also be a contributing factor because of variability in spacing between carbohydrate donor groups and because of variability in distance of closest approach (to the cation) of donor groups in one or more neutral carbohydrate molecules serving as additional ligands. Geometry of the carbohydrate is definitely a determining factor. For example, both sodium alcoholate and potassium alcoholate of D-glucose can associate with a neutral molecule of D-glucose; however, with D-mannose, only potassium alcoholate accommodates an extra ligand. Alcoholates of Li^+ either do not accommodate an additional ligand or are able to do so only with great difficulty. In amylose alcoholate, each glucose unit can conceivably function either as a neutral polyhydroxy ligand or as an alcoholate anion, or both. Inter-residue bonding through a metal ion would stabilise a helical conformation and therefore provide at least a partial explanation for the viscosity behaviour observed by Banks *et al.* (1971). Of the alkali metals, Li^+ would be expected to impart the least stability. Indeed, the observed order of increasing ability of a cation to lower the viscosity number of an amylose solution at pH 12 is $Li^+ < Na^+ < K^+$ (Banks *et al.*, 1971).

Alkaline-earth metal alcoholates of neutral polysaccharides have been little studied. Although the addition of an aqueous mixture of calcium hydroxide and calcium chloride, or barium hydroxide and barium chloride, or strontium hydroxide

and strontium chloride to an aqueous solution of high amylose starch gives an immediate precipitate of an alkaline-earth metal derivative, the stoicheiometry of the reactions has not been investigated.

When Cluskey (1953) investigated the reaction of barium hydroxide with dextran, he found that, as increasing amounts of $Ba(OH)_2$ were added to an aqueous solution of this polysaccharide, the viscosity of the solution became progressively smaller, followed by precipitation of a barium–dextran complex. Addition of HCl to the mixture destroyed the complex and the solution viscosity returned to a level approximating that of the original. Cluskey (1953) proposed that viscosity change and precipitation of the complex resulted from intermolecular and intramolecular bridging by $Ba(OH)_2$ molecules. Conceivably, bridging might also occur by the co-ordination of Ba^{2+} to two anionic oxygens (ionised hydroxyl groups) located on the same chain, as well as on separate chains. The precipitation of barium–amylose complex described in the preceding paragraph is possibly caused by similar intramolecular and intermolecular bonding.

B. *Anionic polysaccharides*

Even at low concentration, polyelectrolytes have a strong affinity for small counterions. To a large extent, especially with univalent cations like those of the alkali metals, this strong association is related to the linear charge density of the polyanion. Magnitude of charge density depends upon spacing of the ionised groups along the polymer chain and is related to the electrical potential that acts upon the counterions and affects counterion distribution between polyion and solution (Smidsrød & Haug, 1971; Liquori *et al.*, 1959). The linear charge density is expressed by the distance between the perpendicular projections of adjacent charged groups on the main axis of the molecule. The larger the distance between ionised groups, the weaker the interaction between counterion and polyion and the smaller, therefore, the degree of ion pairing. The activity coefficient of the counterion increases with decreasing degree of interaction or ion pairing. The chemical nature of the polymer chain seems to be of limited importance with regard to the effect of charge density itself. Also, concentration of polyelectrolyte has only a minor effect, if any, on ion activity (Liquori *et al.*, 1959; Ascoli *et al.*, 1961).

Kohn & Larsen (1972) studied calcium ion activities as a function of degree of polymerisation (DP) and found that polyguluronate and polygalacturonate have higher linear charge densities than polymannuronate. Activity coefficients for Ca^{2+} decrease with increasing DP up to a DP of 30. Beyond DP 30 there appears to be no significant change in the coefficient. The low charge density of polymannuronate probably stems from a more extended chain caused by the *equatorial–equatorial* linkages. X-ray diffraction studies of Li, Na, K and Ca salts of polymannuronate show a helix that is extended almost to the maximum and that contains three uronate units per turn (Mackie, 1971). The molecular repeat distance is about 15 Å. Similar information on calcium polyguluronate and calcium polygalacturonate is

not available at this time. However, the crystalline structures of the calcium salts are probably similar to those of the potassium salts for which data are available (Mackie, 1971). Potassium polyguluronate has a molecular repeat distance of 8·7 Å and a twofold screw axis (i.e. two uronate units per turn). Potassium polygalacturonate has a threefold screw axis and a molecular repeat distance of about 13·0 Å.

Compared with the calcium-ion activity coefficients of calcium polymethacrylate, the calcium-ion coefficients for calcium polyguluronate and pectate are lower (i.e. degree of binding is greater) than what would be expected merely on the basis of linear charge density (Kohn & Larsen, 1972). This difference indicates that linear charge density is not the only factor that controls interaction between polyion and counterions.

In gels, counterion–polyanion interaction is probably stronger than in solution, since about 99% of the calcium ions in calcium alginate gels appear to be bound to the polyanion at 22–25 °C (Katchalsky *et al.*, 1961), whereas in solutions of calcium polyguluronate no more than 92% are bound (Kohn & Larsen, 1972). (Note: Alginate is composed of mannuronate and guluronate units. Blocks of guluronate are responsible for the great affinity of Ca^{2+} for alginate. In solutions of calcium polymannuronate, there is considerably less binding of Ca^{2+} than in solutions of calcium polyguluronate (Kohn & Larsen, 1972).) These percentages are based upon the assumption that the measured activity coefficients ($\gamma_{Ca^{2+}} = 0{\cdot}01$ and 0·08 for alginate gel and polyguluronate solution, respectively) are an accurate reflection of the fraction of cations not involved in ion pairing with the anionic groups of the polymer. This difference in affinity between polyelectrolyte in the gel state and polyelectrolyte in solution may be looked upon as evidence for 'salt bridging' (i.e. the bonding of a polyvalent cation to two anionic groups on separate chains).

For purposes of comparison, the percentages of cation bound to alginate (Katchalsky *et al.*, 1961) at 22–25 °C in dilute solution (~0·01 mole of uronate residue per litre), as determined by Donnan equilibrium measurements, are: Na^+, 60%; K^+, 65%; NH_4^+, 73% and Mg^{2+}, 85%. In alginate *gels*: Ca^{2+}, 99%; Cu^{2+}, 97% and Ag^+, 97%.

The degree to which Ca^{2+} is bound to polymannuronate is essentially the same as the degree to which Ca^{2+} is bound to monomeric D-mannuronate and the activity of Ca^{2+} in these solutions differs little from that in a $CaCl_2$ solution. In contrast, Ca^{2+} binding in solutions of polyguluronate or polygalacturonate is considerably higher than that in solutions of the corresponding monomeric uronate. The respective activity coefficients for Ca^{2+} in 0·0015M solutions of D-galacturonate, D-glucuronate, D-mannuronate, D-guluronate and $CaCl_2$ are 0·72, 0·69, 0·70, 0·74 and 0·75 (Kohn *et al.*, 1968).

The preference for binding Ca^{2+} in a Ca^{2+}–K^+ exchange reaction is largely a function of polyanion geometry. In a competition between alkali metal ions and divalent ions for binding sites on a polymer chain, there may be sites that greatly

prefer divalent ions. The selectivity coefficient (K_K^{Ca}) for polymannuronate is low (4·2) compared with the selectivity coefficients for polyguluronate (70·8) and polygalacturonate (67·8) (Kohn *et al.*, 1968). As discussed in Section IB(1)(a) below, crosslinked pectin has a higher selectivity for Ca^{2+} than does non-crosslinked pectin.

Williams (1971) has suggested that the observed orders of stability for polyanionic complexes of alkali metals and alkaline-earth metals might be largely manifestations of packing problems in which the ratio of cation radius to anion radius is a major factor. In other words, there are sterically preferred ways in which anionic groups can be packed around cations and the size of the hole in which the cation resides can influence complex stability. For example, low complex stability would result if the anionic groups are so large, relative to the size of the cation, that anion–anion contact prevents good contact between cation and all potential donor groups. Low stability might also result if the ligand must adopt a less energetically favourable conformation in order for the cation to chelate with these groups.

There is little reason to doubt that the stability of crystalline complexes is influenced, perhaps even strongly sometimes, by the packing effect described above. However, in solutions, sols or gels, the contribution of a packing effect is less obvious since the structure of a polyelectrolyte solution might not even closely resemble that of a crystal lattice. Perhaps the expression 'steric fit' would be more appropriate than 'packing effect'. Steric fit would apply to both solids and non-solids and would embrace possible situations where packing is not so much an issue as is the ability of a cation to perform certain tasks that are related to cationic bulk and surface area such as: (a) bridging the distance between two or more fixed anionic groups; (b) forming bonds with nearby polar groups, as in inner chelation and (c) accommodating multiple donor groups. The third item applies especially to small cations whose limited surface areas are, for purely steric reasons, unable to accommodate as many bulky donor groups as can larger cations in the same periodic group.

Bregman (1953) has attempted to correlate the order of binding ability of the alkali metal ions with the ability of the cation to polarise such anionic groups as carboxylate and phosphonate on non-carbohydrate polymers. However, the extremely weak polarising ability of an alkali metal cation casts some doubt on the importance of polarisation. The same comment may be made about alkaline-earth metal cations. Although the polarising action of alkali and alkaline-earth metal ions probably contributes to metal–oxygen bond strength, the contribution is probably small compared to that of donor-group basicity.

Briefly, the magnitude of cation binding by a polyanion is influenced by a number of factors:

(a) Linear charge density.
(b) Effective nuclear charge. Polarising ability is related to the magnitude of this factor.

(c) Basicity and polarisability of the anionic donor group.
(d) Nature of the solvent.
(e) Inner chelation. Hydroxyl and other polar groups that are favourably located and suitably oriented for chelation with the metal cation can increase the stability of an ion pair.
(f) Intramolecular chelation where the metal cation is bonded to more than one anionic donor group located on the same polymer chain.
(g) Intermolecular binding of two or more anionic groups by a single cation (formation of salt bridges between separate chains). Such binding possibly occurs to an appreciable extent only where the separate chains are aggregated in microcrystalline bundles (Section I.B(1)(b) below). Conceivably, the folding of a chain may allow different portions of the same macromolecule to associate into similar microcrystalline bundles in which salt bridging can occur.
(h) Steric fit. This factor must be considered in conjunction with factors (e), (f) and (g).

(1) *Carboxylate-containing polysaccharides:* Although strength of binding of an alkali metal ion to an acetate ion decreases with increasing cationic (or crystallographic) radius, as shown by activity coefficient measurements (Robinson & Stokes, 1949), and although strength of binding of an alkaline-earth metal ion to a simple alkanecarboxylate (either with or without an α-hydroxyl group) is similarly related, with few exceptions, to cationic radius (Anon., 1964), the order of affinity of these cations for carboxylate-containing polysaccharides is often just the reverse (Cozzi *et al.*, 1969; Haug & Smidsrød, 1970). This tendency for a carboxylate-containing polysaccharide to bind preferentially the cation (of a given periodic group) with the greater ionic radius has led some workers (Cozzi *et al.*, 1969) to correlate the binding affinity of certain anionic polysaccharides, such as alginate, with the radius of the hydrated cation. (The size of the hydrated cation, which decreases with increasing ionic radius within a given periodic group, is discussed in Section I.B(3) below.) With non-carbohydrate polyelectrolytes, the most common binding sequence is that in which affinity decreases with increasing cationic radius. For example, with polyacrylate and polymethacrylate (either crosslinked or non-crosslinked) the order of decreasing affinity for alkali metal ions is $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$ (Kunin & Fisher, 1962); with Chelex 100 (a Bio-Rad resin containing aryl-$CH_2N(CH_2CO_2^-)_2$ groups) the order for alkali metal ions is $Li^+ > Na^+ > K^+$ and the order for alkaline-earth metal ions is $Ba^{2+} > Ca^{2+}$; but the affinity sequences for Bio-Rex 70 (a carboxylate-containing acrylic polymer resin, also made by Bio-Rad) are $K^+ > Na^+ > Li^+$ and $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$.

The variations in affinity order as described above are not readily explainable. One possible, but not very likely, explanation is that the basicity of the carboxylate donor group varies with the structure of the organic ligand. A strongly basic anionic donor would prefer to bind the cation with the smaller ionic radius and form a preponderant amount of inner-sphere complex. A weakly basic anionic donor might

prefer the cation with the smaller hydrated radius (i.e. larger ionic radius) since such donors would presumably form preponderant amounts of outer-sphere complex. However, it is unlikely that the basicity of a carboxylate group in a polymer is significantly different from that in a simple straight-chain alkanecarboxylate, because donor-group basicity in a straight-chain alkanecarboxylate (as judged by relative magnitudes of acid dissociation constants) varies little with chain length and differs insignificantly from that of cycloalkanecarboxylates. For example, the pK values for acetic acid, propionic acid, *n*-hexanoic acid, cyclopentanecarboxylic acid and cyclohexanecarboxylic acid at 25° are 4·76, 4·87, 4·86, 4·99 and 4·90, respectively (Dean, 1973). Furthermore, even where moderately large variation in pK occurs, as between acetic acid (pK = 4·76), citric acid (pK = 3·13 (Dean, 1973)) and gluconic acid (pK = 3·56 (Cannan & Kibrick, 1938)), there is little or no difference in affinity order for the alkaline-earth metal ions (Cannan & Kibrick, 1938; Dean, 1973).

Where differences in affinity sequences for simple carboxylate ions are encountered, they are almost always caused by the variable position of Mg^{2+}. For acetate ion, the sequence is $Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$ in which the difference in complexing ability between Ca^{2+} and Mg^{2+} is small; however, for the higher homologues, propionate and butyrate, the sequence follows exactly the order of increasing ionic radius: $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+}$ (Cannan & Kibrick, 1938). For α-hydroxy monocarboxylates, such as glycolate (2-hydroxyacetate), lactate (2-hydroxypropionate), glycerate (2,3-dihydroxypropionate) and gluconate, the sequences differ as to the position of Mg^{2+}, which seems to be influenced largely by the number of hydroxyl groups capable of participating as potential donors in inner chelation with an alkaline-earth metal ion. The affinity order for lactate ion is $Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$; for glycerate ion, $Ca^{2+} > Sr^{2+} > Mg^{2+} > Ba^{2+}$ and for gluconate ion, $Ca^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+}$ (Cannan & Kibrick, 1938). The position of Mg^{2+} varies because the affinity of Ca^{2+}, Sr^{2+} and Ba^{2+} increases and that of Mg^{2+} decreases in the order: lactate, glycerate, gluconate. Although the difficultly penetrable hydration shell of Mg^{2+} possibly contributes most towards preventing Mg^{2+} from forming inner chelates as easily as the larger alkaline-earth metal ions, the effects of steric fit cannot be ignored. The small size of the magnesium ion might make chelation with fixed donor groups on many ligands either sterically difficult or sterically impossible.

The binding order for alkaline-earth metal ions in complexation with simple dicarboxylate ions is much the same as that with monocarboxylate ions: $Mg^{2+} > Ca^{2+} > Sr^{2+} \sim Ba^{2+}$ (Cannan & Kibrick, 1938). Even though introduction of one or more hydroxyl substitutents into the ligand increases complex stability for all the ions, it causes the affinity of Mg^{2+} to be intermediate between that of Ca^{2+} and Sr^{2+}. The reason for the position of Mg^{2+} in affinity sequences for hydroxyl-containing dicarboxylates is probably the same as that provided for hydroxyl-containing monocarboxylates.

There is a more plausible explanation for the inverse order of cation affinity of

various carboxylate-containing polysaccharides. Presumably, complexation is predominantly of the inner-complex type and the influence of steric fit on complex stability overrides the effect of ligand basicity and polarisation. It is reasonable to assume that, to some degree at least, intramolecular binding and intermolecular binding (between associated chains) of two anionic groups by an alkaline-earth metal ion are functions of cationic size, or bulk, because of the importance of steric fit in a system where each ligand contains many donor groups and prefers to adopt the energetically most favourable conformation during complexation with counterions. Similarly, cationic bulk should be a determinant in the interaction of anionically bound counterions with hydroxyl or other polar groups located either on neighbouring residues or on separate chains. Inner chelation and intramolecular binding of two anionic groups by a cation are probably not in themselves responsible for the inverse order of affinity for polysaccharides, as evidenced by the close similarity in the orders of affinity of alkaline-earth metal ions in reactions with such diverse unidonor and multidonor anions as acetate, lactate, citrate, oxalate, malonate and malate (Cannan & Kibrick, 1938; Dean, 1973).

Cozzi *et al.* (1969) have evidence from studies of alginic acid, acetylated alginic acid and carboxymethylcellulose that complexation of counterions with hydroxyl groups influences complex stability and cation selectivity in carboxylate-containing polysaccharides. Two hydroxyl groups on a uronate residue are more stabilising than one.

Polyguluronate forms complexes with Sr^{2+} and Ba^{2+} that are stronger than the corresponding complexes of polymannuronate, polygalacturonate, oxidised cellulose, carboxymethylcellulose and polyacrylate (Haug & Smidsrød, 1970). The reason for this difference in binding strength probably lies in an ability of Sr^{2+} and Ba^{2+} to co-ordinate with a greater number of oxygens in polyguluronate than in the other polyanions. Grant *et al.* (1973) have shown from computer model building, at least in intramolecular complexation, that there are a greater number of co-ordination sites for polyguluronate than for polygalacturonate. Their calculations also showed that the potential donor oxygens in polyguluronate are more widely spaced (typically 3·8 Å) than in polygalacturonate ($\sim$3·4 Å), which might explain, from the standpoint of steric fit, why polyguluronate shows a strong preference for Sr^{2+} in a Sr^{2+}–Ca^{2+} exchange reaction whereas polygalacturonate does not (Haug & Smidsrød. 1970).

In the presence of Cu^{2+} ions all carboxylate-containing polymers form gels and the preference for Cu^{2+} in Cu^{2+}–Ca^{2+} exchange reactions is high ($K_{Ca}^{Cu} \geq 8$) for a large number of these polyelectrolytes (Haug & Smidsrød, 1970). This behaviour is not surprising since simple complexes of Cu^{2+} are known to be much more stable than those of Ca^{2+}.

The effects are compared in Table 2 of two hydroxyl groups, one hydroxyl group, and no hydroxyl group on the ability of a cation to displace a carboxylic proton from alginic acid and acetylated alginic acid of different degrees of acetylation (DA). The

greater the decrease in pH, the stronger the binding. Cation selectivity is greatest for alginic acid (which has two hydroxyls per uronic acid unit) and least for totally substituted alginic acid (no hydroxyl group). Carboxymethyl cellulose and monoacetylated alginic acid (each with only one available hydroxyl group) are intermediate in selectivity. From Table 2 it is obvious that the affinity of alkali metal ions for alginic acid decreases in the order $K^+ > Na^+ > Li^+$. However, with monoacetylated alginic acid (DA = 0·93), there is virtually no difference in affinity between these cations.

TABLE 2

EFFECT ON pH OF ADDING CATIONS TO AQUEOUS SUSPENSIONS OF CARBOXYLIC ACID POLYMERS[a]

The pH of mixture before salt addition: 2·97 for alginic acid (AA), 3·32 for acetylated alginic acid (AAA) of DA 0·93, 3·84 for AAA of DA 1·7, and 3·21 for carboxymethylcellulose (CMC). Equivalent ratio of salt to carboxyl group after salt addition = 1·43

Metal salt	*AA*	*AAA* (*DA* 0·93)	*AAA* (*DA* 1·7)	*CMC*
$AgNO_3$	2·42	2·67	2·72	2·82
Tl_2SO_4	2·64	2·80	2·86	3·06
CsCl	2·66	2·80	2·82	2·94
KCl	2·72	2·89	—	—
NaCl	2·80	2·90	—	—
LiCl	2·90	2·90	2·91	3·06
$Pb(NO_3)_2$	2·04	2·24	2·53	2·20
$Ba(NO_3)_2$	2·20	2·48	2·77	2·56
$Sr(NO_3)_2$	2·34	2·52	2·78	2·67
$CuSO_4$	2·40	2·47	2·59	2·56
$Cd(NO_3)_2$	2·41	2·46	2·57	2·58
$Ca(NO_3)_2$	2·44	2·68	2·78	2·66
$BeSO_4$	2·60	2·74	2·76	2·88
$ZnSO_4$	2·61	2·76	2·78	2·85
$CoSO_4$	2·61	2·75	2·78	2·84
$NiSO_4$	2·62	2·76	2·78	2·84
$MnSO_4$	2·72	2·78	2·80	2·85
$MgSO_4$	2·74	2·88	2·96	2·88
$InCl_2$	1·98	2·18	2·23	2·28
$Ce(NO_3)_2$	2·12	2·36	2·46	2·54
$CrCl_3$	2·18	2·41	2·48	2·60

[a] Cozzi *et al.* (1969).

Detailed structures for two crystalline forms of sodium hyaluronate, obtained by Guss *et al.* (1975) by means of x-ray diffraction data and computer model building techniques, provide additional evidence for participation of hydroxyl groups in the binding of metal ions by anionic polysaccharides. Hyaluronic acid is a glycosaminoglycan that is present in the intercellular matrix of most vertebrate connective tissues and in some bacterial capsules. One crystalline form of the sodium salt contains no detectable water and is composed of chains, each of which is a left-handed, four-fold helix of disaccharide units. Intramolecular hydrogen bonds contribute to stabilisation of chain conformation at each glycosidic linkage.

Octahedrally co-ordinated sodium ions are linked to neighbouring polysaccharide chains by $O...Na^+...O$ bridges and neighbouring chains are further linked by hydrogen bonds. Introduction of water molecules into the salt causes a change in crystalline form that is accompanied by some loss of $Na^+...O$ binding and hydrogen bonding.

Because pectins and alginic acid are industrially important, considerable research has been devoted to studying their structure and their chemical behaviour as ion exchangers and gel formers. A better understanding of these polyelectrolytes means a better understanding of the biological function of carboxylate-containing polysaccharides.

(a) Pectic substances: Pectic substances is the collective name given to a group of complex polysaccharides possessing a common backbone of α-1,4-linked D-galactopyranuronic acid residues (Cl chair conformation). Usually present are varying amounts of D-galactose, L-arabinose, L-rhamnose and occasionally trace amounts of other sugars. Composition varies with source and conditions of isolation. Most pectic substances contain about 70–80% of D-galacturonic acid residues and their gel-forming properties arise from the D-galacturonan backbone. A proposed threefold helical structure (Rees, 1969) of sodium pectate is based upon x-ray diffraction data (Fig. 3(a)).

Pectic substances occur widely in plants and are of great import to the food industry because of the unusual ability of their solutions to yield thermoreversible gels in the presence of calcium ion or in the presence of dehydrating agents (e.g. sugars) at a low pH (~3). None of these conditions of pH, dehydrating agent or calcium ion is necessary for gelation if the pectic substance is completely esterified; since commercial pectins are never completely esterified, their gelling properties are sensitive to pH. Adding a suitable acid, such as citric or tartaric acid, produces a low pH that promotes gelation, presumably by suppressing ionisation of carboxyl groups. Without added acid, the ionised carboxyls would cause electrostatic repulsion between the polymer chains and thereby prevent the formation of junction zones necessary for gel network formation.

The function of Ca^{2+} in the gelation of pectic substances has never been clarified. In regard to the possible contribution of complexation to gelation, one expressed view (Rees, 1969) has been that: (a) chelation of Ca^{2+} by suitably arranged carboxylate and hydroxyl groups on separate chains could not be involved because of a contention that hydroxyacids form only weak chelates with alkaline-earth cations and that (b) divalent Ca^{2+} should not be able to bridge two oxyanionic sites located on separate chains. It was emphasised that were the attraction of Ca^{2+} for negatively charged groups as strong as has sometimes been suggested in the literature, $MgSO_4$, $ZnSO_4$ and $Fe_2(SO_4)_3$ would be extremely insoluble in water. Not mentioned in this argument was the low solubility of $CaSO_4$ (0·0154 mole/litre at 25 °C), $CaCO_3$ ($1{\cdot}5 \times 10^{-4}$ mole/litre at 25 °C), or calcium oxalate ($1{\cdot}9 \times 10^{-4}$ mole/litre at 25 °C). Malonate ion also functions as a strong bidentate ligand for calcium ion. Alkanedicarboxylates containing α-hydroxyl groups form chelates that

are more stable than those without hydroxyls. To be sure, there are no proven examples of an alkaline-earth metal cation being bonded to two anionic groups on separate polymer chains in aqueous media. But neither is there proof against such bonding. Present means of analysis have so far been incapable of resolving the question.

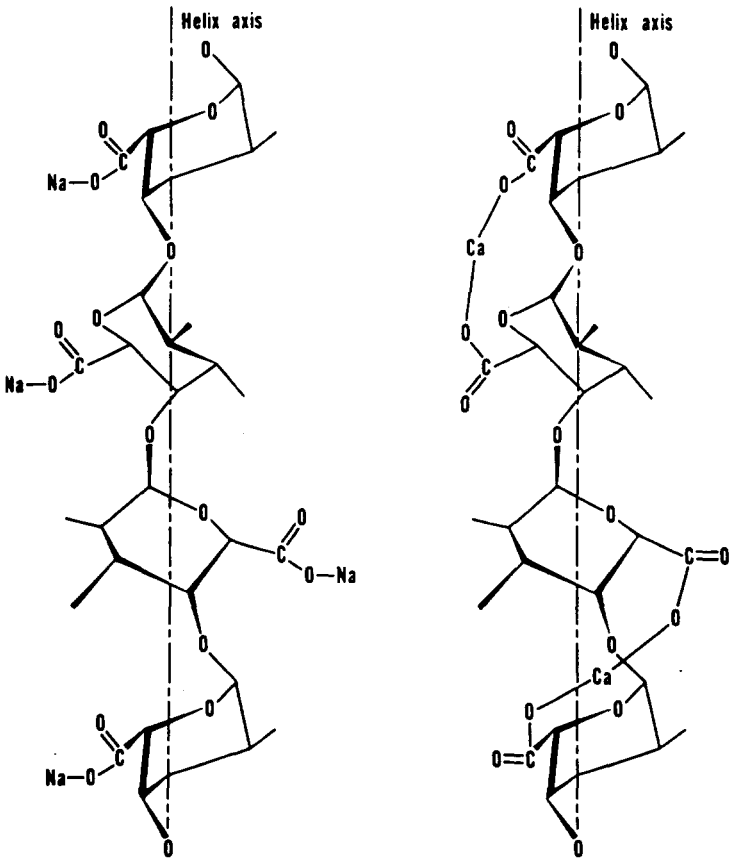


Fig. 3. (a) Probable structure of sodium pectate (Rees, 1969); (b) proposed structure of calcium pectate.

It seems reasonable that if two chains of calcium pectate are sufficiently close to each other, drawn together by those same forces that are operative in forming junction zones in completely esterified pectin (or in pectic acid at low pH), there would be a good possibility for the formation of salt bridges. Hydroxyl groups and

carboxylate groups might both be involved in interchain interactions. Each individual salt bridge need not be strong for it would be only one of a number of similar links along a section of associated chains. The effect of all the individual contributions of calcium bridges on junction-zone stability could be large.

Even if interchain bridging were impossible, there is nothing about the geometry of the pectate molecule that would prevent the formation of intrachain bridges. Figure 3(b) shows a proposed structure of calcium pectate based upon examination of Dreiding molecular models. Even though Ca^{2+} is involved in chelation with two carboxylate groups on adjoining residues, the structure is essentially identical with that of sodium pectate (Fig. 3(a)). In either sodium pectate or calcium pectate, hydroxyl groups are not favourably positioned for inner chelation with the metal ion. Distance between the two anionic oxygens (centre to centre) in calcium pectate is 4·9 (±0·2) Å. This O–O distance is expected on the basis of covalent Ca–O bonds. (The respective atomic radii of Ca and O are 1·74 Å and 0·74 Å; therefore, a covalent Ca–O bond should have a length of 2·48 Å.) Covalent Ca–O bonds occur in crystalline calcium arabonate where the distance between calcium and carboxylate oxygen is about 2·48 (±0·04) Å (Furberg & Helland, 1962).

In examining a molecular model of calcium pectate, Kohn & Furda (1968) observed an apparent O–O distance of 5·5–5·8 Å. This distance is considerably greater than the one I report (4·9 ± 0·2 Å), which is the average of four O–O distances in a model containing five D-galacturonate residues. The high value obtained by Kohn and Furda led them to believe that intramolecular chelation of Ca^{2+} by two carboxylate groups is impossible. My measurements indicate that the carboxylate–carboxylate distance is sufficiently close for chelation in which the Ca–O bonds have a high degree of covalent character.

From an examination of pectate gel structure by means of computer model building, Morris (1973) suggested that a number of polygalacturonate chains associate in such a fashion that the cations are accommodated in regions between the chains. Chain association probably occurs only between homopolymeric chain sections and complete aggregation is prevented by the presence of structural irregularities, such as chain branching or rhamnose segments. These microcrystalline regions (or bundles) of associated chains would constitute the junction zones necessary for gel network formation.

Tibenský (1968) has shown that pectin crosslinked with methylene bridges has a much higher selectivity for Ca^{2+} in Ca^{2+}–K^{+} exchange than has non-crosslinked pectin. Furthermore, the greater the degree of esterification, the lower the selectivity. This condition is what would be expected on the basis of chelation effects. The methylene bridges increase the probability of calcium bridging between anionic sites on separate chains. Monovalent K^{+} cannot form salt bridges between anionic sites. An increase in the degree of esterification of crosslinked pectin decreases the number of anionic sites available for bidentate chelation of Ca^{2+}, whether the chelation is interchain or intrachain.

Calcium ion activities have been determined for solutions (or suspensions) of calcium pectinates of different degrees of esterification (Kohn, 1968). The greater the degree of esterification, the greater is the activity of the Ca^{2+} ions. This behaviour can be interpreted to mean that the probability of inter-residue chelation along the chain diminishes with increased separation of ionised carboxyl groups. However, another interpretation of the higher Ca^{2+} activity would be a decline in ion-pair stability caused by a diminished linear charge density. Undoubtedly, linear charge density is significant, although it may not be the only factor involved.

The activity of Ca^{2+} in a solution of calcium D-galacturonate is about 16% higher than that in a solution of highly esterified pectate (degree of esterification (DE) 95%) (Kohn, 1968). The lower activity of Ca^{2+} in the esterified pectate can be rationalised if the assumption is made that some degree of chain association occurs and that this association allows a significant amount of salt bridging.

Studies of cation activity have shown that in 95% esterified calcium pectate, no more than 39% of the calcium ions are bound to the polyanion. In non-esterified pectate, essentially 100% are bound (Kohn & Furda, 1968).

Coagulability of pectic substances by metal ions is a function of molecular weight, DE and the kind of cation (Deuel & Sohms, 1954; Joslyn & De Luca, 1957). Deuel & Sohms (1954) found that pectinates of low DE (0–10%) are readily coagulated by monovalent cations. Above 10%, this facility does not occur. Ca^{2+} and Mg^{2+} effect coagulation up to 40 to 50% esterification, but not beyond. Cu^{2+} and Al^{3+} will cause coagulation up to 90% esterification. These observations suggest that polyvalent cations differ from monovalent cations largely because of the ability of the former to form salt bridges. The ability of the cation to form bridges and, hence, three-dimensional networks would be expected to increase with increasing cation valency.

(b) Alginic acid: Alginic acid is a long, linear copolymer whose salts are important because of their thickening, suspending, emulsifying, stabilising, gel-forming and film-forming properties (McNeely & Pettitt, 1973). The acid is composed of blocks of β-1,4-linked D-mannopyranuronic acid and α-1,4-linked L-gulopyranuronic acid (Cl and 1C chair conformation, respectively). These blocks may be $(—M)_n$, $(—G—)_n$, or $(—M—G—)_n$, where M and G are, respectively, D-mannuronic acid and L-guluronic acid residues (Haug *et al.*, 1966). The proportions of these block polymers may vary according to the physical requirements of the plant tissue. The physical properties of metal alginates—such as viscosity, gel formation and ion selectivity—depend upon the proportions of mannuronate and guluronate residues in a given sample (Haug *et al.*, 1966); however, the relationship between these properties and molecular structure is poorly understood.

Similar to the gels of metal pectates and pectinates, the gels of metal alginates probably have a structure in which the junction zones (microcrystalline bundles) are composed of associated segments of homopolymer (Fig. 4). In alginates, these homopolymer segments are probably blocks of guluronate units, because polymers

that are rich in guluronate have a greater affinity for divalent ions than do polymers that are rich in mannuronate (Haug, 1959; Haug & Smidsrød, 1965*a* and *b*). High guluronate content increases selectivity of alginate for strontium in mixtures of Sr^{2+} and Ca^{2+} (Haug & Smidsrød, 1967) and for calcium in mixtures of Ca^{2+} and Mg^{2+} (Haug & Smidsrød, 1965*b*). Selectivity of alginates in many ion-exchange reactions is associated solely with guluronate units in the polymers. Both polygalacturonate and polyguluronate have a much higher selectivity for Ca^{2+} than has polymannuronate (Kohn *et al.*, 1968). Because binding of Ca^{2+} by monomeric uronates is weak in relation to binding of Ca^{2+} by polyuronates, calcium-binding properties of polyuronates are necessarily due to the polymeric nature of the polyelectrolyte. Differences in binding property between alginate and pectate are probably caused by differences in stereochemistry.

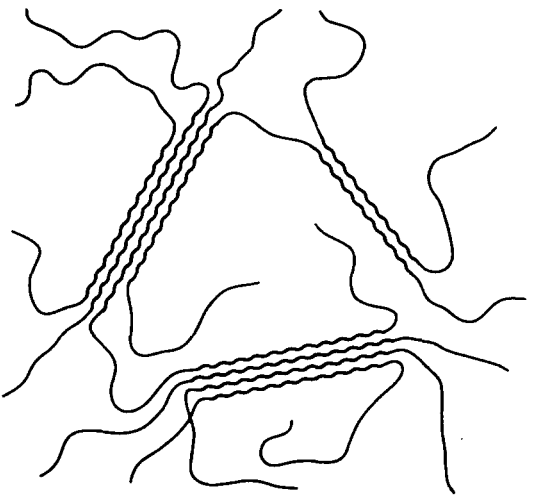


Fig. 4. Alginate gel structure (Morris, 1973).

Significantly, an enzymic conversion of mannuronate units into guluronate units has been reported (Haug & Larsen, 1969). In this conversion, the presence of Ca^{2+} is required, suggesting that the greater affinity of Ca^{2+} for guluronate units is probably involved.

The mechanism of interaction of a metal ion with alginate seems to be one in which the polyanion behaves as a chelating agent. The two vicinal hydroxyl groups at C-2 and C-3 have a significant influence on cation affinity, as shown by thin-layer chromatography and pH studies (Table 2) on acetylated and non-acetylated alginic acid (Cozzi *et al.*, 1969). In the chromatographic investigations, it was demonstrated that the affinity of alginic acid for a metal ion is greater than that of acetylated alginic acid, regardless of cationic charge. The cations studied were Ag^{+}, Tl^{+}, Cu^{2+}, Zn^{2+},

Ni^{2+}, Pb^{2+}, Mg^{2+}, Ba^{2+}, Ga^{3+} and In^{3+}. The importance of hydroxyl groups was also shown by Haug & Smidsrød (1970) who, in ion-exchange studies, obtained a selectivity coefficient K^{Ca}_{Mg} of 40 for polyguluronate and 1·0 for acetylated polyguluronate.

Affinity of alginic acid for cations can be correlated with cation radius and was shown (Cozzi *et al.*, 1969) to decrease as follows for ions of the same periodic group:

$$Cs^+ > K^+ > Na^+ > Li^+ \qquad Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$$
$$Cd^{2+} > Zn^{2+}$$

Affinity for transition-metal cations decreases in the order Cu > Ni, Co, Zn > Mn, which roughly parallels the Irving–Williams order of decreasing chelate stability. Lead(II) is considerably more reactive than Cu(II) (Haug & Smidsrød, 1970).

EPR spectra (Oakes & Child, 1973) show that Cu^{2+} is bound to alginate in preference to Ca^{2+} and that Ca^{2+} is bound more readily than Mn^{2+}. These differences in binding strength might explain the difference in gel strength between alginate gels of Cu^{2+} and Mn^{2+}. Copper ions are bound to the alginate chain in the polysaccharide gel. However, the immediate environment around Mn^{2+} is similar to the bulk liquid water in calcium alginate gels, a similarity indicating only weak bonding of Mn^{2+} to alginate.

Cozzi *et al.* (1969) correlated solubility of a metal alginate with ability of the cation to form intermolecular and intramolecular complexes. With many, but not all, metal ions, chelation involving hydroxyl groups appeared to be necesssary for precipitation. Schweiger (1962) also recognised the importance of hydroxyl groups. He observed that the addition of Ca^{2+} or other divalent cations to an aqueous solution of ammonium diacetyl alginate does not result in the precipitation of metal diacetyl alginate. Thus, two carboxylate groups alone are insufficient for precipitation. (However, it may be argued that steric factors, introduced by *O*-acetyl groups, are responsible for the solubility of metal diacetyl alginate.) Magnesium ion does not cause precipitation, even with non-acetylated alginate (Cozzi *et al.*, 1969).

Angyal (1973) suggested that gelation of calcium alginate may be caused by a type of chelation (Fig. 5). The calcium ion is co-ordinated to oxygen atoms at C-1, C-2 and C-3 of one uronate residue and to a carboxylate oxygen and ring oxygen of a neighbouring residue. Angyal favours a structure in which there is an *ax–eq–ax* sequence of oxygen atoms at the co-ordination site on one of the uronate residues. The divalency of the Ca^{2+} in the Fig. 5 structure could be balanced by co-ordination of the cation with a carboxylate group on a separate chain. Such an association of chains could lead to the kind of junction zone responsible for the gelling properties of calcium alginate.

(2) *Phosphate-containing polysaccharides:* There have been few studies of phosphate-containing polysaccharides for the purpose of determining cation affinities and selectivities. Holló *et al.* (1962) recognised that part of the cation-binding powers of starch granules relates to phosphate content of the starch molecule. In

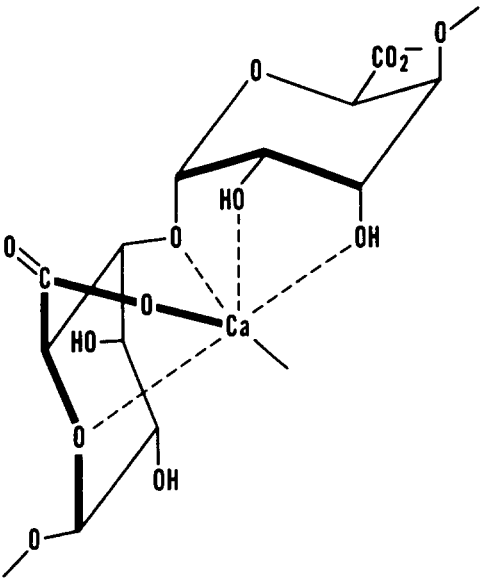
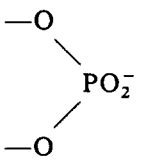


Fig. 5. Possible partial structure of calcium alginate (Angyal, 1973).

potato starch, phosphorus is chemically bound to amylopectin in the form of phosphate groups whereas, in corn or wheat starch, some phosphorus is present not only as chemically bound phosphate groups, but also as phospholipids that can be removed by solvent extraction. Potato starch has a much larger affinity for Ca^{2+} and Mg^{2+} than does solvent-extracted corn starch which possesses a lower phosphorus content. In neutral solution, at low cation concentration and 25 °C, the affinity of potato starch for Ca^{2+} is only about 5 % greater than that for Mg^{2+}, an indication that binding by the phosphate groups is not markedly influenced by difference in hydration spheres of the two cations. With increase in pH of the medium (i.e. with increasing alkalinity), affinity of starch for both cations increases. The affinity for Ca^{2+} increases at a slightly greater rate than that for Mg^{2+}. Undoubtedly, as alkalinity increases, participation of ionised hydroxyl groups becomes increasingly important.

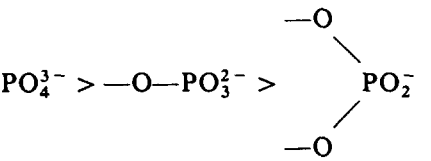
Wettstein *et al.* (1961) prepared crosslinked starch phosphate containing 62 % of



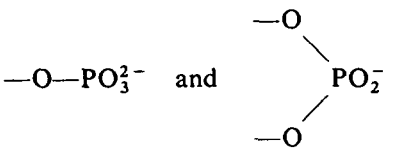
groups and 38 % of —O—PO_3^{2-} groups and found that selectivity for various

divalent cations increased in the order Ca < Ni < Zn < Cu, which follows the Irving–Williams sequence of stability for divalent transition metal ions. Furthermore, selectivity was greater for the divalent phosphate groups than for the monovalent phosphate groups. Although this difference in selectivity cannot be explained because of insufficient data, it might be related to a difference in availability of hydroxyl groups for chelation, as was suggested earlier to explain the selectivity differences between acetylated and non-acetylated alginic acid. The great difference in affinity of phosphate groups for Cu^{2+} and Ni^{2+} permits an easy separation of these two ions by ion-exchange chromatography on cellulose phosphate (Knight, 1959).

The acidity of phosphoric acid esters increases with increasing DE (Kumler & Eiler, 1943): H_3PO_4 < monoester < diester. Basicity of the conjugate bases decreases in the order



which might explain the difference between



with regard to binding affinity. In ion-exchange equilibria, the divalent phosphate group in starch phosphate binds Na^+ in preference to K^+, whereas the univalent phosphate group prefers K^+ over Na^+ (Wettstein *et al.*, 1961). Binding an alkali metal ion to the divalent group probably involves formation of an inner-sphere complex whereas binding to the univalent group possibly involves formation of an outer-sphere complex, the stability of which would increase with decreasing size of the hydrated cation. A hydrated K^+ ion is smaller in size than a hydrated Na^+ ion. The extremely limited amount of data on counterion binding by polysaccharide phosphates permits little additional speculation. Nevertheless, counterion binding by adenosine monophosphate and by linear inorganic polyphosphates (composed of univalent diester units) decreases in the order $Li^+ > Na^+ > K^+$ (Van Wazer & Callis, 1958). The apparent reversal of this order (at least with Na^+ and K^+) in ion-exchange reactions of univalent diester phosphate groups of starch phosphate suggests that relative binding affinities are influenced either by some as yet unrecognised factor which alters the effective basicity of polysaccharide phosphate groups or by steric-fit effects which determine whether cations may chelate easily

with hydroxyl donor groups. Perhaps significantly, the activity coefficient for NaH_2PO_4 is greater than that for KH_2PO_4 (e.g. 0·563 and 0·529, respectively, in a 0·5 molal aqueous solution at 25°) (Robinson & Stokes, 1949). This difference would indicate that the univalent dihydrogen phosphate ion has a greater affinity for K^+ than for Na^+ and suggests that binding is determined by the size of the hydrated cation. Unquestionably, considerably more research is needed in the area of metal–cation complexation with phosphate compounds.

(3) *Sulphate-containing polysaccharides:* On a sulphate-containing polyelectrolyte, fixation of weakly acidic cations often resembles formation of outer-sphere complexes in which the cation is separated from the anionic group by a solvent molecule. The cation and anion are held together in this manner by electrostatic forces; the smaller the radius of the solvated cation, the stronger the bond. A good example of an outer-sphere type of polyelectrolyte complex is the one formed by interaction of cobalt ammine ion $Co(NH_3)_6^{3+}$ with acid mucopolysaccharides in aqueous solution (Mathews, 1960). Ion pairing occurs with no loss of ammonia from the inner co-ordination sphere of the cation.

Stokes & Robinson (1948) and also Kielland (1937) showed that the size of hydrated alkali metal ions decreases in the order $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$; the size of hydrated alkaline-earth metal ions decreases in the order $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+}$. The order of decreasing radius of hydrated alkali metal and alkaline-earth metal ions is exactly the reverse of the order of increasing radius of non-hydrated ions. The affinity of alkali metal and alkaline-earth metal ions for donor groups of low basicity would be expected, in general, to decrease with increasing radius of the hydrated cation.

To illustrate, activity coefficients for alkali metal *p*-toluenesulphonates decrease in the order Li > Na > K (Robinson & Stokes, 1949), which order suggests that binding affinity increases similarly. Furthermore, affinity of sulphonate resins (having little or no crosslinking) for alkali metal ions increases in the order $Li^+ < Na^+ < K^+ < Rb^+ \leq Cs^+$ (Helfferich, 1962). The sulphonate group, like the sulphate group, is weakly basic.

In Cu^{2+}–Ca^{2+} ion-exchange studies, Haug & Smidsrød (1970) found that the selectivity coefficient K_{Ca}^{Cu} is approximately unity for the sulphated polymers fucoidan, dextran sulphate and carrágeenan. Because Ca^{2+} has a low affinity for sulphate polysaccharides (about the same as that of simple alkanecarboxylates), the K_{Ca}^{Cu}-value of ~ 1 must mean that the affinity of Cu^{2+} for sulphated polymers is also low and that Cu^{2+}, like Ca^{2+}, possibly forms weak outer-sphere complexes with sulphate groups. Copper ion forms strong, presumably inner-sphere, complexes with carboxylate groups.

By means of IR spectroscopy, Zundel (1969) obtained considerable information about the location of metal ions with respect to O atoms of sulphonate groups of dried samples of polystyrenesulphonate. The structural similarity between a sulphonate group and a sulphate group suggests that knowledge gained by studies of

sulphonate-containing polymers should be applicable to systems containing sulphated polymers. The fixed sulphonate ion $-SO_3^-$ has a pyramidal structure in which the three S–O bonds are substantially equivalent due to extensive bond resonance. When the cation is located in the near vicinity of the anion, both resonance and pyramidal symmetry of the anion are disturbed. This disturbance, which is detectable by IR spectroscopy, can occur only if the cation is situated asymmetrically with respect to the O atoms of the $-SO_3^-$ ion. Consequently, a cation must be associated with only one of the three oxygen atoms and this association must be in the direction of one of the S–O bonds. Spectra indicate that a cation is attached to every sulphonate group. A divalent cation, such as Mg^{2+}, would be linked to two sulphonate groups, which would account for the membrane-tightening action of polyvalent cations in biological systems. As water is introduced into dry samples of polystyrenesulphonate, cations remain with their anionic groups even at appreciable degrees of hydration (> five water molecules per cation). However, the bond between cation and anion becomes progressively weaker as the degree of hydration increases. The water molecules are located between the cation and neighbouring anionic groups in a network (Fig. 6).

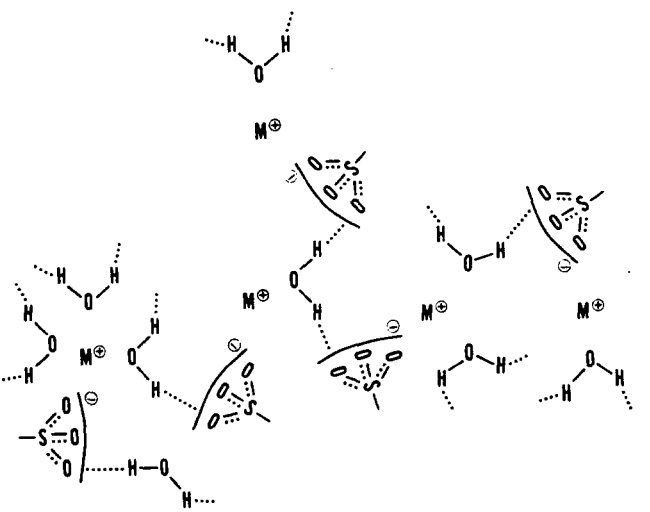


Fig. 6. Positions of metal ions and water molecules in hydrated polystyrenesulphonate (Zundel, 1969).

Cations having strong fields polarise the O–H bond of water molecules and strengthen the hydrogen bonds that link water molecules to neighbouring anionic groups. The stronger the field, the stronger the hydrogen bond. Furthermore, the ability of an O atom of the anionic group to form a hydrogen bond with a water molecule is increased by the cation field. Transition-metal ions interact strongly with

water molecules. However, alkali metal ions have extremely weak fields which allow only a weak network of water molecules between cations and anionic groups.

(a) Dextran sulphate and acid mucopolysaccharides: Satake *et al.* (1972) measured activity coefficients and found the order of decreasing affinity of dextran sulphate (1·35 sulphate groups per hexose residue) for alkali metal ions to be $K^+ > Cs^+ > Na^+ > Li^+$. The position of K^+, relative to Cs^+, has not been explained. Perhaps there are unrecognised steric effects that alter the normally expected sequence. At 0·1M concentration (based upon dissociable groups) and 25 °C, $\gamma_{Li^+} = 0{\cdot}233$, $\gamma_{Na^+} = 0{\cdot}224$, $\gamma_{Cs^+} = 0{\cdot}185$, $\gamma_{K^+} = 0{\cdot}169$ and $\gamma_{Ca^{2+}} \approx \gamma_{Mg^{2+}} = 0{\cdot}03$. These coefficients are probably close approximations to the fraction of cations not bound by the polyanion.

Viscosity measurements (Satake *et al.*, 1972) show that expansion of dextran sulphate chains in solutions of alkali metal salts decreases in the order $Li^+ > Na^+ > K^+ \approx Cs^+$. This order is essentially the same as the order of increasing affinity of metal ions for the polyanion. This parallel relationship is understandable because viscosity (which is a function of chain expansion) should decrease with decreasing charge density of the macromolecule. The lower the negative charge density, the lower the interionic repulsion responsible for polymer expansion. Oddly, measured activity coefficients for Mg^{2+} and Ca^{2+} are almost identical yet light-scattering and viscosity studies show that dextran sulphate expands more in the presence of Mg^{2+} than in the presence of Ca^{2+}. Smidsrød & Haug (1967) reported that the ability of Ca^{2+} to release protons from the acid form of dextran sulphate is slightly greater than that of Mg^{2+}. Apparently Mg^{2+} does have a lower binding affinity than Ca^{2+}, regardless of their similarity in measured activity.

There have been a number of studies of cation binding by sulphated mucopolysaccharides and the possible significance of these reactions in the calcification of cartilage. The chondroitin sulphate component is solely responsible for the cation-binding capacity of cartilage and the cation-binding reactions are of the ion-exchange type (Boyd & Neuman, 1951; Dunstone, 1959 and 1960).

Although selectivity sequencies may vary somewhat according to the composition of the polyelectrolyte, the following orders of increasing cation affinity in aqueous solution are perhaps representative: for chondroitin sulphate A, $K^+ < Na^+ < Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$ (Dunstone, 1962); for chondroitin sulphate B, $K^+ < Na^+ < Mg^{2+} < Ca^{2+}, Sr^{2+}, Ba^{2+}$ (Dunstone, 1962) and for heparin, $Li^+ < Na^+ < Cs^+ < NH_4^+ < Mg^{2+} < Ca^{2+} < Ba^{2+}$ (Jooyahdeh *et al.*, 1974). With the exception of the position of K^+ (relative to Na^+), these are the orders expected for increasing affinity of hydrated metal cations for a sulphated polymer. Although heparin and the chondroitin sulphates contain carboxyl groups in addition to sulphate groups, the ion-exchange behaviour of these substances resembles more the behaviour of a polymer containing only sulphate groups. Undoubtedly, both carboxyl and sulphate groups participate. Lowering pH of the medium to 2·0 considerably diminishes (as much as 90%) the affinity of a cation for chondroitin

sulphate (Mathews, 1960); this action discharges the carboxylate groups and decreases both the number of available binding sites and the linear charge density.

Acid mucopolysaccharides bound in cartilage have an affinity for alkaline-earth metal cations that is different from that of acid mucopolysaccharides in solution. In cartilage the order of increasing affinity is $Ba^{2+} < Sr^{2+}$, Ca^{2+}; in solution the reverse order is observed (Dunstone, 1962). This difference in order is not unexpected since the degree to which steric factors (such as packing effects) are involved in a solid ion-exchanger should differ greatly from that in a solution of polyelectrolyte molecules.

The affinity of an acid mucopolysaccharide for a cation increases with increasing linear charge density (Mathews, 1960). Chondroitin sulphate, which contains one sulphate group and one carboxylate group per disaccharide repeating unit, has less binding power than heparin containing two sulphate groups and one carboxylate group per disaccharide repeating unit. A heparin that contains three sulphate groups has a greater binding power than one containing only two sulphate groups. Hyaluronate, which contains one carboxylate group and no sulphate group per disaccharide repeating unit, ranks below chondroitin sulphate in affinity and in linear charge density.

TABLE 3

STABILITY CONSTANTS OF CALCIUM COMPLEXES OF VARIOUS SULPHATED POLYSACCHARIDES AT pH 7·2 AND $\mu = 0{\cdot}15$[a]

Ligand	*No. of anionic groups per disaccharide unit*		*K*[b]	*log K*
	Sulphate	*Carboxylate*		
Heparin	2	1	123	2·09
Chondroitin sulphate A	1	1	16	1·20
Chondroitin sulphate B	1	1	25	1·40
Bovine nasal cartilage	—	—	15	1·18
Dextran sulphate	0·2	0	22	1·34

[a] Buddecke & Drzenieck (1962).

[b] Determined spectrophotometrically with murexide as competing ligand. The calculated values are based upon the disaccharide unit as ligand.

Buddecke & Drzenieck (1962) have provided stability constants for complexes of calcium with various acid mucopolysaccharides (Table 3). Measurements for these constants were made at physiological concentrations of Ca^{2+} ($\sim 2 \times 10^{-3}$M). The high stability constant for the calcium complex of chondroitin sulphate suggests that simultaneously high concentrations of chondroitin sulphate and calcium in mammalian tissue are causally related and that the chondroitin sulphate behaves as an ion exchanger and fixes extracellular calcium.

Affinity for Na^+ is approximately the same as that for K^+, whether the polyanion is a sulphated polymer or a carboxylate-containing polysaccharide. Potassium ion shows only a slightly greater tendency for ion-pair formation than does sodium ion

(Table 4). Of particular interest is the absence of any obvious relationship between degree of ion-pair formation and solubility of sodium and potassium complexes of acid polysaccharides (either sulphate- or carboxylate-containing polymers). Potassium ions have a strong precipitating effect on sulphate-containing polysaccharides (such as dextran sulphate and carrageenan), whereas sodium ions do not (Smidsrød & Haug, 1967). Yet, as can be seen in Table 4, the difference between Na^+ and K^+ in ability to form ion pairs is small. With carboxylate-containing polysaccharides (e.g. carboxymethyl dextran and alginate) on the one hand the difference between Na^+ and K^+ in precipitating ability is slight. On the other hand, divalent ions in general have a strong precipitating effect on carboxylate-containing polysaccharides. With dextran sulphate, Ca^{2+} and Mg^{2+} have about the same effect as K^+, although the affinity of K^+ for the polyanion is less than that of Ca^{2+} and Mg^{2+} (Smidsrød & Haug, 1967).

TABLE 4
DEGREE OF ION-PAIR FORMATION WITH POTASSIUM AND SODIUM[a]

Polyelectrolyte	*Anionic group*	*Percentage of anionic groups*[b] *bonded to metal cations*	
		Potassium[c]	*Sodium*[c]
Dextran sulphate	Sulphate	81·5	77·5
Carrageenan	Sulphate	38·5	36·5
Carboxymethyldextran	Carboxylate	73·8	68·5
Alginate	Carboxylate	58·8	53·5

[a] Smidsrød & Haug (1967).
[b] Concentration of total anionic groups, ~0·01N.
[c] Determined by cation-selective electrode.

As an explanation for the differences in the precipitating effect of different cations, Strauss *et al.* (1957) have suggested that different ion pairs located on a polymer chain have different solvent affinities; the lower the solvent affinity, the lower the solubility of the polymer. This theory holds for monovalent alkali metal ions but does not hold well for divalent ions. Smidsrød & Haug (1967) picture the decreased solubility effected by divalent ions as being caused by increased interaction between polymer chains. To illustrate, alginate forms strong gels with divalent ions but not with alkali metal ions; carrageenan forms strong gels with K^+, Rb^+ and Cs^+, but only weak gels with Na^+ and Li^+ (Towle, 1973). In the carrageenan complex, ion pairs containing Na^+ and Li^+ are probably strongly solvated, whereas those containing K^+, Rb^+ or Cs^+ are not. Sulphated cellulose also gels in the presence of K^+, Rb^+ or Cs^+, but not in the presence of Na^+ (Schweiger, 1972); gel strength increases in the order $K^+ < Cs^+ < Rb^+$.

(b) Carrageenan: Carrageenan, the hydrocolloid from red marine algae, consists of several components (kappa, lambda, mu, iota and nu), each of which is a sulphated galactan (Towle, 1973). The major components are κ- and λ-carrageenan.

The kappa form, unlike the lambda form, possesses strong gelling characteristics and is predominantly a copolymer of a β-D-galactose-4-sulphate unit joined to a 3,6-anhydro-α-D-galactose unit by means of a β-1,4-linkage; this unit is, in turn, joined to another D-galactose-4-sulphate unit by means of an α-1,3 linkage. Occasionally the 3,6-anhydride unit contains a sulphate group at C-2 or is replaced by an α-D-galactose-6-sulphate unit or an α-D-galactose-2,6-disulphate unit. The exact structure of a κ-carrageenan chain may differ in important details according to the biological source of the sample. The term 'λ-carrageenan' is generally reserved for that component containing α-1,4-linked D-galactose-2,6-disulphate units and β-1,3-linked D-galactose units (some of which contain a sulphate group at C-2) (Rees, 1969).

In the presence of calcium, magnesium, potassium, ammonium, rubidium or caesium salts, κ-carrageenan produces strong, thermally reversible gels (Towle, 1973; Zabik & Aldrich, 1968). Light-scattering experiments show that κ-carrageenan may also gel in the presence of Na^+ or Li^+ (Payens & Snoeren, 1972), but the gels are extremely weak. The gelation mechanism is believed to involve an association of polymer chain segments at junction regions to give a three-dimensional network. Entrained in this network are water molecules, usually constituting more than 99% of the gel. The ordered, microcrystalline junctions are formed by chains intertwining into double helices, which then aggregate by some form of co-operative attraction. In the solid state, the existence of a carrageenan double helix has been shown by x-ray diffraction (Anderson *et al.*, 1969; Arnott *et al.*, 1974); in solutions and gels, its occurrence was revealed by studies of changes in optical rotation with change in temperature (Rees *et al.*, 1969) and by studies of helix-coil transitions of ι-carrageenan segments (Jones *et al.*, 1973).

In general, variation in concentration of polyelectrolyte, irrespective of the type of anionic group, causes little, if any, change in cation activity. This general rule holds for λ-carrageenan, but not for κ-carrageenan. The counterion activity coefficient for the kappa form increases with dilution (Podlas & Ander, 1969). This difference between the two carrageenan forms can be explained by a tendency for the kappa form to be less extended than the lambda form; with dilution, the chain of the kappa form becomes more extended, resulting in a lower linear charge density and, consequently, a larger γ_{cation}. Podlas & Ander (1969) report that the cation activity coefficients for λ-carrageenan are smaller than those for κ-carrageenan when the counterions are the same. This difference can be explained by the greater linear charge density on λ-carrageenan, a condition caused by the greater number of sulphate groups on the lambda form.

These same investigators reported that Na^+ is bound more strongly than K^+ by both kappa and lambda form. This statement is contrary to the results of Smidsrød & Haug (1967) (Table 4) who found that their sample of carrageenan bound K^+ more strongly than Na^+. In any event, the difference in binding between Na^+ and K^+ is small. Schachat & Morawetz (1957) found that carrageenan binds Mg^{2+} more

strongly than Ca^{2+}; this trend, like that found for Na^+ and K^+ by Podlas & Ander (1969), is contrary to what would generally be expected for a sulphated polymer. The situation with Na^+ and K^+ is not without precedence, however, since chondroitin A and B are known to bind Na^+ more strongly than K^+ (Dunstone, 1962).

Iota-carrageenan, like κ-carrageenan, is a gel former. Its structure is basically similar to that of the kappa form, with the exception that it possesses a sulphate group at C-2 of the anhydrogalactose unit. Because X-ray diffraction studies (Arnott *et al.*, 1974) of the calcium salt showed that most inter-helix distances are large, van der Waals forces and direct hydrogen bonding between double helices of this carrageenan cannot, by themselves, stabilise the crystal structure. However, ionic forces are important in holding the structure together. Each cation is co-ordinated to two sulphate groups, each from a different double helix. Each galactose sulphate contributes one oxygen to the co-ordination sphere of each of the two cations. The Ca–O distances are 2·6 and 2·7 Å. The anhydrogalactose sulphate groups are too far from the cations for inner-sphere co-ordination (shortest distance is 4·6 Å).

REFERENCES

ABDEL-AKHER, M., YOUSSEF, A.-M., GHALI, Y. & MICHALINOS, A. N. (1963). Oxidation of polysaccharides with lead tetraacetate. *J. Chem. U.A.R.*, **6**, 107–18.

ACHWAL, W. B. & CHAUGULE, D. A. (1975). Investigations in modified alkaline iron–tartrate complex solvent for cellulose. *Angew. Makromol. Chem.*, **42**, 77–89.

ANDERSON, N. S., CAMPBELL, J. W., HARDING, M. M., REES, D. A. & SAMUEL, J. W. B. (1969). X-ray diffraction studies of polysaccharide sulfates: Double helix models for κ- and ι-carrageenans. *J. Mol. Biol.*, **45**, 85–99.

ANGYAL, S. J. (1973). Complex formation between sugars and metal ions. *Pure Appl. Chem.*, **35**, 131–46.

ANON. (1964). *Stability constants of metal-ion complexes.* Chemical Society, London, Special Publication No. 17.

ANTIKAINEN, P. J. (1974). Spectrophotometric studies on chelate formation. Part II. Chelation of the germanate ion with fructose in aqueous alkaline solutions. *Finn. Chem. Lett.*, **4**, 159–64.

ANTIKAINEN, P. J. & HUTTUNEN, E. (1973). A comparative study on the chelation of pentoses with some inorganic oxyacids. *Suom. Kemistil. B*, **46**, 184–90.

ARNOTT, S., SCOTT, W. E., REES, D. A. & MCNAB, C. G. A. (1974). ι-Carrageenan. Molecular structure and packing of polysaccharide double helices in oriented fibers of divalent cation salts. *J. Mol. Biol.*, **90**, 253–67.

ASCOLI, F., BOTRÉ, C. & LIQUORI, A. M. (1961). On the polyelectrolyte behavior of heparin. I. Binding of sodium ions. *J. Phys. Chem.*, **65**, 1991–2.

BANKS, W. & GREENWOOD, C. T. (1971). Amylose: A non-helical biopolymer in aqueous solution. *Polymer*, **12**, 141–5.

BANKS, W. & GREENWOOD, C. T. (1972). The conformation of amylose in alkaline salt solution. *Carbohydr. Res.*, **21**, 229–34.

BANKS, W., GREENWOOD, C. T., HOURSTON, D. J. & PROCTER, A. R. (1971). Amylose in aqueous solution—A viscometric study. *Polymer*, **12**, 452–66.

BARRY, J. A. HALSEY, G. D., JR. (1963). The interaction and noninteraction of ions with a natural polysaccharide. *J. Phys. Chem.*, **67**, 1698–701.

BELFORD, D. S., MYERS, A. & PRESTON, R. D. (1958). Electron diffraction study of adsorbed metal ions on the surface of cellulose microfibrils. *Nature*, **181**, 1516–18.

BELFORD, D. S. & PRESTON, R. D. (1960). Copper–cellulose complexes. *Nature*, **185**, 911.

BELFORD, D. S., COOK, C. D., NEVARD. E. H. & PRESTON, R. D. (1957). Timber preservation by copper compounds. *Nature*, **180**, 1081–3.

BOYD, E. S. & NEUMAN, W. F. (1951). The surface chemistry of bone. V. The ion-binding properties of cartilage. *J. Biol. Chem.*, **193**, 243–51.

BREGMAN, J. I. (1953). Cation-exchange processes. *Ann. N.Y. Acad. Sci.*, **57**, 125–43.
BUDDECKE, E. & DRZENIECK, R. (1962). Stability constants of the Ca complexes of acid mucopolysaccharides. *Z. Physiol. Chem.*, **327**, 49–64.
CANNAN, R. K. & KIBRICK, A. (1938). Complex formation between carboxylic acids and divalent metal cations. *J. Am. Chem. Soc.*, **60**, 2314–20.
CHAMPETIER, G. & YOVANOVITCH, O. (1951). Alkali starches. *J. Chim. Phys.*, **48**, 587–9.
CLUSKEY, J. E. (1953). *The behavior of dextran, a bacterial polysaccharide, in alkaline solutions.* M.S. Thesis, Bradley University, Peoria, Illinois.
COZZI, D., DESIDERI, P. G. & LEPRI, L. (1969). The mechanism of ion exchange with alginic acid. *J. Chromatogr.*, **40**, 130–7.
CRONSHAW, J., MYERS, A. & PRESTON, R. D. (1958). Chemical and physical investigation of the cell walls of some marine algae. *Biochim. Biophys. Acta*, **27**, 89–108.
DAVIDSON, G. F. & SPEDDING, H. (1958). The absorption of copper by cotton cellulose from sodium cuprate solutions. The Normann compound. *J. Text. Inst.*, **49**, T621–6.
DEAN, J. A. (Ed.) (1973). *Lange's handbook of chemistry*, 5-15–5-35, New York, McGraw-Hill.
DEUEL, H. & SOHMS, J. (1954). Observations on pectic substances. *Adv. Chem. Ser.*, **11**, 62–7.
DUNSTONE, J. R. (1959). Some cation-binding properties of cartilage. *Biochem. J.*, **72**, 465–73.
DUNSTONE, J. R. (1960). Ion-exchange reactions between cartilage and various cations. *Biochem. J.*, **77**, 164–70.
DUNSTONE, J. R. (1962). Ion-exchange reactions between acid mucopolysaccharides and various cations. *Biochem. J.*, **85**, 336–51.
ERLANDER, S. R. & PURVINAS, R. M. (1968). The polyelectrolyte behavior of amylose and its helix-to-coil transition in aqueous alkaline solution. *Staerke*, **20**, 37–45.
FURBERG, S. & HELLAND, S. (1962). The crystal structure of the calcium and strontium salts of arabonic acid. *Acta Chem. Scand.*, **16**, 2372–83.
GRANT, G. T., MORRIS, E. R., REES, D. A., SMITH, P. J. C. & THOM, D. (1973). Biological interactions between polysaccharides and divalent cations: The egg-box model. *FEBS Lett.*, **32**, 195–8.
GRIFFITHS, C. (1949). Locust bean gum. *Manuf. Chem.*, **20**, 321–4.
GRIFFITHS, C. (1952). Locust-kernal gum. *Food*, **21**, 58–9.
GUGLIEMELLI, L. A., DOANE, W. M., RUSSELL, C. R. & SWANSON, C. L. (1972). Starch-cerium (IV) complexes in aqueous media: Formation, isolation, and stability. *Polym. Lett.*, **10**, 415–21.
GUSS, J. M., HUKINS, D. W. L., SMITH, P. J. C., WINTER, W. T., ARNOTT, S., MOORHOUSE, R. & REES, D. A. (1975). Hyaluronic acid: Molecular conformations and interactions in two sodium salts. *J. Mol. Biol.*, **95**, 359–84.
HAUG, A. (1959). Ion exchange properties of alginate fractions. *Acta Chem. Scand.*, **13**, 1250–1.
HAUG, A. & LARSEN, B. (1969). Biosynthesis of alginate. Epimerization of D-mannuronic to L-guluronic acid residues in the polymer chain. *Biochim. Biophys. Acta*, **192**, 557–9.
HAUG, A., LARSEN, B. & SMIDSRØD, O. (1966). A study of the constitution of alginic acid by partial acid hydrolysis. *Acta Chem. Scand.*, **20**, 183–90.
HAUG, A. & SMIDSRØD, O. (1965*a*). The effect of divalent metals on the properties of alginate solutions. II. Comparison of different metal ions. *Acta Chem. Scand.*, **19**, 341–51.
HAUG, A. & SMIDSRØD, O. (1965*b*). Fractionation of alginates by precipitation with calcium and magnesium ions. *Acta Chem. Scand.*, **19**, 1221–6.
HAUG, A. & SMIDSRØD, O. (1967). Strontium-calcium selectivity of alginates. *Nature*, **215**, 757.
HAUG, A. & SMIDSRØD, O. (1970). Selectivity of some anionic polymers for divalent metal ions. *Acta Chem. Scand.*, **24**, 843–54.
HAWORTH, S., JONES, D. M., ROBERTS, J. G. & SAGER, B. F. (1969). Quantitative determination of mixtures of alkyl ethers of D-glucose. Part II. Structural studies of partially methylated cotton cellulose. *Carbohydr. Res.*, **10**, 1–12.
HELFFERICH, F. (1962). *Ion exchange*, 159, New York, McGraw-Hill.
HINOJOSA, O., ARTHUR, J. C., JR. & MARES, T. (1974). Electron spin resonance studies of interactions of ammonia, copper and cupriammonia with cellulose. *J. Appl. Polym. Sci.*, **18**, 2509–16.
HOLLÓ, J., HUSZÁR, J., SZEJTLI, J. & PETHŐ, M. (1962). Recent contributions to the chemistry of starch fractions. XII. Study of the adsorption capacity of starch granules for Ca and Mg ions. *Staerke*, **14**, 343–7.
JACKOBS, J. J., BUMB, R. R. & ZASLOW, B. (1968). Crystalline structure in oriented fibers of KBr-amylose. *Biopolymers*, **6**, 1659–70.
JAYME, G. (1971). Investigations of solutions. A. New solvents. In *Cellulose and cellulose derivatives, Part IV*, (Bikales, N. M. & Segal, L. (eds.)), 381–410, New York, Wiley-Interscience.
JONES, R. A., STAPLES, E. J. & PENMAN, A. (1973). A study of the helix-coil transition of *ι*-carrageenan segments by light scattering and membrane osmometry. *J. Chem. Soc., Perkin Trans.*, **2**, 1608–12.

JOOYAHDEH, F., MOORE, J. S. & PHILLIPS, G. O. (1974). Polyanions and their complexes. Part IX. Binding affinities of inorganic ions to heparin. *J. Chem. Soc., Perkin Trans.*, **2**, 1468–71.

JOSLYN, M. A. & DE LUCA, G. D. (1957). The formation and properties of aluminum pectinates. *J. Colloid. Sci.*, **12**, 108–30.

KATCHALSKY, A., COOPER, R. E., UPADHYAY, J. & WASSERMANN, A. (1961). Counter-ion fixation in alginates. *J. Chem. Soc.*, 5198–204.

KIELLAND, J. (1937). Individual activity coefficients of ions in aqueous solutions. *J. Am. Chem. Soc.*, **59**, 1675–8.

KNIGHT, C. S. (1959). A two-dimensional paper chromatographic method combining ion-exchange and partition techniques. *Nature*, **183**, 165–7.

KOHN, R. (1968). Binding of calcium to carboxyl groups of pectin. *Zucker*, **21**, 468–75.

KOHN, R. & FURDA, I. (1968). Binding of calcium ions to acetyl derivatives of pectin. *Collect. Czech. Chem. Commun.*, **33**, 2217–25.

KOHN, R., FURDA, I., HAUG, A. & SMIDSRØD, O. (1968). Binding of calcium and potassium ions to some polyuronides and monouronates. *Acta Chem. Scand.*, **22**, 3098–102.

KOHN, R. & LARSEN, B. (1972). Preparation of water-soluble polyuronic acids and their calcium salts, and the determination of calcium ion activity in relation to the degree of polymerization. *Acta Chem. Scand.*, **26**, 2455–68.

KUMLER, W. D. & EILER, J. J. (1943). The acid strength of mono and diesters of phosphoric acid. The *n*-alkyl esters from methyl to butyl, the esters of biological importance, and the natural guanidine phosphoric acids. *J. Am. Chem. Soc.*, **65**, 2355–61.

KUNIN, R. & FISHER, S. (1962). Effect of crosslinking on the properties of carboxylic polymers. II. Apparent dissociation constants as a function of the exchanging monovalent cation. *J. Phys. Chem.*, **66**, 2275–7.

LIQUORI, A. M., ASCOLI, F., BOTRÉ, C., CRESCENZI, V. & MELE, A. (1959). On the electrostatic interactions between counterions and macroions in polyelectrolyte solutions. *J. Polym. Sci.*, **40**, 169–78.

MACKIE, W. (1971). Conformations of alginic acids and their salts. *Eur. Biophys. Congr., Proc. 1st*, **4**, 437–9.

MCNEELY, W. H. & PETTITT, D. J. (1973). Algin. In *Industrial gums* (Whistler, R. W. (ed.)), 49–81, New York, Academic Press.

MATHEWS, M. B. (1960). Trivalent cation binding of acid mucopolysaccharides. *Biochim. Biophys. Acta*, **37**, 288–95.

MEYER, K. & ROČEK, J. (1972). One-electron versus two-electron oxidations. Cerium(IV) and cyclobutanol. *J. Am. Chem. Soc.*, **94**, 1209–14.

MORRIS, E. R. (1973). Polysaccharide conformation as a basis of food structure. In *Molecular structure and function of food carbohydrates* (Birch, G. G. & Green, L. F. (eds.)), 125–32, New York, John Wiley.

MURPHY, P. T. & WHISTLER, R. L. (1973). Dextrans. In *Industrial gums* (Whistler, R. L. (ed.)), 513–42, New York, Academic Press.

NORMANN, W. (1906). On copper alkali cellulose. *Chem.-Ztg.*, **30**, 584.

OAKES, J. & CHILD, T. F. (1973). Interactions of Cu^{2+} and Mn^{2+} with agarose and calcium alginate gels by ESR. *Nature (London), Phys. Sci.*, **244**, 107–8.

PAYENS, T. A. J. & SNOEREN, T. (1972). The effect of simple electrolytes on the sol-gel transition of *kappa*-carrageenan. *J. Electroanal. Chem. Interfacial Electrochem.*, **37**, 291–6.

PERLIN, A. S. (1959). Action of lead tetraacetate on the sugars. *Adv. Carbohydr. Chem.*, **14**, 9–61.

PERLIN, A. S. (1964). Ring contraction during the lead tetraacetate oxidation of reducing sugars. *Can. J. Chem.*, **42**, 2365–74.

PODLAS, T. J. & ANDER, P. (1969). Interaction of sodium and potassium ions with κ- and λ-carrageenan in aqueous solutions with and without added salt. *Macromolecules*, **2**, 432–6.

POTTENGER, C. R. & JOHNSON, D. C. (1970). Mechanism of cerium(IV) oxidation of glucose and cellulose. *J. Polym. Sci., Part A-1*, **8**, 301–18.

RAAFLAUB, J. (1951). A photometric method for determining ionized calcium. *Hoppe Seyler's Z. Physiol. Chem.*, **288**, 228–36.

RANGASWAMY, M. & SANTAPPA, M. (1969). Oxidation of alcohols by aqueous ceric ions in perchloric acid. *Indian J. Chem.*, **7**, 473–9.

RAO, V. S. R. & FOSTER, J. F. (1963). Studies of the conformation of amylose in solution. *Biopolymers*, **1**, 527–44.

REES, D. A. (1969). Structure, conformation, and mechanism in the formation of polysaccharide gels and networks. *Adv. Carbohydr. Chem. Biochem.*, **24**, 267–332.

REES, D. A., STEELE, I. W. & WILLIAMSON, F. B. (1969). Conformational analysis of polysaccharides. III. The relation between stereochemistry and properties of some natural polysaccharide sulfates. *J. Polym. Sci., Part C*, **28**, 261–76.

REEVES, R. E. (1951). Cuprammonium–glycoside complexes. *Adv. Carbohydr. Chem.*, **6**, 107–34.

RENDLEMAN, J. A., JR. (1966*a*). Complexes of alkali metals and alkaline-earth metals with carbohydrates. *Adv. Carbohydr. Chem.*, **21**, 209–71.

RENDLEMAN, J. A., JR. (1966*b*). Alkali metal complexes of carbohydrates. I. Interaction of alkali metal salts with carbohydrates in alcoholic media. *J. Org. Chem.*, **31**, 1839–45.

RENDLEMAN, J. A., JR. (1966*c*). Alkali metal complexes of carbohydrates. II. Interaction of bases with carbohydrates in alcoholic media. *J. Org. Chem.*, **31**, 1845–51.

RENDLEMAN, J. A., JR. (1973). Ionization of carbohydrates in the presence of metal hydroxides and oxides. *Adv. Chem. Ser.*, **117**, 51–69.

ROBINSON, R. A. & STOKES, R. H. (1949). Tables of osmotic and activity coefficients of electrolytes in aqueous solution at 25°C. *Trans. Far. Soc.*, **45**, 612–24.

ROSCHIER, R. H. & HYVÄRINEN, S. (1953). Hydroxoanionic compounds of cellulose. *Pap. Puu*, **35**, 1–7.

ROWLAND, S. P., ROBERTS, E. J. & BOSE, J. L. (1971). Availability and state of order of hydroxyl groups on the surfaces of microstructural units of crystalline cotton cellulose. *J. Polym. Sci., Part A*-1, **9**, 1431–40.

ROWLAND, S. P., ROBERTS, E. J. & FRENCH, A. D. (1974). Availability and disposition of hydroxyl groups on surfaces of crystalline cellulose II. *J. Polym. Sci., Polym. Chem. Ed.*, **12**, 445–54.

ROWLAND, S. P., ROBERTS, E. J. & WADE, C. P. (1969). Selective accessibilities of hydroxyl groups in the microstructure of cotton cellulose. *Text. Res. J.*, **39**, 530–42.

RUNDLE, R. E. (1947). The configuration of starch in the starch–iodine complex. V. Fourier projections from X-ray diagrams. *J. Am. Chem. Soc.*, **69**, 1769–72.

RUNDLE, R. E. & BALDWIN, R. R. (1943). The configuration of starch and the starch–iodine complex. I. The dichroism of flow of starch–iodine solutions. *J. Am. Chem. Soc.*, **65**, 554–8.

RUNDLE, R. E. & EDWARDS, F. C. (1943). The configuration of starch in the starch–iodine complex. IV. An X-ray diffraction investigation of butanol–precipitated amylose. *J. Am. Chem. Soc.*, **65**, 2200–3.

RUNDLE, R. E. & FRENCH, D. (1943*a*). The configuration of starch and the starch–iodine complex. II. Optical properties of crystalline starch fractions. *J. Am. Chem. Soc.*, **65**, 558–61.

RUNDLE, R. E. & FRENCH, D. (1943*b*). The configuration of starch in the starch–iodide complex. III. X-Ray diffraction studies of the starch–iodide complex. *J. Am. Chem. Soc.*, **65**, 1707–10.

SATAKE, I., FUKUDA, M., OHTA, T., NAKAMURA, K., FUJITA, N., YAMAUCHI, A. & KIMIZUKA, H. (1972). Interactions of counterions with dextran sulfate in aqueous solutions. *J. Polym. Sci., Polym. Phys. Ed.*, **10**, 2343–54.

SAVIC, J., SAVIC, M. & FILIPOVIĆ, I. (1972). Spectrophotometric determination of stability constants of lactato and β-hydroxypropionato complexes of cobalt(II), nickel(II), and copper(II). *Croat. Chem. Acta*, **44**, 305–15.

SCHACHAT, R. E. & MORAWETZ, H. (1957). The interaction of carrageenin with counterions. *J. Phys. Chem.*, **61**, 1177–9.

SCHWEIGER, R. G. (1962). Acetylation of alginic acid. II. Reaction of algin acetates with calcium and other divalent ions. *J. Org. Chem.*, **27**, 1789–91.

SCHWEIGER, R. G. (1972). Polysaccharide sulfates. I. Cellulose sulfate with a high degree of substitution. *Carbohydr. Res.*, **21**, 219–28.

SENTI, F. R. & WITNAUER, L. P. (1948). Structure of alkali amylose. *J. Am. Chem. Soc.*, **70**, 1438–44.

SENTI, F. R. & WITNAUER, L. P. (1952). X-Ray diffraction studies of addition compounds of amylose with inorganic salts. *J. Polym. Sci.*, **9**, 115–32.

SIDGWICK, N. V. (1950). *Chemical elements and their compounds*, **I**, 450–1, Oxford, Clarendon Press.

SLONEKER, J. H. (1975). Personal communication. Northern Regional Research Center, Peoria, Illinois.

SMIDSRØD, O. & HAUG, A. (1967). Precipitation of acidic polysaccharides by salts in ethanol–water mixtures. *J. Polym. Sci., Part C*, 1587–98.

SMIDSRØD, O. & HAUG, A. (1971). Estimation of the relative stiffness of the molecular chain in polyelectrolytes from measurements of viscosity at different ionic strengths. *Biopolymers*, **10**, 1213–27.

STOKES, R. H. & ROBINSON, R. A. (1948). Ionic hydration and activity in electrolyte solutions. *J. Am. Chem. Soc.*, **70**, 1870–8.

STRAUSS, U. P., WOODSIDE, D. & WINEMAN, P. (1957). Counterion binding by polyelectrolytes. I. Exploratory electrophoresis, solubility, and viscosity studies of the interaction between polyphosphates and several univalent cations. *J. Phys. Chem.*, **61**, 1353–6.

TIBENSKY, V. (1968). Selective cation exchange on cross-linked pectin. *Chem. Zvesti*, **22**, 401–6.

TOWLE, G. A. (1973). Carrageenan. In *Industrial gums* (Whistler, R. L. (ed.)), 83–114, New York, Academic Press.
VAN WAZER, J. R. & CALLIS, C. F. (1958). Metal complexing by phosphates. *Chem. Rev.*, **58**, 1011–46.
WETTSTEIN, F., NEUKOM, H. & DEUEL, H. (1961). Cation exchange on starch phosphate. *Helv. Chim. Acta*, **44**, 1949–56.
WHITAKER, P. M., NIEDUSZYNSKI, I. A. & ATKINS, E. D. T. (1974). Structural aspects of soda-cellulose II. *Polymer*, **15**, 125–7.
WILLIAMS, R. J. P. (1971). Biochemistry of group IA and IIA cations. *Adv. Chem. Ser.*, **100**, 155–73.
ZABIK, M. E. & ALDRICH, P. J. (1968). Gel strength of kappa-carrageenan as affected by cations. *J. Food Sci.*, **33**, 371–7.
ZITCO, V. & BISHOP, C. T. (1966). Oxidation of polysaccharides by lead tetraacetate in dimethyl sulfoxide. *Can. J. Chem.*, **44**, 1749–56.
ZUNDEL, G. (1969). Hydration structure and intermolecular interaction in polyelectrolytes. *Angew. Chem.* (Int. Ed.), **8**, 499–509.

DAVID PEARSON—AN OBITUARY

David Pearson achieved a great deal in his life before he was struck down on 10th September last year.

At an early stage in his career he worked for the Southwark Public Health Laboratory and studied part-time for the BSc at Birkbeck College, London University. He continued to study in his spare time, was awarded the ARIC and then the FRIC on the basis of his work for Branch E in Food and Drugs at Chelsea Polytechnic. He did so well in the course of his work that he was invited to join the lecturing staff for Branch E—again on a part-time basis. This he continued to do for many years and later took a leading part in the teaching of the course when it was transferred to Borough (now South Bank) Polytechnic.

His work in the food industry included a period with Sainsbury's, but eventually he found his true métier when he joined the National College of Food Technology at its inception in 1952. Here he stayed, becoming successively Senior Lecturer and Principal Lecturer, and helped in no small measure in its growth, its entry into the University of Reading in 1966, and in its present status throughout Britain and the world.

During this period he gained his MSc. Later, on the basis of numerous papers on the analysis of food, quality control, and particularly on the quantitative approach to spoilage in meat and fish, he was awarded the DSc by the University of London. He was a founder member of the Institute of Food Science and Technology, of which he became a Fellow in 1965, and took a prominent part in the Editorial Committee of the IFST publication *Journal of Food Technology*.

He was responsible for re-writing Cox's *Chemical Analysis of Foods*, now having run into several editions under his own name, and this, together with his *Laboratory Techniques of Food Analysis*, earned him a world-wide reputation.

He was well liked by all who worked with him in his many different fields of activity and his untimely death at the age of 58 is a great loss to his wife and two children, to the NCFT, the Institute of Food Science and Technology and to his many friends.

M. SPENCER

Fd. Chem. (3) (1978)—
Printed in Great Britain

LIPID AND OTHER CHANGES OCCURRING DURING THE FERMENTATION AND FRYING OF TEMPEH†

S. SUDARMADJI‡ & P. MARKAKIS

Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan 48824, *USA*

(Received: 27 June, 1977)

ABSTRACT

Three phases can be distinguished in the tempeh fermentation of soybeans with Rhizopus oligosporus. *During the first phase a rapid increase in free fatty acid (FFA) content, number of bacteria and temperature is observed, along with a copious growth of the mould. The second phase is characterised by little or no change in FFA content, bacterial and mould growth and by a declining temperature. During the third phase the FFA development and bacterial growth recommence. Organoleptically, tempeh scores best at the end of the first phase (30 h at 32 °C) and keeps its good quality during the second phase (one additional day at 32 °C), but deteriorates rapidly during the third phase. Upon frying in coconut oil, tempeh undergoes a sharp reduction in FFA content with a concomitant increase in the FFA content of the frying oil. While frying alters the percentage composition of the glycerides of tempeh because of coconut oil absorption, the glyceride composition of the frying oil barely changes.*

INTRODUCTION

Tempeh is an Indonesian food prepared from soybeans fermented with *Rhizopus oligosporus* (occasionally other beans or coconut are fermented in a similar way to make tempeh koro, tempeh bongkrek, etc.). After fermentation, the cake-like mass of soybeans and mould mycelia is sliced and either fried or cooked by other means. This food is consumed by millions of people in Indonesia and Surinam. It is also found in the Netherlands. The nut-like flavour of fried tempeh appeals to Europeans and Americans (Hesseltine, 1965). Steinkraus *et al.* (1961) and VanBuren *et al.*

† Michigan Agricultural Experiment Station Journal Article No. 8066.

‡ Present address: Department of Agricultural Technology, Gajah Mada University, Yogyakarta, Indonesia.

Fd. Chem. (3) (1978)—
Printed in Great Britain

(1972) studied the chemical changes occurring during tempeh fermentation and reported an increase in the soluble solids, soluble nitrogen, nitrogen-free extract, ammonia and fibre as the fermentation progressed. Murata (1965), however, considered the differences he found between tempeh and unfermented soybeans with regard to protein, amino acid, moisture, crude fat, ash and fibre contents to be rather slight. Wagenknecht *et al.* (1961) and VanBuren *et al.* (1972) observed an increase in both the acidity of the ether-extractable fraction and the pH of tempeh over those of the unfermented soybeans; they attributed this increased acidity to the enzymatic hydrolysis of glycerides and the increase in pH to the liberation of ammonia. The present study was primarily addressed to the effect of fermentation and frying on the fatty acid composition of tempeh, but observations on the bacterial growth and the organoleptic quality of this food were also included.

MATERIALS AND METHODS

Tempeh was prepared by a method similar to that practised in Indonesia although a pure culture of mould was used in these experiments. Dry soybeans, cultivar Harosoy-63, were soaked overnight and peeled by hand in running water. They were then boiled for 30 min, drained, cooled and inoculated with *R. oligosporus* NRRL 2710 (supplied by the Northern Regional Research Laboratory, Peoria, Illinois) grown on mycological agar slants. The mycelium from one agar slant was suspended in 3 ml of sterilised water and mixed with 300 g of peeled, cooked soybeans. The mix was packed in polyethylene bags with pinholes for air supply, moulded into cakes, 15 × 10 × 2 cm, and incubated at 32 °C. The temperature in the centre of these cakes was measured during incubation by means of thermocouples connected to a Minneapolis-Honeywell recording potentiometer.

Periodically during the incubation, samples of the fermenting product were removed and analysed for moisture, crude fat, free fatty acids, esterified fatty acids and bacterial count. They were also examined organoleptically by the authors. The moisture was determined by drying in a vacuum oven at 70 °C. The crude fat was measured by the Soxhlet method (AOAC, 1970). The free fatty acids were extracted from the crude fat according to Mattick & Lee (1959) and esterified with diazomethane (Schlenk & Gellerman, 1960). The glycerides were transesterified with dimethoxypropane (Mason & Waller, 1964). The methyl esters were separated and quantified by gas liquid chromatography (GLC). A GLC column packed with 10% DEGS-PC yielded a slightly better separation than a 10% SP-1000 column (both from Supelco, Inc.) for the methyl esters of the five main fatty acids of soybeans, palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$). A Perkin-Elmer 900 gas chromatograph was used, with nitrogen as carrier gas and a flame ionisation detector. Temperature programming was applied in the range 100 to 200 °C at 10 °C/min. For total bacterial counting, tempeh samples were

disintegrated with 0·1 % peptone solution in a Waring blender and diluted aliquots of the suspension were plated on Bacto plate count agar (Difco Labs) at 30 °C for 48 h. To prevent the growth of the tempeh mould on the plates, actidione (Upjohn Co.) was incorporated into the medium, at 100 ppm final concentration. The growth of mould on tempeh was followed visually.

RESULTS AND DISCUSSION

The tempeh content in free fatty acids at various times during the fermentation is shown in Fig. 1. There was a rapid increase in total FFA content during the first 30 h, followed by a levelling off, which lasted approximately 24 h, after which a new increase in total fatty acids was observed. The individual acids displayed the same trend of change as the total free fatty acids (Table 1).

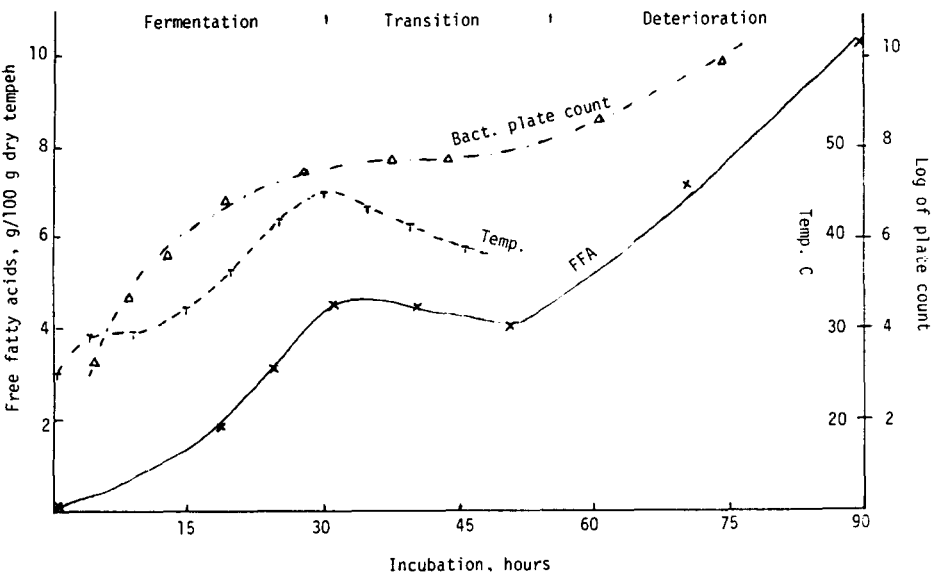


Fig. 1. Free fatty acid (FFA) content, temperature and total bacterial plate count of soybeans incubated with *R. oligosporus* at 32 °C.

The total bacterial count is also shown in Fig. 1. The bacteria of tempeh are mostly spore-forming organisms, the spores of which survived the boiling of the soybeans. *Bacillus licheniformis* and *Bacillus cereus* were identified by K. E. Stevenson, of this Department, in our tempeh (pers. comm.). Their growth profile coincided with that of the tempeh mould and the release of fatty acids. While it is

known that *R. oligosporus* has strong lipolytic activity (Hesseltine & Wang, 1970), it is also possible that the bacteria growing on tempeh may have contributed to the liberation of fatty acids. The likelihood of bacterial participation in the lipolysis is enhanced by the fact that lipolysis is resumed at a later phase of the fermentation when no mould growth is apparent.

TABLE 1
FREE FATTY ACID COMPOSITION OF TEMPEH AT VARIOUS TIMES DURING FERMENTATION (mg of FFA per 100 g moisture-free tempeh)

Time (h)	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	*Total*
0	8	1	6	21	3	39
18	260	82	457	741	162	1702
24	378	154	872	1415	203	3022
30	579	197	1461	2009	287	4533
40	579	247	1449	1858	287	4420
50	464	234	1150	1787	266	3901
70	839	353	2285	3412	505	7394
90	1107	599	3344	4879	749	10678

The temperature profile of the fermentation in the four-day period during which measurements were made showed a rapid increase from the original 25 °C to approximately 45 °C within the first 30 h and then began declining (Fig. 1).

Organoleptic evaluation of the fermenting product revealed that tempeh developed its most appealing flavour, colour and texture after about 30 h of fermentation at 32 °C (incubation temperature). It maintained these desirable attributes for another 24 h at this temperature. Beyond that time, signs of deterioration appeared: loss of pleasant taste, smell of ammonia, darkening of colour, stickiness and collapse of the texture.

On the basis of these observations, three phases may be recognised during the incubation of soybeans with *R. oligosporus: a tempeh fermentation phase* which, at 32 °C, lasts approximately 30 h and during which microbial growth, lipolysis and temperature increase and a product of high sensory quality results; *a transition phase* which lasts about another day and during which microbial growth and lipolysis subside, the temperature decreases and the product maintains its organoleptic properties; and a third phase, *the deterioration phase*, during which bacterial growth and lipolysis reappear and adverse organoleptic changes occur.

Frying resulted in an increase of crude fat in the tempeh. While the 30-h tempeh contained 62·8 % H_2O and 8·7 % crude fat, the fried tempeh had only 2·2 % moisture and 26·5 % crude fat. Frying at 180 °C for 4 min resulted in decreasing concentrations of all five free fatty acids present in soybeans (Table 2). The decrease in linolenic acid was appreciably lower (18 %) than that in other acids (55 to 70 %). In an effort to explain this difference, tempeh was heated in an oven at 200 °C for

TABLE 2
FREE FATTY ACID COMPOSITION OF TEMPEH BEFORE AND AFTER FRYING AT 180 °C FOR 4 MIN (mg FFA per 100 g moisture-free tempeh)

	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	*Total*
Before frying	631	337	1397	1764	352	4481
After frying	186	152	553	574	253	1718

20 min. The oven-heated tempeh contained 37% more free linolenic acid than the unheated tempeh, while the increase in other free fatty acids varied from 10 to 29%. Apparently, during frying, linolenic acid was also released in greater quantities than the other acids, thereby mitigating the migration effect of this acid to the frying medium. Since the coconut oil used in this experiment was refined and practically free of free fatty acids, no new free fatty acids were expected or found in the fried tempeh.

Table 3 shows the free fatty acid composition of coconut oil after heating at 180 °C and frying tempeh in it. While the lower MW fatty acids (C_8 to C_{14}) must have been formed from coconut glycerides by hydrolysis, at least part of the higher MW free fatty acids (C_{16}, C_{18}) must have originated from the tempeh, since there was a considerable reduction in the free fatty acid content of tempeh as a result of frying.

TABLE 3
FREE FATTY ACID COMPOSITION OF COCONUT OIL AFTER HEATING AND FRYING TEMPEH IN IT FOR TWO HOURS (mg per 100 g oil)

$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	*Total*
0·6	0·5	8·2	4·3	6·6	4·6	7·3	9·0	0·7	41·8

The composition of the glycerides of tempeh and coconut oil before and after frying is shown in Table 4. It is apparent that coconut oil was absorbed by the tempeh, resulting in considerable change in the percentage distribution of the glyceridic fatty acids of the tempeh: increase in caprylic ($C_{8:0}$), capric ($C_{10:0}$), lauric ($C_{12:0}$) and myristic ($C_{14:0}$) and decrease in oleic (18:1), linoleic (18:2) and linolenic (18:3). Dilution by coconut oil must have contributed to the substantial decrease in

TABLE 4
PERCENTAGE DISTRIBUTION OF THE FATTY ACIDS IN THE GLYCERIDE PORTION OF TEMPEH AND COCONUT OIL BEFORE FRYING (bf) AND AFTER FRYING (af)

	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Tempeh (bf)	0·0	0·0	0·0	0·0	0·0	8·9	3·4	29·7	51·0	7·0
Tempeh (af)	0·0	3·3	3·3	29·8	11·1	8·9	3·7	16·5	20·4	3·0
Coconut oil (bf)	0·7	6·7	5·4	46·1	18·4	9·2	3·8	7·5	2·2	0·0
Coconut oil (af)	0·6	6·2	5·3	47·4	18·2	9·7	3·8	6·9	1·9	0·0

the glycerides of unsaturated fatty acids in the tempeh after frying. On the other hand, little change in the glyceride composition of the coconut oil was observed, as the exchange between oils was chiefly one way, from frying medium to tempeh.

REFERENCES

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (1970). Official Methods of Analysis. Washington, DC, USA.

HESSELTINE, C. W. (1965). A milenium of fungi, food and fermentation.

HESSELTINE, C. W. & WANG, H. C. (1970). Fermented soybean foods. Workshop, University of Iowa, USA.

MASON, M. E. & WALLER, G. R. (1964). Dimethoxypropane induced transesterification of fats and oils in preparation of methyl esters for gas chromatographic analysis. *Anal. Chem.*, **3**, 583.

MATTICK, L. R. & LEE, F. A. (1959). A note on the method for the extraction of free fatty acids from lipid materials. *Food Res.*, **24**, 451.

MURATA, K. (1965). Nutritional value of tempeh. Osaka City University, Japan.

SCHLENK, H. & GELLERMAN, J. L. (1960). Esterification of fatty acids with diazomethane on small scale. *Anal. Chem.*, **41**, 173.

STEINKRAUS, K. H., VANBUREN, J. P. & HAND, D. B. (1961). *Studies on tempeh—An Indonesian fermented soybean food.* Publication 843:275, NAS-NRC, Washington, DC, USA.

VANBUREN, J. P., HACKLER, L. R. & STEINKRAUS, K. H. (1972). Solubilization of soybean tempeh constituents during fermentation. *Cer. Chem.*, **49**, 208.

WAGENKNECHT, A. C., MATTICK, L. R., LEWIN, L. M., HANEL, D. B. & STEINKRAUS, K. H. (1961). Changes in soybean lipids during tempeh fermentation. *J. Food Sci.*, **26**, 373.

LIPASE FROM *VICIA FABA MINOR*

DENNIS G. A. DUNDAS,† H. MICHAEL HENDERSON† & N. A. MICHAEL ESKIN‡

† *Department of Food Science.* ‡ *Department of Foods and Nutrition. University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada*

(Received: 26 July, 1977)

ABSTRACT

Lipase was partially purified from small faba beans by ethanol precipitation and Sephadex gel filtration and characterised by disc gel electrophoresis, isoelectric focusing and molecular weight determination in the presence of sodium dodecyl sulphate. Kinetic studies demonstrated that the properties of the enzyme conformed generally to those of lipases from other sources. The isoelectric point was pH 4·8, and electrophoresis at pH 9·3 revealed one lipase band in the R_f 0·25–0·31 region. The molecular weight was 210,000 ± 20,000. The possible importance of lipase is discussed with respect to the degradation of small faba bean lipids and to chemical changes occurring during storage of processed faba beans.

INTRODUCTION

The small faba bean, or horse bean, *Vicia faba* L. var. *minor* (Peterm.) Beck, is widely cultivated in many countries, including the majority of the nine member-states of the European Economic Community, and is becoming an increasingly important field crop for the Canadian Prairies. Processed faba beans become rancid very soon after mechanical disruption of the tissues, representing storage and acceptability problems. The rapid development of rancidity is due to the relatively high proportion of unsaturated fatty acids present in the lipid fraction, especially linoleic acid. This acid is particularly susceptible to oxidation, due to the presence of an active lipoxygenase (Eskin & Henderson, 1974; 1976) which is highly specific towards unsaturated free fatty acids. The majority of faba bean fatty acids occur in triglycerides or phospholipids so that an investigation into the means of release of free fatty acids from the triglyceride fraction appeared to be warranted. The presence of lipase (triacyl glycerol acylhydrolase, EC 3.1.1.3) had been previously

Fd. Chem. (3) (1978)—
Printed in Great Britain

suspected in the faba bean, through the observation that the total free fatty acids content increased markedly during an accelerated storage study under controlled conditions of faba beans in flour or protein concentrate form (Hinchcliffe *et al.*, 1977).

The purpose of this investigation was the detection of lipase in the faba bean and the partial purification and biochemical characterisation of the enzyme.

EXPERIMENTAL

Source material

Small faba beans (*Vicia faba* L. var. *minor* cv. Ackerperle) were obtained from the Department of Plant Science, University of Manitoba. The beans were harvested in 1975 and subsequently stored at room temperature. Porcine pancreatic lipase was obtained from Sigma Chemical Co., St Louis, Mo., USA.

Enzyme assay

The lipase assay was a modification of the titratable acidity procedure of San Clemente & Vadehra (1967). Lipase activity was determined by measuring the rate of hydrolysis of a triglyceride emulsion by potentiometric titration, lipolysis being followed by continuous titration with 0·01N NaOH at pH 8·5 and at 37°C. The emulsified substrate (5% olive oil or corn oil or 5·0–40·0 mM tributyrin, stabilised with 10% gum arabic solution by high speed blending for 1–3 min) was introduced into the reaction vessel, with 5·0 mM $CaCl_2$, 1·0M NaCl and 10·60 mM sodium deoxycholate or sodium taurocholate, to give a final volume of 15·0 ml. Enzyme inhibitors, mercuric chloride and *p*-chloromercuribenzoate, were included in the reaction mixtures in the appropriate experiments. The assay mixture was continuously bubbled with CO_2-free nitrogen. The pH was adjusted to 8·5, after which 1·0–5·0 ml of enzyme preparation were added and the pH readjusted to 8·6. Zero time was recorded when the pH meter nulled at pH 8·5 and the rate of base addition was determined for 5–10 min. Control experiments demonstrated that non-enzymic hydrolysis of the substrate was negligible below pH 10. The final assay volume of the emulsion did not exceed three times the initial volume, to avoid complete dissolution of the emulsified substrate by excessive dilution (Downey & Andrews, 1965).

One unit of lipase activity was equivalent to one micromole of free fatty acid produced per minute at 37°C.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as the standard, or by ultraviolet absorption at 280 nm.

Partial purification

Lipase was partially purified from crude extracts, containing 0·9% sodium chloride, of dehulled faba bean acetone powder, by 30–50% ethanol precipitation. The precipitate was dissolved in a minimal amount of water, stored at 4 °C and employed for kinetic studies.

The ethanol-precipitated fraction was further purified by Sephadex G-100 gel filtration at 4 °C. The column dimensions were 2·5 × 30 cm. The eluant was 10·60 mM sodium deoxycholate in sodium phosphate buffer, 0·01 M, pH 7. The elution flow rate was approximately 20 ml/h and the protein in the effluent was continuously monitored at 280 nm. The effluent was collected in 5 ml fractions, for the determination of enzyme activity.

Disc electrophoresis on polyacrylamide gel

Electrophoresis was carried out according to the procedure of Davis (1964), with an anionic gel system (7·5% polyacrylamide gel, pH 9·3) described in the Polyanalyst Electrophoresis Apparatus manual published by Buchler Instruments Inc., Fort Lee, New Jersey, USA. The gels were modified by doubling the concentrations of riboflavin and of ammonium persulphate. Electrophoresis was at 4 °C at 5 mA/tube for 1 h. Owing to the low activity of lipase in the extracts, relatively large amounts of sample (500 μg protein) had to be applied to each gel.

Detection of lipase and protein band patterns

Total protein was determined by immersion in Amido Black for 1 h, followed by electrophoretic destaining. For lipase determination, the gels were flooded with freshly-prepared buffered solutions containing 0·04% α-naphthyl acetate and 0·01% Fast Blue B salt (Lawrence *et al.*, 1967). The gels were developed in a mixture of ethanol:10% acetic acid (= 3:2) for 2 h at 37 °C. Lipase activity was shown as dark red bands.

Isoelectric focusing

This was carried out, according to the procedure of Wrigley (1968), in 7·5% polyacrylamide gel, over the pH range 3–10 employing LKB Ampholine carrier ampholyte No. 8141 (LKB Produkter AB, Bromma, Sweden). The enzyme sample (500 μg protein) was incorporated into the gel mixture prior to photopolymerisation.

Molecular weight determination

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed according to Dunker & Rueckert (1969).

RESULTS

Partial purification

An 8·6-fold purification, with a 79 % yield, of faba bean lipase was obtained from ethanol precipitation (Table 1).

Gel filtration of the ethanol fraction on the Sephadex column resulted in a 40-fold purification of the enzyme, with a yield of 25 %. A single lipase activity peak was detected immediately upon elution of the void volume and considerable non-lipase protein, as a separate peak, was detected following the lipase peak (Fig. 1).

TABLE 1
PARTIAL PURIFICATION OF LIPASE FROM *Vicia faba minor*

Fraction	*Specific activity (units/mg protein)*[a]	*Yield* (%)	*Purification*
Crude extract	0·110	100	1·0
Ethanol pptn.	0·946	79	8·6
Sephadex G-100	4·40	25	40·0

[a] One unit of lipase activity is equivalent to one micromole of free fatty acid produced per minute at 37 °C.

Kinetic studies

A linear relationship between enzyme concentration and the amount of free fatty acids liberated was evident with respect to all determinations carried out. The enzyme was more active towards tributyrin than to olive oil or corn oil, indicating a possible preference for glycerides of short-chain fatty acids over glycerides of long-chain fatty acids. The K_m value for tributyrin was 22·0 mM. The enzyme was active over approximately 2·5 pH units, with the optimum at pH 8·5. It was stable at 37 °C over a pH range of 6·5–9 for 10 min, while after 30 min the enzyme was completely stable only at pH 7. The enzyme was completely inactivated by exposure to 65 °C for 2 min.

Faba bean lipase was activated by sodium chloride, up to maximum activity in a solution with an ionic strength of 0·6. Sodium deoxycholate and sodium taurocholate promoted greater enzyme activity, which attained a maximum value at a bile salt concentration of 12·0 mM.

The enzyme was inhibited by mercuric chloride, with approximately 60 % of the original activity being non-competitively inhibited by 5·0 mM $HgCl_2$. The enzyme was non-competitively inhibited by 10·0 mM *p*-chloromercuribenzoate, with a 20 % reduction in activity.

Electrophoresis

Disc electrophoresis on polyacrylamide gel at pH 9·3 of the faba bean lipase crude extract revealed a major lipase band at R_f 0·30 and a faint minor lipase band at R_f

0·74. These correspond to the protein bands at R_f 0·31 and 0·75, respectively, other protein bands being inactive with respect to lipase. The band pattern of the ethanol fraction consisted of one enzyme band at R_f 0·31, with protein bands at R_f 0·31, 0·50 and 0·62. Electrophoresis of the lipase peak eluted from the Sephadex column resulted in one enzyme band, and a corresponding protein band, at R_f 0·25.

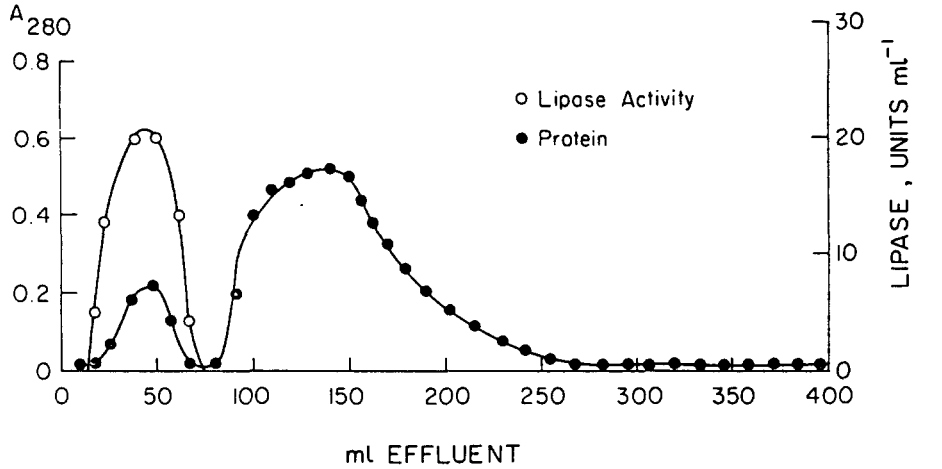


Fig. 1. Gel filtration of *Vicia faba minor* lipase on Sephadex G-100 in the presence of 10·60 mM sodium deoxycholate.

Corresponding results with porcine pancreatic lipase indicated one enzyme activity band with a slightly greater electrophoretic mobility.

Isoelectric point

Gel electrofocusing over a pH range of 3–10 indicated that the enzyme possessed one isoelectric point at pH 4·8.

Molecular weight

Disc gel electrophoresis (5% polyacrylamide gel, pH 7·2) in the presence of 0·1% sodium dodecyl sulphate revealed one lipase band of which the molecular weight was 210,000 ± 20,000 (four determinations). The molecular weight of porcine pancreatic lipase was 180,000 ± 5000 (four determinations).

DISCUSSION

The detection and partial purification of an active lipase in *V. faba minor* has been achieved. Part of the lipid degradation in the faba bean may therefore be outlined as

follows, to incorporate the observations of Eskin & Henderson (1974, 1976) on lipoxygenase:

$$\text{Triglycerides} \xrightarrow[H_2O]{\text{lipase}} \text{free fatty acids (especially 18:2)} \xrightarrow[O_2]{\text{lipoxygenase}} \text{hydroperoxides}$$

Hinchcliffe *et al.* (1977) analysed, by thin-layer chromatography, faba bean lipids exclusive of free fatty acids, and found that approximately two-thirds of the lipids were present as phospholipid and about 30% as triglyceride. Present investigations are therefore concerned with the possible production of unsaturated free fatty acids from the degradation of faba bean phospholipids.

The presence of lipase could account in part for the marked increase in the total free fatty acid content observed by Hinchcliffe *et al.* (1977) during twelve weeks of accelerated storage conditions for faba bean flour and protein concentrate. It is suggested that lipolysis control in faba beans may be desirable and could probably be achieved by a heat treatment stage during processing to inactivate the enzymes while minimising deterioration in product quality. The soluble form of faba bean lipase was shown to be relatively heat labile, being completely inactivated on exposure to 65°C for two minutes.

The biochemical characteristics of *V. faba minor* lipase, determined in this study, have been shown to conform generally with those of lipases from other sources. A substrate preference for glycerides of short-chain fatty acids, over glycerides of long-chain fatty acids, has also been shown for castor bean lipase (Ory *et al.*, 1962). The pH optimum of 8·5 is identical with that of peanut alkaline lipase (Sanders & Pattee, 1975) and contrasts with the pH optimum of 4·3 for castor bean acid lipase (Ory *et al.*, 1962), so that *V. faba minor* lipase may be characterised as an 'alkaline lipase'. Activation by sodium chloride and by bile salts has been confirmed in this study. Benzonana & Desneulle (1968) demonstrated that optimum activation of pancreatic lipase was attained in the presence of either 7 mM NaCl or 12 mM deoxycholate or 1·7 mM taurocholate. Faba bean lipase was shown to be activated by comparatively large amounts of sodium chloride and of taurocholate.

Inhibition of faba bean lipase by mercuric chloride and by *p*-chloromercuribenzoate has been demonstrated in this study. Similar inhibitory effects by these two compounds were demonstrated for lipase from *Puccinia graminis tritici* (Knoche & Horner, 1970) and for castor bean lipase (Ory *et al.*, 1962). The partial and non-competitive inhibition may indicate that sulphydryl groups are not involved in the active site of the enzyme but may be necessary to maintain the integrity of the enzyme structure.

The results obtained in this study from electrophoresis, electrofocusing and molecular weight determination provide evidence that *V. faba minor* lipase is a single homogeneous esterase, capable of hydrolysing α-naphthyl acetate, and may be isolated as a high molecular weight form. This molecular weight is in close

agreement with molecular weights of 200,000 for one rat adipose tissue lipase fraction (Downey & Andrews, 1965), of greater than 200,000 for a lipase from *Vernonia anthelmintica* seed (Olney *et al.*, 1968) and of 250,000 for a Micrococcus lipase (Lawrence *et al.*, 1967). All these lipases may possess an inherently high molecular weight or may be aggregated forms of the enzyme, or the lipase may be closely associated with inactive high molecular weight material, probably lipoprotein. Electrophoretic and isoelectric homogeneity has also been shown for Micrococcus lipase (Lawrence *et al.*, 1967) and for the lipase of *Chromobacterium viscosum* (Sugiura & Isobe, 1974). This may indicate that these two microorganisms, as well as *V. faba minor*, produce a single low-specific esterase.

The use of sodium deoxycholate as the eluant in gel filtration did not have the expected effect of disaggregation of the high molecular weight lipase (Chorvath & Fried, 1970). There was no evidence that deoxycholate brought about a conversion of the high molecular weight lipase to a form of apparently lower molecular weight. The gel filtration procedure resulted in a 40-fold purification of the enzyme, most probably due to the separation of considerable inactive protein which had not been directly associated with lipase.

ACKNOWLEDGEMENT

Financial assistance from the National Research Council of Canada is gratefully acknowledged.

REFERENCES

BENZONANA, G. & DESNEULLE, P. (1968). Action of some effectors on the hydrolysis of long-chain triglycerides by pancreatic lipase. *Biochim. Biophys. Acta*, **164**, 47–58.

CHORVATH, B. & FRIED, M. (1970). Partial purification of an extracellular leptospiral lipase. *J. Bacteriol.*, **102**, 879–80.

DAVIS, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.*, **121**, 404–27.

DOWNEY, W. K. & ANDREWS, P. (1965). Gel filtration applied to the study of lipases and other esterases. *Biochem. J.*, **94**, 642–50.

DUNKER, A. K. & RUECKERT, R. R. (1969). Observations on molecular weight determinations on polyacrylamide gel. *J. Biol. Chem.*, **244**, 5074–80.

ESKIN, N. A. M. & HENDERSON, H. M. (1974). Lipoxygenase in *Vicia faba minor*. *Phytochem.*, **13**, 2713–16.

ESKIN, N. A. M. & HENDERSON, H. M. (1976). A study of lipoxygenase from small faba beans. *Proc. IV Int. Congr. Food Sci. Technol.*, 1974, Vol. 1, 263–9.

HINCHCLIFFE, C., MCDANIEL, M., VAISEY, M. & ESKIN, N. A. M. (1977). The flavor of faba beans as affected by heat and storage. *Can. Inst. Food Sci. Technol. J.*, **10**(3), 181–4.

KNOCHE, H. W. & HORNER, T. L. (1970). Some characteristics of a lipase preparation from the uredospores of *Puccinia graminis tritici*. *Plant Physiol.*, **46**, 401–5.

LAWRENCE, R. C., FRYER, T. F. & REITER, B. (1967). The production and characterization of lipases from a Micrococcus and a Pseudomonad. *J. Gen. Microbiol.*, **48**, 401–18.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–75.

OLNEY, C. E., JENSEN, R. G., SAMPUGNA, J. & QUINN, J. G. (1968). The purification and specificity of a lipase from *Vernonia anthelmintica* seed. *Lipids*, **3**, 498–502.

ORY, R. L., ST. ANGELO, A. J. & ALTSCHUL, A. M. (1962). The acid lipase of the castor bean. Properties and substrate specificity. *J. Lipid Res.*, **3**, 99–105.

SAN CLEMENTE, C. L. & VADEHRA, D. V. (1967). Instrumental assay of microbial lipase at constant pH. *Appl. Microbiol.*, **15**, 110–13.

SANDERS, T. H. & PATTEE, H. E. (1975). Peanut alkaline lipase. *Lipids*, **10**, 50–4.

SUGIURA, M. & ISOBE, M. (1974). Studies on the lipase of *Chromobacterium viscosum*. III. Purification of a low molecular weight lipase and its enzymatic properties. *Biochim. Biophys. Acta*, **341**, 195–200.

WRIGLEY, C. W. (1968). Gel electrofocusing—A technique for analyzing multiple protein samples by isoelectric focusing. *Science Tools* (*LKB*), **15**, 17–23.

TRANSFORMATION OF CARBOHYDRATE AND NITROGEN COMPOUNDS IN POTATO TUBERS DURING DRYING: THE INFLUENCE OF VIRUS DISEASES

G. LISIŃSKA, G. SOBKOWICZ & W. LESZCZYŃSKI

The Agricultural University, Department of Storage and Food Technology, Wrocław, Poland

(Received: 7 September, 1977)

ABSTRACT

The influence of virus diseases on the concentrations of starch, total sugars, reducing sugars and nitrogen compounds was investigated in fresh and dried potato tubers. Virus disease hinders carbohydrate transformation and enhances nitrogenous transformation.

INTRODUCTION

It was found in previous studies (Lisinska *et al.*, 1976) that potato tubers infected with virus diseases had different properties from uninfected control samples. Thus tubers from fields infected with viruses contained less dry matter and starch but more nitrogen compounds. Moreover, tubers infected with the viruses *X* and Y^o contained smaller amounts of reducing sugars than controls.

Differences between the chemical composition of healthy tubers and tubers infected with the *X* virus were also noted by Krzymańska & Hoppe (1969). Allison (1953) examined the influence of virus infection on the content of amino acids in potato tubers and found that the tubers of infected plants contained more glutamine, glutaminic acid, aspartic acid and asparagine.

Bawden (1956) claims that virus diseases cause the changes in enzymic activity responsible for the transformation of carbohydrate and nitrogen compounds.

The authors' views, mentioned above, suggest that virus diseases may cause constant lesions of the enzymatic apparatus and, consequently, the biochemical changes in the infected tubers during drying may be slightly different from the changes in healthy tubers.

Fd. Chem. (3) (1978)—
Printed in Great Britain

The aim of this work was to compare the extent of transformation of carbohydrate and nitrogen compounds during drying of tubers infected with various types of virus.

MATERIALS AND METHODS

The tubers of four potato varieties were used in this study: Prosna, Noteć, Nysa and Wyszoborski. They were delivered by the Institute of Potatoes in Młochów from the field experiments carried out in the vegetative seasons of 1971 and 1972. During the experiments in four fields the potatoes were artificially infected with the viruses *X*, *Y°*, *Y"* and *L*. One field suffered a natural infection with the virus *S* and there was also a control field with healthy potatoes.

Fifty tubers were selected from each sample. They were washed, cleaned and cut into two parts lengthwise, one for the analysis of the fresh potatoes and the other for the drying of potato slices, 3–4 mm thick, at room temperature.

In the fresh and dried material the following components were determined after crushing: dry matter, starch, reducing sugars, total sugars, total nitrogen, protein nitrogen, amide nitrogen, amino acid nitrogen and amino acids and sugars. The methods of determination were the same as in previous work (Lisińska *et al.*, 1976).

The results (the mean results of two parallel repetitions) are shown in the accompanying figures.

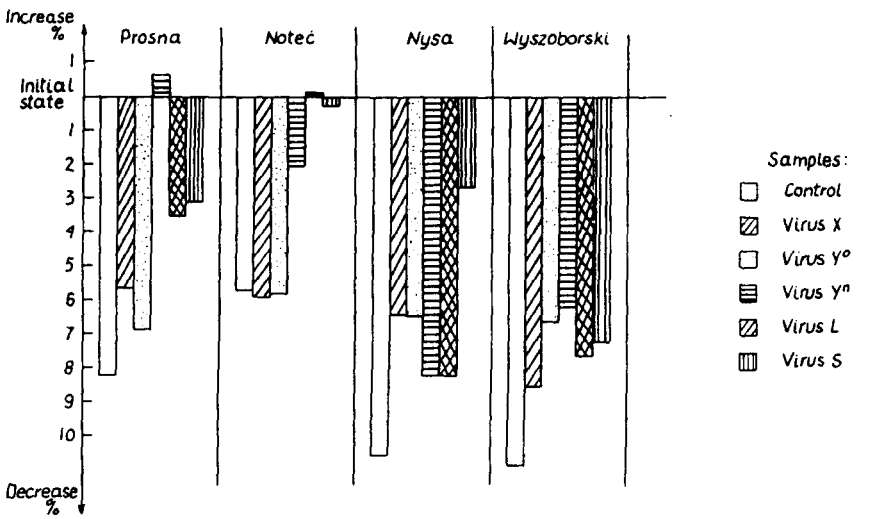


Fig. 1. Changes in the starch content of dried potatoes expressed in per cent, in relation to the starch content in fresh material. Vegetative season 1971.

RESULTS AND DISCUSSION

Figures 1 and 2 show the decrease in starch content during the drying of potato tubers, expressed in per cent, in relation to the quantity of starch in the fresh material. In all the control samples from the vegetative seasons of 1971 and 1972 the decrease in starch content was greater than in the samples infected with different types of virus. During the drying of the control samples of the Nysa and the Wyszoborski potato tubers of 1971, the quantity of starch decreased about 11 %. In infected potatoes of the same variety the decrease in the starch content was 2·5 % to 8·5 %.

No significant changes in starch content were observed during drying in the potato tubers of the Prosna variety from both vegetative seasons infected with virus Y^n or in the tubers of the Noteć variety infected with virus *L*.

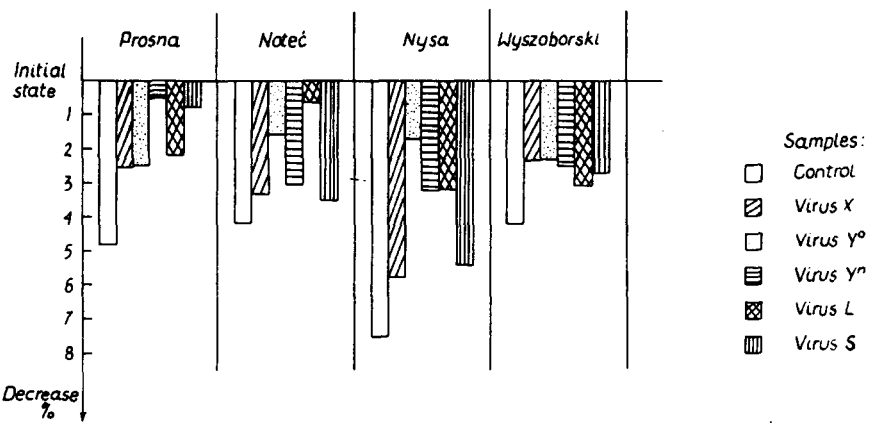


Fig. 2. Changes in the starch content of dried potatoes, expressed in per cent, in relation to the starch content in fresh material. Vegetative season 1972.

Figures 3 to 6 show changes in the content of total sugars and reducing sugars in dried potato tubers. The changes are shown in relation to the content of these components in fresh potatoes. The increase in total sugars content (Figs. 3 and 4) during drying of all the samples of the Wyszoborski variety was nearly twice as great as in samples of the other varieties. In the samples of one variety, the tubers which were not infected with virus diseases reacted the most extensively on drying; in these samples the increase in total sugars content was the highest.

In dried tubers the reducing sugars content (Figs. 5 and 6) increased 3–75 % compared with that of fresh tubers. The increase in the content of reducing sugars during drying differed with variety, vegetative season and kind of virus disease. The greatest quantity of reducing sugars was contained in the dried potatoes of the

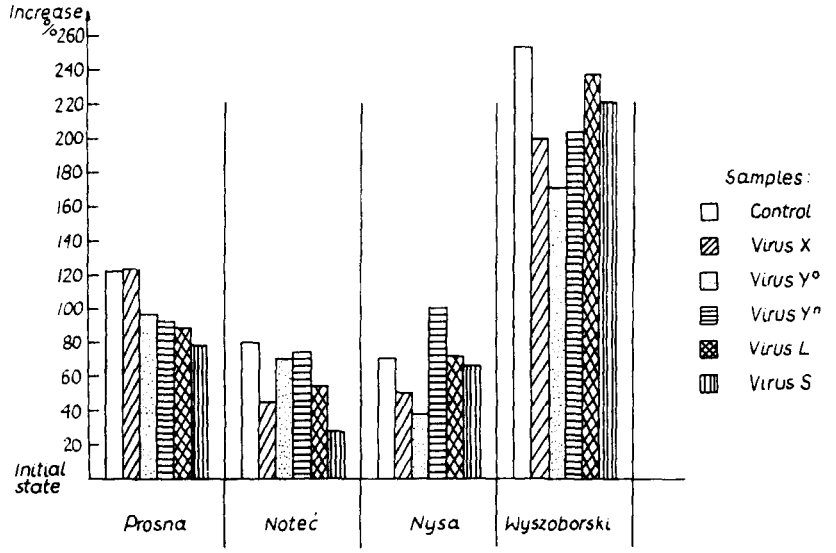


Fig. 3. Changes in the total sugars content of dried potatoes, expressed in per cent, in relation to the contents of these sugars in fresh material. Vegetative season 1971.

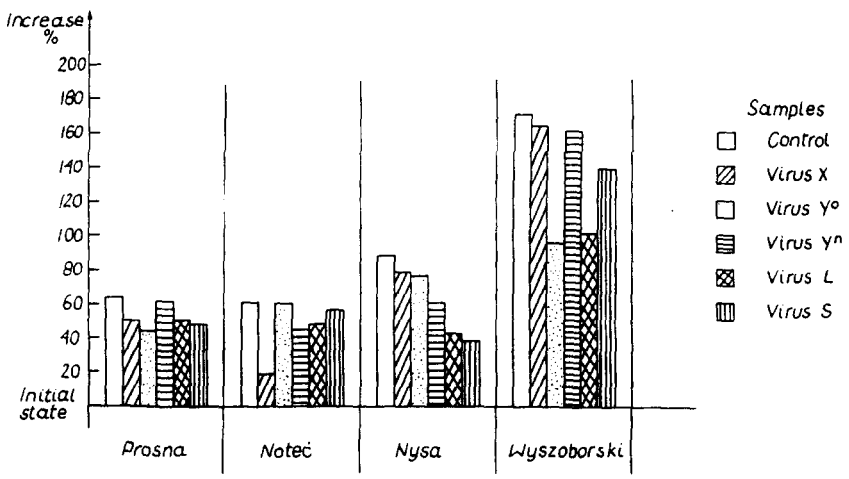


Fig. 4. Changes in the total sugars content of dried potatoes, expressed in per cent, in relation to the contents of these sugars in fresh material. Vegetative season 1972.

Wyszoborski variety infected with virus Y^n. The content of this component increased more than 50% in the sample mentioned in 1971 and 75% in 1972 in comparison with the quantity of reducing sugars in fresh potatoes.

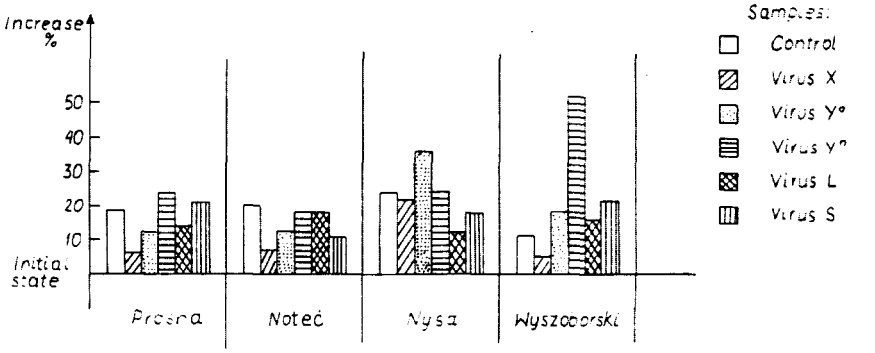


Fig. 5. Changes in the reducing sugars content of dried potatoes, expressed in per cent, in relation to the contents of these sugars in fresh material. Vegetative season 1971.

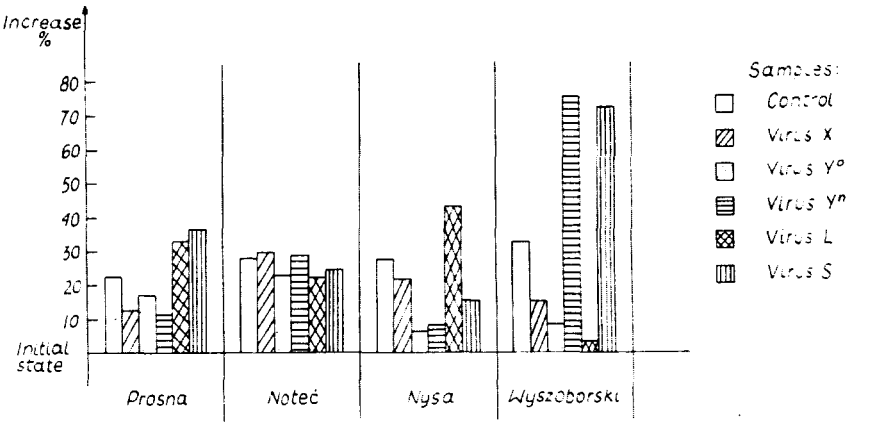


Fig. 6. Changes in the reducing sugars content of dried potatoes, expressed in per cent, in relation to the content of these sugars in fresh material. Vegetative season 1972.

Figure 7 shows a chromatogram of the sugars contained in the extracts from fresh and dried potato tubers of the Wyszoborski variety, infected with various virus diseases. The sugars content in fresh potatoes of the control sample and the infected samples was identical. After drying saccharose, fructose and glucose, as well as other sugars, were observed in all the samples. Two of these sugars were identified as maltose and ribose. Chromatograms of the sugars contained in the tubers of other varieties of the potatoes examined were similar to those shown in Fig. 7.

Figures 8 to 13 show the changes in the content of nitrogen compounds in dried potato tubers in relation to their content in fresh potatoes.

The content of protein nitrogen (Figs. 8 and 9) decreased during the drying of tubers. The decrease of protein nitrogen in dried tubers reached 50% of this

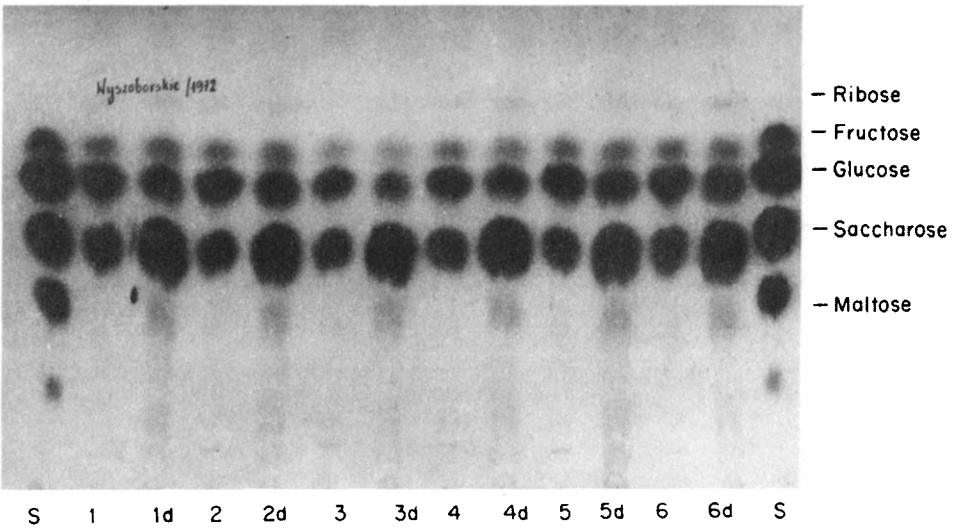


Fig. 7. Chromatogram of sugars contained in extracts from fresh and dried potato tubers: S—standard; 1-6 fresh potatoes; 1d-6d dried potatoes; 1,1d—the control sample; 2,2d—virus *X*, 3,3d—virus *Y*^{n}, 4,4d —virus *L*; 5,5d—virus *S*.

component's content in fresh material. In the potato tubers from the control fields smaller changes were observed in the protein nitrogen content during drying than in the potato tubers infected with various virus diseases.

In most of the samples examined the content of amide nitrogen (Figs. 10 and 11) and of amino acid nitrogen (Figs. 12 and 13) increased in dried potatoes compared with fresh potatoes. Changes in the content of free amino acids and amides during the drying of tubers varied in the samples examined according to the variety of

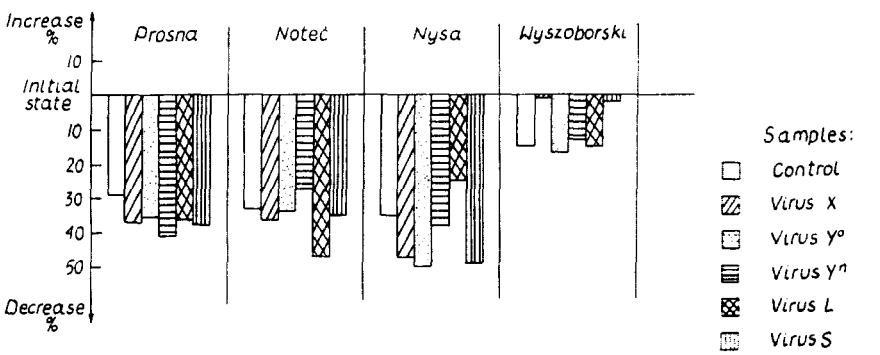


Fig. 8. Change in the protein nitrogen content of dried potatoes, expressed in per cent, in relation to the protein nitrogen content of fresh material. Vegetative season 1971.

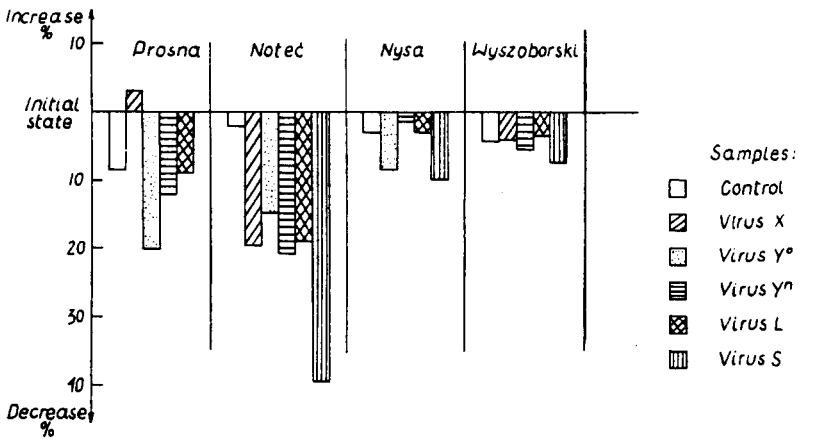


Fig. 9. Change in the protein nitrogen centent of dried potatoes, expressed in per cent, in relation to the protein nitrogen content of fresh material. Vegetative season 1972.

potato and kind of virus disease. The highest increase of amide nitrogen content was noted in the Nysa variety infected with the *X* virus—e.g. 40% in 1971 and 67% in 1972. On the other hand, the greatest amount of amino acid nitrogen was contained in the dried tubers of the same variety attacked by virus disease *S* and coming from the vegetative season of 1972. In the tubers of this sample the content of amino acid nitrogen increased 67% during drying.

The quantitative chromatographic analysis of free amino acids and amides confirmed the results obtained by chemical determinations of these components'

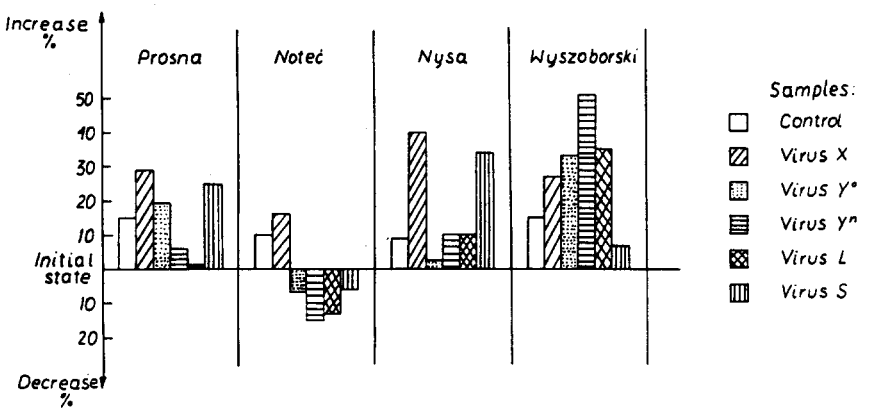


Fig. 10. Changes in the amide nitrogen content of dried potatoes, expressed in per cent, in relation to the amide nitrogen content of fresh material. Vegetative season 1971.

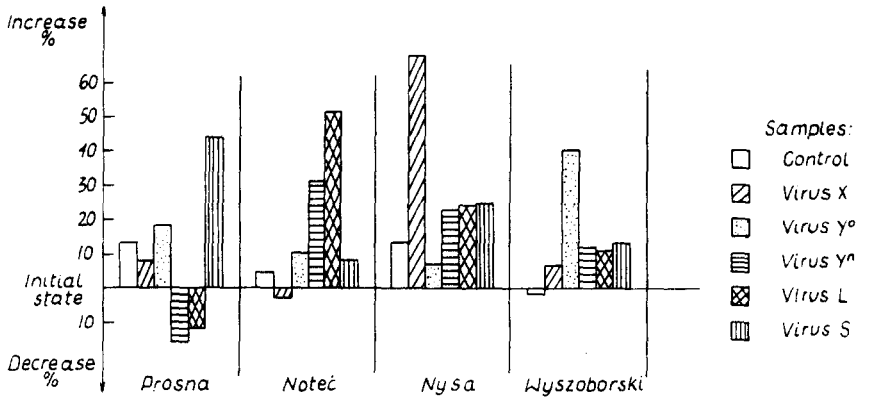


Fig. 11. Changes in the amide nitrogen content of dried potatoes, expressed in per cent, in relation to the amide nitrogen content of fresh material. Vegetative season 1972.

contents. Actually, more aspartic acid, alanine, serine and glutamine was contained in the dried than in fresh potatoes. However, a conspicuous influence of virus diseases on the amino acid and amide compositions in the dried tubers was not observed.

DISCUSSION

The results of the research show that the infection of potatoes with virus diseases influenced the speed of the carbohydrate and nitrogen compounds' changes in potatoes during drying. The experiment also confirmed that the decrease of starch content was more extensive during drying of tubers from the control fields than of tubers from the diseased plants. This accounts for the decrease of the activity of the enzymes responsible for the transformation of starch into sugar, and vice versa, in the infected tubers. Many authors (Bawden, 1956; Krzymańska & Hoppe, 1969; Rebowska, 1966; Wynd, 1943) have claimed that in diseased potatoes synthesis capacity and the accumulation of the reserve matter is lowered during the vegetative season. According to Kryzmańska & Hoppe (1969) this accounts for the reduction of photosynthesis as well as the speed of synthesis and carbohydrate transportation in the diseased plants.

During the drying of diseased potato tubers the increase of amide and amino acid nitrogen was greater than in healthy tubers. There are many studies (Allison, 1953; Andreae & Thompson, 1950; Bawden, 1956; Bershtein *et al.*, 1960; Leszczyński *et al.*, 1976) which confirm that plants infected with viruses contain more nitrogen components than healthy plants. Allison (1953) and Andreae & Thompson (1950)

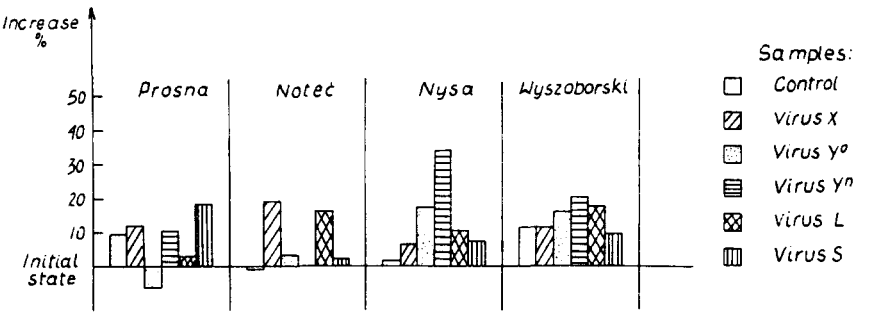


Fig. 12. Changes in the amino acid nitrogen content of dried potatoes, expressed in per cent, in relation to the amino acid nitrogen content of fresh material. Vegetative season 1971.

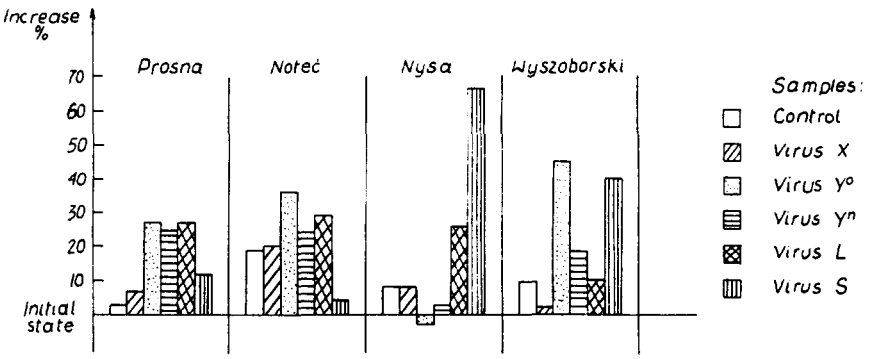


Fig. 13. Changes in the amino acid nitrogen content of dried potatoes, expressed in per cent, in relation to the amino acid nitrogen content of fresh material. Vegetative season 1972.

proved that the diseased tubers contained twice or three times as much amino acids as healthy tubers. These data show that virus diseases do not decrease the activity of enzymes responsible for the changes in nitrogen compounds in plants but may even increase it. Thus it is evident that the more extensive processes of protein decomposition to amides and amino acids in dried potato slices from the infected fields was due to virus disease during the vegetative season.

CONCLUSIONS

The following conclusions can be drawn on the basis of the results obtained in the course of this study:

(1) The infection of potatoes with virus diseases influenced biochemical

processes taking place during the drying of sliced potato tubers at room temperature.

(2) Changes in the content of starch and total sugars in the diseased tubers being dried were less extensive than in healthy tubers.

(3) The influence of the potato infection on the changes in the nitrogen compounds during drying of potato tubers varied according to the variety of potatoes and the kind of virus disease. However, the increase of the amino acids and amides, as well as the decrease of protein nitrogen, in infected potato tubers during their drying was greater than in healthy tubers.

REFERENCES

ALLISON, R. N. (1953). Effect of leaf roll virus infection on the soluble nitrogen composition of potato tubers. *Nature*, **171**, 573.

ANDREAE, W. A. & THOMPSON, K. L. (1950). Effect of leaf roll virus infection on the amino acid composition of potato tubers. *Nature*, **166**, 72–3.

BAWDEN, F. C. (1956). *Plant viruses and virus diseases*. Chronica Botanica Company. Waltham, Mass., USA.

BERSHTEIN, B. J., LEOUTEVA, YU. A. & OKANENKO, A. S. (1960). The effect of various types of degeneration of potato on concentration of amino acids in the tubers. *Doklady Akademii Nauk*, **134**(4), 976–9.

KRZYMAŃSKA, J. & HOPPE, W. (1969). Effect of virus *X* infection on the biochemical changes in potato tubers. *Biuletyn JOR*, **44**, 147–54.

LESZCZYŃSKI, W., LISIŃSKA, G. & SOBKOWICZ, G. (1976). Effect of virus diseases on the chemical composition of potato tubers. *Materiały VII Sesji Naukowej Komitetu Technologii i Chemii Żywności PAN Warszawa* 15–16 *czerwiec*, 32–3.

LISIŃSKA, G., SOBKOWICZ, G. & LESZCZYŃSKI, W. (1976). Transformation of carbohydrate and nitrogen compounds in potato tubers during drying. Part I. The influence of tritox applied in cultivation of potatoes upon transformation of carbohydrate and nitrogen compounds during potato drying. *Food Chemistry*, **1**, 57–65.

REBOWSKA, Z. (1966). Problem of plant viruses. *Postepy Nauk Rolniczych*, **6**, 6–16.

WYND, F. L. (1943). Metabolic phenomena associated with virus infection in plants. *Botanical Review*, **9**, 395–465.

REVIEW—VITAMIN C LOSSES FROM PEAS DURING BLANCHING IN WATER

J. D. SELMAN

Department of Chemical Engineering, University of Technology, Ashby Road, Loughborough, Leics. LE11 3TU, Great Britain

(Received: 4 October, 1977)

ABSTRACT

The literature relating to losses of vitamin C from peas during blanching in water was reviewed in an attempt to elucidate the mechanisms involved in such losses. No conclusions could be drawn due to the variations between experiments and the poor control of the important parameters. Although a more detailed study of this topic has now been completed, it was concluded that there may be other food processing operations which, despite much experimental work, have in fact received only limited study.

INTRODUCTION

Many workers have studied the overall weight loss and specific solute losses during vegetable blanching. Solutes lost into the blanch water consist largely of sugars, soluble protein, water-soluble vitamins and minerals. Appreciable fresh weight losses may also occur due primarily to water loss. Of recent studies reviewed by Lee (1958) and Feaster (1960), most show the retention of a given solute as a range of values for a particular vegetable after a given blanching treatment. Many of these results have been obtained from experiments using commercial equipment and have provided a useful indication of the resulting nutrient content of the product. However, it is evident that most workers have failed to control or even to record factors which could influence the loss of solutes or the accuracy of the data. Thus results obtained by different workers cannot always be compared, and clear indications of how the solutes are lost are not readily apparent.

Fd. Chem. (3) (1978)—
Printed in Great Britain

To illustrate differences in blanching studies one vegetable has been selected for a review of the reported losses of one solute, namely vitamin C. Peas were chosen for study because of their economic importance as a processed commodity and the fact that an 'average' portion of fresh or frozen peas (approximately 50–80 g) could provide a significant proportion of the recommended daily intake of vitamin C (30 mg/day).

VITAMIN C LOSSES FROM PEAS DURING BLANCHING

To illustrate the wide range of conditions under which studies on peas have been made, some of the more important variables are summarised from selected papers in Table 1.

Laboratory blanching studies were made by Fenton *et al.* (1936) in which samples of peas and cooking water were removed for analysis in proportional quantities to maintain the ratio. The peas were then plunged immediately into iced 8% trichloracetic acid and macerated to extract the ascorbic acid (AA). It was found that the greatest loss of AA occurred during the first 2 min of cooking. This was the time taken for the water to return to boiling point after the peas had been added. Fenton *et al.* suggested that this large initial loss might have been due to the combined leaching and enzymic oxidation of AA during the initial heating period, the oxidation being effected by the oxygen in the tissues which had not yet escaped due to expansion. It would therefore have been desirable to take measurements during the first 2 min, but this was not done.

The rate of loss of AA after the water had returned to boiling was much lower and decreased almost to zero, most of this loss being accounted for in the water. After 14 min in boiling water, Thomas Laxton peas had lost 58% of the initial AA content, 48% being in the water. Alderman peas boiled for 13 min lost 47%, 40% being in the water. However, the mechanisms involved cannot be accounted for from the data although the slow rate of loss towards the end of the cooking time appears to have been largely diffusive.

Jenkins *et al.* (1938) used only one pea-growing site to minimise variations in the pea samples due to soil and climate. Ascorbic acid estimation was based on the method of Bessey & King (1933) but the extracting solutions contained 2% metaphosphoric acid and 8% trichloroacetic acid. Metaphosphoric acid in the extractant gave a clearer extract and allowed more sensitive titration. Jenkins *et al.*'s blanching data are given in Table 2 and suggest that the rate of loss was uniform during the first 128 sec but decreased from 128 to 153 sec. In view of the results of Fenton *et al.* (1936), it would have been useful to have blanched for longer times, but retention was of the same order as that reported by Fenton *et al.* Strict comparison of these results is not valid because of the different conditions of blanching. Todhunter & Sparling (1938) blanched peas and froze them within six hours. The

TABLE 1

RELEVANT EXPERIMENTAL DETAIL OF REVIEWED LITERATURE

Reference	*Cultivar (Fr = Freezing C = Canning)*	*Harvesting method*	*Maturity index*	*Sieve size of peas*	*Initial ascorbic acid[a] content of peas (mg/100 g F.wt)*	*Reference for method of vit. C estimation*	*Type of blanching equipment*	*Product/water ratio*
Fenton *et al.* (1936)	Thomas Laxton, Alderman (Fr)	Hand	When succulent	Unsized	23·0 24·0	Bessey & King (1933)	Batch, laboratory scale	1:1·3
Jenkins *et al.* (1938)	Thomas Laxton Alderman Telephone (Fr)	Vined	Not given	5	24·0	Mack & Tressler (1937)	Scott pipe blancher	Not given
Todhunter & Sparling (1938)	Telephone (Fr)	Vined	Not given	6 (after thawing)	Not given	Musulin & King (1936)	Not given	Not given
Adam *et al.* (1942)	Surprise, Lincoln, Charles 1 (C)	Not given	Not given	Not given	34·2 26·8 28·0	Not given	Batch, laboratory scale	1:3
Guerrant *et al.* (1947)	Perfection (C)	Not given	Not given	4	19·6 (fancy) 25·3 (standard)	Wagner *et al.* (1947*b*)	Laboratory scale rotary	1:10
Wagner *et al.* (1947*a*)	Alaska, Sweet (C)	Vined	Not given	3, 4, 5	122–142, 131–139 dry weight basis	Wagner *et al.* (1947*a*)	Not given	Not given
Wagner *et al.* (1947*b*)	Alaska (C)	Vined	[b]TR < 105 and TR > 150	(1, 2, 3) 4, (5, 6) 7	127·0 (Dry weight basis from ungraded pea sample)	Wagner *et al.* (1947*b*)	Commercial rotary	Not given
Feaster *et al.* (1949)	Climax (C)	Vined	Not given	4	18·9–19·2 (standard) 23·2–23·5 (extra standard)	Feaster *et al.* (1949)	Commercial rotary	Not given
Heberlein *et al.* (1950)	Prince of Wales (C)	Vined	TR < 105 and TR = 116–145	Unsized	Not given	Assoc. Vit. Chem. (1947)	Laboratory scale rotary	Not given
Bedford & Hard (1950)	Hundredfold (Fr)	Laboratory sheller	Not given	Not given	26·6–31·2	Loeffler & Ponting (1942)	Agitated baskets	1:18·2
Hartzler & Guerrant (1952)	Freezonian (Fr)	Hand	Not given	Not given	21·0	Hartzler & Guerrant (1952)	Not given	1:1
Holmquist *et al.* (1954)	Alaska, Prince of Wales (C)	Not given	TR > 95	(1 and 2) 3 (4 and 5)	Not given	Not given	Not given	Not given
Varoquaux (1971)	Hybris Juwel	Not given	Not given	Not given	Not given	AOAC (1965)	Batch, laboratory scale	1:10
Morrison (1972)	6 Cultivars (Fr) (see Table 7)	Vined	TR = 105	Unsized	See Table 7	AOAC (1970)	Commercial rotary	1:4

[a](F = Fresh). [b](TR = Tenderometer reading).

TABLE 2
ASCORBIC ACID RETAINED IN PEAS AFTER BLANCHING AND COOLING IN WATER (FROM JENKINS *et al.*, 1938)

Blanch time (*sec*)	*Ascorbic acid retained*[a] (*mg*/100 *g F.wt.*)	(*mg*/100 *g D.wt.*)
0	25	111
60	21	95
85	20	87
128	17	78
153	16	73

[a] F = Fresh. D = Dry.

peas were graded (size six) after thawing and AA analyses made at this stage but none were made on fresh peas, so that losses during the process could not be assessed.

Adam *et al.* (1942) blanched peas in water or steam and determined AA in the peas only. The loss of AA in three cultivars after blanching is shown in Table 3. They suggested that the large initial losses of weight, volume and AA were due to the expulsion of some of the cell contents as turgor was lost. However, they did not explain the difference in observed volume change between Surprise and Lincoln peas

TABLE 3
PERCENTAGE LOSS OF ASCORBIC ACID AFTER BLANCHING (FROM ADAM *et al.*, 1942)

Blanch time (*min*)	*% loss of ascorbic acid* *Surprise*	*Lincoln*	*Charles I*
1 (water)	38	29	38
3 (water)	49	34	50
6 (water)	56	40	55
3 (steam)	28	16	25

after steam blanching, nor the increase in weight of the Charles I peas after water blanching. Also they did not attempt to explain the similarity of the AA losses from Surprise and Charles I peas despite the fact that the initial values for the two cultivars differed; nor did they offer reasons for the high retention in the Lincoln peas. All their data refer to peas cooled in water for 1 min after blanching, and hence no conclusions can be made concerning the mechanism of loss.

Ascorbic acid loss after blanching was reported by Guerrant *et al.* (1947) for four times at three temperatures (Table 4). At the lower blanch temperatures the rate of heat penetration into the pea will be slower and consequently the period during which enzyme activity could occur would be longer. Relatively greater loss due to enzymic oxidation might therefore be expected at the lower temperatures, which did not occur. At the same time the rate of cell disorganisation would also be slower so

TABLE 4
PERCENTAGE LOSS OF ASCORBIC ACID FROM SIZE 4 PEAS AFTER BLANCHING (FROM GUERRANT *et al.*, 1947)

Blanch time (min)	3	6	9	12
Blanch temperature (°C)				
82·2	22·9	30·3	37·4	45·5
87·8	28·1	34·1	42·4	52·4
93·3	32·4	34·8	53·4	59·2

that the net loss of cell contents from the tissues would occur more slowly than at higher temperatures, which might affect the rate of vitamin C loss. Net fresh weight losses were also recorded but no clear trends were apparent. Guerrant *et al.* (1947) also studied losses in serial blanching in which five successive 6 min immersions were made in the same blanch water at 87·8 °C (Table 5). The proportion of AA lost tended to decrease with repeated use of the same blanch water. However, the amount of AA presumed to have been oxidised varied from 9·1 to 17·2%. This suggests that, as the peas had been size graded, the rate of heat penetration may have differed in successive blanchings. It is uncertain, therefore, whether the reported trends are real.

TABLE 5
PERCENTAGE LOSS OF ASCORBIC ACID AFTER SERIAL BLANCHING PEAS (FROM GUERRANT *et al.*, 1947)

Immersion No.	*% loss of ascorbic acid*		
	Total	*In blanch water*	*Oxidised*
1	33·0	23·9	9·1
2	32·5	16·7	15·8
3	33·0	18·4	14·6
4	30·4	18·9	11·5
5	28·3	11·1	17·2

Wagner *et al.* (1947*a*) gave figures for the AA losses from Alaska and Sweet peas blanched in 18 different canneries during two seasons. Each cannery had its own series of operations and blanching specifications but little detailed information was given. During the first season the AA was extracted using a solution containing 3% metaphosphoric acid and 8% acetic acid. However, they reported serious discrepancies in the results obtained and assumed that some oxidation had occurred during the extraction procedure. Subsequently they used a mixture of 6% metaphosphoric acid and 8% acetic acid which apparently gave more consistent results. Some of the discrepancies could well have arisen from variations in the process specifications of each cannery. The AA content for the fresh peas was expressed only on a dry weight basis and no figure for the moisture content was given. Ascorbic acid loss for the various canneries was: Alaska 21–36%, Sweet 15–37%. The smaller, more tender peas lost a greater percentage of vitamins than

the more mature, and AA was lost to a greater extent than other vitamins. Explanation of the relative losses is not simple—they may be due to the relative susceptibilities of the vitamin to oxidation, destruction and leaching. The distribution of the vitamin between the testa and cotyledon tissue could also affect the relative losses. Further losses of AA occurred during the post-blanch cooling period, but no figures were given. Certainly the solute loss from peas that have been both blanched and cooled in water is likely to be greater than that incurred during blanching alone.

In a more detailed study, with vined Alaska peas, Wagner *et al.* (1947*b*) gave values for AA contents after blanching but the value given for fresh peas was that for an ungraded sample (Table 6). They concluded that the higher temperature tended

TABLE 6
ASCORBIC ACID CONTENT OF ALASKA PEAS AFTER BLANCHING (FROM WAGNER *et al.*, 1947*b*)

Blanch time (*min*)	*Blanch temperature* (°*C*)	*Ascorbic acid* (*mg*/100 *g D.wt*)[a]		
		Sizes 1, 2, 3	*Size* 4	*Sizes* 5, 6
2·5	76·7–82·2	117·0	88·5	87·3
8·0	76·7–82·2	89·0	70·2	66·0
2·5	93·3	124·0	106·0	87·3
8·0	93·3	86·5	75·5	66·0

[a] D = Dry.

to decrease AA loss, and that the more mature peas tended to lose less AA than the smaller, younger peas. However, the actual figures reported do not bear out such conclusions and percentage losses based on the only reported value of AA content in the fresh peas also do not agree with their general conclusions. In fact, no clear trends were apparent in this data. The experimental detail in the latter two papers is sparse and only two blanch times were used. In fact, the shortest time of 2·5 min was longer than the time at which the greatest initial weight loss had been reported by previous workers to occur. The data offered no clues as to the mechanisms of solute and water loss during blanching.

Feaster *et al.* (1949) blanched peas at 87·8–93·3 °C for two blanch times. The observed losses were low in comparison with those reported elsewhere (1–24 % after 4·5 min), but it is difficult to account for this. The results do not help the study of the mechanisms of loss because of the range of the blanch temperature and the lack of information about the experimental conditions.

Heberlein *et al.* (1950) used 6 % or 3 % metaphosphoric acid as the extractant for the fresh or blanched peas, respectively. With immature peas blanched at 96 °C there was no loss after 1 min. This was in complete disagreement with other reported losses after 1 min blanching at a similar temperature and their methods seem to have been inaccurate.

Bedford & Hard (1950) compared the effects of two methods of cooling: (i)

cooling for 30 sec in water at 15·6 °C and (ii) cooling in a stream of air at 21·1 °C for 10 min. The AA extracting solution contained only 1 % metaphosphoric acid and this may not have been sufficient to stabilise the AA as Wagner *et al.* (1947*a*) suggested that even a 3 % solution was inadequate. The air-cooled peas had a higher AA content than the water-cooled peas after a given blanching treatment which accords with the findings of Wagner *et al.* (1947*a*), showing that further loss of AA occurs during water cooling. However, loss during blanching did not tend to increase with blanch time and the results were inconsistent. For example, in 1946 the AA loss was the same after 1 and 1·5 min at 100 °C, whereas in 1947 the figure for the loss after 1·5 min blanching was smaller than that for the loss after 1 min blanching. Such inconsistencies may well have been due to the extractant used.

Hartzler & Guerrant (1952) found that the greatest loss of AA during blanching occurred after 30 sec, but that losses after 1·5 and 3 min were much smaller. This was contrary to most findings and it seems possible that the extractant (0·5 % oxalic acid) was too dilute to stabilise the AA effectively.

Blanching peas for 5 min at 96·1 °C in water, Holmquist *et al.* (1954) noted that AA loss (21–40 %) was greater than during steam blanching at 100 °C. Their samples were drawn from two cultivars which may account in part for the variability of the data. Smaller losses were noted by Varoquaux (1971), i.e. 3 % and 11 % after 1 and 4 min blanching, respectively, but cultivars were mixed and the maturity index, size and initial AA content were not given.

More recently, account has been taken of the equally antiscorbutic dehydroascorbic acid, which is present in peas (and which is formed by oxidation of AA). Both were estimated by Morrison (1972, 1974) using a fluorimetric technique (AOAC, 1970) and reported as 'total' vitamin C. Although studies were made of the effects of maturity, cultivar and post-vining delay following various processes including commercial freezing, cleaning, blanching and cooking, the individual contents for the two forms were reported in only a few experiments.

Morrison found that the loss of vitamin C from peas shelled in a miniature viner was greater than that from hand shelled peas during blanching. Similarly, the loss of vitamin C during blanching commercially vined peas was greater than the loss from miniature vined peas. This strongly suggests that increasing damage done to peas prior to blanching tends to increase the loss of vitamin C during blanching. However, whether the loss was due to increased leaching or oxidation was not ascertained for the amounts lost into the water were not determined. Six pea cultivars (TR = 105) were separately blanched for 1 min at 96–98 °C. The initial vitamin C contents and the losses after blanching are shown in Table 7. The importance of cultivars was evident; whether the initial content determined the amount lost was not clear. Moreover, the peas were not graded so that the data cannot be adequately interpreted. The observed losses were of the same order as in most earlier studies, being lower than those of Adam *et al.* (1942) and higher than those of Varoquaux (1971).

TABLE 7
VITAMIN C CONTENT OF SIX PEA CULTIVARS AND % LOSS AFTER BLANCHING FOR 1 MIN AT 97°C IN WATER (FROM MORRISON, 1974)

Cultivar	*Initial vitamin C content (mg/100 g D.wt)*[a]	*% vitamin C loss*
Sparkle	104·6	16·6
Swift	138·6	23·3
Swan	124·8	9·6
Scout	99·1	27·7
Puget	104·4	19·6
Dark Skinned Perfection	120·0	19·4

[a] D = Dry.

CONCLUSIONS

In this review attention is directed to some of the important factors which may affect the AA content of peas and its retention during processing. These include cultivar, size, maturity, mechanical damage (shelling and harvesting equipment), blanch method, time and temperature and analytical methods. Post-blanch cooling should be treated as a separate operation. Failure to control or specify many of these conditions and the use of unsatisfactory methods of chemical estimation have meant that results from apparently similar studies may not be directly compared, extrapolation or interpretation often being impossible. From the scientific point of view this lack of comprehensive definition, together with a failure to apply elementary statistical tests of significance, reduces understanding of the chemical and physical changes involved in blanching almost to guesswork.

What is required is a study under carefully controlled conditions, initially to elucidate the main mechanisms involved in mass transfer from vegetables during blanching in water, and then to obtain accurate data on the changes of the vitamin C content of peas during blanching. Such a study, covering several of the important aspects, has now been completed, the conclusions of which will be published shortly (Selman, 1977). However, it is important to stress that the blanching operation is only one of many food processing operations which, despite much experimental work, may in fact have received only limited study.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Professor E. J. Rolfe and Mr S. Green of the National College of Food Technology.

REFERENCES

ADAM, W. B., HORNER, G. & STANWORTH, J. (1942). Changes occurring during the blanching of vegetables. *J. Soc. Chem. Ind. Lond.*, **61**, 96–9.

AOAC (1965). *Official methods of analysis* (10th Ed.), Association of Official Analytical Chemists, Washington DC.

AOAC (1970). *Official methods of analysis* (11th ed.), Association of Official Analytical Chemists, Washington DC.

ASSOCIATION OF VITAMIN CHEMISTS, INC. (1947). *Methods of vitamin assay*. Interscience Publishers Inc., New York.

BEDFORD, C. L. & HARD, M. M. (1950). The effect of cooling method on the ascorbic acid and carotene content of spinach, peas and snap beans, preserved by freezing. *Proc. Am. Soc. hort. Sci.*, **55**, 403–9.

BESSEY, O. A. & KING, C. G. (1933). The distribution of vitamin C in plant and animal tissues and its determination. *J. biol. Chem.*, **103**, 687–98.

FEASTER, J. F. (1960). In: *Nutritional evaluation of food processing*. (Harris, R. S. & Von Loesecke, H. (Eds.)), 109. John Wiley and Sons, New York.

FEASTER, J. F., MUDRA, A. E., IVES, M. & TOMPKINS, M. D. (1949). Effect of blanching time on vitamin retention in canned peas. *Canner*, **108**(1), 27–30.

FENTON, F., TRESSLER, D. K. & KING, C. G. (1936). Losses of vitamin C during the cooking of peas. *J. Nutr.*, **12**(3), 285–95.

GUERRANT, N. B., VAVICH, M. G., FARDIG, O. B., ELLENBERGER, H. A., STERN, R. M. & COONEN, N. H. (1947). Effect of duration and temperature of blanch on vitamin retention by certain vegetables. *Ind. Engng Chem. ind. Edn.*, **39**, 1000–7.

HARTZLER, E. R. & GUERRANT, N. B. (1952). Effect of blanching and of frozen storage of vegetables on ascorbic acid retention and on the concomitant activity of certain enzymes. *Fd Res.*, **17**, 15–23.

HEBERLEIN, D. G., PTAK, L. R., MEDOFF, S. & CLIFCORN, L. E. (1950). Quality and nutritive value of peas as affected by blanching. *Fd Technol., Champaign.*, **4**(3), 109–14.

HOLMQUIST, J. W., CLIFCORN, L. E., HEBERLEIN, D. G. & SCHMIDT, C. F. (1954). Steam blanching of peas. *Fd Technol., Champaign.*, **8**, 437–45.

JENKINS, R. R., TRESSLER, D. K. & FITZGERALD, G. A. (1938). Vitamin C content of frozen vegetables. VIII Frozen Peas. *Fd Res.*, **3**, 133–40.

LEE, F. A. (1958). The blanching process. *Adv. Fd Res.*, **8**, 63–109.

LOEFFLER, H. J. & PONTING, J. D. (1942). Ascorbic acid—rapid determination in fresh, frozen or dehydrated fruits and vegetables. *Ind. Engng Chem. Analyt.* Edn., **14**, 846–9.

MACK, G. L. & TRESSLER, D. K. (1937). Vitamin C in vegetables—VI: A critical investigation of the Tillmans method for the determination of ascorbic acid. *J. biol. Chem.*, **118**, 735.

MORRISON, M. H. (1972). *Studies on the Vitamin C and thiamin contents of peas before and after processing*. M.Sc. Thesis, University of Strathclyde.

MORRISON, M. H. (1974). The vitamin C and thiamin contents of quick frozen peas. *J. Fd Technol.*, **9**(4), 491–500.

MUSULIN, R. R. & KING, C. G. (1936). Metaphosphoric acid in the extraction and titration of vitamin C. *J. biol. Chem.*, **116**, 409–13.

SELMAN, J. D. (1977). *Studies on vegetable blanching*. Ph.D. Thesis, University of Reading.

TODHUNTER, E. N. & SPARLING, B. L. (1938). Vitamin values of garden-type peas preserved by frozen pack method. I. Ascorbic acid (vitamin C). *Fd Res.*, **3**, 489–98.

VAROQUAUX, P. (1971). Losses of soluble matter during blanching of peas. *C.r. Seanc. Acad. Agric. Fr.*, **57**(11), 949–57.

WAGNER, J. R., STRONG, F. M. & ELVEHJEM, C. A. (1947*a*). Effect of commercial canning operations on the ascorbic acid, thiamin, riboflavin and niacin contents of vegetables. *Ind. Engng Chem. ind. Edn.*, **39**, 985–90.

WAGNER, J. R., STRONG, F. M. & ELVEHJEM, C. A. (1947*b*). Effects of blanching on the retention of ascorbic acid, thiamin, and niacin in vegetables. *Ind. Engng Chem. ind. Edn.*, **39**, 990–3.

CHEMICAL EVALUATION OF NUTRITIVE VALUE OF SOYA PAPS AND PORRIDGES, THE NIGERIAN WEANING FOODS

O. U. EKA

Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria

(Received: 10 November, 1977)

ABSTRACT

The nutritive value of paps and porridges prepared using millet, guinea corn and maize was assessed by chemical analysis before and after supplementation with soya bean milk or flour. Millet porridge fortified with soya bean milk had the highest amounts of protein and essential amino acids.

The nutrient status of the fortified paps and porridges was comparable with that of a commercial weaning food, cerelac. The value of the soya bean paps and porridges as weaning foods for Nigerian children is discussed.

INTRODUCTION

A number of different foods are consumed in Nigeria. Little, however, is known about their chemical composition and nutritive values. Several investigators have carried out biochemical studies on certain Nigerian foods (Oyenuga & Amazigo, 1957; Bassir, 1964; Bassir & Loebel, 1968; Oke, 1965; Malik, 1965; Akinrele, 1966, 1970; Oke & Tella, 1968; Akinrele & Edwards, 1971; Umoh, 1973; and Umoh & Bassir, 1977). Some of these workers (Malik, 1965; Akinrele, 1966; Umoh, 1973; and Umoh & Bassir, 1977) have studied the losses in nutrients during preparation. Their studies have been almost entirely restricted to foods consumed in the southern parts of Nigeria. If the prevention and eradication of malnutrition is to be achieved at a national level, there is a need to extend such biochemical studies to foods consumed in the northern parts of the country.

A drastic change from the traditional diets and eating habits to a more modern European diet may not provide the right solution to nutritional problems in Nigeria.

Fd. Chem. (3) (1978)—© Applied Science Publishers Ltd, England, 1978
Printed in Great Britain

As a result of the change in eating habits accompanying the increasing urbanisation and socio-economic awareness of some Nigerians, improved nutrition may come about or, because of a too rapid change in traditional eating habits, disease may ensue (Adadevoh, 1972).

Lack of adaptation is suggested as a particular cause of disease. That is, defects in the absorption, digestion and metabolism of the newly-presented food may be responsible for disease. For example, it has recently been shown that the absorption of lactose (the sugar of milk and milk products) is impaired in Nigerians, especially those from the non-dairy areas whose intake of milk and milk products ceases after weaning (Olatunbosun & Adadevoh, 1971).

Milk as a therapeutic diet in the treatment of the protein deficiency syndrome may, because of its lactose content, give rise to symptoms of intolerance with abdominal pain and diarrhoea. Thus, cow's milk may not be the best protein replacement or supplementation diet in the Nigerian context. It follows that local food products must be relied upon to provide adequate nutrition. This therefore calls for extensive analyses of local agricultural products and especially for determination of their essential nutrients.

It is to be realised that new foods could be developed as a result of extensive analyses of the local food ingredients and diets. For example, three sources of high quality protein foods have now been recommended for Nigerians after nutritional studies. One of these, 'Soy Ogi', a corn and soya bean protein enriched food, has been developed over the last ten years by the Federal Institute of Industrial Research, Oshodi (Akinrele *et al.*, 1970; Akinrele & Edwards, 1971). The other, 'Gari' fortified with soya, has also been studied (Bassir, 1964; Bassir & Loebel, 1968; Akinrele, 1970). These foods have been shown to be suitable on chemical evaluation of their nutrient compositions, biological protein quality and, in some cases, clinically, for their therapeutic qualities. A third source, leaf protein, is obtained from green leaves and compares favourably with commercial protein supplements and 'Soy Ogi' (Oke, 1969; Fafunso, 1971). It is a high quality protein source and has recently been shown to have therapeutic efficacy in the management of protein–calorie malnutrition (Olatunbosun & Oke, 1972).

These three protein sources, especially 'Soy Ogi' and leaf protein, compare favourably with known commercial protein supplements and have a greater advantage since they are potentially cheaper (Akinrele & Edwards, 1971). The objectives of a public health nutrition programme should give priority to the effective utilisation of the locally available agricultural resource to provide a cheap and adequate local protein food or supplement.

Eka & Edijala (1972) showed that guinea corn and millet paps were low in protein. It was recommended that the paps be enriched with cow's milk or taken with other foods rich in protein. Kay *et al.* (1975) described methods of using soya bean to improve the protein content of West African diets and thus prevent kwashiorkor. Kay *et al.* (1977) showed how to prepare soya bean milk and flour without a beany

and bitter taste. Eka & Kay (1977) carried out chemical analyses of some soya bean meals prepared using traditional Nigerian methods.

The present series of investigations was carried out to evaluate, by chemical analysis, the nutritive value of cereal paps and porridges supplemented with soya bean flour or soya bean milk.

EXPERIMENTAL

Preparation of soya pap and porridge for analysis

The soya beans and cereal samples were supplied by the Institute for Agricultural Research, Ahmadu Bello University, Zaria, Nigeria. The paps and porridges were prepared using traditional methods as described by Eka & Edijala (1972). The soya bean flour and milk were prepared following methods outlined by Eka & Kay (1977).

Studies were carried out on the following types of paps and porridges:

(a) Paps and porridges prepared from millet, maize and guinea corn.
(b) Paps and porridges prepared from millet supplemented with soya bean milk or soya bean flour.
(c) Cerelac: a Nigerian commercial infant food.

In the preparation of the pap, the cereal grains were cleaned and washed and then soaked in water and kept at room temperature (20–30 °C) for 16 h. The grains were then separated from the water and ground into a paste using a mill. The paste was suspended in three volumes of water, mixed and sieved through a muslin cloth. The filtrate was allowed to stand at room temperature for 24 h, so that the starch settled at the bottom of the container. The water layer was decanted off and the starch used for preparing the pap. In preparing the pap, 4·5 g wet starch was suspended in 20·5 ml of water and 1 g of sucrose was added. The suspension was heated with constant stirring and cooked for about 3 to 5 min to form a thick, viscous paste known as pap.

Soya bean flour was prepared by crushing and sieving soya beans previously boiled for 30 min and then dried. Soya bean milk was prepared by stirring one part of the flour with three volumes of cold water and filtering through a muslin cloth.

The filtrate was boiled for 10 min, 20·5 ml of the milk being equivalent to 1·3 g dry milk solids. In preparing pap supplemented with soya bean milk, the starch paste was cooked with the milk instead of water. The soya bean porridge was prepared from the flour by boiling a suspension of 2·1 g of the flour in 20·5 ml of water and adding 1 g of sucrose. The porridge supplemented with soya bean milk was similarly prepared but the milk was used in place of water. Porridge supplemented with soya bean flour was prepared by mixing 2·1 g of cereal flour with 0·9 g of soya bean flour and cooking in 20·5 ml of water containing 1 g of sucrose.

Pap supplemented with soya bean flour was prepared using 4·5 g of wet starch paste, 0·9 g of dry soya bean flour, 20·5 ml of water and 1 g of sucrose. The cerelac samples were purchased from the local supermarket at Zaria, Nigeria.

Analysis of the samples

The methods of sample treatment and analysis were similar to those recommended by the Association of Official Analytical Chemists (AOAC, 1970). In all cases the diet was dried at a suitable temperature (i.e. 60–80 °C) and then ground into a powder, the dried powder being preserved for subsequent analysis. The ash and organic matter were determined by incineration of a known weight of the sample until ash was obtained. The organic matter was obtained by subtracting the weight of the ash from the weight of the dry material.

The lipid was estimated by exhaustive extraction of a known weight of the material with fat solvent (petroleum ether, boiling point 60–80 °C) using a Soxhlet apparatus. The protein was determined by the macro-Kjedahl method (AOAC, 1970). The carbohydrate content was obtained by the difference method, that is, by subtracting the total protein and lipid from the organic matter. Crude fibre was estimated using the procedure described by Joslyn (1970).

The elemental composition was also determined using AOAC methods (AOAC, 1970). Sodium and potassium were determined by flame photometric methods.

Calcium, magnesium, zinc, iron and copper were estimated using an atomic absorption spectrophotometer. The amino acid analysis was carried out using an automatic amino acid analyser (Beckman 120°). The procedure followed was that described by Oyeleke (1977).

RESULTS

Table 1 shows the proximate chemical composition of the food samples expressed as an average of three determinations. The moisture contents of the paps were high but comparable to that of the prepared cerelac food. The millet supplemented with soya bean milk showed the highest ash content but the value was quite close to that in cerelac. The crude protein in the millet pap supplemented with soya bean milk was high, almost double the amount in cerelac. The non-supplemented cereal paps and porridges were comparatively low in crude protein. The total carbohydrate was high in all the samples and ranged between 70 % and 92 % of dry material. Crude fibre was determined in two samples. The petroleum ether extract ranged between 3 % and 5 % of the food samples and the difference among the food samples was marginal.

Table 2 shows the mineral element content of the food samples. Cerelac showed the highest amount of potassium, sodium and calcium but copper was not detected. Millet porridge supplemented with soya bean milk was rich in most of the elements

TABLE 1
CHEMICAL COMPOSITION OF THE FOOD SAMPLES

Sample	*g/100 g wet weight*	*g/100 g dry weight*				
	Moisture	*Ash*	*Crude protein*	*Carbohydrate*	*Crude fibre*	*Ether extract*
Guinea corn pap	78·3	0·49	4·29	91·8	—	3·4
Maize pap	77·9	0·23	5·55	90·6	—	3·6
Millet pap	84·8	0·50	6·95	88·4	—	4·2
Millet pap supplemented with soya bean flour	72·4	1·18	15·85	78·7	—	5·2
Millet pap supplemented with soya bean milk	76·4	1·75	19·16	73·7	—	5·4
Cerelac	71·0	2·06	10·50	81·6	—	4·7
Guinea corn porridge	86·3	0·97	5·16	90·8	—	3·1
Maize porridge	85·7	1·52	7·22	87·2	—	4·1
Millet porridge	86·3	1·13	8·05	87·4	0·03	3·4
Millet porridge supplemented with soya bean flour	80·9	1·51	15·92	78·4	—	4·2
Millet porridge supplemented with soya bean milk	80·7	2·23	22·93	70·4	0·04	4·4

— = Not determined.

determined. Iron was present in all the samples, the range being between 20 mg/100 g and 60 mg/100 g.

Table 3 shows the amino acid composition of the food samples—lysine was lower in the cereal paps than in the cerelac. The quantity of lysine in the millet supplemented with soya bean milk was about 50 % that in the cerelac. Apart from the comparatively low lysine content, the millet pap supplemented with soya bean milk was comparable in amino acid content to cerelac.

TABLE 2
MINERAL ELEMENT CONTENT OF THE FOOD SAMPLES (mg/100 g DRY MATTER)

Sample	*K*	*Na*	*Zn*	*Mg*	*Ca*	*Cu*	*Fe*
Guinea corn pap	60	60	11·0	26	20	2·0	36·5
Maize pap	30	60	12·5	18	28	1·5	25·5
Millet pap	70	50	12·0	24	24	0	32·0
Millet pap supplemented with soya bean flour	130	30	13·0	84	88	0	28·0
Millet pap supplemented with soya bean milk	440	30	15·0	130	94	0·5	44·5
Cerelac	1360	600	13·5	70	292	0	23·5
Guinea corn porridge	180	30	30·5	128	40	1·5	25·0
Maize porridge	310	35	32·5	212	44	1·5	36·0
Millet porridge supplemented with soya bean flour	260	100	34·5	184	96	2·5	56·5
Millet porridge	260	130	33·5	146	56	1·5	38·0
Millet porridge supplemented with soya bean milk	580	100	35·0	240	110	2·5	51·5

TABLE 3
AMINO ACID COMPOSITION OF THE FOOD SAMPLES (mg AMINO ACID/g SAMPLE)

Amino acid	*Millet pap*	*Millet porridge*	*Millet porridge supplemented with soya bean flour*	*Millet porridge supplemented with soya bean milk*	*Cerelac*
Lysine	0·58	0·63	1·83	3·56	7·64
Histidine	1·08	1·18	2·16	3·60	2·52
Ammonia	2·11	2·40	4·99	4·93	3·46
Arginine	1·29	0·53	1·87	5·51	3·81
Tryptophan	—	0·21	0·98	1·12	—
Aspartic acid	5·79	8·30	16·31	31·59	9·40
Threonine	2·73	3·94	0·22	10·39	5·27
Serine	3·63	4·67	7·19	12·86	7·12
Glutamic acid	18·98	22·75	30·11	56·35	41·88
Proline	5·49	5·34	9·44	14·20	14·62
Glycine	1·54	2·93	4·58	9·38	3·12
Alanine	6·47	8·26	9·01	13·81	4·18
Valine	4·12	5·54	7·58	13·68	7·91
Methionine	1·72	2·26	2·26	3·84	3·10
Isoleucine	3·90	4·58	7·47	13·07	6·36
Leucine	10·14	11·75	14·69	24·11	12·98
Tyrosine	3·00	2·94	4·89	8·08	5·32
Phenylalanine	4·91	5·52	7·84	14·10	6·90

DISCUSSION

The high moisture content of the food samples is bound to influence the quantity that must be consumed in order to meet the specific energy or nutrient demand. In a weaning food high moisture content is an asset and to meet the nutrient requirement the frequency of feeding can be worked out. All the food samples contained high amounts of carbohydrate in terms of dry weight. When the content is considered in terms of wet weight and the recommended daily requirement it becomes clear that a large quantity of the prepared foods is required in order to meet the daily calorie requirement.

The dietary allowance recommended by the Food and Agriculture Organisation (FAO, 1972) shows that children aged 0–1 year and 2–6 years require 1000 kcal and 1500 kcal per day, respectively. To meet this requirement, a child aged one year or less will have to consume 1550 g of millet porridge supplemented with soya bean milk. The main limitation on using porridge as a weaning diet for children up to two years old is its coarseness. Porridge may be recommended for children of 2–6 years old while pap supplemented with soya bean milk is suitable for younger infants. The petroleum ether extract of the food samples showed that cerelac was richer in this fraction. The amount was almost twice that in millet pap supplemented with soya bean milk. This may raise the energy value of the cerelac and thus affect the quantity that must be consumed in order to meet the recommended daily intake of calories.

The supplementation of the cereal meal with soya bean products improved the protein quantity and quality of the cereal foods.

It has been shown that proteins in a mixture of cereals and pulses have a higher growth-promoting capacity than that of cereals or pulses alone (Srikantia, 1975). The sulphur-containing amino acids such as methionine and the aromatic amino acids such as tryptophan are limiting amino acids in legumes (Srikantia, 1975). Legumes are, however, comparatively rich in lysine, the major limiting amino acid in cereals. The combination of cereals and legumes can therefore result in an amino acid balance in the diet.

Acid hydrolysis of protein is known to destroy a large amount of tryptophan and cystine. The negligible or low amounts of these two amino acids may be attributed, at least in part, to the method of hydrolysis. The study of Akinrele & Edwards (1971) has shown that 'Soy Ogi', a maize-based pap, is a complete protein food suitable for weaning children. Millet pap or porridge supplemented with soya bean milk compares favourably with 'Soy Ogi' and other infant foods, including cerelac, with regard to the amino acid composition of their protein, which is of fundamental importance in determining their nutritional quality.

Most of the food samples seem to be adequate in the mineral elements determined. There was no detectable copper in a few of the food samples (Table 2). Iron was high in all the samples and would meet the recommended daily allowance of 12 mg/100 g dry matter (Oke, 1972; Lutham, 1969). The actual amount of the mineral elements available to the consumer may depend on various factors such as the limitation of absorption by phytic acid or oxalic acid present in the foods.

CONCLUSIONS

Soya paps and porridges would serve as weaning foods for children on the basis of their nutrient contents which are comparable to those of 'Soy Ogi' and the other available commercial foods such as cerelac and Lactogen.

The amount of money spent on kwashiorkor cases in paediatric hospitals would be reduced significantly if the supplemented foods could be produced cheaply and made readily available to all areas in Nigeria where malnutrition cases occur.

ACKNOWLEDGEMENTS

The author is grateful to Miss Paulette Faryna of the Agricultural Extension and Research Liaison Service for preparing the food samples and also to Miss Edisua Oku and Mr Lawal for helping with some of the analyses.

REFERENCES

ADADEVOH, B. K. (1972). Nutrition problems and agricultural resources. *Nigerian, Med. J.*, **2**, 122.

AKINRELE, I. A. (1966). A biochemical study on traditional methods of preparation of Ogi. Ph.D. Thesis, University of Ibadan, Nigeria.

AKINRELE, I. A. (1970). Nutrient enrichment of gari. *W. African J. Biol. Appl. Chem.*, **10**, 19.

AKINRELE, I. A., ADEYINKA, O., EDWARDS, C. C. A., OLATUNJI, F. O., DINA, J. A. & KOLEOSO, O. A. (1970). The development and production of 'Soy-Ogi', *Fed. Inst. Industr. Report (Nigeria)*, **42**, 177.

AKINRELE, I. A. & EDWARDS, C. C. A. (1971). An assessment of the nutritive value of maize-soy mixture, 'Soy-Ogi' as a weaning food in Nigeria. *British J. Nutr.*, **26**, 177.

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (1970). *Methods of analysis* (11th ed.). Washington, DC.

BASSIR, O. (1964). Improving the level of nutrition. *W. Afr. J. Biol. Appl. Chem.*, **7**, 32.

BASSIR, O. L. & LOEBEL, W. (1968). Feasibility studies on a plant protein diet as a relief for animal protein shortage in child nutrition. *W. Afr. J. Biol. Appl. Chem.*, **11**, 53.

EKA, O. U. & EDIJALA, J. K. (1972). Chemical composition of some traditionally prepared Nigerian foods. *Nigerian J. Sci.*, **6**, 177.

EKA, O. U. & KAY, T. (1977). Chemical evaluation of the nutrient status of soya bean meals prepared using traditional methods. *Samaru, Agric. Newsletter*, **19**, 76.

FAFUNSO, M. A. (1971). The biochemistry and technology of the proteins of some leafy vegetables in Western Nigeria. Ph.D. Thesis, University of Ibadan, Nigeria.

FOOD AND AGRICULTURAL ORGANISATION (FAO) (1972). *Caloric requirements*, FAO Nutritional Studies No. 15 (5th ed.), Rome.

FOOD AND AGRICULTURAL ORGANISATION (FAO) (1969). *Protein requirements*. FAO Nutritional Studies No. 16 (2nd ed.), Rome.

JOSLYN, M. A. (1970). *Methods in food analysis*, 2nd Ed., Academic Press, New York, London.

KAY, T., IFEACHO, C. L., ONOWU, G. M., UDENZE, V., NNAMANI, G., ANAZONWU-BELLO, J. N., IFENU, F., MODEBE, V. & EMEMBOLU, M. (1975). Use of soya bean to improve the protein content of diet in West Africa and thus prevent kwashiokor. *J. Trop. Paediatrics Environ. Child Health*, **21**, 45.

KAY, T., OGUNSOLA, V. A. & EKA, O. U. (1977). The prevention of beany taste development and elimination of bitter taste in preparing soya bean food in the rural community of Nigeria. *Samaru. Agric. Newsletter*, **19**, 11.

LUTHAM, M. C. (1969). *Human nutrition in tropical Africa*, FAO, Rome, 67.

MALIK, Z. R. (1965). Nutritional changes in some Nigerian foods during dietary preparation and their effect on mammalian body. Ph.D. Thesis, University of Ibadan, Nigeria.

OKE, O. L. (1965). Chemical studies on some Nigerian foodstuff—'Lafun'. *W. Afr. J. Biol. Appl. Chem.*, **8**, 53.

OKE, O. L. (1969). Leaf protein as a solution to protein shortage in developing countries. *J. Nutr. Dietetics*, **6**, 37.

OKE, O. L. & TELLA, A. (1968). Studies on the nutritional significance of leaf proteins in the rat. *West Afr. Pharmacist*, **10**, 2.

OKE, O. L. (1972). A nutrition policy for Nigeria. *World Rev. Nutr. Dietetics*, **14**, 1.

OLATUNBOSUN, D. A. & ADADEVOH, B. K. (1971). Lactose deficiency in Nigeria. *Am. J. Dig. Dis.*, **16**, 909.

OLATUNBOSUN, D. A. & OKE, O. L. (1972). Chemical trial of leaf protein supplements in the management of protein calorie malnutrition. *WACMR. 1st Med. Res. Seminar Abstract*, 40.

OYELEKE, O. (1977). Assessment of the nutritive value of two varieties of sorghum grain, *Sorghum bicolor* (L), Ph.D. Thesis, Ahmadu Bello University, Zaria, Nigeria.

OYENUGA, V. A. & AMIZIGO, G. E. (1957). Hydrocyanic acid content of cassava. *W. Afr. J. Biol. Appl. Chem.*, **1**, 3.

OYENUGA, V. A. (1968). *Nigerian foods and feeding-stuff*. (3rd ed.), Ibadan University Press.

SRIKANTIA, S. G. (1975). Use of legumes and green leaf vegetables in feeding infants and young children. *PAG. Compendium*, **2**, 1231.

UMOH, J. B. (1973). Changes in the nutritive values of some Nigerian diets after cooking by certain South-Eastern Nigerian traditional methods. Ph.D. Thesis, University of Ibadan, Nigeria.

UMO, I. B. & BASSIR, O. (1977). Nutrient changes in some traditional Nigerian foods during cooking. Part 1: Vitamin changes. *Fd. Chem.*, **2**, 155.

CHEMORECEPTION OF SWEET-TASTING DIPEPTIDE ESTERS: A THIRD BINDING SITE

A. VAN DER HEIJDEN, L. B. P. BRUSSEL & H. G. PEER

Unilever Research Duiven, Zevenaar, The Netherlands

(Received: 10 November, 1977)

ABSTRACT

The question whether dipeptide esters with the general formula $COO^- . C . CNH_3^+ . CO . NH . CR . CO . OCH_3$ *are sweet or not cannot be explained solely by the presence of two binding sites* (NH_3^+ *and* COO^-) *acting together as the so-called AH–B system.*

On the basis of data collected at this laboratory and elsewhere, a third binding site is postulated, located at the centre of the bulky R constituent. The position of this third site is compared with those proposed for the sweet nitroaniline series by Kier (1972) *and for sugars by Shallenberger & Lindley* (1977).

INTRODUCTION

Shallenberger & Acree (1967) proposed that perception of sweetness is due to intermolecular hydrogen bonding between the sweet compounds and the receptor site. The common unit in the sweet compounds was described as an AH–B system in which A and B are electronegative groups and H is hydrogen. The distance AH–B needs to be about 0·3 nm for sweetness response; this was confirmed in the case of, for example, sugars, amino acids, saccharin and nitroanilines. In sweet-tasting dipeptide esters (Mazur *et al.*, 1969) the AH–B system is easily identified as the NH_3^+ and COO^- groups of the L-aspartic acid moiety.

Lately it has become clear that, apart from the AH–B distance and the nature of AH and B, a third molecular feature is essential for perception of sweetness. Deutsch & Hansch (1966) indicated the importance of a hydrophobic area. A study by Kier (1972) led to the prediction of the presence—and the approximate location—of a

Fd. Chem. (3) (1978)—
Printed in Great Britain

third structural feature, X, capable of a dispersion bonding with a receptor feature. Höltje & Kier (1974) explained the different sweetness levels in a series of nitroanilines with the aid of model interaction energy calculations. The carbon atom of the phenyl ring to which the side chain is attached was defined as the first atom of the X moiety. They found the optimum X-receptor distance to be 0·425 nm.

Recently, Shallenberger & Lindley (1977) postulated that in sugars like D-glucose the hydrophobic site (denoted as γ) is centred on the C_6 carbon atom. The close proximity of the γ and X features in relation to the respective AH–B systems is striking. Closer study of the structures of sweet-tasting dipeptide esters has suggested a similar positioning of a third binding site for these compounds.

CONFORMATION OF THE SWEET-TASTING DIPEPTIDE ESTERS

The sweetness of L-aspartyl-L-phenylalanine methyl ester (L-Asp-L-Phe-OMe; Aspartame) and some other dipeptide esters was first reported by Mazur *et al.* (1969). Since then, many sweet dipeptide esters have been discovered (e.g. Fujino *et al.*, 1973; Mazur *et al.*, 1970, 1973; Ariyoshi *et al.*, 1974; Brussel *et al.*, 1975). There are three space-filling and/or interacting centres in Aspartame (Fig. 1) and similar dipeptide esters, namely:

> the NH_3^+ and the COO^- groups of the L-aspartic moiety; they comprise the AH–B system when they are not arranged in the trans-conformation;
> the ester group of the L-phenylalanine moiety; increasing the size of the ester group seems only to decrease relative sweetness (Mazur *et al.*, 1969);
> the side chain R (in Aspartame CH_2-ϕ); the length and the size of R greatly influence the relative sweetness (Brussel *et al.*, 1975).

Recently, Lelj *et al.* (1976) studied the conformations of Aspartame in aqueous solutions in the pH range 3·5 to 11·7. The Aspartame molecule can be seen as a combination of two rotamer series, D_I, D_{II}, D_{III} and F_I, F_{II}, F_{III}. The authors concluded that of all nine possible combinations, the F_ID_{II} conformation is the one which interacts with the receptor site of the taste bud. The three rotamers with D_I were excluded because of the trans-arrangement of the NH_3^+ and the COO^- groups,

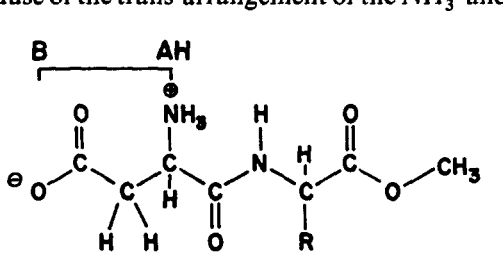


Fig. 1. Aspartame: L-aspartyl-L-phenylalanine methyl ester (R = CH_2-ϕ).

the distance between which exceeds the critical distance of the AH–B system. The authors then restricted themselves to frequently occurring conformations and based their ultimate choice between F_ID_{II} and $F_{II}D_{II}$ on the former as this conformation has similar space-filling properties to the sweet D-leucine molecule.

We consider it premature, however, to assume that Aspartame and D-leucine interact at the same receptor site. Furthermore, space-filling molecular models show

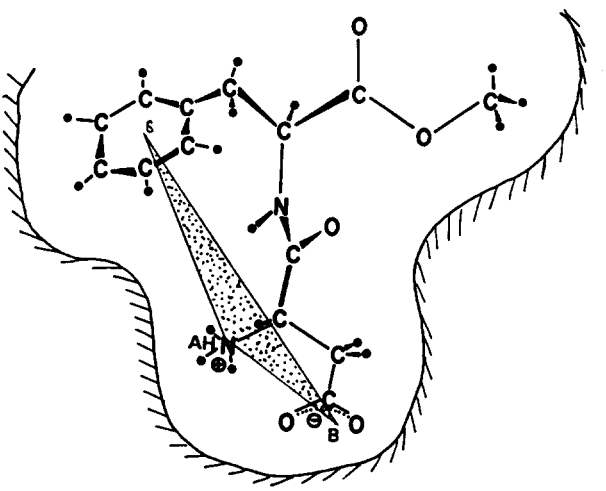


Fig. 2. Proposed conformation of Aspartame and the receptor site.

that, with a F_ID_{II} conformation of Aspartame, the space-filling structure of the side chain R cannot have any influence on sweetness response. For, if the NH_3^+ and COO^- groups are attached to the receptor site, then the R group falls exactly in line with the projection of the rest of the molecule (see Lelj *et al.*, 1976, Figs. 5 and 6). We have demonstrated, however, that the length of the side chain R *is* a limiting factor for perceived sweetness (Brussel *et al.*, 1975). Reconsidering the data of Lelj *et al.* (1976) we conclude that, stereochemically, the $F_{II}D_{II}$ conformation (Fig. 2) is most likely to interact with the receptor site. This conclusion is sustained by findings on the influence of the length and size of the ester groups (Mazur *et al.*, 1969, 1973; Ariyoshi *et al.*, 1974; Ariyoshi, 1976) and those of the R substituents (Brussel *et al.*, 1975; Mazur *et al.*, 1969, 1973; Ariyoshi, 1976) on the sweetness of all sweet-tasting L-Asp-L-amino acid esters, L-Asp-D-amino acid esters and aspartic acid amides (Mazur *et al.*, 1970).

In the $F_{II}D_{II}$ conformation the ester and the R groups serve as 'locking groups', the length and the size of which influence the binding constant, the probability of hitting, and/or the residence time, and hence determine relative sweetness. When R is too long (> 0·88 nm, Brussel *et al.*, 1975) the molecule simply does not fit into the

receptor site (this is not the case with the $F_I D_{II}$ conformation proposed by Lelj *et al.*, 1976). When R is too short ($< \sim 0{\cdot}48$ nm, Brussel *et al.*, 1975) the ineffective 'locking' prevents any significant sweetness sensation.

THE THIRD BINDING SITE

In Fig. 3 the two very similar triangles indicate the positions of the third binding site as defined by Kier (1972) and Shallenberger & Lindley (1977) with respect to the AH–B system. In view of the large influence of the R substituent on the relative

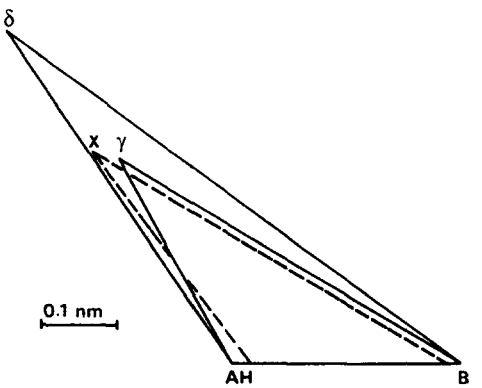


Fig. 3. Location of the third binding sites in: (X) nitroanilines (Kier, 1972); (γ) sugars (Shallenberger & Lindley, 1977) and (δ) dipeptide esters (this paper). Distances in nm: AH-δ $\sim 0{\cdot}52$; B-δ $\sim 0{\cdot}74$; AH-B $\sim 0{\cdot}3$; AH-X $\sim 0{\cdot}35$; B-X $\sim 0{\cdot}55$; AH-B $\sim 0{\cdot}26$; AH-γ 0·314; B-γ 0·525; AH-B 0·3.

sweetness of sweet-tasting dipeptide esters, we would like to postulate that a third binding site for these esters is located within this R group. The size of the ester group of the L-phenylalanine moiety also has an influence on the perception of sweetness, but its effect on relative sweetness is not so pronounced as that of R.

We define the third binding site, δ, in the sweet-tasting dipeptide ester series as the centre of the bulky R substituent, e.g. the centre of the phenyl ring in Aspartame (Fig. 2), the carbon atom of the *t*-butyl group attached to sulphur in L-Asp-L-Cys (S-t.But)-OMe; the centre of the fenchyl group in L-Asp-Ama-(fenchyl)-OMe, etc. (see Brussel *et al.*, 1975). The dipeptide esters are not such rigid molecules as the nitroanilines studied by Kier (1972) or the sugars examined by Shallenberger & Lindley (1977). Therefore, the distances shown in Fig. 3 between AH and B and the third binding site ($\sim 0{\cdot}52$ and $\sim 0{\cdot}74$ nm, respectively) are less exact than those calculated by Kier (1972) and Shallenberger & Lindley (1977).

Although the triangles in Fig. 3 have been derived from three completely different types of sweet molecules, they show a great similarity. Furthermore, the location of

δ justifies our suggestion that it is the $F_{II}D_{II}$ conformation which interacts with the receptor site. It is now clear why L-Asp-D-Phe-OMe is not sweet as in this case the bulky phenylalanine group prevents the molecule fitting into the receptor site. When we compare the relative sweetness of some sweet-tasting L-Asp-D-amino acid esters (Ariyoshi *et al.*, 1974), it is striking that this is generally higher for larger ester groups (now occupying the R position). In these compounds we define δ as the carbon atom of the ester group attached to oxygen.

Besides the length and size of R the hydrophobic character will also help determine relative sweetness. At the moment, however, we consider the correct fit of R as the most important factor.

REFERENCES

ARIYOSHI, Y. (1976). The structure-taste relationships of aspartyl dipeptide esters. *Agr. Biol. Chem.*, **40**(5), 983–92.

ARIYOSHI, Y., YASUDA, N. & YAMATANI, T. (1974). The structure–taste relationships of the dipeptide esters composed of L-aspartic acid and β-hydroxy amino acids. *Bull. Chem. Soc. Japan*, **47**(2), 326–30.

BRUSSEL, L. B. P., PEER, H. G. & VAN DER HEIJDEN, A. (1975). Structure–taste relationship for some sweet-tasting dipeptide esters. *Z. Lebensm. Unters. Forsch.*, **159**, 337–43.

DEUTSCH, E. W. & HANSCH, C. (1966). Dependence of relative sweetness on hydrophobic bonding. *Nature*, **211**(5044), 75.

FUJINO, M., WAKIMASU, M., TANAKA, K., AOKI, H. & NAKAJIMA, N. (1973). L-Aspartyl-aminomalonic acid diesters. *Naturwissenschaften*, **60**, 351.

HÖLTJE, H. D. & KIER, L. B. (1974). Sweet taste receptor studies using model interaction energy calculations. *J. Pharm. Sci.*, **63**, 1722.

KIER, L. B. (1972). A molecular theory of sweet taste. *J. Pharm. Sci.*, **61**, 1394.

LELJ, F., TANCREDI, T., TEMUSSI, P. A. & TONIOLO, C. (1976). Interaction of α-L-aspartyl-L-phenylalanine methyl ester with the receptor site of the sweet taste bud. *J. Amer. Chem. Soc.*, **98**, 6669.

MAZUR, R. H., SCHLATTER, J. M. & GOLDKAMP, A. H. (1969). Structure–taste relationships of some dipeptides. *J. Amer. Chem. Soc.*, **91**, 2684.

MAZUR, R. H., GOLDKAMP, A. H., JAMES, P. A. & SCHLATTER, J. M. (1970). Structure–taste relationships of aspartic acid amides. *J. Med. Chem.*, **13**(6), 1217.

MAZUR, R. H., REUTER, J. A., SWIATEK, K. A. & SCHLATTER, J. M. (1973). Synthetic Sweeteners. 3. Aspartyl dipeptide esters from L- and D-alkylglycines. *J. Med. Chem.*, **16**(11), 1284.

SHALLENBERGER, R. S. & ACREE, T. E. (1967). Molecular theory of sweet taste. *Nature*, **216**, 480–2.

SHALLENBERGER, R. S. & LINDLEY, M. G. (1977). A lipophilic-hydrophobic attribute and component in the stereochemistry of sweetness. *Fd. Chem.*, **2**, 145.

ESTIMATING CALCIUM IN MECHANICALLY DEBONED POULTRY MEAT BY OXIDIMETRY AND ATOMIC ABSORPTION SPECTROPHOTOMETRY

A. C. GERMS & H. STEUNENBERG

Processing Department, Spelderholt Institute for Poultry Research, Ministry of Agriculture and Fisheries, Beekbergen, The Netherlands

(Received: 12 November, 1977)

ABSTRACT

In samples taken over six months from 68 lots of mechanically deboned poultry meat, from several Dutch meat producers, average calcium content by atomic absorption spectrophotometry (AAS) was 2·36 g/kg. As AAS requires expensive equipment, laboratories less well equipped need something less sophisticated. A series of ten meat samples with increasing calcium content was analysed by two oxidimetric methods and by AAS. For in-plant quality control to a maximum calcium content of 2·5 g/kg the oxidimetric methods seemed sufficiently precise and repeatable because a confidence interval of 0·40 g/kg (P = 0·95) was found for analysis on duplicate subsamples. The relationship between the oxidimetric method of Peter (1966) and AAS could not be explained completely, whereas the oxidimetric method of AOAC corresponded well with AAS. There was a significantly ($\alpha = 0{\cdot}01$) higher correlation ($r = 0{\cdot}9996$) between the AOAC method and AAS, so that the AOAC method is preferred more than that of Peter.

INTRODUCTION

In recent years, it has become feasible to separate meat from poultry backs, necks and wings by utilising mechanical deboners. A major factor in acceptance of mechanically deboned meat is the amount of bone particles remaining in the meat. On this subject, the ISO working document 34/6 N 125 (1975) states: 'Bone splinters can cause damage or discomfort if eaten. On the other hand, bone can contribute disproportionately to the weight of the product. Methods are therefore needed for controlling by analysis the total amount of adventitious bone in food products and

Fd. Chem. (3) (1978)—
Printed in Great Britain

the size distribution of larger pieces of bone. Two types of procedure are described in the literature. These are direct methods of separating bone, for weighing or examination, and indirect methods based on analysing for a known component of bone. Direct methods usually rely on digestion of non-bone material by alkaline or enzymic digestion. Indirect methods are usually based on estimating the content of calcium, a characteristic component of bone. It seems likely that simple and effective methods can be agreed without too much difficulty for the relevant chemical analyses but problems are seen in agreeing how to interpret the figures in terms of an equivalent weight of native bone'.

According to Beschikking (1977) in the Netherlands, maximum contents of 2·5 and 10 g/kg are allowed in such meat for calcium and bone, respectively. The factor 4 between the two contents does not correspond with one of the factors 6·25 and 4·55 recommended by the US Department of Agriculture for chickens and for hens and turkeys, respectively (Grunden & MacNeil, 1973). Calcium content of bone is known to vary with the age of the animal and with the type of bone (Field *et al.*, 1974). However, the total amount of bone would not be important if the consumer were only concerned to avoid products with an excessive mineral content (Linke *et al.*, 1974). If the authorities want to test the bone content by a direct method, the more rapid calcium analysis may be used as a screening method to indicate which samples should be analysed by the direct method (Arneth, 1977).

Calcium content can be analysed precisely by atomic absorption spectrophotometry (AAS), but this method requires expensive equipment, so that less well equipped laboratories need something less sophisticated. The EDTA method of estimating calcium has been compared with AAS for the analysis of mechanically deboned poultry meat by Grunden & MacNeil (1973). They found a high correlation ($r = 0{\cdot}99$) between the two methods. However, all values with EDTA were slightly higher than with AAS. According to Grunden & MacNeil, these higher values could be attributed to the presence of magnesium. Kamm & Coffin (1968) estimated calcium in such meat by an oxidimetric method.

Peter (1966) has outlined how calcium can be estimated in foodstuffs by the oxidimetric method with sufficient precision. The magnesium present would not be analysed as calcium in his method. To precipitate the calcium, an excess of oxalic acid is used. The magnesium oxalate formed and the excess of oxalic acid not bound by the calcium and magnesium are removed by washing the precipitate with hot water. In this way, only the oxalic acid bound by the calcium would be titrated with potassium permanganate. In the AOAC method, only a slight excess of oxalic acid is used, by adding oxalic acid to a certain pH. With a dilute NH_4OH solution, the excess of oxalic acid is removed.

In this investigation, calcium was estimated by AAS in mechanically deboned poultry meat marketed by several Dutch producers. A statistical comparison was made between the two simple routine oxidimetric methods and the AAS method, analysing a range of samples with increasing calcium content.

MATERIAL AND METHODS

Poultry meat was obtained over half a year from five Dutch processors and deboned by four different types of machine (Jack Prince, Paoli, Beehive and Bibun). Two subsamples (duplicates) of each of the meat samples were ashed and the ash dissolved in dilute HCl. In these solutions calcium content was estimated by AAS (Varion Techtron, 1972). Subsamples were ashed by the Dutch standard method NEN 3441 (ISO Recommendation R 936), but prescribed addition of magnesium acetate was omitted.

With the resulting data, ten samples forming a series with increasing calcium content were chosen. In a first experiment, calcium content was estimated in duplicate by AAS and by an oxidimetric method (Peter, 1966) in the solutions obtained from the ashes of these samples (Table 1). Magnesium content was also analysed in duplicate in these solutions by AAS.

In a second experiment, the same samples were analysed in the same way by the two methods for calcium and by the AOAC (1970) method 14014. Instead of the indicator, a pH meter was used in the latter method to add oxalic acid to pH 4·4–4·6.

TABLE 1

CALCIUM AND MAGNESIUM CONTENTS BY TWO METHODS IN A SERIES OF MECHANICALLY DEBONED POULTRY MEATS WITH INCREASING CALCIUM CONTENT* (x = MASS FRACTION)

Sample	x $(Ca)/g.kg^{-1}$				x $(Mg)/g.kg^{-1}$	
	AAS		*Oxidimetric Peter* (1966)		*AAS*	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
1	1·1[a]	1·2[a]	1·7[a]	1·0[a]	0·20[a]	0·22[a]
	1·2[b]	1·2[b]	1·0[b]	1·1[b]	0·20[b]	0·22[b]
2	1·3	1·5	1·4	1·4	0·24	0·25
	1·4	1·4	1·2	1·4	0·24	0·25
3	2·0	2·0	2·3	2·0	0·30	0·29
	1·9	2·0	1·9	2·3	0·30	0·29
4	2·2	2·3	1·4	2·6	0·24	0·23
	2·2	2·3	2·1	3·0	0·24	0·23
5	3·0	3·1	2·7	3·6	0·24	0·24
	3·0	3·1	3·4	4·5	0·24	0·24
6	3·7	3·6	3·3	3·4	0·20	0·20
	3·7	3·6	3·0	3·4	0·20	0·20
7	4·7	4·7	5·2	5·1	0·29	0·31
	4·8	4·7	4·7	5·3	0·28	0·31
8	5·1	5·0	5·1	6·3	0·27	0·29
	5·1	5·0	4·9	6·0	0·28	0·29
9	5·7	5·6	5·5	5·2	0·29	0·30
	5·6	5·6	5·8	5·7	0·29	0·30
10	6·2	6·0	6·6	6·7	0·25	0·27
	6·4	6·0	6·4	6·5	0·25	0·27

*Estimates in quadruplicate: ashing in duplicate (indicated by (*a*) and (*b*)) and calcium and magnesium content in duplicate in each ash (indicated by [a] and [b]).

RESULTS AND DISCUSSION

The results of analysis of the poultry meats marketed by several Dutch producers are listed in Table 2. The content in 68 samples for calcium was 2·36 g/kg—that is, just below the maximum allowed in the Netherlands for mechanically deboned meat. In one period, plant B produced meat with far too high a calcium content. The other plants produced meat with a sufficiently low calcium content.

TABLE 2

AVERAGE CALCIUM CONTENT BY ATOMIC ABSORPTION SPECTROPHOTOMETRY OF MECHANICALLY DEBONED POULTRY MEAT (n = NUMBER OF SAMPLES; $\bar{x}$ = AVERAGE MASS FRACTION; $\bar{\bar{x}}$ = GENERAL AVERAGE MASS FRACTION)

Plant	$\bar{x}$ $(Ca)/g.kg^{-1}$	n	*Type of deboner*
A	1·1	12	Jack Prince
B	3·8	19	Bibun
B	2·1	7	Bibun
C	1·5	8	Paoli
D	2·4	8	Bibun
E	2·0	14	Beehive
$\bar{\bar{x}}$	2·36	68	

Experiment 1

With the resulting data, ten samples forming a series with increasing calcium content were chosen in order to compare calcium contents by the oxidimetric method of Peter with those by AAS. The results—and those for magnesium—are given in Table 1.

In order to compare the values statistically, the results of the first experiment were also transformed by an arc sine function. However, this transformation did not improve the results of an analysis of covariance for the relationships between the methods. The values were therefore not transformed in the second experiment.

The coefficient of correlation between y (oxidimetric calcium content) and x (AAS calcium content) was 0·990. The regression y upon x could be described by $y = 1{\cdot}056x - 0{\cdot}05$ with a s_E (standard error of the regression) of 0·29 g/kg. However, the slope and intercept did not differ significantly ($\alpha = 0{\cdot}05$) from 1 and 0, respectively. Further, s_E multiplied by $\sqrt{2}$ approached the s_2 of 0·43 g/kg found for the oxidimetric method. The two methods therefore corresponded satisfactorily. Hence introduction of magnesium in the analysis of covariance did not improve ($\alpha = 0{\cdot}05$) the relationship between the methods. Multiplication of s_E by $\sqrt{2}$ was necessary, because this standard deviation was obtained from the averages of the samples based on four measurements for each method, whereas s_2 was calculated from the averages of the duplicates (a) and (b), each based on two measurements.

The precision (internal variation between aliquots [a] and [b]) expressed in terms of

standard deviation (s_1), was found to be 0·05 and 0·30 g/kg for the AAS method and Peters' oxidimetric method, respectively. In practice, with one measurement in each of the duplicates, the repeatability, calculated from the duplicates (a) and (b) and also expressed as standard deviation, would be 0·05 and 0·34 g/kg, respectively.

Experiment 2

The results from the first experiment raised the question whether the AOAC (1970) method 14014 would have a precision and repeatability approaching those of AAS more closely. The repeatability and precision in the second experiment for the

TABLE 3

PRECISION AND REPEATABILITY (EXPRESSED AS STANDARD DEVIATIONS s_1 AND s_2, RESPECTIVELY) OF ATOMIC ABSORPTION SPECTROPHOTOMETRIC AND OXIDIMETRIC METHODS WITH THE CONFIDENCE INTERVAL $\Delta\bar{x}$ ($P = 0{\cdot}95$) FOR CALCIUM IN DUPLICATE

Method	$s_1/g.kg^{-1}$	$s_2/g.kg^{-1}$ [a]	$\Delta\bar{x}/g.kg^{-1}$
AAS	0·05	0·05	0·23
Peter	0·13	0·09	0·40
AOAC	0·13	0·09	0·39

[a] Corrected to one measurement per subsample.

oxidimetric methods (Table 3) indicated that no errors had been made in weighing and ashing and that the samples were homogeneous. A better result was achieved than in the first experiment for the precision and repeatability of the method of Peter. The precision and repeatability (expressed as standard deviation) of the AAS method were significantly ($\alpha = 0{\cdot}01$) better than those of the oxidimetric methods. They turned out to be independent of calcium content as there was no significant ($\alpha = 0{\cdot}05$) correlation between the difference between duplicates and the calcium content. This meant that the confidence interval $\Delta\bar{x}$ ($P = 0{\cdot}95$) of the averages of the duplicates per meat sample could be calculated. The results (Table 3) show that both oxidimetric methods are sufficiently precise and repeatable for in-plant quality control to 2·5 g/kg.

From the calcium contents estimated by the three methods, the slopes and intercepts for the linear relationships between the oxidimetric methods and AAS were obtained from analysis of covariance as the coefficients of correlation and the standard deviations for the regressions (Table 4).

The coefficient of correlation (r) and the standard deviation (s_E) for the relationship between the method of Peter and AAS did not differ significantly ($\alpha = 0{\cdot}05$) from those in the first experiment. In contrast with Experiment 1, the relationship was not explained satisfactorily because the standard deviation, 0·09 g/kg, obtained for the oxidimetric method was significantly ($\alpha = 0{\cdot}01$) less than the value 0·22 g/kg obtained by multiplying the s_E of the regression by $\sqrt{2}$. There

remains something to be explained in the relationship because only the linear relation was found by analysis of covariance and no significant quadratic or higher relations.

The coefficients of correlation and s_E for the relationship between the AOAC method and AAS were significantly ($\alpha = 0{\cdot}01$) better than those for the relationship between the method of Peter and AAS. The standard deviation of the AOAC

TABLE 4

RELATIONSHIPS BETWEEN OXIDIMETRIC METHODS AND ATOMIC ABSORPTION SPECTROPHOTOMETRY (AAS) (y_1 = METHOD OF PETER (1966); y_2 = AOAC METHOD; x = AAS; r = COEFFICIENT OF CORRELATION; s_E = STANDARD ERROR OF THE REGRESSION)

Regression	*Slope*	*Intercept*	r	s_E
y_1 upon x	1·047	0·11	0·997	0·16
y_2 upon x	1·015	−0·11	0·9996	0·05

method corresponded with that obtained by multiplying by $\sqrt{2}$ the s_E for the relationship between this method and AAS. This relationship was therefore explained completely. The AOAC method is therefore preferable to the method of Peter. Since the slope and intercept of the regression line did not differ significantly ($\alpha = 0{\cdot}05$) from 1 and 0, respectively, the results of the AOAC method correspond completely with those by AAS.

CONCLUSIONS

The average calcium content of mechanically deboned poultry meat from several Dutch processors was 2·36 g/kg.

The magnesium content did not influence the oxidimetric method of Peter for calcium.

The precision and repeatability of AAS were significantly ($\alpha = 0{\cdot}01$) better than those of the oxidimetric methods of Peter and AOAC.

For each of the three methods, precision and repeatability were independent of the calcium content ($\alpha = 0{\cdot}05$).

For in-plant quality control to a maximum calcium content of 2·5 g/kg, the oxidimetric methods were sufficiently precise and repeatable.

The relationship between the oxidimetric method of Peter and AAS was not completely explained, in contrast with the relationship between AAS and the oxidimetric method of AOAC.

The AOAC method had a significantly ($\alpha = 0{\cdot}01$) higher correlation with AAS than the method of Peter.

The oxidimetric method of AOAC is preferable to that of Peter.

The results of the AOAC method correspond completely with those of AAS.

ACKNOWLEDGEMENT

Thanks are due to Mr P. J. W. van Schagen for statistical assistance.

REFERENCES

AOAC (1970). *Official methods of analysis of the Association of Official Analytical Chemists* (11th ed.), 213, Washington, Association of Official Analytical Chemists.

ARNETH, W. (1977). Ein Verfahren zur halbautomatischen Calciumbestimmung in einem Proteinhydrolysat. *Fleischwirtschaft*, **57**, 100–2.

BESCHIKKING UITVOER VLEESPRODUKTEN (1977). *Nederlandse Staatscourant*, 24 March.

FIELD, R. A., RILEY, M. L., MELLO, F. C., CORBRIDGE, M. H. & KOTULA, A. W. (1974). Bone composition in cattle, pigs, sheep and poultry. *J. Anim. Sc.*, **39**, 493–9.

GRUNDEN, L. P. & MACNEIL, J. H. (1973). Examination of bone content in mechanically deboned poultry meat by EDTA and atomic absorption spectrophotometric methods. *J. Food Sc.*, **38**, 712–13.

ISO (1975). Working document 34/6 N 125: *Meat and meat products—Method(s) for determination of bone content*, November.

KAMM, L. & COFFIN, D. E. (1968). Determination of the bone content of comminuted chicken. *Can. Inst. Food Technol. J.*, **1**, 83–5.

LINKE, H., ARNETH, W. & BEM, Z. (1974). Ein Verfahren zur mechanisierten Restfleischgewinnung unter hygienischen, analytischen und lebensmittelrechtlichen Gesichtspunkten. *Fleischwirtschaft*, **54**, 1653–660.

NEN (1968). 3441. Vlees en vleesprodukten. Bepaling van as. Rijswijk, Nederlands Normalisatie Instituut.

PETER, G. (1966). Uber die Bestimmung von Calcium und Kalium in Lebensmitteln mit veränderlichen Matrix. II Bestimmung von Calcium durch Titration mit Kaliumpermanganat. *Z. Anal. Chem.*, **220**, 421–6.

VARION TECHTRON (1972). *Analytical methods for flame spectroscopy.*

CHANGES IN THE HYDROCYANIC ACID CONCENTRATION DURING TRADITIONAL PROCESSING OF CASSAVA INTO 'GARI' AND 'LAFUN'

A. O. KETIKU, I. O. AKINYELE, O. O. KESHINRO & O. O. AKINNAWO

Department of Human Nutrition, University of Ibadan, Ibadan, Nigeria

(Received: 18 November, 1977)

ABSTRACT

Two sets of 48 cassava plants, harvested at three different times, were processed using one of the traditional methods to determine the effectiveness of hydrocyanic acid reduction to innocuous levels in the cassava products 'gari' and 'lafun'. Dry matter content of the cassava was 37·6%; this changed significantly with the stages of processing to 84·5% in 'gari'. This change was due mainly to pressing and roasting. Starch content remained constant during 'gari' processing. Hydrocyanic acid was significantly reduced from an initial concentration of 90·1 mg/kg fresh grated pulp to 25·8 mg/kg in 'gari'. The fermentation step was found to be most effective in the reduction. Dry matter content during 'lafun' processing changed from 39·3% to 86·5% due mainly to sun drying for 96 h. Hydrocyanic acid was significantly reduced from 165·5 mg/kg in fresh grated pulp to 19·6 mg/kg in 'lafun'. Soaking in static water was responsible for three-quarters of the decrease in hydrocyanic acid.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a hardy crop which thrives and gives yields in soils where other food crops would produce little. It is a major supplementary food for people in tropical areas as a source of calories. In Nigeria, cassava is cultivated as an integral part of the food production system and the tuber at harvest is processed locally into 'gari', cassava flour ('lafun'), 'fufu', 'kpokpo gari' and even starch for consumption. In a survey of Southern Nigeria, Nicol (1952) found that 25–56% of the dietary calories came from cassava.

The toxic nature of cassava due to the presence of cyanogenic glycosides which, on hydrolysis, yield hydrocyanic acid (HCN) is well documented (Jones, 1959; Wood,

Fd. Chem. (3) (1978)—
Printed in Great Britain

1965; Oke, 1968). The cyanogenic glycosides are distributed throughout the cassava plant, but the concentration varies greatly between varieties and also with climate, edaphic and cultural conditions. Linamarin and methyllinamarin constitute the only glycosides in cassava yielding HCN. The acid has been incrimated in the aetiology of tropical ataxic neuropathy, a group of nervous disorders diagnosed in patients who have been subsisting for a long time on a diet of cassava products (Oshuntokun, 1969).

Most of the steps involved in the traditional method of processing cassava root tubers into various cassava products aim at reducing the concentration of linamarin and HCN to innocuous levels. These steps include fermentation, pressing, roasting and cooking. The efficacy of the processing techniques is, however, not known since HCN values are usually quoted for the final cassava products without any reference to the HCN level in the original cassava tuber. This, in effect, means that a processing technique that successfully reduces the HCN of a low toxicity cassava to innocuous levels in the product may not be as efficient with a cultivar of high HCN toxicity, thus leaving a higher amount of residual HCN in the final product.

This paper presents the results of a study aimed at determining the effectiveness of traditional methods in eliminating or reducing to innocuous levels the HCN content of cassava root tubers processed into 'gari' and 'lafun', the most widely consumed cassava products in Nigeria.

MATERIALS AND METHODS

'Gari' preparation

The cassava tubers used in the preparation of 'gari' were one year old at the time of harvest from a local farm. Forty-eight cassava plants were numbered on the farm and randomly allocated to three periods of harvest with one week intervals between harvests.

After each harvest, the tubers were transported from the farm to the laboratory where they were washed free of any adhering soil, followed by weighing. Total weight of the tubers for the 48 plants amounted to 16 kg. Peeling of the tubers was carried out by making an incision with a knife from the top to the bottom of each tuber followed by the removal of the peel by hand. The pulps were then grated mechanically by a mill to form a mash from which samples were taken for starch, dry matter and hydrocyanic acid determination. The mash was then transferred into a covered basket and left in front of a house under a tree to ferment for 48 h. Samples for analysis were taken at 24 h and 48 h, after which the mash was transferred into a jute bag preparatory to pressing. The jute bag was then placed in a flat container and two large stones were put on top of the bag to achieve pressing. This step lasted 48 h and samples for analysis were also taken at 24 and 48 h. The remaining mash was

then roasted in a clay oven to make 'gari'. Samples were taken from the resulting 'gari' for analysis.

'Lafun' preparation

Another set of 48 cassava plants, of the same age and from the same farm as those used for 'gari' preparation, were numbered and randomly allocated to three harvest periods with only a week's interval between harvests. The harvested tubers for each period were treated as for 'gari' and the pulps obtained after peeling were then divided into four groups of equal weight. One group was grated with a hand grater to form mash which was analysed for dry matter and HCN. The remaining three groups were immersed in equal volumes of static water sufficient to completely cover the pulp which was contained in plastic buckets of the same size. After five days the water was discarded and the peeled tubers were each mashed manually. The contents of one bucket were taken to the laboratory for analysis whilst those of the other two buckets were sun dried for 48 h and 96 h, respectively. Dry matter and HCN were estimated on the dried samples. The 'lafun' experiments began one week after the last harvest for the 'gari' experiments.

In both the 'gari' and 'lafun' preparations, dry matter was determined in an air oven according to the AOAC method (AOAC, 1970). Starch was estimated by the method of McCready *et al.* (1950) and HCN was determined using the iodimetric method as described by Knowles & Watkins (1950).

RESULTS AND DISCUSSION

The results of the mean changes in dry matter, starch and HCN during 'gari' processing are presented in Table 1 whilst those for 'lafun' processing are shown in Table 2. During 'gari' processing, the mean dry matter content of the mash increased from an initial 37·6% to 40·7% during fermentation and 50·3% after pressing the

TABLE 1

MEAN CHANGES[1] AND STANDARD ERRORS OF THE MEAN FOR DRY MATTER, STARCH AND HYDROCYANIC ACID CONTENT OF CASSAVA DURING 'GARI' PREPARATION

Item	*Dry matter*[2] (%)	*Starch*[2] (*g*/100 *g*)	*Hydrocyanic acid*[2] (*mg/kg fresh grated pulp*)
Starting mash	$37{\cdot}6^{a} \pm 0{\cdot}5$	$87{\cdot}7^{a} \pm 3{\cdot}1$	$90{\cdot}1^{a} \pm 2{\cdot}7$
24 h fermentation	$40{\cdot}9^{a} \pm 0{\cdot}5$	$82{\cdot}7^{a} \pm 3{\cdot}2$	$73{\cdot}2^{b} \pm 7{\cdot}1$
48 h fermentation	$40{\cdot}7^{a} \pm 1{\cdot}3$	$85{\cdot}4^{a} \pm 3{\cdot}2$	$55{\cdot}3^{c} \pm 5{\cdot}2$
24 h pressing	$48{\cdot}7^{b} \pm 2{\cdot}8$	$80{\cdot}7^{a} \pm 2{\cdot}8$	$36{\cdot}0^{d} \pm 6{\cdot}2$
48 h pressing	$50{\cdot}3^{b} \pm 1{\cdot}0$	$79{\cdot}4^{a} \pm 6{\cdot}4$	$32{\cdot}8^{d} \pm 5{\cdot}3$
Roasting	$84{\cdot}5^{c} \pm 0{\cdot}9$	$86{\cdot}2^{a} \pm 2{\cdot}8$	$25{\cdot}8^{e} \pm 2{\cdot}1$

[1] Means in each column with a dissimilar letter are significantly different according to Duncan's Multiple Range Test ($P < 0{\cdot}01$).

[2] Average of nine determinations (triplicate for each harvest period).

fermented mash by the application of weights to the wet material contained in sacks. The dry matter content after fermentation was not significantly different from that in the mash but after pressing it became highly significant ($P < 0.01$). The dry matter content of 50·3 % obtained after pressing agrees with the reported figure of 50 % for that stage of processing (Jones, 1959; Sturtevant, 1969; Coursey, 1973). After this initial dewatering, the dry matter content following roasting was 84·5 %,

TABLE 2

MEAN CHANGES[1] AND STANDARD ERRORS OF THE MEAN FOR DRY MATTER AND HYDROCYANIC ACID CONTENT OF CASSAVA DURING 'LAFUN' PREPARATION

Item	*Dry matter*[2] (%)	*Hydrocyanic acid*[2] *(mg/kg fresh grated pulp)*
Mash	$39·3^{a} \pm 0·07$	$165·5^{a} \pm 1·30$
5-day soaking	$39·9^{a} \pm 0·53$	$35·9^{b} \pm 2·93$
5-day soaking + 48 h drying	$86·5^{b} \pm 2·30$	$25·5^{c} \pm 3·20$
5-day soaking + 96 h drying	$86·2^{b} \pm 0·12$	$19·6^{c} \pm 2·10$

[1] Means in each column with a dissimilar letter are significantly different according to Duncan's Multiple Range Test ($P < 0·01$).
[2] Average of nine determinations (triplicates for each harvest period).

significantly different from those obtained from the other stages of processing. This final product, containing 15·5 % moisture, is considered safe for storage without risk of spoilage. Ingram & Humphries (1972) have, however, suggested that the water content of the final product should be 12 %. This was found to be impracticable with this traditional processing method due to the humid conditions existing locally.

The quantities of starch present at the different stages of processing, expressed on a dry matter basis (Table 1) were not significantly different and would indicate that limited changes were occurring to alter the quantity of starch during processing. The observed differences in starch concentrations at the different stages of processing were therefore due largely to individual variations amongst harvests or a possible hydrolysis of starch to oligosaccharides with processing.

HCN, expressed in milligrammes per kilogramme mash, was found to decrease significantly ($P < 0·01$) with each stage of processing. The mean decrease from the starting mash to the finished 'gari' was $90·1 \pm 2·7$ to $25·8 \pm 2·1$ mg/kg. This represented a 71·4 % decrease with fermentation for 48 h accounting for 38·6 %, 48-h pressing for 25 % and the final roasting step for only 7·8 % of the observed decrease. This breakdown indicates that the detoxication process is most effective during the fermentation and pressing stages. The residual HCN of 25·8 mg/kg can be considered innocuous when measured against the guide of acute toxicity established by Koch (1933), Bolhuis (1954) and de Bruijn (1971). The rate of cyanide decrease was also significantly ($P < 0·01$) different amongst harvest periods (Fig. 1). The rates of HCN decrease in starting mash for harvest periods I and III were not significantly different but were significantly higher than that for harvest period II. A

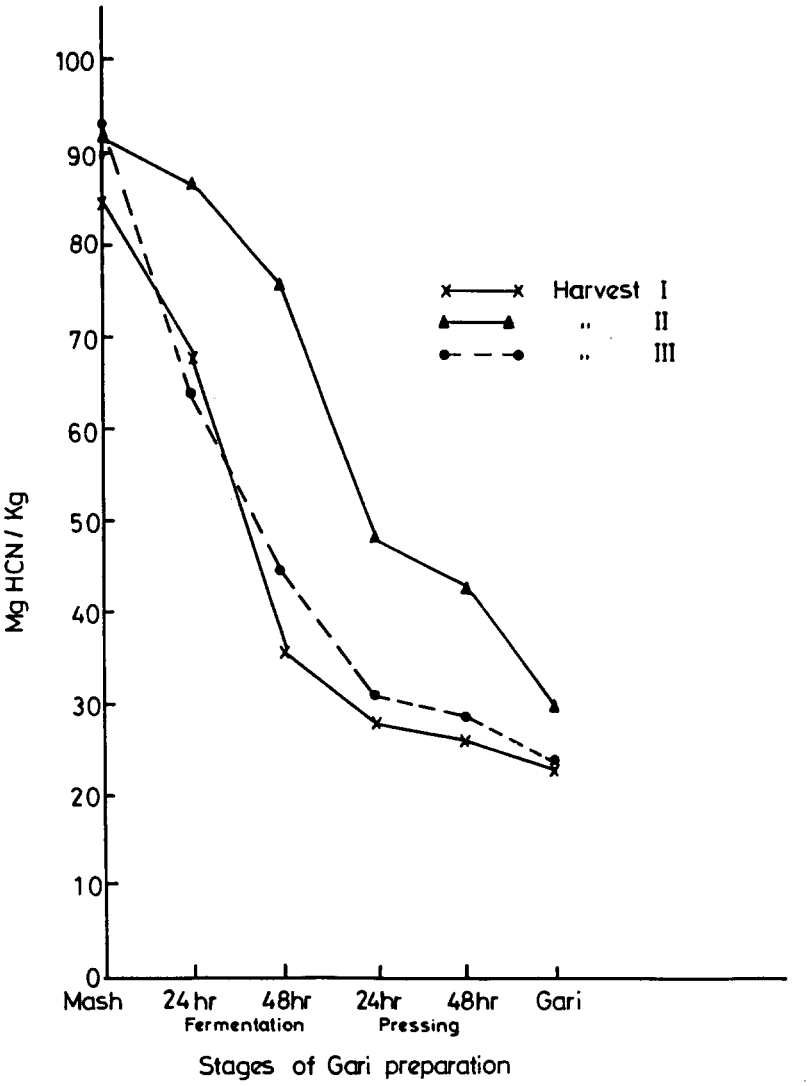


Fig. 1. HCN changes during 'gari' preparation.

possible explanation for this difference could be that the initial grating of the fresh cassava in harvest period II was not thorough enough, thereby preventing total tissue damage. This might have been the case since the mill broke down a few times during the grating of the cassava in harvest period II. It has been reported that there is always a marked difference in the extent of linamarase action on the cyanoglycosides, depending on the amount of tissue damage (Butler *et al.*, 1973).

The HCN level of 25·8 mg/kg obtained in this study for 'gari' represents 28·6% of initial cyanide and falls within the range quoted by Oke (1968). It is lower than values reported by Akerele *et al.* (1962) for mechanised 'gari'. Whilst the level of HCN remaining in 'gari' is considered safe, it is still appreciable. The cases of poisoning sometimes reported (Oshuntokun, 1973) arise from the total dependence of people in certain areas of Nigeria on 'purupuru' which contains five times as much HCN and is different in mode of preparation from the 'gari' described in this study. It is, however, still possible for poisoning to arise from consumption of carelessly prepared products or unusually high HCN material prepared by those familiar with less toxic varieties. Problems may also arise if the 'gari' is prepared in such a way as to contain active enzymes capable of hydrolysing linamarin. An important additional aspect to consider is the extent to which HCN will be retained as cyanhydrins by reacting with carbonyl groups in various compounds, especially carbohydrates. It has been suggested that these cyanhydrins dissociate in the intestine with the absorption of the cyanide in the bloodstream (Wood, 1966; de Bruijn, 1971). Finally, there may also be individual susceptibility to HCN poisoning while abnormal gut microflora might lead to an unusually high release of hitherto unhydrolysed linamarin after ingestion.

During 'lafun' processing the dry matter content was found to be constant through the soaking step but significantly ($P < 0·01$) increased after 48 h sun drying to 86·5% (Table 2). This increase remained unchanged after 96 h sun drying. The residual moisture of 13·5–13·8% was found to be satisfactory for effective storage.

The different stages of processing were effective and significant ($P < 0·01$) in reducing the HCN in 'lafun' to an innocuous level. The mean reduction was from 165·5 ± 1·30 to 19·6 ± 2·1—a percentage reduction of 88·14% in the HCN present initially in the pulp. Soaking in static water considerably reduced the HCN from 165·5 ± 1·30 to 35·9 ± 2·9 mg/kg sample which represented a 78·3% decrease. There are several factors that could have been responsible for the effectiveness of this soaking step in eliminating most of the HCN. It is possible that extraneous microflora could have brought about fermentation or that autolytic hydrolysis of the linamarin might have taken place with consequent dissolution of HCN in the medium. Pressing the soaked pulp manually also helped to eliminate water containing some liberated HCN. Sun-drying for 96 h further reduced the HCN in the 'lafun' by an additional 9·38%.

Sun drying has been reported to reduce the HCN level in cassava flour from 39 mg/kg (Paula & Rangel, 1939); this was higher than the findings in this study in which sun drying reduced the HCN level from 35·9 mg/kg to 19·6 mg/kg. The rates of HCN reduction were similar among the three harvest periods (Fig. 2). This would indicate that the method employed for processing 'lafun' is less susceptible to variability in its effectiveness. It is doubtful if the 'gari' processing method would have reduced the HCN content (165·5 mg/kg) in the fresh cassava used in 'lafun' preparation to the same low level of 19·6 mg/kg. This is indicated by the fact that

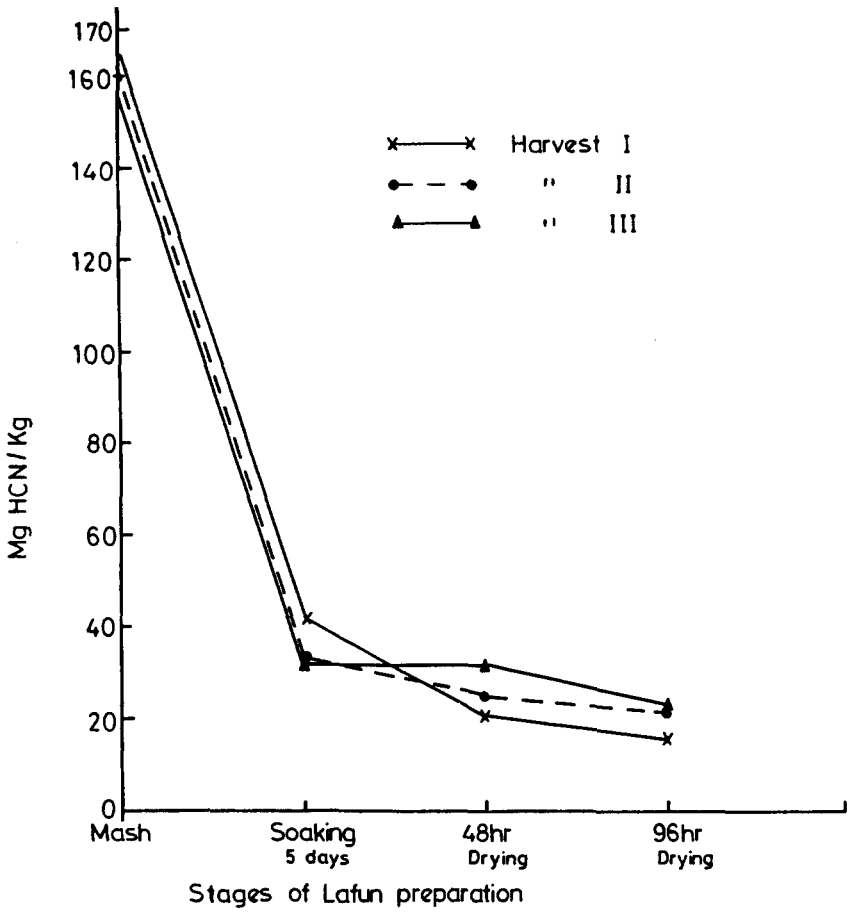


Fig. 2. Changes in HCN during 'lafun' preparation.

HCN from cassava of a lower toxicity (90·1 mg/kg) used for 'gari' preparation was only reduced to 25·8 mg/kg.

CONCLUSIONS

It is evident from this study that the traditional methods of preparation of 'gari' and 'lafun' tested were effective in reducing the HCN content of the final product to innocuous levels. However, further information is needed on the variations in the traditional processing methods in Nigeria and, in addition, an effort should be made to quantify the amounts of unhydrolysed linamarin present in the final product and its degree of toxicity when ingested in the absence of active linamarase. Finally, the

pathway of the detoxication of the residual HCN in the body should be followed to ascertain the amount of HCN accumulation in the blood over a specified period of time.

ACKNOWLEDGEMENT

The authors express their sincere gratitude to Professor A. Omololu, Head, Department of Human Nutrition, University of Ibadan, for providing the funds and facilities for this study.

REFERENCES

AKERELE, I. A., COOK, A. S. & HOLGATE, R. A. (1962). The manufacture of gari from cassava in Nigeria. *Proc. 1st Int. Congress Fd. Technology, London*, 633–44.

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (AOAC) (1970). *AOAC official methods of analysis* (11th edn.), Washington, DC.

BOLHUIS, G. G. (1954). The toxicity of cassava roots. *Neth. J. Agric. Sci.*, **2**(3), 176–85.

BRUIJN, G. H. DE. (1971). *Etude du caractere cyanogenetique du manioc*, Veenman and Zonen, Wageningen.

BUTLER, G. W., REAY, P. F. & TAPPER, B. A. (1973). Physiological and genetic aspects of cyanogenesis in cassava and other plants. In: *Chronic cassava toxicity*, Int. Develop. Res. Centre Monogr. IDRC-OIOe, 65–71.

COURSEY, D. G. (1973). Cassava as food: Toxicity and technology. In: *Chronic cassava toxicity*, Int. Develop. Res. Centre Monogr. IDRC-OIOe, 27–36.

INGRAM, J. S. & HUMPHRIES, J. R. O. (1972). Cassava storage—A review. *Trop. Sci.*, **14**(2), 131–48.

JONES, W. O. (1959). *Manioc in Africa*. Stanford University Press, Stanford, Calif., USA.

KNOWLES, F. & WATKINS, J. E. (1950). *A practical course in agricultural chemistry*, 2nd edn., Macmillan, London.

KOCH, L. (1933). *Cassaveselectie*, Veenman and Zonen, Wageningen.

MCCREADY, R. M., GUGGOLZ, S. V. & OWENS, H. S. (1950). Determination of starch and amylose in vegetables. *Anal. Chem.*, **22**, 1156.

NICOL, B. M. (1952). The nutrition of Nigerian peasants. *Brit. J. Nutr.*, **6**, 1.

OKE, O. L. (1968). Cassava as food in Nigeria. *Wld. Rev. Nutr. Diet*, **9**, 227–50.

OSHUNTOKUN, B. O. (1969). Chronic cyanide intoxication and a degenerative neuropathy in Nigeria. Ph.D. Thesis, University of Ibadan.

OSHUNTOKUN, B. O. (1973). Ataxic neuropathy associated with high cassava diets in West Africa. In: *Chronic cassava toxicity*, Int. Develop. Res. Centre Monogr. IDRC-OIOe, 127–38.

PAULA, R. D. DE G. & RANGEL, J. (1939). HCN or poison of bitter or sweet Manioc. *Rev. Alimentar.* (Rio de Janeiro), **3**(29), 215–17.

STURTEVANT, W. C. (1969). History and ethnography of some West Indian starches. In: *The domestication and exploitation of plants and animals* (Ucko, P. J. & Dimbleby, G. W. (Eds.)), Duckworth, London.

WOOD, T. (1965). The cyanogenic glucoside content of cassava and cassava products. *J. Sci. Fd. Agric.*, **16**(b), 300–5.

WOOD, T. (1966). The isolation properties and enzymic breakdown of linamarin from cassava. *J. Sci. Fd. Agric.*, **17**(1), 85–90.

USE OF THE BOEHRINGER REFLOMAT FOR RAPID DETERMINATION OF D-GLUCOSE CONCENTRATION IN MIXTURES OF FOOD SUGARS

G. G. BIRCH, Y. M. GALLALI & M. W. KEARSLEY

National College of Food Technology, University of Reading, Weybridge, Surrey, Great Britain

(Received: 19 October, 1977)

Although the Boehringer Reflomat (Irmscher *et al.*, 1974; Katterman *et al.*, 1974; Schliack & Gutsche, 1971) has been established as a useful routine tool for the determination of D-glucose in blood samples, no account seems to be available of its use in food analysis. Since the method employs the principle of glucose oxidase determination there would appear to be no obvious difficulty in applying it to a purified solution of food sugars and indeed the latter should be less subject to interference by associated metabolites than the former. However, the instrument is designed to work with a precision of only ± 3–5% in a biological fluid of fairly standard composition and it is possible that in food samples which differ markedly from blood in their composition, the reproducibility might be even less acceptable. Furthermore, the glucose oxidase utilised in this technique contains some maltase which might very well interfere with analyses of glucose syrup mixtures. With this in mind we have examined the instrument for the analysis of some typical mixtures of food sugars with the aim of particularly investigating errors which could arise in samples containing glucose syrups and maltose in high proportions (Table 1).

The results show standard deviations of between 0 and 7·61, the highest values occurring with estimations in mixtures containing glucose and fructose, and in soft drink preparations, the latter containing acid, minerals and pigments in addition to sugars. It might be inferred from the results in Table 1 that the higher standard deviations could be attributed to the non-sugar components in these preparations because the enzyme system employed in the Reflomat is well established as specific for D-glucose and without action on other monosaccharides such as fructose, galactose or pentoses. Normally, oligosaccharides such as maltose and other maltodextrins which occur in glucose syrups (Birch *et al.*, 1970) are not encountered in blood due to their hydrolysis in the brush border region of the small intestine (McMichael, 1973). However, they now constitute a high proportion of sugars,

Fd. Chem. (3) (1978)—

TABLE 1
QUANTITIES OF D-GLUCOSE ESTIMATED (mg/100 ml)

Samples D-Glucose	Added	100·0	110·0	120·0	130·0	140·0
	Recovered	105·0	112·0	120·0	125·0	138·0
		105·0	107·0	121·0	125·0	144·0
		105·0	113·0	121·0	131·0	144·0
		102·0	112·0	120·0	131·0	138·0
		102·0	108·0	121·0	131·0	138·0
		102·0	108·0	121·0	131·0	130·0
	Mean	104·0	110·0	121·0	129·0	140·0
	(% Difference)	(+4·0)	(0·0)	(+0·8)	(−0·8)	(0·0)
	Standard deviation	1·58	2·38	0·41	2·83	2·94
		A	B	C	D	E
D-Glucose + maltose		100·0	110·0	120·0	130·0	140·0
		111·0	119·0	121·0	132·0	140·0
		112·0	117·0	129·0	129·0	139·0
		109·0	117·0	126·0	126·0	140·0
		109·0	117·0	126·0	126·0	140·0
		111·0	119·0	121·0	129·0	139·0
	Mean	110·0	118·0	125·0	128·0	140·0
	(% Difference)	(+10·0)	(+7·3)	(+4·2)	(+6·2)	(0·0)
	Standard deviation	1·26	1·00	3·16	2·28	0·63
D-Glucose + 25 DE G/S		101·0	112·0	123·0	134·0	145·0
		112·0	119·0	122·0	129·0	139·0
		112·0	117·0	122·0	127·0	137·0
		109·0	119·0	124·0	127·0	135·0
		108·0	119·0	126·0	128·0	138·0
		108·0	119·0	126·0	127·0	140·0
	Mean	110·0	119·0	124·0	128·0	138·0
	(% Difference)	(+8·9)	(+6·4)	(+0·8)	(−4·5)	(−4·8)
	Standard deviation	1·84	0·89	1·79	0·89	1·73
D-Glucose + 43 DE G/S	Added	102·0	114·0	126·0	138·0	150·0
	Recovered	112·0	119·0	126·0	132·0	137·0
		111·0	119·0	126·0	130·0	143·0
		111·0	121·0	126·0	129·0	142·0
		111·0	123·0	131·0	135·0	137·0
		114·0	123·0	127·0	133·0	143·0
	Mean	112·0	121·0	127·0	132·0	140·0
	(% Difference)	(+9·8)	(+6·1)	(+0·8)	(−4·3)	(−6·7)
	Standard deviation	1·18	1·79	1·95	1·90	2·83

TABLE 1—*contd.*

Samples						
D-Glucose + 62 DE G/S		103·7	117·4	131·1	145·0	158·5
		114·0	122·0	133·0	139·0	158·0
		108·0	122·0	134·0	142·0	155·0
		111·0	121·0	127·0	153·0	153·0
		112·0	119·0	143·0	153·0	169·0
		111·0	121·0	143·0	146·0	155·0
	Mean	111·0	121·0	136·0	147·0	158·0
	(% Difference)	(+7·0)	(+3·1)	(+3·7)	(+1·4)	(−0·3)
	Standard deviation	1·95	1·10	4·73	3·97	2·56
D-Glucose + maize G/S (29·5 DE)		102·5	115·0	122·5	140·0	152·5
		112·0	119·0	126·0	132·0	149·0
		111·0	119·0	121·0	139·0	145·0
		107·0	114·0	126·0	131·0	142·0
		107·0	120·0	126·0	131·0	142·0
		110·0	117·0	123·0	135·0	145·0
	Mean	109·0	118·0	124·0	134·0	143·0
	(% Difference)	(+6·3)	(+2·6)	(+1·2)	(−4·3)	(−6·2)
	Standard deviation	2·10	2·14	2·10	3·10	2·37
D-Glucose + potato G/S (47·2 DE)	Added	103·0	115·5	128·5	141·0	153·8
	Recovered	114·0	116·0	129·0	139·0	142·0
		108·0	117·0	127·0	139·0	142·0
		111·0	116·0	132·0	137·0	144·0
		112·0	117·0	132·0	137·0	147·0
		111·0	117·0	129·0	139·0	146·0
	Mean	111·0	117·0	130·0	137·0	144·0
	(% Difference)	(+7·8)	(+1·3)	(+1·2)	(−2·8)	(−6·4)
	Standard deviation	1·95	0·63	1·95	2·19	2·04
D-Glucose + Rice G/S (22·2 DE)		104·0	118·0	132·0	146·0	160·0
		112·0	119·0	132·0	139·0	152·0
		109·0	121·0	129·0	140·0	153·0
		116·0	119·0	123·0	145·0	151·0
		116·0	119·0	126·0	145·0	151·0
		113·0	120·0	129·0	142·0	152·0
	Mean	113·0	120·0	128·0	142·0	152·0
	(% Difference)	(+8·7)	(+1·8)	(−3·0)	(+0·7)	(−5·0)
	Standard deviation	2·65	0·89	3·10	2·50	0·77

TABLE I—*contd.*

Samples						
D-Glucose + Wheat G/S		102·0	114·0	126·0	138·0	150·0
		099·0	109·0	120·0	129·0	139·0
		101·0	111·0	119·0	129·0	140·0
		101·0	124·0	126·0	139·0	143·0
		104·0	124·0	126·0	139·0	143·0
		102·0	117·0	128·0	132·0	140·0
	Mean	101·0	117·0	124·0	134·0	141·0
	(% Difference)	(−0·9)	(+2·6)	(−1·6)	(−2·3)	(−6·0)
	Standard deviation	1·67	5·33	3·61	4·56	1·44
D-Glucose + H.F-G/S	Added	104·8	120·0	134·0	148·8	163·5
	Recovered	106·0	116·0	126·0	139·0	149·0
		114·0	116·0	126·0	139·0	158·0
		111·0	116·0	143·0	154·0	162·0
		109·0	115·0	143·0	154·0	162·0
		110·0	118·0	134·0	146·0	164·0
	Mean	110·0	116·0	134·0	146·0	159·0
	(% Difference)	(+5·0)	(−3·3)	(0·0)	(−1·9)	(−2·8)
	Standard deviation	2·61	1·00	7·61	5·97	5·37
D-Glucose + Drink 'A'		110·0	130·0	150·0	170·0	190·0
		106·0	124·0	142·0	168·0	191·0
		106·0	129·0	144·0	164·0	200·0
		110·0	140·0	144·0	160·0	190·0
		114·0	140·0	142·0	168·0	200·0
		111·0	135·0	146·0	168·0	192·0
	Mean	109·0	134·0	144·0	166·0	195·0
	(% Difference)	(−0·9)	(+3·1)	(−4·0)	(−2·4)	(+2·6)
	Standard deviation	3·1	6·29	1·55	3·22	3·87
D-Glucose + Drink 'L'		101·3	113·0	124·0	134·2	146·5
		104·0	114·0	124·0	139·0	145·0
		114·0	119·0	126·0	139·0	145·0
		109·0	119·0	127·0	139·0	149·0
		114·0	119·0	126·0	140·0	149·0
		113·0	123·0	127·0	139·0	145·0
	Mean	111·0	119·0	126·0	139·0	149·0
	(% Difference)	(+9·6)	(+5·3)	(+1·6)	(+3·6)	(+1·7)
	Standard deviation	3·87	2·56	1·10	0·63	4·38

All figures are in mg of D-glucose/100 ml. Columns A, B, C, D, E are mixtures of D-glucose (100 mg) and 10, 20, 30, 40, 50 mg, respectively of the specified sugars. Figures listed as *added* are the actual amounts of D-glucose in these mixtures, determined by the CIRF procedure (1963).
G/S = Commercial acid hydrolysed glucose syrup.
H.F-G/S = Commercial high fructose glucose syrup.
Other glucose syrups (G/S) are laboratory samples prepared from the sources specified.
Drink 'A' = a laboratory soft drink formulation made from glucose syrup, citric acid, water, lemon flavour and tartrazine.
Drink 'L' = a commercial soft drink.

syrups, confectionery, jams and soft drinks and it is important that such substances should not interfere with the determination of D-glucose in food analysis. The results listed in Table 1 show a reproducibility of $\pm 10\%$ which suggests that the method, in its present form, is not totally unsuitable for food analysis. It is possible that the variable recoveries obtained are in part due to the different viscosities of the food sugar samples compared with blood samples, since this would affect rate of diffusion of constituents on to the test strips. Alternatively, some of the rare food sugars present in glucose syrup, and in particular maltose, might actually interfere with the glucose oxidase determination.

ACKNOWLEDGEMENT

We thank Advanced Sweeteners (London) Ltd, for the kind gift of a Boehringer Reflomat.

REFERENCES

BIRCH, G. G., GREEN, L. F. & COULSON, C. B. (1970). *Glucose syrups and related carbohydrates*, Applied Science Publishers Ltd, London.

IRMSCHER, K., ANFT, W. & KATTERMANN, R. (1974). Quantitative Schnellbestimmung der Glucose im Blut und Blutplasma mit einem Remission Photometer (Reflomat). *Verhandlungsberichte vom 9. Kongress der deutschen Diabetes-Gesellschaft, Travemunde.*

KATTERMANN, R., NAROSKA, I. & BUSCH, E. W. (1974). Quantitative glucose-bestimmung mit dem 'BM-Testmeter'. *Z. klin. Chem. klin. Biochem.*, **12**, 43.

MCMICHAEL, H. B. (1973). Carbohydrate intolerance. In: *Molecular structure and function of food carbohydrates.* (Birch, G. G. & Green, L. F. (Eds.)). Applied Science Publishers Ltd, London.

SCHLIACK, V. & GUTSCHE, H. (1971). Epidemiologie des Diabetes mellitus. In: *2nd Intern. Donau-Symposium uber Diabetes mellitus* (Magyar, I. & Beringer, A. (Eds.)). Wiener Med. Akademie, Wien, S. 471.

CIRF (1963). *Standard Analytical Methods of the Member Companies of the Corn Industries Research Foundation.* Washington DC.

BOOK REVIEWS

World Food Resources: Actual and Potential. By Michael Allaby. Applied Science Publishers Ltd, London. vii + 418 pp. Bibliography and index. Price: £15·00.

Mr Allaby brings together an exceptionally wide range of issues that he considers relevant to a discussion of world food resources and has made a valiant, although not always entirely successful, attempt to avoid superficiality by the incorporation of a great deal of recent data. His statistics relate to the late 1960s and early 1970s for the most part, and are discussed sensibly, with the use of a number of economic and other applied scientific concepts. However, the tabular form is somewhat unexciting in such a long book, especially when compared, for example, with Borgstrom's *World Food Resources*. Mr Allaby's book contains a great deal more detail than Borgstrom's, or Pirie's *Food Resources*, but does not have the unity or easy flow of either of these shorter texts. Many of the chapters seem written in isolation, and if the book were to be valuable as a reference work, it would benefit from a more compehensive index.

Chapters 1 and 2 comprise a quarter of the book and discuss agricultural production and causes of supply and price variations, growth of population and the demand for food, and review the current situation in various regions and countries. Although the data is helpful and as recent as can be expected, one is not always sure that Mr Allaby does justice to the complexity of some of the economic concepts he rightly incorporates.

Chapter 3 considers food strategies, as outlined in World Food Congress Plans, defines and discusses malnutrition and reviews the successes and limitations of *the* Green Revolution and others like it.

Chapters 4 to 8 and Chapter 10 are brief reviews of world resources available for crop and animal, including fish, production; energy accounting in agriculture; and trends in, and effects on agricultural production of, climatic changes.

Chapter 9, and Chapters 11 to 13, look at the prospects for improvement in agricultural production and the economic consequences of international aid in attempting to increase developing countries' agricultural output, give a review of recent economic developments, especially in terms of economic power in international bargaining, and suggest possible developments in the near future.

Fd. Chem. (3) (1978)—
Printed in Great Britain

Each chapter has a useful list of references and there is a bibliography of about 50 items preceding the index.

Despite its wide range, the book spends little time discussing food between the farm and consumer. Possible improvements in distribution and processing are rarely touched on, and international trade treated in only a limited fashion—for example, when considering the disadvantageous effects of price reductions on indigenous farming communities and the UNCTAD IV proposals.

Mr Allaby succeeds in producing a general book with more statistical information than many written on the world food situation. He appears aware, also, of some of the limitations of his material and what can be drawn from it.

J. A. BURNS

World Review of Nutrition and Dietetics. Vol. 28: Some Aspects of Human and Veterinary Nutrition. Edited by G. Bourne, Atlanta, Ga. xii + 258 pp., 21 Figs., 37 Tables. Bound, 1978. Price: SFr./DM 161.–/ approx. US $71·75.

This is Volume 28 of a well established and valuable reviewing journal in the field of nutrition at present published twice yearly. The last three volumes have been subtitled; this one is headed *Some Aspects of Human and Veterinary Nutrition.* These volume titles do little to define narrow subject fields within the main topic. The Editorial Board and the contributors are truly international. Volume 28 contains five review articles (which is a smaller number than usual): 'Parenteral nutrition' (110 pages, 409 references), 'Biochemistry and physiology of magnesium (31 pages, 141 references), 'Bone growth and development in protein–calorie malnutrition' (44 pages, 296 references), 'Hepatocarcinogens in Nigerian foodstuffs' (21 pages, 110 references) and 'Carcass evaluation of cattle, sheep and pigs' (25 pages, 49 references).

All the articles are carefully subdivided and their contents printed at the beginning of each review, as well as at the beginning of the volume. Each article is well documented with an extensive and up-to-date reference list. They are not all edited to a uniform format.

The titles are not always indicative of the contents and it is a surprise that the review entitled 'Biochemistry and physiology of magnesium' in a periodical of this title is largely devoted to human aspects and almost completely ignores magnesium deficiency in ruminant animals, an ever present and economically significant phenomenon of developed agriculture which has stimulated the production of an appreciable amount of original scientific research data.

There is a subject index. As with the immediately preceding volumes, there is a cumulated contents list and author index (in this case for Volumes 5 to 27). It is unfortunate that this information is not given on the spine. There is no subject index for the 23 volumes.

Each chapter has a useful list of references and there is a bibliography of about 50 items preceding the index.

Despite its wide range, the book spends little time discussing food between the farm and consumer. Possible improvements in distribution and processing are rarely touched on, and international trade treated in only a limited fashion—for example, when considering the disadvantageous effects of price reductions on indigenous farming communities and the UNCTAD IV proposals.

Mr Allaby succeeds in producing a general book with more statistical information than many written on the world food situation. He appears aware, also, of some of the limitations of his material and what can be drawn from it.

J. A. BURNS

World Review of Nutrition and Dietetics. Vol. 28: Some Aspects of Human and Veterinary Nutrition. Edited by G. Bourne, Atlanta, Ga. xii + 258 pp., 21 Figs., 37 Tables. Bound, 1978. Price: SFr./DM 161.–/ approx. US $71·75.

This is Volume 28 of a well established and valuable reviewing journal in the field of nutrition at present published twice yearly. The last three volumes have been subtitled; this one is headed *Some Aspects of Human and Veterinary Nutrition.* These volume titles do little to define narrow subject fields within the main topic. The Editorial Board and the contributors are truly international. Volume 28 contains five review articles (which is a smaller number than usual): 'Parenteral nutrition' (110 pages, 409 references), 'Biochemistry and physiology of magnesium (31 pages, 141 references), 'Bone growth and development in protein–calorie malnutrition' (44 pages, 296 references), 'Hepatocarcinogens in Nigerian foodstuffs' (21 pages, 110 references) and 'Carcass evaluation of cattle, sheep and pigs' (25 pages, 49 references).

All the articles are carefully subdivided and their contents printed at the beginning of each review, as well as at the beginning of the volume. Each article is well documented with an extensive and up-to-date reference list. They are not all edited to a uniform format.

The titles are not always indicative of the contents and it is a surprise that the review entitled 'Biochemistry and physiology of magnesium' in a periodical of this title is largely devoted to human aspects and almost completely ignores magnesium deficiency in ruminant animals, an ever present and economically significant phenomenon of developed agriculture which has stimulated the production of an appreciable amount of original scientific research data.

There is a subject index. As with the immediately preceding volumes, there is a cumulated contents list and author index (in this case for Volumes 5 to 27). It is unfortunate that this information is not given on the spine. There is no subject index for the 23 volumes.

The series has two disadvantages, first, because of the very wide range of coverage, the specialist reader is likely to find a rather small proportion of reviews of interest in his precise field, however significant they may be, and, secondly, the cost, at about $71·75, is high by the standards of many other journals in this subject area and is not completely offset by the fact that each volume is bound in high quality hard covers ready for shelving.

NIGEL JENKINS

ANNOUNCEMENTS

THIRD WORLD MEAT CONFERENCE IN FLORENCE, 1978

OPIC—The Permanent International Meat Office—will hold its third World Conference in Florence, Italy from 25th to 28th September, 1978, chaired by Mr Peter J. Lardinois, the former Vice President of the European Community.

The President of OPIC, Dr Elio Ragno of Italy, has indicated that some 1200 participants are expected to attend the Conference, coming from all over the world and representing government agencies, scientific, industrial, farming and trade organisations.

In 1976, the world production of all kinds of meat amounted to 120 million tons. If, for the immediate future, there is no forecast of shortage of meat this is due only to economic factors. Inflation increased costs of production, processing and distribution have raised meat prices to high levels both in industrialised and emerging countries. The link between the increase in prices and the pattern of meat consumption is starkly underlined by statistical evidence: slightly more than one-third of the world's population eats three-quarters of the world's meat production. This and other worldwide problems will be on the agenda of the Conference which includes: world meat policy; problems of meat production, processing and consumption; the health aspects of the increase of production and of broader regulation of cattle and the meat trade.

The organisation of the third World Meat Conference has been entrusted to CIM (Consorzio Italiano fra Macellatori Industriali), OPIC's Italian founding member. The headquarters of the organising committee are in Rome, at Via Aureliana 25.

TORRY RESEARCH STATION JUBILEE CONFERENCE

In connection with the fiftieth anniversary of the establishment of Torry Research Station, an International Conference on Fish Science and Technology will be held in Aberdeen, Scotland, from 23 to 27 July, 1979.

Fd. Chem. (3) (1978)—
Printed in Great Britain

The aims of the Conference will be twofold: first to review the state of advancement in various areas of fish science and technology and, secondly, to provide a forum for the presentation of the latest findings in research and development.

The first aim will be achieved through fifteen plenary lectures given by leading authorities. There will be two general lectures on the theme 'Response to Change', four lectures on fish technology topics (handling methods, preservation methods, utilisation and new products, quality improvement and maintenance) and nine lectures on fish science (quality assessment, proteins and structure, lipids, other organic components, nutrition, biological factors, microbiology, physical properties and processes, engineering). The formal lectures will combine a retrospective account of developments over the past fifty years with appraisals of the current position and of probable future trends.

The second aim will be achieved by inviting scientists and technologists to read and discuss accounts of original work. Time will be available to present about thirty-five papers which will be selected on the basis of interest and topicality. All the lectures and papers will be published.

The Conference will last four days in two equal halves separated by one day devoted to visits. On this middle day, Torry Research Station will be open to visitors. Post-Conference tours will be arranged to places of interest.

Further details and application forms will be available from Torry Research Station, PO Box 31, 135 Abbey Road, Aberdeen AB9 8DG, Scotland, towards the end of 1978.

METHOD FOR THE ESTIMATION OF AVAILABLE CARBOHYDRATE IN FOODS

ANNE C. DEAN

Laboratory of The Government Chemist, Cornwall House, Stamford Street, London, SE1 9NQ, Great Britain

(Received: 20 October, 1977)

ABSTRACT

Several types of foods have been analysed for available carbohydrate, comprising free sugars, starch, dextrins and glycogen. The foods examined were meat pie, fish fingers, raw and cooked liver, canned soup, baked beans, yoghourt, cereal, green beans and biscuit. Free sugars were extracted from the foods with 80% (v/v) ethanol. After deproteinisation and deionisation, an aliquot of the purified extract was injected into the Technicon autoanalyser. In this system sugars are separated chromatographically as ionised borate complexes on an anion exchange column at 53°C. Following elution, sugars are determined by reaction with orcinol in sulphuric acid at 95° by measurement of the absorbance at 420 nm of the resulting yellow solutions.

Starch, dextrins and glycogen, if present, were determined in the insoluble residue remaining after extraction of the free sugars. The use of the enzyme glucamylase allows the quantitative transformation of starch to glucose by a stepwise cleavage of ((α-D-(1 → 4) and (α-D-(1 → 6)) glucosidic linkages. The glucose was estimated with the specific enzyme glucose oxidase by colorimetry at 420 nm in the autoanalyser.

INTRODUCTION

Recent interest in the role of sugars, especially sucrose (Yudkin, 1967) and unavailable carbohydrate (Burkitt, 1975), in human health and nutrition has made it desirable to determine more accurately the quantity of available (Southgate, 1969*a*) and unavailable (Southgate, 1969*b*) carbohydrate in the diet. This paper describes methods for the determination of available carbohydrate comprising glucose, fructose, sucrose, maltose, lactose, starch and glycogen in a meat product, vegetables, liver, cooked fish, a dairy product and cereals.

Fd. Chem. (3) (1978)—
Printed in Great Britain

EXPERIMENTAL

Preparation of foodstuffs

With the exception of cereals which were pulverised, all other food products were freeze-dried and ground.

Paper chromatography

Aqueous extractions of freeze-dried samples (10% w/v) were prepared for chromatography (paper, Schleicher and Schuell, 2043b Mg 1) (Hehl, 1973) for qualitative examination of the sugars present. Solvent: 1—propanol: ethyl acetate: water; 7.1.2. Visualising reagent: 4% aniline in methanol (10 ml) 4% diphenylamine in methanol (10 ml) and 2 ml syrupy phosphoric acid. Chromatograms were dipped in the reagent and dried in an oven at 80 °C until spots appeared.

Extraction of free sugars

Freeze-dried material (1–2 g) was weighed into a cellulose thimble and extracted in a Soxhlet apparatus with 80% (v/v) ethanol (100 ml) for 3 h. The pH of the extract should be neutral and no inversion of sucrose occurred at this stage. After identification and estimation of the relative quantities of the constituent sugars by paper chromatography, a sugar not present in the extract was added as an internal standard. Mannose was added to samples which did not contain fructose, but since these two sugars are incompletely separated, rhamnose was added if fructose was present. The ethanolic solutions of the sugars were concentrated under reduced pressure at 40 °C in a rotary evaporator. After weighing, sugar syrups were dissolved in water for deproteinisation.

Deproteinisation (*Laidlaw & Reid*, 1952)

The sugar extract in distilled water (100 ml) was heated to 95 °C on a boiling water bath and equal volumes of barium hydroxide (saturated, *ca.* 10 ml) and cadmium sulphate (equinormal) solutions were added simultaneously. The mixture was cooled immediately and filtered through a bed of Celite filter-aid on a sintered glass funnel (porosity 4) with suction. The filtrate was concentrated to syrup under reduced pressure in a weighed flask and reweighed. The syrup was dissolved in water (10 ml) to be deionised, or a suitable aliquot was taken.

Deionisation

Preparation of the resin: Biodeminrolit (500 g, supplier Hopkin and Williams) was stirred in distilled water (2·5 litres) and, to minimise losses of sugars by absorption on the resin, the liquid was kept saturated by the addition of solid carbon dioxide for approximately 8 h. The resin was filtered on a Buchner funnel, rinsed with distilled water and stored in an air-tight jar until required. When required, the resin was poured in a slurry into a column (24 × 1 cm) fitted with a sintered glass disc

(porosity 1). The sugar solution, or a suitable aliquot, was allowed to percolate through the column until the meniscus was level with the resin surface. The sugars were then eluted with 70 ml of water at a flow rate of 2 ml/min. The eluate was concentrated under reduced pressure, made up to a suitable volume (50 ml) and an aliquot taken for injection in the Technicon autoanalyser.

SUGAR CHROMATOGRAPHY USING THE TECHNICON CARBOHYDRATE ANALYSER SC 1 (FOISSAC, 1967 AND SMITH & McALLAN, 1969)

The flow system used is shown in Fig. 1, using a positive displacement flow rate of 0·75 ml/min through the resin bed of Chromobeads S. All buffer solutions were

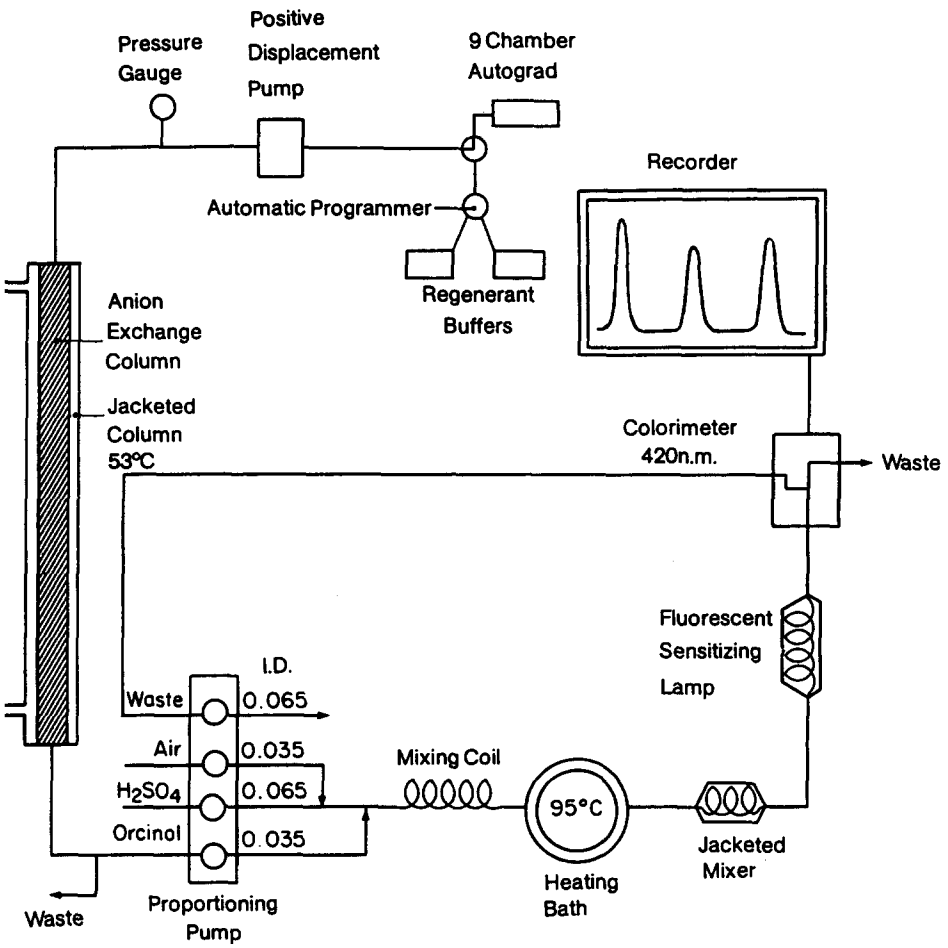


Fig. 1. Sugar chromatography flow system.

filtered before use through Whatman filter tubes size B2. A gradient was prepared from five buffer solutions for use in the nine chamber autograd, as described in Table 1. The elution of sugars with this gradient took eight hours. The resin was regenerated overnight for 14 h with 10% potassium tetraborate and equilibrated for $1\frac{1}{2}$ h before use with buffer No. 1 with the aid of an automatic programmer. The column was re-packed when resolution decreased, normally after six months of continuous use. The resin was removed from the column and suspended in 0·4M

TABLE 1

Solutions for the gradient	
Buffer solutions	*Autograd*
1. 0·1M H_3BO_3, pH 8·0	Chambers A and B, 50 ml of 1
2. 0·1M H_3BO_3 + 0·05M NaCl, pH 8·0	Chamber C, 25 ml of 1, 25 ml of 2
3. 0·1M H_3BO_3 + 0·1M NaCl, pH 8·0	Chamber D, 20 ml of 3, 30 ml of 4
4. 0·2M H_3BO_3, pH 8·0	Chamber E, F and G, 25 ml of 4, 25 ml of 5
5. 0·2M H_3BO_3 + 0·2M NaCl, pH 9·5	Chamber H, J, 50 ml of 5

sodium chloride. Compacted particles were separated by decantation. The resin was re-packed and eluted with 0·4M sodium chloride for 8 h followed by 10% potassium tetraborate until free from chloride. A normal gradient and regeneration cycle was carried out before use. The purification of the colour reagent (1 g orcinol in 70% v/v sulphuric acid, 1000 ml) was as follows.

Purification of orcinol

Orcinol (50 g) was refluxed in toluene (750 ml) with Norit charcoal (2 g) for 2 h. The mixture was filtered while hot through a fluted filter paper (Whatman No. 1). After an oil had settled out from the clear filtrate the supernatant liquid was carefully decanted and allowed to cool with constant agitation in an ice-bath to a temperature between 8° and 10 °C. The crystals obtained were filtered on a Buchner funnel under vacuum and dried over paraffin wax shavings in a desiccator in the dark.

Sugar chromatography

After deionisation, an aliquot of the sugar solution (1 ml) was diluted with boric acid solution 0·2M (1 ml) 10 min before injection. Using a microsyringe, 100–500 μl of this mixture were normally injected, containing not more than 50 μg of each sugar. A calibration for each sugar was prepared by injecting standard sugar solutions at three different concentrations in the range 0–50 μg. Quantities of sugars present in sample extracts were calculated by comparison of peak areas with the calibration obtained with standard sugar solutions at a column temperature of 53 °C. The recovery of sugars was calculated from the amount of internal standard

found compared with the known amount added to the sample and the results were corrected accordingly. Recoveries were normally in the range 90–100% but, with ageing of the pump tubes, could rise to 110%. These were renewed and recalibrated every six weeks. The intensity of colour produced in the reaction of sugars with orcinol/sulphuric acid is very dependent on the temperature and concentration of the sulphuric acid. A typical calibration in a 15 mm cell at 420 nm in terms of the relative peak area per microgramme of sugar injected was as follows: Sucrose 0·200, maltose 0·178, lactose 0·171, rhamnose and mannose 0·188, galactose 0·168, fructose 0·106 and glucose 0·263.

Starch and/or glycogen determination

The residue remaining after extraction of the free sugars with 80% ethanol was dried in a vacuum oven at 30°C to constant weight. The dried sample was finely ground if necessary in a pestle and mortar. Starch was determined following enzymic hydrolysis to glucose. Acid hydrolysis was used if modified starches were thought to be present and the liberated glucose estimated by the use of glucose oxidase or a reducing sugar method (Somogyi, 1951).

Acid hydrolysis of starch to glucose (*Pirt & Whelan*, 1951)

The residue insoluble in 80% ethanol (500 mg) containing up to 100 mg of starch was treated with 1·5N sulphuric acid solution (10 ml) in a boiling water bath for 3 h. The hydrolysate was cooled, neutralised with sodium hydroxide in the presence of phenolphthalein until just pink and brought back to colourless with one drop of acid. Hydrolysates were diluted to contain not more than 70 mg of glucose/100 ml, filtered (Postlip paper A) and analysed for glucose content in the Technicon autoanalyser II using Boehringer's glucose oxidase (GOD-PERID) colorimetric reagent for blood glucose.

Enzymic hydrolysis of starch to glucose (*Thivend* et al., 1972)

The residue (< 500 mg containing ⩾ 100 mg of starch) was heated on a boiling water bath with water (3 ml) for 1 h; or, if a cereal starch, autoclaved at 121°C for 60 min. Acetate buffer (2·5 ml, 0·15M pH 4·8) was added followed by 5 ml of glucamylase suspension in distilled water (2·0 g/50 ml *Rhizopus delemar* (Sigma)). A drop of toluene was added and the suspension was incubated at 50°C for 2 h with gentle shaking. After dilution to 50 ml the solution was filtered, if necessary, and analysed for glucose content (Marks, 1959) in the Technicon autoanalyser as described below. Standard digests of potato, wheat and corn starch (supplier, BDH) were always analysed concurrently with samples as a check on the enzyme activity.

Determination of glucose

The analytical system used is shown in Fig. 2. The colour reagent used was Boehringer's glucose oxidase (GOD-PERID, Cat. No. 15756).

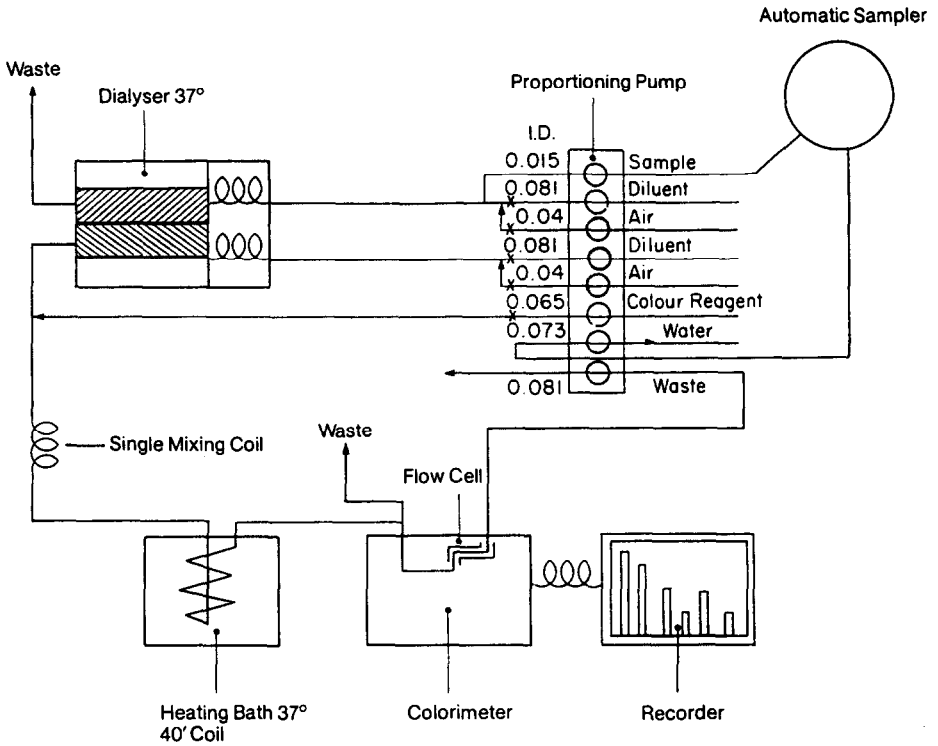


Fig. 2. Flow system for the determination of starch by the autoanalyser.

Preparation of the colour reagent: The enzyme, buffer and colour reagent are supplied in a solid form by the manufacturers: one bottle is sufficient to make one litre of reagent. The solid was dissolved with gentle swirling in 500 ml of water and made up to one litre with glass distilled water. The solution was stable for six weeks if kept in a dark bottle in a refrigerator.

Preparation of the diluent: Sodium chloride (9·0 g AR) was dissolved in glass distilled water and made up to one litre with the addition of the surfactant Brij 35 solution (0·5 ml).

The glucose solutions for analysis were placed in the sampler on the autoanalyser using a two minute wash, one minute sample rate timing can. Peak heights obtained from samples on the recording chart were compared with those from freshly prepared glucose standards in the range 0–100 mg/100 ml. The starch content of the original foodstuff was calculated from the amount of glucose liberated on hydrolysis by multiplying this figure by 0·9. The relative error in the starch determination was 5%.

RESULTS AND DISCUSSION

The values obtained for the available carbohydrate in the proximate analysis of a range of foods are given in Table 2.

This method was developed for the analysis of available carbohydrate in foods for the preparation of tables of food composition. Figures for carbohydrate given in nutritional tables have often been obtained by subtracting the sum of the other constituents (protein, moisture, fat and ash) from 100. The figure thus obtained comprises available and unavailable carbohydrate and is not accurate for the calculation of calorific value. The use of a specific enzyme for the hydrolysis of starch to glucose, instead of acid hydrolysis, avoids any hydrolysis of unavailable carbohydrate and gives a more accurate figure for starch. Acid hydrolysis is only necessary if modified starches are thought to be present which are incompletely hydrolysed by glucamylase.

The Technicon sugar chromatography autoanalyser system was used except that an improved bubble pattern was obtained using acidflex tubes of internal diameter 0·065 mm for the acid/orcinol and waste flow streams and standard pump tubes of internal diameter 0·035 mm for the air and column effluent.

Smith & McAllan (1969) found that the temperature (53 °C) at which the resin was held affected the sensitivity of the colour reaction with fructose and the disaccharide palatinose. This was possibly due to isomerisation of the sugars since two peaks were obtained on elution, the second peak emerging with and immediately after glucose on the chromatogram. In addition, reagent peaks appeared after the glucose position even if only elution buffer was passed through the column. In the present work the reagent peaks referred to by these authors when passing elution buffer alone through the column were obtained; fructose was found to give a linear response at 35 °C and no second peak appeared during an 8-h run when using up to 50 μg of fructose. The response for fructose was found to be higher and linear at 40 °C but maltose and lactose were difficult to resolve at 40 °C. Sugar mixtures containing sucrose, maltose, lactose, fructose and glucose could only be resolved at 53 °C, although sucrose, fructose and glucose were satisfactorily resolved at 40 °C and the response was very reproducible. It was found necessary to add an internal standard to each sample extract immediately after Soxhlet extraction to account for mechanical losses in filtration and deproteinisation procedures and variation due to ageing of the pump tubes.

Comparative analysis of the sugar mixtures by reducing with cuprimetric reagents was possible if only one reducing sugar were present. The results obtained from the autoanalyser for sweet biscuit (sucrose = 21·2%, glucose = 0·5%) agreed with those obtained by Lane and Eynon titration (sucrose = 21·6%, glucose = 0·7%) (Lane & Eynon, 1923). Results obtained by Lane and Eynon titration were found to be less reproducible than those obtained using the autoanalyser when reducing sugars were present at levels below 5% in the fresh foodstuff.

TABLE 2
PROXIMATE COMPOSITION OF SOME FOODS (%)

	Meat pie	*Fish fingers (fried)*	*Liver (Calves')* Raw	Cooked	*Canned mushroom soup*	*Baked beans*	*Yoghourt (nut)*	*Corn flakes*	*Green beans (cooked)*	*Sweet biscuit*
Available carbohydrate										
Sugars										
Sucrose	ND	ND	ND		0·26	3·2	9·7	2·4	0·3	21·6
Maltose	ND	ND	ND		0[b]	0	0	0·4	0	0
Lactose	ND	ND	ND		0·42	0	3·2	0	0	0
Fructose	ND	ND	ND		0·08	0·8	1·0	2·9	0·6	0
Galactose	ND	ND	ND		0	0	1·3	0	0	0
Glucose	ND	ND	ND		0·02	1·0	0·9	1·6	0·4	1·4
Starch (and Glycogen)	24·1	15·5	1·7	6·6	2·8	4·6	0·05	68·2	1·3	44·2[a]
Unavailable carbohydrate[c]	ND	ND	ND	ND	ND	7·0	ND	9·5	3·4	1·6
Water[d]	36·8	55·6	69·7	52·6	89·2	73·6	73·4[e]	3·0	90·7	2·7
Protein[d]	9·9	13·5	20·1	27·0	0·9	5·1	5·3[e]	7·9	1·9	6·1
Fat[d]	27·0	13·1	7·2	13·2	3·7	0·5	2·6[e]	1·6	ND	22·3
Ash	ND	2·6	ND	ND	1·5	2·0	ND	3·6	0·4	1·4
Totals	98·0	100·0	99·0	99·0	99·0	98·0	98·0	101·0	99·0	101·0

[a] Determined by acid hydrolysis and reducing powder (Somogyi, 1951) probably contains a modified starch.
ND = Not determined.
[b] The limit of detection for sugars is 0·02%.
[c] Southgate (1969).
[d] *Methods of Test for Meat and Meat Products*, BS 4401, 1969, 1970.
[e] *Methods for the Chemical Analysis of Dried Milk*, BS 1968.

Two methods (Gutman, 1970; Friedemann *et al.*, 1967) of extracting starch from foods prior to determination were tried; the first, an extraction with dimethyl sulphoxide (4 v) and 8N hydrochloric acid (1 v) (Gutmann, 1970) did not give reproducible results and the other (Friedemann *et al.*, 1967) was found to be laborious in practice. Enzymic hydrolysis of the starch to glucose, without prior separation of the starch, and estimation of the liberated glucose was found to be preferable. Glucamylase from *Rhizopus delemar* (Sigma) was found to give reproducible results. Some variation in activity between batches was experienced with glucamylase from Aspergillus species (Koch-Light). Modified starch, particularly starch acetates, are incompletely hydrolysed by glucamylase. When modified starches were thought to be present in the foodstuff, acid hydrolysis was used to hydrolyse the starch to glucose which was determined by reducing powder (Somogyi, 1951). Errors occur in this determination, however, if partial breakdown of any unavailable carbohydrate present occurs during the hydrolysis. Glucose syrups and malt extracts, which are present in some breakfast cereals, were partially extracted by 80% ethanol and as the maltodextrins were not estimated in the sugar chromatogram the value obtained for starch and dextrins was low. These samples were therefore digested with glucamylase without prior extraction of free sugars and a value for total glucose, maltose, dextrins and starch in the sample obtained from the glucose released. From this value was subtracted the amount of glucose and maltose in the sample obtained by analysis of the 80% ethanolic extract by chromatography of the borate-esters, to give a value for starch and dextrins in the sample. Sucrose was the only free sugar found to give a positive interference in the glucose oxidase estimation of unextracted samples. This was probably due to a trace impurity of invertase in the glucamylase and was negligible unless a large amount of sucrose was present in the sample. Incomplete release of glucose on digestion with glucamylase occurred in some cereal samples, most probably due to protein carbohydrate cross linkages. The use of pancreatin (20 mg), in addition to glucamylase, was found to release the starch for enzymic digestion.

ACKNOWLEDGEMENT

The Government Chemist is thanked for permission to publish this paper.

REFERENCES

BURKITT, D. P. (1975). Diet and disease: Dietary fibre. *Royal Soc. of Health Journal*, **95**(4), 186–8.

FOISSAC, L. (1967). Automatic liquid chromatography applied to research and control in the food industry. *Chimie Analytique*, **49**(12), 649–56.

FRIEDEMANN, T. E., WITT, N. F. & NEIGHBOURS, B. W. (1967). Determination of starch and soluble carbohydrates. I. Development of method for grains, stock feeds, cereal foods, fruits and vegetables. *J.A.O.A.C.*, **50**(4), 944–58.

GUTMANN, I. (1970). New enzymatic methods for the determination of maltose, starch and lactate in food chemistry. *Z. Anal. Chem.*, **252**, 234–6.

HEHL, G. (1973). Determination of carbohydrates in plants. *Z. Anal. Chem.*, **19**, **266**, 268–72.

LAIDLAW, R. A. & REID, R. G. (1952). Analytical studies on the carbohydrates of grasses and clovers. (1) Development of methods for the estimation of the free sugar and fructosan contents. *J. Sci. Fd. Agric.*, **3**, 19–25.

LANE, J. H. & EYNON, L. (1923). Determination of reducing sugars by means of Fehling's solution with methylene blue as internal indicator. *J. Soc. Chem. Ind.*, **42**, 32T–37T.

MARKS, V. (1959). An improved glucose-oxidase method for determining blood, CSF and urine glucose levels. *Clinica Chimica Acta*, **4**, 395–400.

Methods for the chemical analysis of dried milk (1968). BS. 1743.

Methods of test for meat and meat products (1969). Pt. 2; (1970) Pts. 3 and 4. BS. 4401.

PIRT, S. J. & WHELAN, W. J. (1951). The determination of starch by acid hydrolysis. *J. Sci. Fd. Agric.*, **2**, 224–8.

SMITH, R. H. & MCALLAN, A. B. (1969). *Automation in analytical chemistry*, Technicon. International Symposium, London, pp. 207–12.

SOMOGYI, M. (1951). A reagent for the copper-iodometric determination of very small amounts of sugar. *J. Biol. Chem.*, **119**, 771–6.

SOUTHGATE, D. A. T. (1969*a*). Determination of carbohydrates in foods. I. Available carbohydrate. *J. Sci. Fd. Agric.*, **20**, 326–30.

SOUTHGATE, D. A. T. (1969*b*). Determination of carbohydrates in foods. II. Unavailable carbohydrates. *J. Sci. Fd. Agric.*, **20**, 331–5.

THIVEND, P., MERCIER, C. & GUILBOT, A. (1972). *Methods in carbohydrate chemistry* (Whistler, R. L. & Bemiller, J. N. (Eds.)), Vol. VI, 100–5.

YUDKIN, J. (1967). Evolutionary and historical changes in dietary carbohydrates. *Am. J. Clin. Nutr.*, **20**, 108–15.

TOMATO VOLATILE COMPONENTS: EFFECT OF PROCESSING

VICTOR SIESO & JEAN CROUZET

Laboratoire de Biochimie Appliquée, Université des Sciences et Techniques du Languedoc, 34060—Montpellier Cedex, France

(Received: 28 July, 1976)

ABSTRACT

The effect of processing on the volatile components present in tomato products has been studied by combined gas chromatography–mass spectrometry. The conditions involved in the preparation of canned juice do not seriously affect the heavy components, while the most volatile (hexanal, hexenal, hexenols) decrease or disappear. After a prolonged treatment (of tomato paste) these components also disappear and heat-induced products are found (aromatic compounds and furan byproducts). Three compounds (furfural, linalyl acetate and 6-methyl-5-hepten-2-one) are more important. Some of the volatile compounds of the fresh fruit are present, along with heat-induced products, in water condensed from the evaporator. These results are confirmed by the study of the compounds isolated from fresh juice heated at 100 °C in a closed system. The increase of trans-2-*hexenal and decrease of* trans-2-*hexen*-1-*ol are discussed on the basis of possible pathways by which these products are formed in tomato.*

INTRODUCTION

During the last ten years the volatile components of fruits and vegetables have been extensively studied (Buttery *et al.*, 1971, Johnson *et al.*, 1971). The latter reference is a review concerning tomato, the fruit considered in this study.

Most literature concerning the effect of processing on the volatile components of tomato is less extensive and more dispersed. Nelson & Hoff (1969) have reviewed the studies published up until this time; these authors have shown that the concentrations of acetaldehyde, acetone, methanol and hexenal were modified during the processing. They confirm that methyl sulphide found by Miers (1966) is a heat induced product. Buttery *et al.* (1971) report that heating caused an increase in

Food Chem. (2) (1977)—
Printed in Great Britain

the relative amounts of α terpineol and linalol. Reymond (1971) points out that chromatograms of vacuum steam distillates of tomato pastes processed by different heat pretreatments (cold break, activated break, hot break) are different in the hexenols and hexenals regions. Seck *et al.* (1976) have observed in a commercial tomato juice the decrease or disappearance of the volatile components of the aroma of fresh tomatoes, especially *cis*-3-hexenal, and the formation of heat-induced products (furan derivatives and aromatic compounds).

The purpose of the present work is to establish these findings by studying the influence of processing on volatile tomato components under controlled conditions.

EXPERIMENTAL

Materials

Tomatoes (ROMA VF variety) were grown in an open field at the experimental station of Puyricard 13, France. Fresh juice was prepared by cold break of fruits and refining. Canned juice was obtained by hot break (80 °C), refining and pasteurisation of the cans in boiling water for 10 min. Tomato paste (28° Brix) was prepared by vacuum concentration at 63–65 cm pressure with the product at 55–60 °C in a pilot unit. Before canning the paste is heated at 94 °C and the cans immediately cooled with fresh water.

The water condensed from the concentrator unit was collected and extracted with methylene chloride.

Fresh juice was heated at 100 °C for 10 min (after thermal equilibration) in a closed system constituted by a 6 litre reactor fitted out with a reflux condenser and a mechanical stirrer. The fresh juice was frozen at −20 °C and conserved at this temperature for one to five months. The canned juice and tomato paste were stored at +4 °C for the same period.

Isolation of volatile components

The volatile components were obtained by stripping in a cyclone apparatus working under vacuum (Cobb, 1969). The extraction of 1 litre of juice or reconstituted juice (in the case of the tomato paste study) was carried out over a 2 h period at 30 °C under 80 mm pressure. The traps were cooled with ice water and liquid nitrogen successively. The condensed fractions in the two traps were extracted with methylene chloride, the organic extract was dried over a small quantity of anhydrous sodium sulphate and filtered. Most of the excess methylene chloride was then removed under a stream of nitrogen to yield a concentrate (2 ml from about 30 litres of initial product).

Analytical procedures

Analytical gas–liquid chromatography was carried out on a 10 ft × 0·25 in o.d.

stainless steel column packed with 1·5% Carbowax 20 M on Chromosorb W 60–80 mesh. The temperature was programmed from 50 to 170 °C at 2 °C per min and maintained. For preparative purposes a 10 ft × 0·5 in o.d. stainless steel column packed with 20% OV-17 on Chromosorb W, AW-DCMS was used. Fractions were collected in dry ice-methanol cooled capillary glass U-tubes.

Combined gas chromatography–mass spectrometry was carried out with a GIRDEL 3000 chromatograph coupled with a VARIAN MAT CH 5 mass spectrometer by a Watson Biemann separator. The source temperature was 250 °C and the ionising energy 70 eV. The column was the same as that used for analytical purposes. Authentic samples of chemical compounds were obtained from commercial supply houses or were synthesised by other laboratories or by ourselves using established methods.

RESULTS AND DISCUSSION

Before undertaking the study of the effect of processing on volatile tomato components, it was necessary to have a reference by studying the volatile components extracted from the fruit in the conditions utilised in the present work.

The chromatogram obtained upon separation of tomato volatile components (variety ROMA V.F.) on Carbowax 20 M 1·5% is presented in Fig. 1. Identified compounds are listed in Table 1 in order of their elution. All these products except two (carvone and citronellol) are known as volatile tomato components. Carvone (peak 47) was identified by its mass spectra (Stenhagen *et al.*, 1970) and its presence confirmed by determination of its retention time (RT). This product is probably formed by oxygenation of linalol during the crushing of the fruit or during the extraction process in the cyclone.

The peak 48–49, besides exhibiting characteristic fragments of geranial, showed the possible presence of citronellol (Cornu & Massot, 1975).

The presence of *trans*-2-hexen-1-ol tentatively identified by Seck & Crouzet (1973) was confirmed by determination of its RT. From the fractions isolated by preparative GC it was possible to detect by mass spectrometry the presence of the following compounds (Table 2): citronellyl acetate (Cornu & Massot, 1975), linalyl acetate (Stenhagen *et al.*, 1970), a product yielding a mass spectral peak at m/e:99 characteristic of some δ lactones (Urbach *et al.*, 1973) identified as δ octalactone and cuminyl alcohol (Von Sydow, 1963). The two first components have been previously described in tomato by Shah *et al.* (1969) by functional group analysis, retention time and IR spectra, and the last two are tentatively identified among the tomato volatile components for the first time.

Figure 2 shows the chromatogram obtained for the volatile components isolated from the canned juice. We can note the disappearance of the peak characteristic of hexanal while peaks of pentanol (peak 15) and of *cis*-3-hexen-1-ol (peak 28) are

considerably reduced. The quantities of *trans*-2-hexanal (peak 14) and *trans*-2-hexen-1-ol (peak 29) in the mixture are more important.

On the other hand, the furfural derivatives, especially methyl-5-acetyl-2 furan (peak 41) and furfuryl alcohol (peak 44) are more important than in the fresh juice. We can also note the appearance of ethyl benzene (peak 6) and of a trimethyl benzene (peak 20). These products are known as thermal products formed during the degradation of carbohydrates (Fragerson, 1969).

TABLE 1
IDENTIFICATION OF VOLATILE COMPONENTS ISOLATED FROM TOMATO PRODUCTS (Figs. 1 to 5)

Peak No.	*Compounds*	*Means of identification*	*Mass spectral data* m/e
1	Ethanol	RT	
2	Chloroform	MS–RT	
3	Propanol	MS–RT	
4	Toluene	MS–RT	
5	Hexanal	MS–RT	
6	Ethyl benzene	MS	
7	*p*-xylene	MS–RT	
8	*m*-xylene	MS–RT	
9	*o*-xylene	MS–RT	
10	1-pentene-3-ol	MS–RT	
11	butanol	MS–RT	
12	propyl benzene	MS	105, 119. 134, 91, 97
13	3-methyl butanol	MS–RT	
14	*trans*-2-hexenal	MS–RT	
15	pentanol	MS–RT	
16	*p*-cymene	MS–RT	
17	1-methyl-4-ethyl benzene	MS	105, 120, 77, 39, 91
18	styrene	MS–RT	104, 103, 78, 57, 77
19	acetoin	MS	
20	trimethyl benzene	MS	
21	benzyl methyl ketone	MS	134, 91, 57, 43
22	4-heptanol	MS–RT	
23	diethylbenzene	MS	105, 119, 134, 91, 97
24	2-methyl-2 pentanol	MS–RT	
25	6-methyl-5-hepten-2-one	MS–RT	
26	hexanol	MS–RT	
27	2-heptanol	MS	
28	*cis*-3-hexen-1-ol	MS–RT	
29	*trans*-2-hexen-1-ol	MS–RT	
30	2-isobutylthiazol	MS–RT	

From the fractions obtained by preparative GC we have tentatively identified the 2-methyl butenal (Table 2); this compound has been previously identified in roasted products (Kinlin *et al.*, 1972; Vitzthum *et al.*, 1975).

The chromatogram of tomato paste is given in Fig. 3. In this figure we can observe that the less volatile compounds have disappeared while many peaks are more important than in the chromatogram obtained for the fresh juice (Fig. 1).

Three peaks are particularly important: linalyl acetate (peak 37) observed in the

TABLE 1—*contd.*

Peak No.	*Compounds*	*Means of identification*	*Mass spectral data* m/e
31	2-octenal	MS–RT	
32	benzyl ethyl ketone	MS	148, 105, 43, 91, 77
33	furfural	MS–RT	
34	6-methyl-5-hepten-2-ol	MS–RT	
35	2-acetyl furan	MS–RT	
36	benzaldehyde	MS–RT	
37	linalyl acetate	MS–RT	
38	linalol	MS–RT	
39	5-methyl furfural	MS–RT	
40	*p*-tolualdehyde	MS–RT	91, 119, 120, 39, 65
41	5-methyl-2-acetyl furan	MS–RT	
42	δ butyrolactone	MS	
43	phenylacetaldehyde	MS–RT	
44	furfuryl alcohol	MS–RT	
45	α-terpineol	RT	
46	4-isopropyl phenyl methanol	MS	91, 77, 79, 132, 135, 150
47	carvone	MS–RT	82, 54, 108, 93, 58
48	geranial	MS–RT	81, 55, 82, 68, 95
49	citronellol	MS	41, 69, 65, 67, 81
50	geranyl acetone	MS	
51	geraniol	MS–RT	
52	2,4-decadienal	MS	
53	guaiacol	MS–RT	
54	benzyl alcohol	MS–RT	
55	2,6-di-methyl-2,6-undecadien-10-one	MS	
56	2-phenyl ethanol	MS–RT	
57	β-ionone	MS–RT	
58	*p*-anisaldehyde	MS–RT	
59	isopropyl anisol	MS	
60	eugenol	MS–RT	

fresh juice, furfural (peak 33) found in the compounds isolated from heated tomatoes (Buttery *et al.*, 1971) and from canned juice (Seck *et al.*, 1976) and 6-methyl-5-hepten-2-one (peak 25). Cole & Kapur (1957) have found that this last product is formed during lycopene oxidation.

TABLE 2

COMPOUNDS IDENTIFIED BY MASS SPECTROMETRY IN FRACTIONS ISOLATED BY PREPARATIVE GAS CHROMATOGRAPHY

Tomato product	*Compounds*	*Mass spectral data* m/e
Fresh juice	citronellyl acetate	43, 41, 69, 81, 28
Fresh juice	linalyl acetate	41, 93, 69, 43, 45, 136
Fresh juice	δ-octalactone	99, 42, 43, 71, 142
Fresh juice	cuminyl alcohol	150, 135, 107, 109, 91
Canned juice	2-methyl-butenal	55, 29, 84, 27, 39
Heated juice	vinyl ketone	41, 55, 70
Heated juice	2-ethyl-6-vinyl pyrazine	134, 133, 51, 52, 53

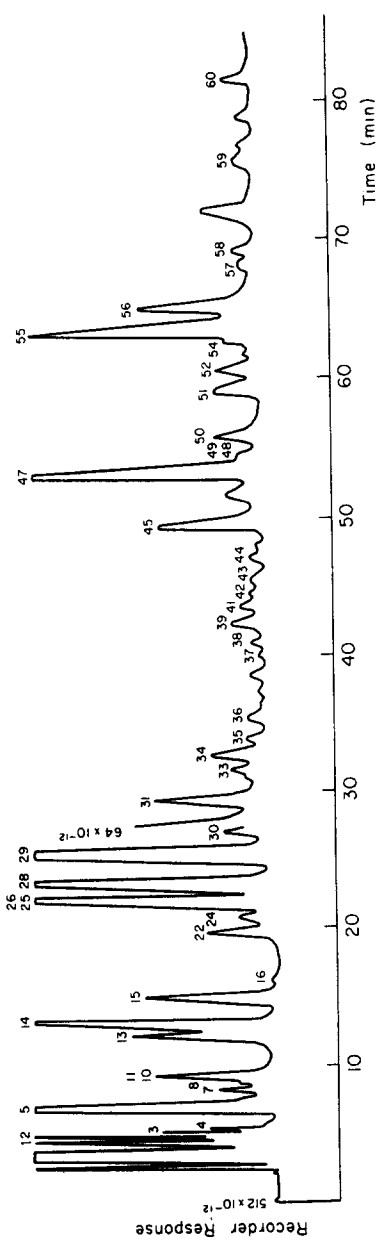


Fig. 1. Gas chromatogram of volatile components of fresh tomato juice.

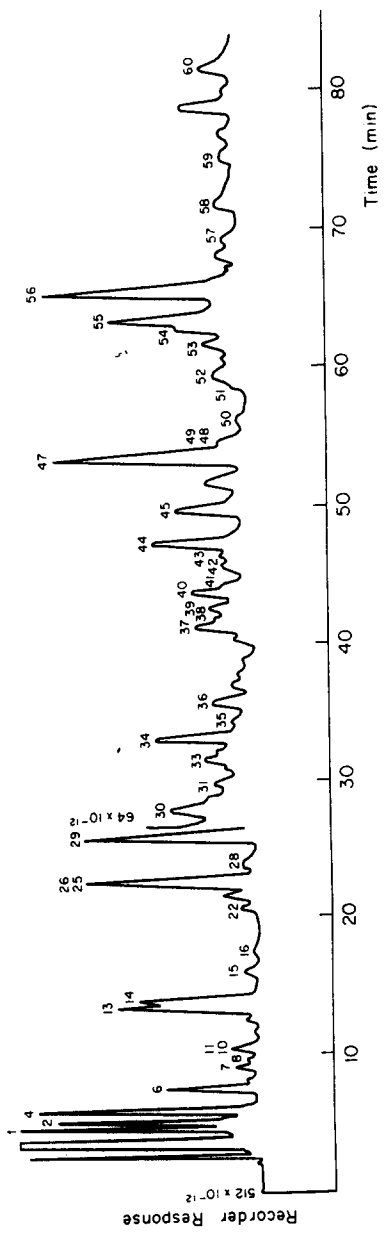


Fig. 2. Gas chromatogram of volatile components of canned tomato juice.

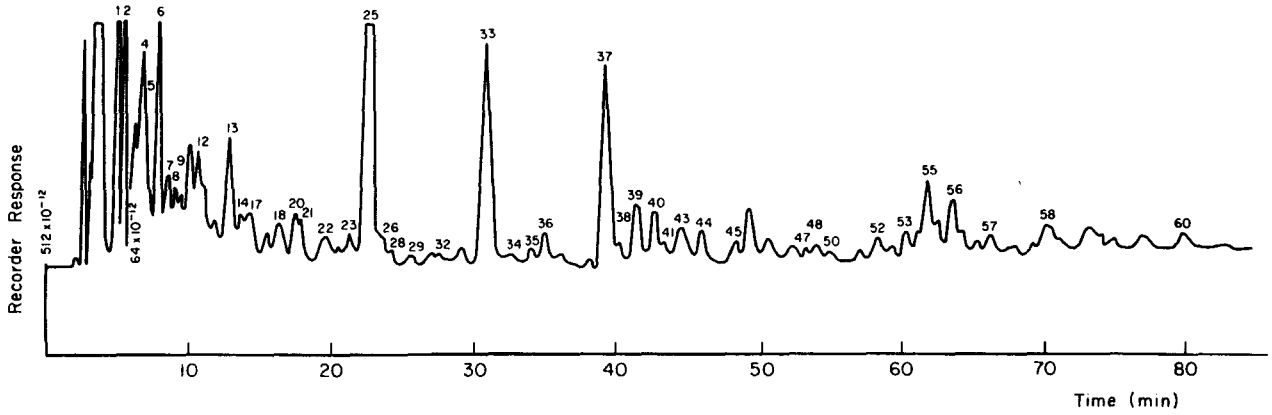


Fig. 3. Gas chromatogram of volatile components of tomato paste.

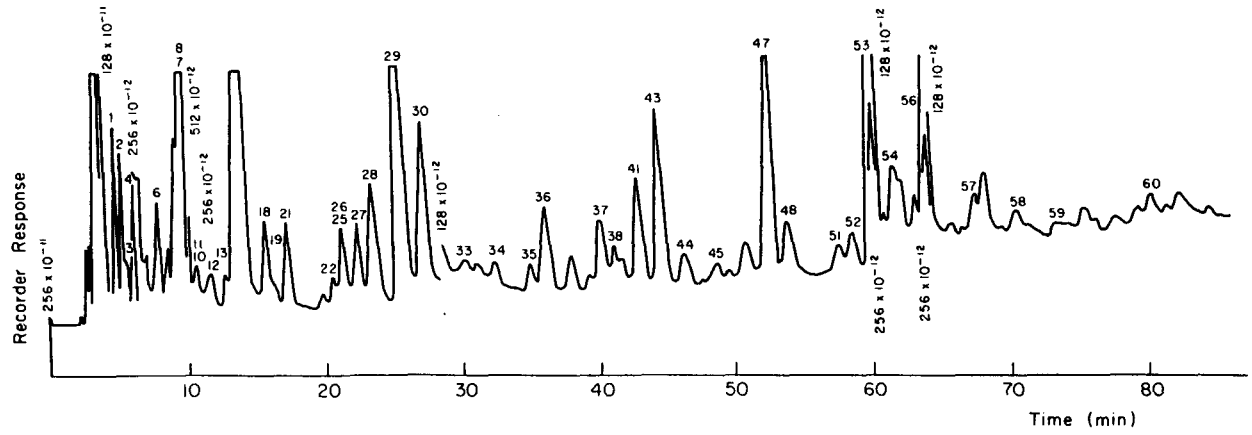


Fig. 4. Gas chromatogram of volatile components isolated from condensed water in the concentrator unit.

Mass spectral data indicate the possible presence of the following compounds: 1-methyl-4-ethyl benzene (peak 17) (Cornu & Massot, 1975); *n* propyl benzene (peak 12) (Cornu & Massot, 1975); a diethyl benzene (peak 23); benzyl ethyl ketone (peak 32) (Cornu & Massot, 1975); styrene (peak 18) (Jouret *et al.*, 1972) also identified by its RT. These products may be formed by heating during the concentration process; the styrene, also found by Jouret *et al.* (1972) during wine making, proceeds from the decarboxylation of cinnamic acid.

In condensed water (Fig. 4), we note the presence of several compounds involved in the aroma of fresh tomato: 3-methyl butanol, *trans*-2-hexenal, *cis*-3-hexen-1-ol, *trans*-2-hexen-1-ol, 2-isobutylthiazol, stripped during the concentration process. Beside these compounds we note the appearance or a marked increase of benzene derivatives: styrene already isolated from the tomato paste, toluene, *o*-, *m*- and *p*-xylene, benzaldehyde, phenylacetaldehyde and 2-phenylethanol. Furan by-products already found in tomato juice (Cobb, 1969) and especially furfural and 5-methyl-2-acetylfuran are found in this fraction.

On the other hand we have isolated from the condensed water the following compounds: a compound (peak 46) identified as an isopropyl phenyl methanol *m/e*:150 (M), *m/e*:132 (M–18), *m/e*:135 (M–15), *m/e*:91 (tropilium ion), *m/e*:77 and 79 (aromatic ring), *m/e*:43 (isopropyl) and a compound (peak 21) identified as benzyl methyl ketone, *m/e*:134(M), *m/e*:91 (tropilium ion), *m/e*:57 (CH_3–CO–CH_2^+), *m/e*:43 (CH_3–CO^+). These aromatic compounds are certainly induced by heat treatment during the concentration process.

We have tried to establish the effect of heat on the formation of volatile components by heating fresh tomato juice at 100 °C during 10 min (after thermal equilibrium) in a closed system (Karlsson-Ekstrom & Von Sydow, 1973).

Under these conditions we have found (Fig. 5) in the processed product the volatile compounds of the fresh products unchanged by the treatment. On the other hand we can see that heavy compounds are more important than in the case of fresh juice, especially furan derivatives, benzaldehyde, phenylacetaldehyde and 2,6-dimethyl-2,6-undecadien-10-one.

Among the compounds formed under these conditions we have found after fractionation by preparative chromatography a product characterised by S.M. fragments at *m/e*:41 (isopropyl), *m/e*:55 (CH_2=CH–C≡O^+), *m/e*:70

$$(CH_2{=}CH{-}\underset{\underset{O}{\|}}{C}{-}CH_2 + H)$$

tentatively identified as a vinyl ketone and 2-ethyl-6-vinylpyrazine (Friedel *et al.*, 1971) (Table 2). Pyrazines are typical heat-induced products. Ryder (1966) has previously identified two of them in tomato products as 2,6-dimethylpyrazine and 2-methylpyrazine.

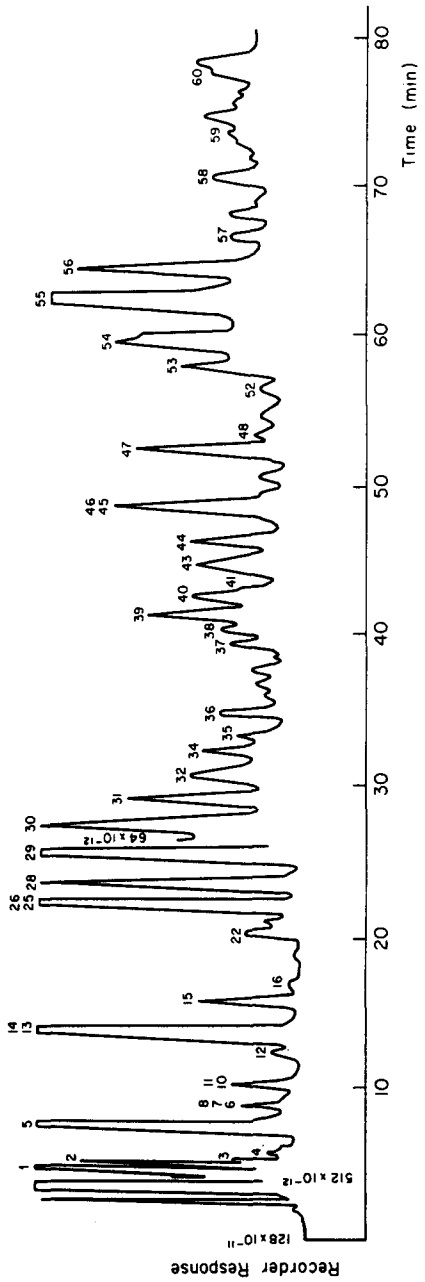


Fig. 5. Gas chromatogram of volatile components isolated from tomato juice heated at 100°C for 10 min.

The relative proportions of the most characteristic products present in fresh and heated juice were determined (Table 3). The area of each peak was estimated and the values so obtained divided by the sum of the area of all peaks present in the chromatogram (the solvent and products with lower retention times were not involved in this calculation).

TABLE 3
RELATIVE AMOUNTS OF THE MOST CHARACTERISTIC COMPOUNDS PRESENT IN FRESH AND HEATED JUICE (100 °C for 10 minutes)

Compounds	*Fresh juice*	*Heated juice*
Toluene	0·47	0·73
Hexanal	11·4	12·7
Xylenes	0·93	1·08
Trans-2-hexenal	9	19·5
Pentanol	3·8	3·25
Trimethyl benzene		
6-methyl-5-heptene-2-one	0	0·51
and *n* hexanol	9·7	11·2
Cis-3-hexenol	12·4	10·4
Trans-2-hexenol	30	21·8
2-isobutylthiazol	1·37	1·08
Furfural	0·14	0·42
2-acetyl furan	0·05	0·14
Benzaldehyde	0·07	0·30
Linalol	0·03	0·19
5-methyl furfural	0·13	0·47
5-methyl-2-acetylfuran	0·07	0·15
Phenyl acetaldehyde	0·05	0·66
Furfuryl alcohol	0·09	0·4
α-terpineol and isopropyl-4-phenyl alcohol	0·5	1·3
Carvone	2·05	0·79
2,6-dimethyl-2,6-undecadien-10-one	1·04	2·45
2-phenyl ethanol	0·44	1·02

The data obtained for the heated product confirm the qualitative findings, i.e. increase of relative amounts of aromatic and furan compounds of 2,6 dimethyl-2,6-undecadien-10-one. The increase of relative amount of terpineol and linalol reported by Buttery *et al.* (1971) is confirmed.

On the other hand we observed an increase of the amount of *trans*-2-hexenal in relation to a decrease of *trans*-2-hexen-1-ol whereas the amount of *cis*-3-hexen-1-ol is constant. This result can be explained if we consider the possible pathways by which these products are formed (Fig. 6) (Kazeniac & Hall, 1970; Jadhav *et al.*, 1972; Stone *et al.*, 1975; Sieso *et al.*, 1976).

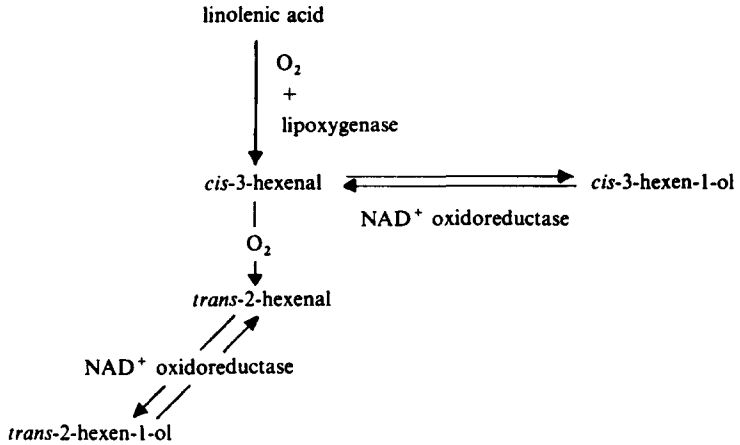


Fig. 6. Formation of hexenals and hexenols from linolenic acid.

Cis-3-hexenal formed during crushing of the fruit is in part reduced to *cis*-3-hexen-1-ol by the action of NAD^+ oxidoreductase, and in part isomerised to *trans*-2-hexenal which is in turn reduced to *trans*-2-hexen-1-ol.

We can suppose that NAD^+ oxidoreductase is always under activated form in the fresh juice (Nicolas, 1975) and that the reduction reactions occur during the stripping of volatile compounds and during the first steps of heating. Under these conditions *cis*-3-hexenal is completely reduced or isomerised. The reduction rate of *trans*-2-hexenal (lower than that of *cis*-3-hexenal) (Stone *et al.*, 1975) and the enzyme denaturation during heat increase explain the decrease of the relative amount of *trans*-2-hexen-1-ol and the increase of the relative amount of *trans*-2-hexenal in the heated product. It may be supposed that non-enzymatic oxidation at 100 °C is not a likely cause of the increase of *trans*-2-hexenal at the expense of *trans*-2-hexene-1-ol, because such a reaction would also lead to an equal decrease in the amount of *cis*-3-hexene-1-ol.

ACKNOWLEDGEMENTS

The authors are indebted to Mr J. Motemps, who provided the tomatoes and facilities to prepare tomato products and to Mr P. Dubois and J. Rigaud, INRA Dijon for expert gas chromatography–mass spectrometry.

REFERENCES

BUTTERY, R. G., SEIFFERT, R. M., GUADANI, D. G. & LING, L. C. (1971). Characterization of additional volatile components of tomato, *J. Agric. Fd Chem.*, **19**, 524–9.

COLE, E. R. & KAPUR, N. S. (1957). Stability of lycopene, I. Degradation by oxygen. II. Oxidation during heating of tomato pulp, *J. Sci. Fd Agric.*, **8**, 360–5, 366–8.

COBB, W. Y. (1969). Quantification of flavorful food components using isotope dilution, *J. Fd Sci.*, **34**, 466–9.

CORNU, A. & MASSOT, R. (1975). *Compilation of mass spectral data*, 2nd edn., Heyden, London.

FRAGERSON, I. S. (1969). Thermal degradation of carbohydrates, *J. Agric. Fd Chem.*, **17**, 747–50.

FRIEDEL, P., KRAMPL, V., RADFORD, T., RENNER, J. A., SHEPARD, F. W. & GIANTURCO, M. A. (1971). Some constituents of aroma complex of coffee, *J. Agric. Fd Chem.*, **19**, 530–2.

JADHAV, S., SINGH, B. & SALUNKHE, D. K. (1972). Metabolism of unsaturated fatty acids in tomato fruit: linoleic and linolenic acids as precursors of hexanal. *Plant and Cell. Physiol.*, **13**, 449–59.

JOHNSON, A. E., NURSTEN, H. E. & WILLIAMS, A. A. (1971). Vegetable volatiles: a survey of components identified: part II. *Chem and Ind. (London)*, **43**, 1212–24.

JOURET, C., MOUTONNET, M. & DUBOIS, P. (1972). Formation du vinylbenzène lors de la fermentation alcoolique du raisin. *Ann. Technol. Agric.*, **21**, 69–72.

KARLSSON-EKSTROM, G. & VON SYDOW, E. (1973). Aroma of black-currants. VII Influence of processing parameters on the aroma of black-currants, *Lebensm. Wiss. Technol.*, **5**, 165–9.

KAZENIAC, S. J. & HALL, R. M. (1970). Flavor chemistry of tomato volatiles, *J. Fd Sci.*, **35**, 519–30.

KINLIN, T. E., MURALIDHARA, R., PITTET, A. O., SANDERSON A. & WALDRAT, J. P. (1972). Volatile components of roasted filberts, *J. Agric. Fd Chem.*, **20**, 1021–8.

MIERS, J. C. (1966). Formation of volatile sulfur compounds in processed tomato products, *J. Agric. Fd Chem.*, **14**, 419–23.

NELSON, P. E. & HOFF, J. E. (1969). Tomato volatiles: effect of variety, processing and storage time, *J. Fd Sci.*, **34**, 53–7.

NICOLAS, M. (1975). *Alcool deshydrogénase de tomate. Purification et étude de quelques propriétés*, Thèse de Spécialité. Université de Montpellier.

REYMOND, D. (1971). Analytical evaluation of food quality, *Fd Tech.*, **25**, 78–82.

RYDER, W. S. (1966). Progress and limitations in the identification of flavor components. In *Flavor Chemistry*, ed. Gould, R. F. pp. 70–93, American Chemical Society, Washington.

SECK, S. & CROUZET, J. (1973). Constituants volatils de *Lycopersicon esculentum*, *Phytochemistry*, **12**, 2925–30.

SECK, S., CROUZET, J. & PIVA, M. T. (1976). Influence des traitements thermiques sur la nature des constituants volatils de la tomate, *Ann. Technol. Agric.*, **25**, 85–95.

SHAH, B. M., SALUNKHE, D. K. & OLSON, L. E. (1969). Effects of ripening process on chemistry of tomato volatiles, *J. Amer. Soc. Hort. Sci.*, **94**, 171–6.

SIESO, V., NICOLAS, M., SECK, S. & CROUZET, J. (1976). Constituants volatils de la tomate: mise en évidence et formation par voie enzymatique du *trans*-hexène-2-ol, *Agr. Biol. Chem.*, **40**, 2349–53.

STENHAGEN, E., ABRAHAMSON, S. & MACLAFFERTY, F. W. (1970). *Atlas of mass spectra data*, New York, Interscience Publishers.

STONE, E. J., HALL, R. M. & KAZENIAC, S. J. (1975). Formation of aldehydes and alcohols in tomato fruits from U-^{14}C-labeled linolenic and linolic acids, *J. Fd Sci.*, **40**, 1138–41.

VON SYDOW, E. (1963). Mass spectrometry of terpenes II. Monoterpene alcohols, *Act. Chem. Scand.*, **17**, 2504–12.

URBACH, C., STARK, W. & NOBUHARA, A. (1973). Low resolution mass spectra of some unsaturated δ-lactones, *Agr. Biol. Chem.*, **36**, 1217–28.

VITZTHUM, O. G., WERKHOFF, P. & HUBERT, P. J. (1975). New volatiles constituents of black tea aroma, *J. Agric. Fd Chem.*, **23**, 999–1003.

METABOLIC EVALUATION OF RICE PROTEIN†

BIENVENIDO O. JULIANO

Chemistry Department, International Rice Research Institute, Los Baños, Laguna, Philippines

(Received: 31 October, 1977)

ABSTRACT

Rice protein occurs in milled rice as discrete particles called protein bodies, 1–4 μm in size. Very little variation in lysine content of rice protein has been observed at any protein level. Rat assays for protein quality by protein efficiency ratio, slope-ratio technique and nitrogen balance showed that the relative protein value of milled rices (6–15 % protein) ranged from 42 to 82 % of milk and egg protein. True digestibility of rice protein in rats ranged from 94 to 100 % and biological value ranged from 68 to 75 %. An increase in protein content results in an increase in the utilisable protein of the milled rice. Similar results were obtained with nitrogen-balance studies in Man. True digestibility is 79 to 85 %, biological value ranging from 59 to 67 % and NPU from 47 to 55 %.

Cooking reduces the digestibility but improves the biological value of milled rice protein in growing rats, resulting in similar net protein utilisation for raw and cooked rice. Lysine digestibility remains almost complete with cooking. A 15 % fraction of cooked-rice protein is also indigestible in Man and is expelled as intact faecal protein particles.

INTRODUCTION

The Genetic Evaluation and Utilisation (GEU) programme on protein content of the International Rice Research Institute (IRRI) seeks the improvement of the protein content of milled rice (*Oryza sativa* L.) from its present level of 7 % to about 9 % (Juliano & Beachell, 1975; Coffman & Juliano, 1976). The project includes a study of the effect of protein content on the properties of the grain and its protein

† Invited paper presented at the ASEAN Subcommittee on Protein Second Workshop on Metabolic Evaluation, Bangkok, Thailand, held from 23 to 26 August, 1977.

Fd. Chem. (3) (1978)—
Printed in Great Britain

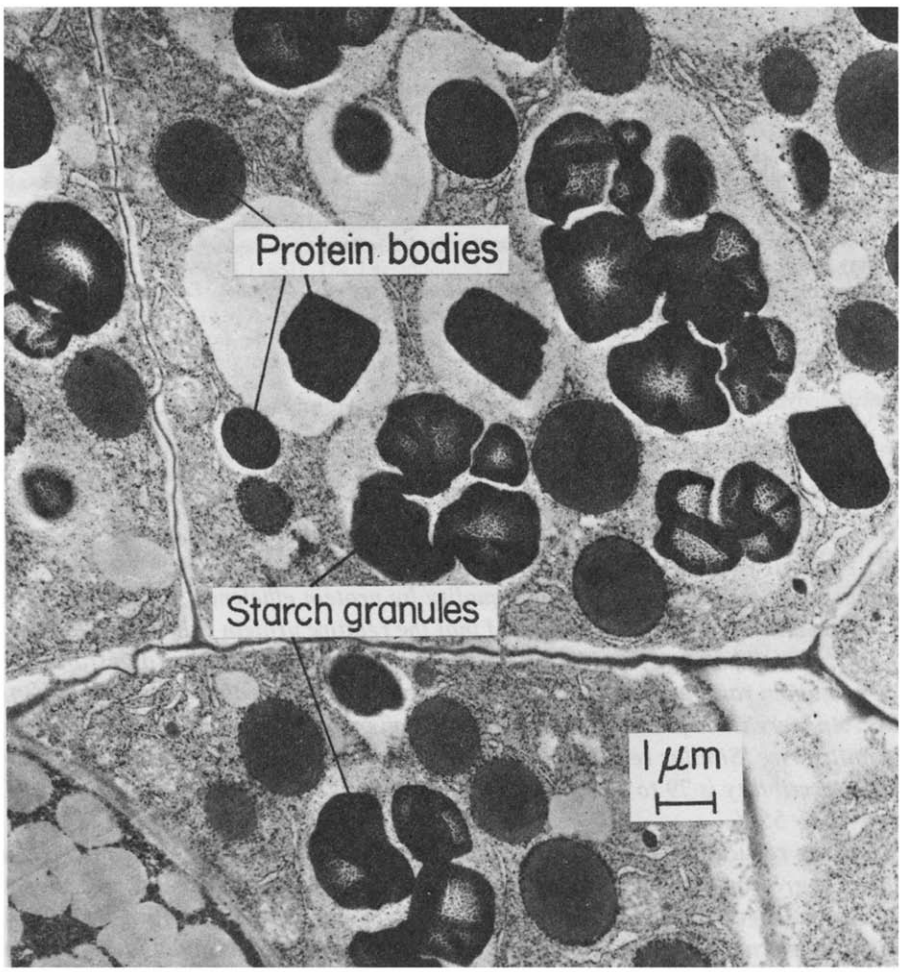


Fig. 1. Three types of protein bodies in the subaleurone layer of IR26 rice grain 10 days after flowering (Harris & Juliano, 1977; IRRI, 1976).

and, in co-operative studies, the effect of protein content on the nutritional properties of the protein. In this paper IRRI's present knowledge on the nutritional value of rice protein is briefly summarised.

PHYSICOCHEMICAL STUDIES

Rice protein exists almost exclusively as single-membrane protein bodies, 1 to 4 μm in size, in the endosperm (Harris & Juliano, 1977; Juliano, 1972). Rice protein

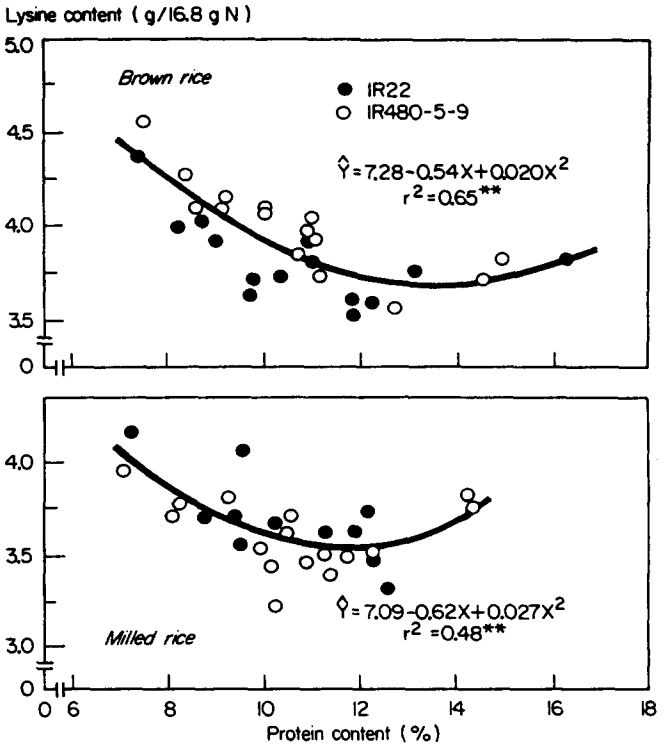


Fig. 2. Relationship between protein content and lysine content of protein in IR22 and IR480-5-9 brown and milled rice (Juliano *et al.*, 1973). Samples above 13% protein have low grain weight.

bodies, therefore, have the same amino acid composition as whole milled rice protein. In the subaleurone layer, in addition to the usual type, crystalline-type protein bodies have been identified which are absent in the inner endosperm cells (Harris & Juliano, 1977; Bechtel, 1976) (Fig. 1).

Milled rice protein is made up of 5% albumin (water soluble), 10% globulin (salt soluble), <3% prolamin (alcohol soluble) and ≥80% glutelin (alkali soluble) (Juliano, 1972). The proportion of lysine, the first limiting amino acid, in the protein tends to decrease as protein content increases but no further decrease occurs above 10% protein (Juliano *et al.*, 1973) (Fig. 2). The increase in protein content is due mainly to the glutelin fraction (Cagampang *et al.*, 1966).

Protein distribution in the endosperm becomes more even with an increase in protein content, as demonstrated by histological and chemical means (Juliano *et al.*, 1973). The increase in protein content is the result of a greater number of protein bodies (Harris & Juliano, 1977).

Juliano *et al.* (1973) were unsuccessful in obtaining any high lysine mutants of rice; only ±0·5 percentage point range of mean lysine content was obtained at any

protein level. This is probably because the amount of low-lysine protein, prolamin, in rice protein is already low and the high-lysine mutants of barley, corn and sorghum so far are the result of a decrease in the relatively high prolamin content of their protein.

EVALUATION IN EXPERIMENTAL ANIMALS

The factor 6·25 is used in metabolic evaluation to convert Kjeldahl nitrogen of rice to protein instead of the usual 5·95 (Juliano, 1972) to make the rice diet isonitrogenous with the standard diet. Most of the data available on the nutritive value of rice protein were obtained with growing rats. Co-operative nutritive studies were initiated in 1969 to evaluate the effect of an increase in protein content of rice on protein quality using the major rat assays available. The protein efficiency ratio (PER) or net protein ratio (NPR) at the standard protein level of 10% cannot be run as the usual protein level of rice is 7% (Bressani *et al.*, 1971). The use of a 90% rice diet in comparison with casein at the same protein level gave a poor correlation between PER and the amino acid score because of the dependence of PER on dietary protein level. The 5% protein PER or NPR values ranked the samples in the same order as amino acid score (Bressani *et al.*, 1971) (Table 1).

Slope-ratio methods for protein quality estimation have been proposed based on three or more dietary protein levels (Hegsted *et al.*, 1968; Hegsted, 1969). Relative nutritive value (RNV) based on the ratio of growth response to rice to growth response to lactalbumin (Hegsted & Juliano, 1974) or casein (McDonald, 1974) or egg (Murata *et al.*, 1978) tended to follow the amino acid score data (FAO, 1973) (Table 2). The use of the same protein level for the diets as in sets II and IV, rather than the same rice level, resulted in a narrower range of RNV. Good correspondence was also obtained among RNV based on gain in body weight, in carcass nitrogen and in body water as a function of nitrogen intake (Bressani *et al.*, 1971; Hegsted & Juliano, 1974; Murata *et al.*, 1978) (Table 3).

TABLE 1

EFFECT OF PROTEIN CONTENT ON PROTEIN QUALITY OF MILLED RICE BASED ON 'PROTEIN EFFICIENCY RATIOS' AND 'NET PROTEIN RATIOS' AT 5% PROTEIN DIETS IN GROWING RATS (BRESSANI *et al.*, 1971)

Protein source	*Protein content* (%)	*Lysine* (*g*/16 *g N*)	*Amino acid score* (%)	*PER*	*Relative quality*[a] (%)	*NPR*	*Relative quality*[a] (%)
Intan	5·97	4·07	74·0	2·44	83·2	3·60	80·4
IR8	7·69	3·59	65·3	2·09	71·2	3·25	72·5
IR8	10·2	3·50	63·6	1·85	63·1	3·01	67·2
BPI-76-1	15·2	3·19	58·0	1·46	49·8	2·43	54·2
Casein	86·2	7·46	—	2·20	75·0	3·36	75·0

[a] Based on PER or NPR of casein at the same dietary protein level as 75%.

TABLE 2
EFFECT OF PROTEIN CONTENT ON PROTEIN QUALITY OF MILLED RICE BASED ON VARIOUS SLOPE-RATIO ASSAYS (WEIGHT GAIN) IN GROWING RATS

Protein source	*Protein content* (% *N* × 6·25)	*Lysine* (*g*/16 *g N*)	*Amino acid score* (%)	*Relative nutritive value* (%) *I*[a]	*II*[b]	*III*[c]	*IV*[d]
Intan	5·97	4·07	74·0	78	77	82	—
Commercial	6·67	3·36	61·1	—	—	—	51
IR8	7·69	3·59	65·3	69	72	63	—
IR22	7·88	3·75	68·2	78	—	—	—
IR22	10·0	3·87	70·4	77	—	—	—
IR8	10·2	3·50	63·6	68	67	—	—
IR480-5-9	10·3	3·54	64·4	—	—	57	—
IR480-5-9	11·0	3·18	57·8	—	—	—	48
IR1103-15-8	11·6	3·65	66·4	65	—	—	—
IR480-5-9	11·8	3·34	60·7	53	—	—	—
IR480-5-9	12·3	3·34	60·7	54	—	—	—
BPI-76-1	15·2	3·19	58·0	46	60	42	—

[a] Based on 0, 28, 56 and 84% rice diets and lactalbumin slope as 100% (Hegsted & Juliano, 1974).
[b] Based on 0, 1, 2, 3, 4 and 5% protein diets and casein as 75% (Bressani *et al.*, 1971).
[c] Based on 2, 5 and 8% protein diets and casein as 75% (McDonald, 1974).
[d] Based on 0, 4, 8, 12 and 15% protein diets and egg as 100% (Murata *et al.*, 1978).

Net protein utilisation (NPU) values corresponded closely to amino acid scores in the nitrogen balance assay utilising a constant calorie (10 g dry matter) and nitrogen intake (150 mg N) and separate nitrogen analysis of faeces and urine of the rat (Eggum & Juliano, 1973; 1975) (Table 4). Samples of low-protein varieties Intan and IR8 require the addition of casein in order to achieve the desired dietary protein level required (Eggum & Juliano, 1973). Brown rice protein is less digestible than milled rice protein. Amino acid digestibility of rice protein has also been determined (Eggum & Juliano, 1973). The use of destarched cooked rice, which allowed the *ad*

TABLE 3
COMPARATIVE RNV DATA OBTAINED FOR MILLED RICES BASED ON GAIN OF BODY WEIGHT, CARCASS N AND BODY WATER

Sample	*Protein content* (% *N* × 6·25)	*Lysine* (*g*/16 *g N*)	*Amino acid score* (%)	*Body wt*	*RNV* (%) *Carcass N*	*Body water*
Intan[a]	5·97	4·07	74·0	77	69	—
IR8[a]	7·69	3·59	65·3	72	67	—
IR8[a]	10·2	3·50	63·6	67	51	—
Commercial[b]	8·30	—	—	50	—	—
Rice flour[b]	19·1	—	—	44·7	44·8	44·2
Commercial[c]	6·67	3·36	61·1	51	46	51
IR480-5-9[c]	11·0	3·18	57·8	47·8	45·3	43·5

[a] Based on slope for casein as 75% (Bressani *et al.*, 1971).
[b] Based on slope for lactalbumin as 100% (Hegsted *et al.*, 1968).
[c] Based on slope for egg as 100% (Murata *et al.*, 1978).

TABLE 4
EFFECT OF PROTEIN CONTENT ON PROTEIN QUALITY OF MILLED RICE BASED ON NITROGEN BALANCE IN GROWING RATS (EGGUM & JULIANO, 1973, 1975; MURATA *et al.*, 1978).

Protein source	*Protein content* (% $N \times 6{\cdot}25$)	*Lysine* (g/16 g *N*)	*Amino acid score* (%)	*True digest-ibility* (%)	*Biological value* (%)	*NPU* (%)
Milled rice						
Intan	5·97	4·07	74·0	100·1	75·2	75·3
IR8	7·69	3·59	65·3	96·2	73·1	70·3
IR8	10·2	3·50	63·6	95·4	68·4	65·2
IR22	10·0	3·87	70·4	98·5	69·7	68·7
IR1103-15-8	11·6	3·65	66·4	95·9	74·3	71·1
IR480-5-9	11·8	3·34	60·7	94·5	67·9	64·2
BPI-76-1	15·2	3·19	58·0	94·4	70·1	66·2
Brown rice IR480-5-9	10·9	3·59	65·3	90·8	70·8	64·2
Milled rice						
IR8 0 kg N	8·14	3·57	64·9	99·2	69·5	68·9
IR8 120 kg N	9·90	3·41	62·0	98·0	69·2	67·8
IR480-5-9 0 kg N	9·90	3·50	63·6	99·8	71·0	71·0
IR480-5-9 60 kg N	11·4	3·38	61·5	100·6	68·4	68·8
IR480-5-9 90 kg N	13·0	3·33	60·5	100·1	67·7	67·8
Commercial rice	6·67	3·36	61·1	—	—	56[a]
IR480-5-9	11·0	3·18	57·8	—	—	63, 56[a]

[a] Based on carcass N analysis (Murata *et al.*, 1978)

libitum feeding to be carried out using 10% protein diets, resulted in a narrower range of NPU based on carcass nitrogen (Murata *et al.*, 1978) (Table 4). High-protein lines (Table 5) have similar NPU values to low-protein rices (Table 4).

Recently, McLaughlan (1976) reported a single-dose rat assay called relative nitrogen utilisation (RNU) at a 8–10% dietary protein level. RNU of rice measured at 6·6% protein level was 64% of lactalbumin in comparison with 47% by PER, 68% by NPR and 70% by RNV.

In rats, cooking the milled rice resulted in a significant drop in true digestibility but significantly increased the biological value of the protein such that NPU was significantly higher for cooked rice than for raw rice in two of three samples (Eggum

TABLE 5
NITROGEN BALANCE OF MILLED RICE OF HIGH PROTEIN LINES IN GROWING RATS. AGR. RES. LAB., COPENHAGEN AND IRRI, 1975–1977

Protein source	*Protein content* (% $N \times 6{\cdot}25$)	*Lysine* (g/16 g *N*)	*Amino acid score* (%)	*True digest-ibility* (%)	*Biological value* (%)	*NPU* (%)
IR480-5-9	11·2	3·42	62·2	100·4	66·8	67·1
IR480-5-9, destarched	49·4	3·26	59·3	94·7	65·4	61·9
IR2031-724-2	10·2	3·54	64·4	99·9	66·5	66·4
IR2153-338-3	12·2	3·56	64·7	98·5	69·9	68·8

TABLE 6

MEAN PROPERTIES OF PROTEIN AND NITROGEN BALANCE IN GROWING RATS FED RAW AND COOKED, FREEZE-DRIED MILLED SAMPLES OF THREE RICES (EGGUM *et al.*, 1977)

Property	*Raw milled rice*[a]		*IR480-5-9 Parboiled milled rice*		*LSD* (5%)
	Uncooked control	*Cooked, freeze-dried*	*Uncooked control*	*Cooked, freeze-dried*	
Crude protein (% N × 6·25)	8·93	9·04	10·4	9·90	NS
Lysine (g/16 g N)	3·60	3·51	3·60	3·71	NS
Amino acid score (%)	65·5	63·8	65·5	67·5	NS
True digestibility[b] (%)	99·7	88·6	94·7	85·1	0·9
Biological value[b] (%)	67·7	78·2	70·4	80·1	1·1
NPU[b] (%)	67·5	69·2	66·7	68·3	1·4
Utilisable protein (%)	6·02	6·24	6·94	6·76	0·14

[a] Mean of IR29, IR32 and IR480-5-9 milled rices.
[b] Six rats per diet.

et al., 1977) (Table 6). Determination of amino acid digestibility verified that lysine digestibility was least affected by cooking (Eggum *et al.*, 1977) (Table 7). Cooking had no effect on the digestibility of starch and the total energy of milled rice. Difference in protein content has no adverse effect on the digestibility of rice in rats. The undigested 'protein' was calculated to be poorer in lysine, histidine and arginine than cooked rice protein (Table 7).

TABLE 7

MEAN CONTENT AND PERCENTAGE DIGESTIBILITY IN GROWING RATS OF PROTEIN, SELECTED AMINO ACIDS, STARCH AND ENERGY OF RAW AND COOKED IR29 AND IR480-5-9 MILLED RICE AND AMINO ACID COMPOSITION (BY DIFFERENCE) OF UNDIGESTED 'PROTEIN' OF COOKED RICE (EGGUM *et al.*, 1977)

Constituent	*Content (g/16 g N)*		*True digestibility (%)*		*Cooked undigested 'protein' (g/16 g N)*
	Raw	*Cooked*	*Raw*	*Cooked*	
Arginine	8·06	8·04	98·8	95·0	2·8
Cysteine	2·18	2·18	99·5	82·0	3·6
Histidine	2·20	2·20	99·4	96·8	0·6
Isoleucine	4·28	4·32	99·6	88·4	4·3
Leucine	8·68	8·80	99·6	85·6	11·8
Lysine	3·15	3·14	99·9	99·4	0·2
Methionine	2·55	2·56	99·2	90·7	2·0
Phenylalanine	5·24	5·26	99·2	86·8	6·1
Threonine	3·42	3·48	99·6	90·7	2·8
Tryptophan	1·36	1·37	99·8	87·4	1·6
Tyrosine	5·28	5·34	100·0	86·0	7·1
Valine	5·88	5·88	99·3	88·0	6·3
Protein (%)	9·92	10·67	100·0	89·4	—
Starch (%)	76·72	79·85	99·9	99·9	—
Energy (Kcal/g dry matter or %)	4·20	4·16	96·8	95·4	—

The assays of protein quality of milled rice in rats showed that utilisable protein increases with an increase in protein content since the percentage drop in protein quality was just a fraction of the percentage increase in protein content. Earlier, Blackwell *et al.* (1966) showed similar growth rates in Long-Evans rats fed 5·6% protein diets from Intan (6·2% protein, 68% amino acid score) and BPI-76 (10·3% protein, 62% amino acid score) precooked milled rice, again indicating that the nutritive value of rice parallels protein content.

Preliminary assays of these rice samples have been carried out on the meadow vole (*Microtus pennsylvanicus*) but the results did not correlate with amino acid score since a number of voles did not gain weight, reflecting variability within the colony (Worall & Elliott, 1974). Microbiological assays in *Streptococcus zymogenes* based on growth (Mickelsen & Makdani, 1973) and methionine availability (Ford, 1977) showed little differentiation among the rices.

HUMAN STUDIES

Most of the human studies carried out on metabolism of rice protein are based on nitrogen balance and used cooked rice exclusively. True digestibility of rice protein ranged from 79 to 85% (Parthasarathy *et al.*, 1964; Huang & Tung, 1971; Bressani, 1972; Roxas *et al.*, 1975) (Table 8). The digestibility value of 85% is closer to the value obtained for cooked rice in rats than the 100% for raw rice (Table 6). High-protein flours from outer layers of milled rice are reported to be less digestible than protein of whole grain milled rice (Graham, 1971). Viteri *et al.* (1971) report 90% protein digestibility for wheat flour and 85% for milled rice. Although a diet in

TABLE 8
NITROGEN BALANCE STUDIES IN CHILDREN FED MILLED RICE DIETS

Property	*Parthasarathy* et al. (1964)		*Huang & Tung* (1971)	*Bressani* (1972)		*Roxas* et al. (1975)
Nationality of subjects	Indian		Chinese	Guatemalan		Filipino
Age of subjects (years)	8–9	8–9	1	—	—	1·5–2
Caloric intake (Kcal/kg/day)	82	81	91	—	—	100
Amino acid score of diet (%)	82	82	—	—	—	62
N intake (mg/kg/day)	215	207	188	235	320	314
True digestibility (%)	82·6	82·5	81[b]	83·8	79·1	82[b]
Biological value (%)	64·1	66·6	63[b]	66·0	59·3	74[b]
Net protein utilisation (%)	52·9	54·9	52[b]	55·3	46·9	60[b]
Relative protein value (%)	76[a]	76[a]	73[c]	91[d]	81[d]	85[c]

[a] Based on skim milk protein with NPU of 69·7% and 71·8%, respectively.
[b] Calculated based on 21 mg/kg body weight daily endogenous faecal N loss and 53 mg/kg body weight daily endogenous urinary N loss (Huang & Tung, 1972).
[c] Based on milk protein with NPU of 71%.
[d] Based on milk protein with NPU of 60·8% and 57·7%, respectively.

which wheat flour and milk contributed equally to dietary protein had a higher protein digestibility (97%) than a mixture of IR20 milled rice and milk (88%) in three Filipino children, the wheat–milk diet had a lower biological value (64 versus 78%) and NPU (62 versus 69%) than the rice–milk diet (IRRI, 1976).

Reported biological values of rice protein in children are 59–74% (Table 8). Some of the data were calculated based on endogenous faecal and urinary nitrogen losses of 1-year-old Chinese children of 21 mg and 53 mg/kg body weight, respectively reported by Huang & Tung (1972). These are slightly lower than the values of Viteri *et al.* (1971) of 24·6 ± 8·6 mg and 65·1 ± 14·9 mg endogenous faecal and urinary nitrogen losses in young Guatemalan children.

NPU of rice protein ranged from 47 to 60% (Table 8). Corresponding relative protein value (RPV) of milled rice ranged from 73 to 91% based on NPU of milk protein as 100%. Knapp *et al.* (1973) reported similar nitrogen retention of milk and rice in 6-months-old infants—78 ± 13 mg N retained for a range of 319–338 mg N intake of milk and 73 ± 11 mg N retained for a range of 309–318 mg N/kg body weight/day rice intake.

Addition of lysine and threonine to the diet improved the biological value and NPU of rice protein without any effect on digestibility (Parthasarathy *et al.*, 1964; Huang & Tung, 1971; Bressani, 1972). RPV increased three percentage points as a result of lysine fortification but increased three to twelve percentage points by lysine and threonine addition. In three Latin-American infants (6–9 months-old) supplementation of rice with lysine, threonine and potassium improved nitrogen retention in only two of the three children (Kaye *et al.*, 1961). The improvement of protein quality in Man by lysine/threonine supplementation is generally less than that shown in rats (Hegsted & Juliano, 1974). Chen *et al.* (1967) showed that lysine is the first limiting amino acid in rice protein for nitrogen retention in human adults.

Using the slope-ratio technique, Inoue *et al.* (1973) calculated an NPU of 33·1% for rice at maintenance energy in young (20- to 27-year-old) men (Table 9). At excess energy NPU of rice increased to 49·7% but NPU of egg protein also increased. RPV of milled rice was 75% of egg at the maintenance energy level and 79% at the excess energy level. True digestibility of rice protein was 85·8 ± 1·3% of egg protein. Relative biological value of rice protein was estimated as nearly 100% of egg protein in the excess group and 80–90% in the non-excess group.

As part of IRRI's GEU protein programme, the practical effect on nitrogen balance was studied by the replacement of low-protein rice with high-protein rice in rice diets wherein the contribution of rice in the diet is kept constant. Co-operative work by Clark *et al.* (1971) showed an increase in nitrogen retention and percent apparent retention by replacing Bluebonnet rice with 8·24% protein with BPI-76-1 rice with 15·2% protein (Table 10). Apparent digestibility of the protein was the same for the two rice samples, even though BPI-76-1 had a lower amino acid score than Bluebonnet rice.

Similar results were subsequently obtained with Filipino children fed a mixture of

TABLE 9
SLOPE-RATIO OF NITROGEN BALANCE VERSUS NITROGEN INTAKE FOR EGG AND MILLED RICE AT EXCESS AND MAINTENANCE ENERGY IN YOUNG JAPANESE MEN[a] (INOUE *et al.*, 1973)

Property	*Excess energy*		*Maintenance energy*	
	Rice	*Egg*	*Rice*	*Egg*
Subjects (numbers)	10	6	14	11
Energy intake (Kcal/kg)	57 ± 2	57 ± 2	45 ± 2	45 ± 2
Slope	0·469	0·538	0·268	0·411
Intercept	−38·3	−36·2	−32·0	−37·0
N intake for maintenance (mg/kg)	81·6	67·3	119·3	90·1
N requirements[b] (mg/kg)	92·5	73·2	139·0	104·2
NPU (%)[c]	49·7	62·8	33·1	44·1
RPV (%)	79	100	75	100

[a] Nitrogen intake of 50–120 mg/kg body weight for the rice diet and 45–100 mg/kg for the egg diet.
[b] Nitrogen intake at maintenance plus one SD.
[c] From tangents established between endogenous nitrogen loss and nitrogen intakes corresponding to the requirements.

milled rice and surgeon fish (*Acantharus bleakeri*) (Roxas *et al.*, 1975) and milled rice and mung bean (*Vigna radiata* (L.) Wilczek) (Roxas *et al.*, 1976) diets (Tables 11 and 12). Correction of the data for endogenous losses (Huang & Tung, 1972) showed some drop in biological value of rice–fish protein with an increase in protein content of rice which parallels the decrease in amino acid score of the diet (Table 11). However, such a decrease in biological value was noted only in one of the two rice–mung bean diets (Table 12). In all three cases, however, utilisable protein was higher in the diets containing high-protein rice.

Nitrogen balance data are really comparable only when done at the same nitrogen intake, because protein quality is affected by protein level in the diet (FAO, 1973). Because of the absence of such human data for the protein quality of low-protein and high-protein rices, co-operative balance studies are under way in children using IR480-5-9 (11% protein) and IR32 (7% protein) rice with Dr G. G. Graham and W. C. MacLean, Jr, Johns Hopkins University/Instituto de Investigacion Nutricional, Miraflores, Peru and with Dr C. Ll. Intengan, Food and Nutrition

TABLE 10
NITROGEN BALANCE IN SIX MALE STUDENTS AND ONE FEMALE STUDENT FED MILLED RICE DIET (CLARK *et al.*, 1971)

Property	*Bluebonnet*	*BPI-76-1*
Protein content (% N × 6·25)	8·24	15·2
Lysine (g/16 g N)	3·76	3·09
Amino acid score (%)	68·4	56·2
Dietary N intake (mg/kg body wt)	96·2	176·1
Daily faecal N (mg/kg body wt)	22·2	38·7
Daily urine N (mg/kg body wt)	70·4	116·8
Daily retained N (mg/kg body wt)	3·6	20·6
Apparent digestibility (%)	76·9	78·0
Apparent retention (%)	3·7	11·7

TABLE 11

AMINO ACID SCORES AND NITROGEN BALANCE OF MILLED RICE–SURGEON FISH DIETS FED TO FILIPINO CHILDREN USING MILLED RICE DIFFERING IN PROTEIN CONTENT (ROXAS *et al.*, 1975)

Property	*IR8 rice*	*IR480-5-9 Rice*		*Rice–milk*
Protein (N × 6·25) of rice (%)	7·7	10·3	11·9	
Lysine of rice (g/16 g N)	3·78	3·54	3·99	
Children (number)[a]	12	11	12	
Lysine in diet (g/16 g N)	5·44	4·99	4·66	
Amino acid score of diet (%)[b]	99	91	85	
Daily N intake (mg/kg body wt)	187	231	254	250
True digestibility (%)[c]	84	85	85	87
Biological value (%)[c]	80	73	69	83
NPU (%)[c]	67	62	59	72
RPV (%)[d]	94	87	83	100

[a] Ten children partook of all the three diets.
[b] Based on 5·5 g lys/16 g N as 100%.
[c] Based on published obligatory faecal N loss of 21 mg/kg body wt and obligatory urinary N loss of 53 mg/kg body wt (Huang & Tung, 1972).
[d] Based on NPU of milk and egg protein of 71% as 100%.

Research Institute, Manila, using casein as the reference protein. Dr P. C. Huang is also estimating protein quality in adults of the IR480-5-9 milled rice by the slope-ratio method at the National Taiwan University, Taipei, in comparison with a Chinese rice variety, Chianong No. 8, and egg as the reference protein.

Preliminary co-operative growth studies in 1975 for five months with Dr S. M. Pereira, Christian Medical College and Hospital, Vellore, Tamil Nadu, India, showed no differences in the increase in height and weight of pre-school children (3–6

TABLE 12

AMINO ACID SCORES AND NITROGEN BALANCE OF MILLED RICE–MUNG BEAN DIETS FED TO FILIPINO CHILDREN COMPARED WITH DIET OF MILLED RICE AND MILK (ROXAS *et al.*, 1976)

Property	*Rice–milk*[a]	*Rice–mung bean (100:18·6)*		*Rice–milk*[a]	*Rice–mung bean (100:27·7)*	
		IR8	*IR480-5-9*		*IR8*	*IR480-5-9*
Protein (N × 6·25) of rice (%)	7·4	7·5	11·4	7·4	7·5	11·4
Lysine of rice (g/16 g N)	—	3·50	3·25	—	3·50	3·25
Children (number)	4	4	4	5	5	5
Lysine of diet (g/16 g N)	5·94	4·92	4·39	5·94	5·40	4·98
Amino acid score of diet (%)[b]	100	89	80	100	98	90
Daily N intake (mg/kg body wt)	192	197	256	196	235	294
True digestibility (%)[c]	87	78	83	90	85	85
Biological value (%)[c]	83	76	73	75	64	58
NPU (%)[c]	72	59	61	68	55	50
RPV (%)[d]	100	83	86	96	77	70

[a] Equal contribution of milled rice and powdered milk to dietary N.
[b] Based on 5·5 g lys/16 g N as 100%.
[c] Based on published obligatory faecal N loss of 21 mg/kg body wt and obligatory urinary N loss of 53 mg/kg body wt (Huang & Tung, 1972).
[d] Based on NPU of milk and egg protein of 71% as 100%.

years old) fed a diet of 80% rice using IR480-5-9 (9% protein) and Kichili (6% protein) rices. A one-year feeding trial is scheduled for later this year using IR2153-338-3 grown in Hyderabad as the high-protein rice. The interpretation of the results of this growth study would require the data on the protein quality in children of low-protein and high-protein rices.

Finally, IRRI has been studying, since 1975, the nature of the indigestible protein of cooked milled rice in Man. Faecal protein particles derived from cooked rice have been isolated from Japanese adults by Tanaka *et al.* (1975) and such particles were also demonstrated in Filipino children fed cooked rice (IRRI, 1977). Protein faecal particles are insoluble in 0·1N NaOH but soluble in 98–100% formic acid. Their SDS-polyacrylamide electrophoretic pattern is similar to that of *in vitro* indigestible raw rice protein (IRRI, 1977). Transmission electron microscopy indicates that the faecal protein particle is the central 10–15% by volume of the protein bodies of cooked rice.

ACKNOWLEDGEMENTS

Part of this work was supported by Contract No. NO1-AM-7-0726 from the National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health (USA) and by a grant from the Rockefeller Foundation.

REFERENCES

BECHTEL, D. B. (1976). Pers. comm. US Grain Marketing Research Center, Manhattan, Kansas.

BLACKWELL, R. Q., YANG, T. H. & JULIANO, B. O. (1966). Effect of protein content in rice upon growth rates of rats fed high-rice diets (abstract). *Proc. 11th Pacific Sci. Congress*, **8** (Nutr. Pacific Area), 15.

BRESSANI, R. (1972). El valor nutricional del arroz en comparacion con el de otros cereales en la dieta humana de America Latina. In: *Politicas Arroceras en America Latina*, 1–20, Cali, Colombia, Centro Internacional de Agricultura Tropical.

BRESSANI, R., ELIAS, L. G. & JULIANO, B. O. (1971). Evaluation of the protein quality of milled rices differing in protein content. *J. Agr. Food Chem.*, **19**, 1028–34.

CAGAMPANG, G. B., CRUZ, L. J., ESPIRITU, S. G., SANTIAGO, R. G. & JULIANO, B. O. (1966). Studies on the extraction and composition of rice proteins. *Cereal Chem.*, **43**, 145–55.

CHEN, S. C.-S., FOX, H. M. & KIES, C. (1967). Nitrogenous factors affecting the adequacy of rice to meet the protein requirements of human adults. *J. Nutr.*, **92**, 429–34.

CLARK, H. E., HOWE, J. M. & LEE, C.-J. (1971). Nitrogen retention of adult human subjects fed a high protein rice. *Am. J. Clin. Nutr.*, **24**, 324–8.

COFFMAN, W. R. & JULIANO, B. O. (1976). Current status of breeding high protein rice. In: *Improving the nutrient quality of cereals II* (Wilcke, H. L. (Ed.)) 150–3. Washington, DC, US Agency for Int. Dev.

EGGUM, B. O. (1973). *A study of certain factors influencing protein utilization in rats and pigs.* Copenhagen, Agricultural Research Laboratory Publ. No. 406. 173 pp.

EGGUM, B. O. & JULIANO, B. O. (1973). Nitrogen balance in rats fed rices differing in protein content. *J. Sci. Food Agr.*, **24**, 921–7.

EGGUM, B. O. & JULIANO, B. O. (1975). Higher protein content from nitrogen fertiliser application and nutritive value of milled-rice protein. *J. Sci. Food Agr.*, **26**, 425–7.

EGGUM, B. O., RESURRECCION, A. P. & JULIANO, B. O. (1977). Effect of cooking on nutritional value of milled rice in rats. *Nutr. Rep. Int.*, **16**, 649–55.

FOOD AND AGRICULTURE ORGANIZATION (1973). *Energy and protein requirements.* FAO Nutr. Meet. Rep. Ser. No. 52, 63.

FORD, J. E. (1977). Pers. comm. Nat. Inst. for Res. in Dairying, Reading, England.

GRAHAM, G. G. (1971). Research to produce new or improved products. Review of Centrally Funded AID Research, Washington, DC, US Agency for Int. Dev. (mimeographed).

HARRIS, N. & JULIANO, B. O. (1977). Ultrastructure of endosperm protein bodies in developing rice grains differing in protein content. *Ann. Biol.*, **45**, 1–5.

HEGSTED, D. M. (1969). Nutritive value of cereal proteins in relation to human needs. In: *Protein-enriched cereal foods for world needs* (Milner, M. (Ed.)) 38–48. Am. Assoc. Cereal Chemists, St Paul, Minn.

HEGSTED, D. M. & JULIANO, B. O. (1974). Difficulties in assessing the nutritional quality of rice protein. *J. Nutr.*, **104**, 772–81.

HEGSTED, D. M., NEFF, R. & WORCESTER, J. (1968). Determination of the relative nutritive value of proteins. Factors affecting precision and validity. *J. Agr. Food Chem.*, **16**, 190–5.

HUANG, P.-C. & TUNG, T.-C. (1971). Growth and nitrogen balance of infants on rice diets supplemented with lysine and threonine. *J. Formosan Med. Assoc.*, **70**, 398–404.

HUANG, P.-C. & TUNG, T.-C. (1972). Protein requirements of infants of about one year of age. Paper presented at 9th Int. Nutr. Congr., Mexico.

INOUE, G., FUJITA, Y. & NIIYAMA, Y. (1973). Studies on protein requirements of young men fed egg protein and rice protein with excess and maintenance energy intakes. *J. Nutr.*, **103**, 1673–87.

INTERNATIONAL RICE RESEARCH INSTITUTE (1976). Annual Report for 1975. Los Baños, Philippines, 111–25.

INTERNATIONAL RICE RESEARCH INSTITUTE (1977). Annual Report for 1976. Los Baños, Philippines, 63–72.

JULIANO, B. O. (1972). The rice caryopsis and its composition. In: *Rice chemistry and technology* (Houston, D. F. (Ed.)), 16–74. Am. Assoc. Cereal Chemists, St Paul, Minn.

JULIANO, B. O., ANTONIO, A. A. & ESMAMA, B. V. (1973). Effects of protein content on the distribution and properties of rice protein. *J. Sci. Food Agr.*, **24**, 295–306.

JULIANO, B. O. & BEACHELL, H. M. (1975). Status of rice protein improvement. In CIMMYT/Purdue Univ., *High-quality protein maize*, 457–69. Dowden, Hutchinson and Ross, Inc., Stroudsburg, Pa.

KAYE, R., BARNESS, L. A., VALYASEVI, A. & KNAPP, J. (1961). Nitrogen balance studies of plant proteins in infants. In National Academy of Sciences—National Research Council. *Meeting protein needs of infants and children*, 297–312. Washington, DC.

KNAPP, J., BARNESS, L. A., HILL, L. L., KAYE, R., BLATTNER, R. J. & SLOAN, J. M. (1973). Growth and nitrogen balance in infants fed cereal proteins. *Am. J. Clin. Nutr.*, **26**, 586–90.

MCDONALD, B. E. (1974). Pers. comm. Dept. Foods and Nutr., Univ. of Manitoba, Winnipeg, Canada.

MCLAUGHLAN, J. M. (1976). The relative nitrogen utilization method for evaluating protein quality. *J. Assoc. Offic. Anal. Chemists*, **59**, 42–5.

MICKELSEN, O. & MAKDANI, D. (1973). Pers. comm. Dept. Food Science and Human Nutrition. Michigan State Univ., East Lansing.

MURATA, K., KITAGAWA, T. & JULIANO, B. O. (1978). Protein quality of a high-protein milled rice in rats. *Agr. Biol. Chem.*, **42**, 565–700.

PARTHASARATHY, H. N., JOSEPH, K., DANIEL, V. A., DORAISWAMY, T. R., SANKARAN, A. N., NARAYANA RAO, M., SWAMINATHAN, M., SREENIVASAN, A. & SUBRAHMANYAN, V. (1964). The effect of supplementation of a rice diet with lysine, methionine and threonine on the digestibility coefficient, biological value and net protein utilization of the proteins and on the retention of nitrogen in children. *Can. J. Biochem.*, **42**, 385–94.

ROXAS, B. V., INTENGAN, C. LL. & JULIANO, B. O. (1975). Effect of protein content of milled rice on nitrogen retention of Filipino children fed rice–fish diet. *Nutr. Rep. Int.*, **11**, 393–8.

ROXAS, B. V., INTENGAN, C. LL. & JULIANO, B. O. (1976). Protein content of milled rice and nitrogen retention of preschool children fed rice–mung bean diets. *Nutr. Rep. Int.*, **14**, 203–7.

TANAKA, Y., HAYASHIDA, S. & HONGO, M. (1975). The relationship of the feces protein particles to rice protein bodies. *Agr. Biol. Chem.*, **39**, 515–8.

VITERI, F. E. & BRESSANI, R. (1972). The quality of new sources of protein and their suitability for weanling and young children. *Bull. World Health Org.*, **46**, 827–43.

VITERI, R., BRESSANI, R. & ARROYAVE, G. (1971). Fecal and urinary nitrogen losses. Expert Committee FAO-ESN Document FAO/WHO/PR/78b (iii). Cited in *PAG Bull.*, **5**(3), (1975), 30.

WORALL, D. & ELLIOTT, F. (1974). Pers. comm. Dept. Crop and Soil Sci., Michigan State Univ., East Lansing.

INFLUENCE OF pH AND TEMPERATURE UPON CALCIUM ACCUMULATION AND RELEASE BY BOVINE SARCOPLASMIC RETICULUM*

TOYOTERU KANDA,† A. M. PEARSON‡ & R. A. MERKEL

Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan 48824, *USA*

(Received: 29 July, 1976)

ABSTRACT

Sarcoplasmic reticulum (SR) was prepared from fresh beef sternomandibularis *muscle and shown to be relatively free from contamination by lysosomes, sarcolemma and mitochondrial membranes.* Ca^{2+} *accumulation by SR from fresh and cold-shortened muscle was* 51 *and* 39 *nmoles/mg protein, respectively. The* Ca^{2+} *accumulating ability of fresh SR vesicles decreased with lowering of pH* (7·3, 6·8, 6·2, 5·5 *and* 5·0) *at all temperatures* (0, 15 *and* 38 °C). *Lowering the temperature from* 38 *to* 0 °C *at pH* 6·6 *resulted in the release of* 48% *of the total accumulated* Ca^{2+}, *whereas the corresponding value on lowering the temperature from* 38 *to* 15 °C *at the same pH was only* 12%. *Thus, low temperatures accelerate the release of* Ca^{2+} *by SR. Although simultaneously lowering pH and temperature also increased* Ca^{2+} *release by SR, the amount of* Ca^{2+} *released was less than if pH and temperature were altered independently. The findings are discussed in the light of explaining cold shortening.*

INTRODUCTION

Locker & Hagyard (1963) first demonstrated that beef muscle shortened on pre-rigor exposure to cold and that shortening was associated with decreased muscle tenderness. Although it was later shown that holding the muscle at 15 °C prevented shortening and cold-induced toughening (Marsh & Leet, 1966; Marsh *et al.*, 1968), the mechanism of cold shortening has not been unequivocally established. Weiner & Pearson (1966) reported that EDTA (ethylenediamine tetraacetic acid) injection inhibited postmortem shortening of muscle, whereas it was shown later that

* Michigan Agricultural Experiment Station Journal Article No. 7544.
† Present address: The Lion Dentifrice Co. Ltd, No. 3 1-Chome, Honjo, Sumida-Ku, Tokyo, Japan.
‡ Author to whom requests for reprints should be addressed.

Food Chem. (2) (1977)—
Printed in Great Britain

microinjections of Ca^{2+} increased pre-rigor muscle shortening and muscle toughness (Pearson *et al.*, 1973); all of which indirectly suggested that release of Ca^{2+} ions by sarcoplasmic reticulum (SR) at low temperatures may be responsible for cold shortening and the associated toughening effect on muscle. A recent report by Buege & Marsh (1975) presented evidence that the mitochondria may be directly involved in cold shortening through release of Ca^{2+} ions under postmortem anaerobic conditions, but that the SR could be indirectly involved due to a decrease in calcium uptake as a consequence of low temperatures.

The present investigation was undertaken to determine the influence of pH and temperature upon Ca^{2+} release by the SR. This was accomplished by saturating freshly isolated and purified SR vesicles with $^{45}Ca^{2+}$ ion and measuring the accumulation and release at different pH values and temperatures. Ca^{2+} accumulation and release were also measured in SR isolated from fresh (pre-rigor immediately post mortem) and from cold shortened (24 h at 0 °C) bovine *sternomandibularis* muscle.

MATERIALS AND METHODS

Preparation of sarcoplasmic reticulum (SR)

Sternomandibularis muscle was removed from beef carcasses immediately following death by exsanguination. All external fat and connective tissue were dissected from the muscle prior to use. SR vesicles were prepared immediately after trimming, or else the trimmed muscles were held at either 15 or 0 °C for 24 h prior to isolating the SR.

SR vesicles were prepared as described by Meissner & Fleisher (1971), which was carried out at 0 °C according to the diagram shown in Fig. 1. A total of 3 ml of crude SR was placed on top of a discontinuous gradient containing 5mM HEPES (0·3M sucrose and 10mM *N*-2-hydroxyethylpiperazine-*N'*-1-ethensulphonic acid) buffer (pH 7·4) with different percentages of sucrose in the different layers as shown in Fig. 2. The sucrose concentration in percent (w/w) was adjusted using a Valentine refractometer. After application of 3 ml of the crude SR, the tubes were spun for 2·5 h at 23,500 rpm in an SB-283 rotor in an IEC-preparative ultracentrifuge.

Vesicle fractions were carefully removed from the gradient with a pipette. The top 4·8 ml of the gradient was removed and discarded. The next 3·9 ml fraction was collected and diluted with 2 volumes of 5mM HEPES buffer (pH 7·4), which was added in four equal parts over a period of 30–45 min to minimise osmotic shock. The fraction was then centrifuged for 1 h at 35,000 rpm using the SB-283 rotor in the IEC-preparative ultracentrifuge. The pellet was resuspended in a solution containing 0·3M sucrose and 2·5 ml HEPES buffer and stored at 0 °C until used. Some of the resuspended SR was frozen using dry ice–acetone and stored at −20 °C for stability studies.

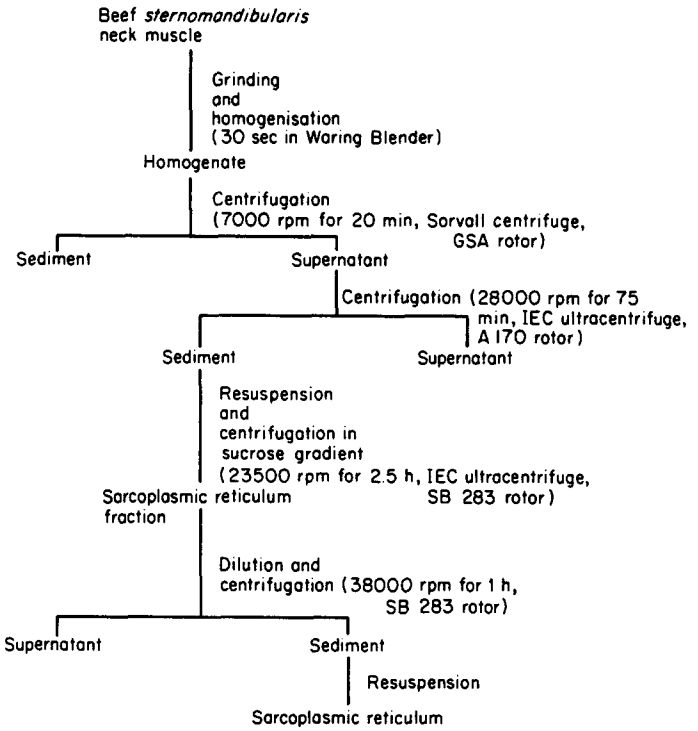


Fig. 1. Flow sheet showing procedure for isolation of sarcoplasmic reticulum.

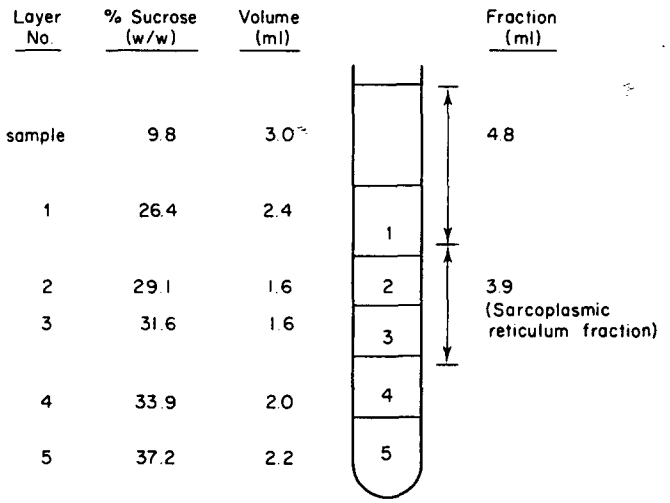


Fig. 2. Fractionation of sarcoplasmic reticulum on a sucrose gradient.

pH measurements

Fresh trimmed muscle was stored at 0 °C for pH measurements. Small portions of the stored muscle were removed after 0, 0·5, 1, 3, 5, 10 and 24 h, homogenised in 5 volumes of distilled–deionised water and the pH was measured using an expanded scale pH meter (Radiometer-type PHM-26, Copenhagen).

Ca^{2+} accumulation and Ca^{2+} release

Ca^{2+} accumulation and Ca^{2+} release were determined using a reaction mixture containing 100mM KCl, 10mM $MgCl_2$, 5mM ATP, 10mM histidine and 0·1mM $CaCl_2$. The reaction mixture was first adjusted to pH 7·3 and $^{45}CaCl_2$ was added to give a final count of 80,000 cpm/ml. Then Ca^{2+} accumulation was determined by placing 3 ml of the reaction mixture in a test tube and adding 0·1 N HCl to adjust to the desired pH (either pH 6·6, 6·2, 5·8, 5·6 or 5·0). Following temperature equilibration at 38, 15 or 0 °C, the reaction was initiated by adding 40–80 μg of SR/ml of reaction mixture. The reaction was allowed to proceed for 3 min and terminated by filtration through a Millipore filter, type GS (average pore size 0·22 μ) as described by Martonosi & Feretos (1964). Ca^{2+} accumulation was calculated from the difference in radioactivity between the reaction mixture without added SR (control) and that containing added SR. Accumulation of Ca^{2+} at each adjusted pH value was compared to that at pH 7·3.

Ca^{2+} release was determined in two tubes containing 3 ml of reaction mixture equilibrated to 38 °C. The reaction was initiated by adding 40–80 μg/ml of SR protein and allowed to continue for 3 min at 38 °C. The reaction was terminated in one tube by passing through a Millipore filter as described above, whereas the other tube was transferred from 38 °C to either a 15 or 0 °C constant temperature water-bath, to lower the temperature of the reaction mixture, and incubated for 10 min. After incubation the reaction was stopped by passing the reaction mixture through the Millipore filter. Ca^{2+} release was calculated from the amount of Ca^{2+} bound by the SR vesicles before and after changing the pH and/or temperature.

$^{45}Ca^{2+}$ radioactivity was counted on either a TRI-CARB scintillation spectrometer (Packard Instruments) or a Nuclear-Chicago, Mark I, model 6894 liquid scintillation counter after mixing the filtrates with PCS scintillation liquid (Amersham–Searle Corporation). The following counting conditions were used: (1) Packard TRI-CARB scintillation spectrometer: window setting = 50–1000 and gain = 11·5%; (2) Nuclear-Chicago Mark I; upper window setting = 9·9, lower = 0·5 and attenuator—c-550.

The concentration of $CaCl_2$ solution in the reaction mixture and the endogenous Ca^{2+} in the SR were analysed by atomic absorption spectroscopy as described by Duggan & Martonosi (1970) using a Perkin–Elmer atomic absorption spectrometer, Model 303. The instrument was calibrated with a standard Ca^{2+} solution (1–10 ppm) in the presence of 10% trichloroacetic acid and 1% of $LaCl_3$.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951) using 1 ml of the appropriately diluted SR preparation and comparing with a standard curve prepared from bovine serum albumen.

Enzyme assays for purity of SR

The succinate–cytochrome *c* reductase activity of the SR preparations was measured by the procedure of Tisdale (1967), which served as a marker to detect the presence of mitochondrial enzymes. Contamination of the SR preparation by the sarcolemma was monitored by the method of Mitchell & Hawthorne (1965). Acid phosphatase activity of the SR preparation was used as a marker enzyme for the presence of lysosomes using a diagnostic kit from Sigma Chemical Company.

RESULTS

Separation of SR on sucrose gradient

The density gradient profiles of SR vesicles prepared from fresh and cold-shortened muscle are shown in Fig. 3. The fresh muscle preparations usually had a broad continuous band in the upper half of the gradient and a narrower, distinct band at the interface between 33·9 and 37·2% sucrose solutions, whereas cold-shortened muscle differed only in exhibiting a marked reduction in the width of the latter band.

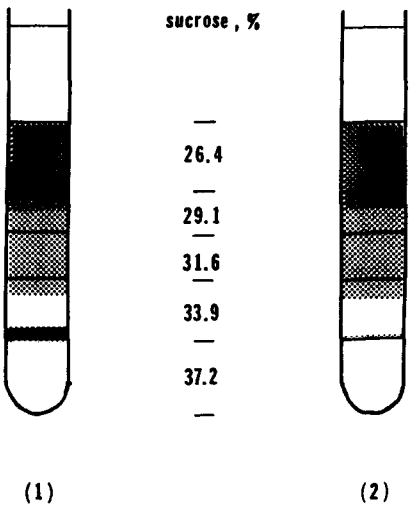


Fig. 3. Density gradient profiles of the sarcoplasmic reticulum from fresh muscle (1) and from cold-shortened muscle (2). Note the distinct differences in the width of the bands.

However, the density gradient profile for vesicles prepared from muscle held for 24 h at 15 °C exhibited the same pattern as the vesicles from cold-shortened muscle, thus indicating that cold-shortening *per se* was not responsible for the differences between vesicles prepared from fresh and cold-shortened muscle. Results suggest that the differences in the profiles are associated with post mortem holding times and may be due to autolysis.

The yield of SR vesicles was 42 ± 8 μg/g for fresh beef muscle, 25 ± 4 μg/g for cold-shortened beef muscle and 16 μg/g for beef muscle stored for 24 h at 15 °C. On the other hand, fresh rabbit *longissimus* muscle yielded 380 μg/g of SR vesicles. The much higher yield for rabbit as compared to beef muscle may be due in part to the larger amount of connective tissue in the beef *sternomandibularis* muscle, which could reduce the efficiency of removal of the SR vesicles, or perhaps be associated with more extensive development of SR in white muscle (rabbit) as compared to red beef muscle (Peachey, 1970). The higher yield of SR vesicles from cold-shortened beef muscle as compared to that of the same beef muscle held for 24 h at 15 °C may be due to a greater amount of proteolysis at the higher temperature.

Purity of SR preparations

The specific activity of succinate–cytochrome *c* reductase was 0·007 μmoles of cytochrome *c* reduced/min/mg of protein. Using the reducing rate of 0·5 μmoles/min/mg of protein for purified mitochondria (Meissner & Fleisher, 1971), the specific activity obtained in this study indicated only 1·4% contamination by mitochondria.

Acid phosphatase activity of the SR preparations was 0·015 μmoles/P_i/min/mg protein. This value may be compared with values of 0·002 to 0·005 μmoles/P_i/min/mg protein for rabbit SR reported by Meissner & Fleisher (1971). Using these values, results indicated that the SR preparations used in this study were only slightly contaminated with lysosomes.

The amount of 5′-nucleotidase activity was negligible for the preparations utilised in this investigation, indicating that there was negligible contamination by the sarcolemma.

Although an attempt was made to remove any myofibrillar protein contaminating the SR preparation by extraction with a solution containing 0·6 M KCl, 0·3 M sucrose and 10 mM histidine (pH 7·3), the extraction resulted in the inactivation of the Ca^{2+}-accumulating ability of the vesicles. Therefore, no attempt was made to purify the preparation by removal of the myofibrillar proteins.

Influence of SR protein concentration on Ca^{2+} accumulation

The relationship between protein concentration of the SR and accumulation of Ca^{2+} is shown in Fig. 4. The graph shows that the accumulation of Ca^{2+} by the SR is linear between 16 and 80 μg protein/ml of reaction mixture, although the curve did not extrapolate to zero. Since the amount of accumulated Ca^{2+} was approximately

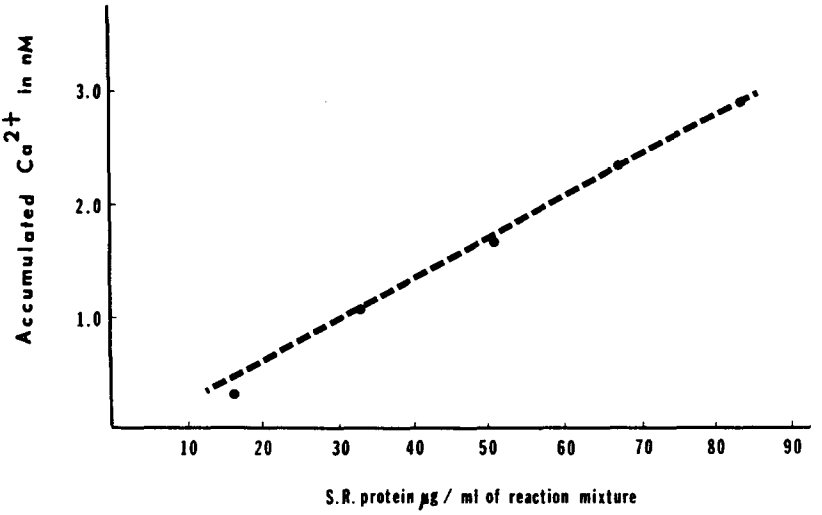


Fig. 4. Ca^{2+} accumulation by sarcoplasmic reticulum as a function of protein concentration. Carried out in HEPES buffer (pH 7·3) at 38 °C.

proportional to protein concentration between 40 and 80 µg, the measurement of Ca^{2+} accumulation and release was made within this range.

Stability of SR vesicles

A number of investigators (Ebashi & Lipmann, 1962; Muscatello *et al.*, 1962; Lee *et al.*, 1965; Eleter & Inesi, 1972) have shown that SR vesicles isolated from muscle immediately post mortem lose their activity during storage at 0 °C and neutral pH (7·0–7·4). However, the activity of isolated vesicles was fairly stable for 90 min storage at 0 °C in 0·3M sucrose and 2·5mM HEPES buffer (Fig. 5).

Figure 6 shows the stability of isolated SR at pH 7·3 in HEPES buffer. The graph shows that the vesicles lost their activity rapidly, even at −20 °C. Thus, determination of Ca^{2+}-release and Ca^{2+}-accumulation by the vesicles was measured immediately after isolation.

Ca^{2+}-accumulation by SR

The amount of endogenous Ca^{2+} bound by the SR was 16 nmoles/mg of protein for beef *sternomandibularis* muscle, which compares fairly well to a value of 35 nmoles Ca^{2+}/mg protein for rabbit muscle SR reported by Meissner *et al.* (1973) but is far different from the value of 500 nmoles Ca^{2+}/mg protein reported by Chevallier & Butow (1971) for rabbit SR. The differences observed may be associated with variations in the isolation procedures and/or differences between species.

Figure 7 shows Ca^{2+} accumulation of SR as a function of reaction time and

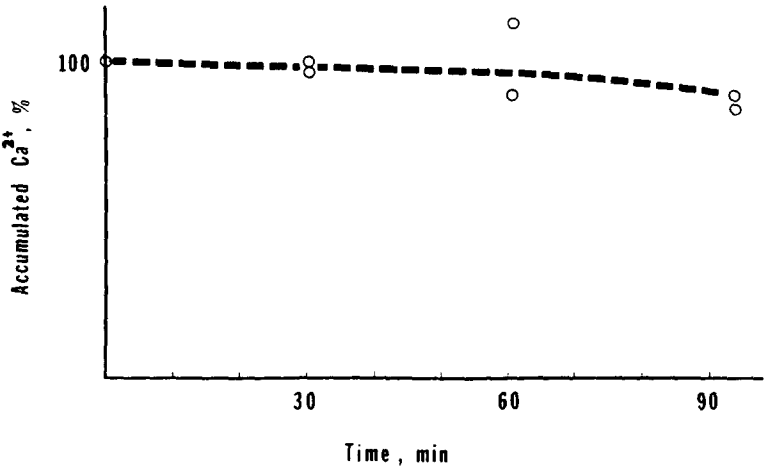


Fig. 5. Stability of sarcoplasmic reticulum vesicles at pH 7·3 (HEPES buffer) and 0 °C.

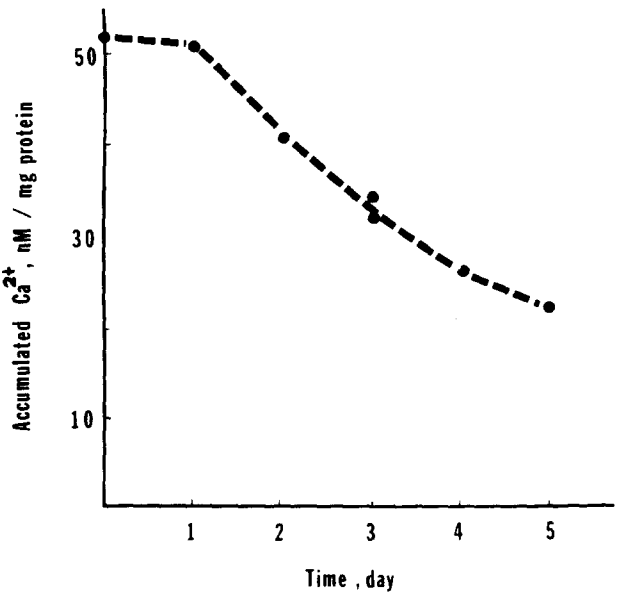


Fig. 6. Stability of sarcoplasmic reticulum vesicles at pH 7·3 (HEPES buffer) and −20 °C.

temperature. The plot shows that Ca^{2+} accumulation begins immediately upon addition of the SR, and that the SR is completely saturated within 1 min. At both 0 and 15 °C the accumulated Ca^{2+} is gradually released with the passage of time. By 20 min approximately 8 and 15 % of the accumulated Ca^{2+} was released by the SR at 15

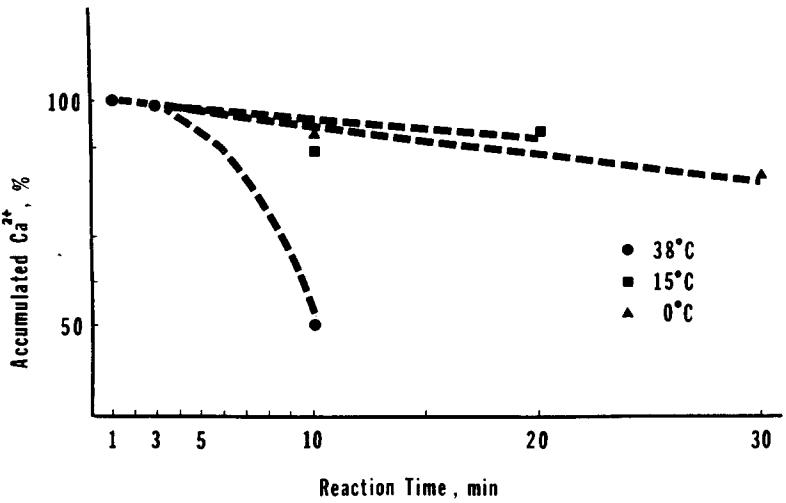


Fig. 7. Ca^{2+} accumulation of sarcoplasmic reticulum vesicles as a function of reaction time (pH 7·3).

and 0°C, respectively. At 38°C, however, the SR vesicles lost 50% of the accumulated Ca^{2+} within 10 min. This is in agreement with the findings of Inesi *et al.* (1973), who reported that raising the temperature above 35°C markedly reduced Ca^{2+} accumulation by the SR. Ca^{2+} saturated SR is extremely unstable to temperatures in the range of 30–50°C (Johnson & Inesi, 1969; Sreter, 1969), which may explain the shortening phenomenon in bovine *sternomandibularis* muscle observed at temperatures above 30°C.

TABLE 1

Ca^{2+} ACCUMULATION OF SR VESICLES FROM FRESH MUSCLE, COLD-SHORTENED MUSCLE AND MUSCLE STORED FOR 24 h AT 15°C

SR source	*Accumulated Ca^{2+} (nM/mg protein)*[a]
Fresh muscle	50·7 ± 2·6
Cold-shortened muscle	39·0 ± 1·3
Muscle stored 24 h at 15°C	0

[a] Ca^{2+} determinations were performed for 3 minutes, at pH 7·3 and 38°C. Each value represents the average of four determinations for two different muscle preparations.

Table 1 summarises the Ca^{2+} accumulation of SR vesicles prepared from fresh muscle, from cold-shortened muscle and from muscle stored 24 h at 15°C. Fresh muscle SR vesicles accumulated 50·7 nmoles of Ca^{2+}/mg of protein, whereas cold-shortened muscle SR vesicles retained about 75% of the Ca^{2+} accumulating ability of fresh SR. SR vesicles prepared from muscle stored at 15°C for 24 h completely lost their ability to bind Ca^{2+} ions. The decrease in Ca^{2+} accumulation with post

mortem time agrees with earlier reports by Greaser *et al.* (1967) and Goll *et al.* (1971).

The pH changes in beef *sternomandibularis* muscle held at 0 °C are shown in Fig. 8. During the first 3 h period, the pH declined rapidly to 6·4, and then dropped more slowly to 5·8 by 24 h. The rapid drop in pH coincident with a fast decline in temperature may accelerate shortening of muscle.

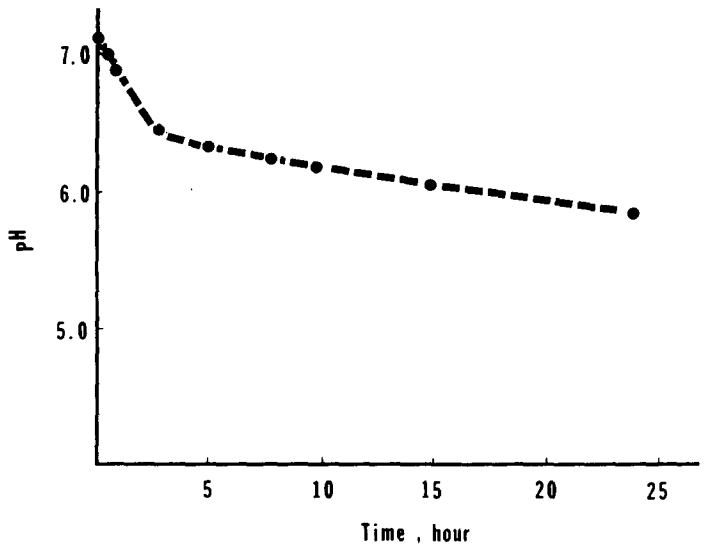


Fig. 8. Changes in pH of beef *sternomandibularis* muscle during storage at 0 °C.

Figure 9 shows the effect of pH upon Ca^{2+} accumulation by the SR at 0, 15 and 38 °C. At pH 7·3, SR vesicles accumulated only about 25 and 75 % as much Ca^{2+} at 0 and 15 °C, respectively, as at 38 °C. At pH 5·0, however, Ca^{2+} accumulation was only 10 nmoles/mg of protein at all three temperatures. As the pH was increased, the differences between different temperatures gradually widened. Maximum Ca^{2+} accumulation (50 nmoles Ca^{2+}/mg of protein) in the pH range of 5·0–7·3 occurred at the highest pH, which is equivalent to that of living muscle (Bate-Smith, 1948). On the other hand, Sreter (1969) found maximum Ca^{2+} accumulation for rabbit muscle to occur at pH of 5·6–6·5 and to be about 5-fold higher than the values for beef *sternomandibularis* in the present study.

Ca^{2+} release by SR

The effects of temperature and pH upon Ca^{2+} release by fresh bovine muscle SR is shown in Fig. 10. The graph shows that temperature did not affect the release of Ca^{2+} at physiological pH (7·3). At pH 6·6, however, about 48 % of the bound Ca^{2+} was released by lowering the temperature from 38 to 0 °C. At the same pH (6·6),

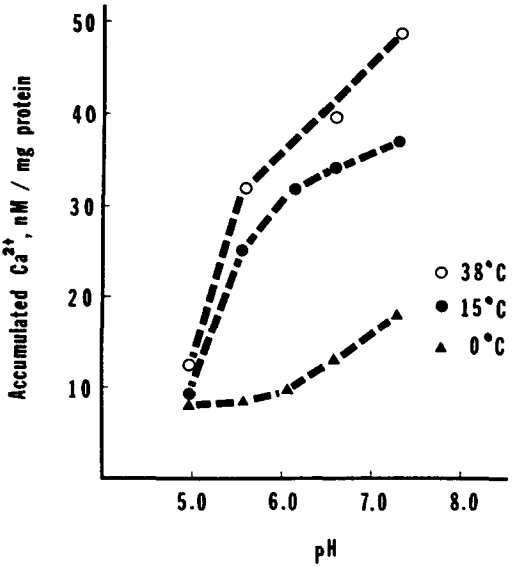


Fig. 9. Ca^{2+} accumulation of sarcoplasmic reticulum vesicles at different pH values and temperatures.

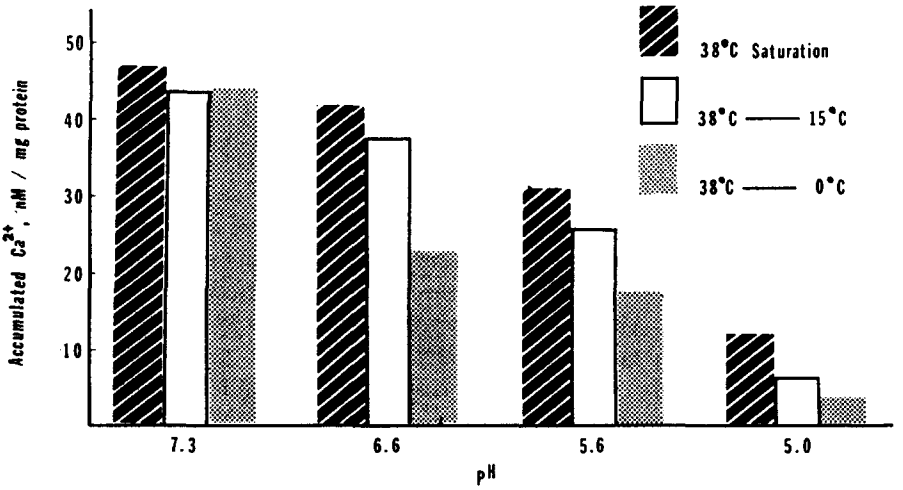


Fig. 10. Effect of pH and temperature on Ca^{2+} release from saturated sarcoplasmic reticulum vesicles.

lowering the temperature from 38 to 15 °C resulted in the release of only 12 % of the total bound Ca^{2+}. Thus, both temperature and pH influenced Ca^{2+} release from SR.

Table 2 shows the amount of Ca^{2+} released from SR vesicles on simultaneously

TABLE 2
CALCIUM RELEASE FROM SATURATED SR VESICLES ON SIMULTANEOUSLY CHANGING pH AND TEMPERATURE[a]

Final pH	*Temperature*	*Released Ca^{2+} (nM mg protein)*
6·6	38 → 0 °C	9·3 ± 6·3
5·6	38 → 0 °C	23·2 ± 0·1
5·0	38 → 0 °C	29·3 ± 5·6

[a] Saturated SR vesicles had 50·7 ± 2·6 nM Ca^{2+}/mg protein.

changing pH and temperature. Results indicate that lowering of pH and temperature simultaneously increases Ca^{2+} release from SR, and in effect was equivalent to cold-shortening. However, the amount of Ca^{2+} released from SR on simultaneously lowering pH and temperature was less than when either temperature or pH were lowered independently. Since the normal physiological changes occurring in intact muscle include a drop in both temperature and pH, the amount of Ca^{2+} accumulation and release may be less in pre-rigor post mortem muscle than was found in altering either pH or temperature alone. Nevertheless, Ca^{2+} release did occur on lowering pH and temperature together.

The amount of Ca^{2+} released on lowering the temperature from 38 to 0 °C was least at pH 6·6, but increased with each successive drop in pH. This demonstrates that SR releases more and more Ca^{2+} as the pH declines. Since there is a rather consistent drop in pH until rigor mortis occurs (Pearson *et al.*, 1973; Briskey, 1964) conditions causing maximum Ca^{2+} release (i.e. a simultaneous drop in pH and temperature) occur in pre-rigor post mortem muscle.

DISCUSSION

The results of this study indicate that both pH and temperature influence Ca^{2+} accumulation and release by bovine SR, which may be related to the phenomenon of cold shortening. Fresh SR preparations accumulated more Ca^{2+} than cold-shortened SR, which supports the hypothesis of Buege & Marsh (1975) that exposure to cold may decrease the ability of bovine SR to accumulate Ca^{2+}. Conversely, both cold temperatures and low pH values decreased Ca^{2+} accumulation and increased Ca^{2+} release by SR, which would support the theory that the leakage of Ca^{2+} by SR under such conditions is responsible for cold-shortening.

The present study indicates that simultaneously lowering the pH and temperature results in less Ca^{2+} being released by SR than when pH and temperature are lowered independently. However, conditions existing during normal chilling of pre-rigor muscle (a simultaneous drop in pH and temperature) may be sufficient to cause enough Ca^{2+} release to cause cold-shortening, as some 82% of the Ca^{2+} was released under such conditions in the present study.

Although the results of the present study do not prove whether or not cold temperatures and low pH are responsible for cold-shortening, they do show that such conditions increase Ca^{2+} release. Nevertheless, results suggest that the decreased Ca^{2+} accumulation by SR at cold temperatures and low pHs are at least contributing factors to cold-shortening. The question as to whether or not Ca^{2+} release by mitochondria (Buege & Marsh, 1975) or Ca^{2+} release by the SR is actually responsible for cold-shortening was not resolved in this study. The data obtained in this study alone are convincing for the role of SR in cold-shortening, but do not offer an explanation for the fact that red muscles shorten much less under aerobic than under anaerobic conditions (Buege & Marsh, 1975). Further work will be required to resolve the relative roles of the SR and mitochondria in cold-shortening of pre-rigor post mortem muscle.

ACKNOWLEDGEMENT

The research reported herein is a portion of the MS thesis submitted by the senior author to Michigan State University in partial fulfillment of the requirements for this degree. The authors also gratefully acknowledge the assistance of The Lion Dentifrice Co. Ltd, Tokyo, Japan for providing support to the senior author while completing the requirements for the MS degree.

REFERENCES

BATE-SMITH, E. C. (1948). The physiology and chemistry of rigor mortis with special reference to the aging of beef. *Adv. Rood Res.*, **1**, 1–38.

BRISKEY, E. J. (1964). Etiological status and associated studies of pale, soft, exudative porcine musculature. *Adv. Food Res.*, **13**, 89–178.

BUEGE, D. R. & MARSH, B. B. (1975). Mitochondrial calcium and postmortem muscle shortening. *Biochem. Biophys. Res. Commun.*, **65**, 478–82.

CHEVALLIER, J. & BUTOW, R. A. (1971). Calcium binding to the sarcoplasmic reticulum of rabbit skeletal muscle. *Biochemistry*, **10**, 2733–7.

DUGGAN, P. F. & MARTONOSI, A. (1970). Sarcoplasmic reticulum. IX. The permeability of sarcoplasmic reticulum membranes. *J. Gen. Physiol.*, **56**, 147–53.

EBASHI, S. & LIPMANN, F. (1962). Adenosine-triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. *J. Cell Biol.*, **14**, 389–97.

ELETER, S. & INESI, G. (1972). Phase changes in the lipid moieties of sarcoplasmic reticulum membranes induced by temperature and protein conformational changes. *Biochim. Biophys. Acta*, **290**, 178–85.

GOLL, D. E. STROMER, M. H., ROBSON, R. M., TEMPLE, J., EASON, B. A. & BUSCH, W. A. (1971). Tryptic digestion of muscle components simulates many of the changes caused by postmortem storage. *J. Anim. Sci.*, **33**, 963–82.

GREASER, M. L., CASSENS, R. G. & HOEKSTRA, W. G. (1967). Changes in oxalate-stimulated calcium accumulation in particulate fractions from postmortem muscle. *J. Agr. Food Chem.*, **15**, 1112–17.

INESI, G., BLANCHET, S. & WILLIAMS, D. (1973). *Organization of Energy Transducing Membranes*, pp. 93–136. M. Nakano and L. Packer, eds., University Park Press, Baltimore, Maryland.

JOHNSON, P. N. & INESI, G. (1969). The effect of methyl-xanthines and local anesthetics on fragmented sarcoplasmic reticulum. *J. Pharm. Expt. Ther.*, **169**, 308–12.

LEE, K. S., TANAKA, K. & YU, D. H. (1965). Studies on adenosine triphosphatase, calcium uptake and relaxing activity of the microsomal granules from skeletal muscle. *J. Physiol.*, **179**, 456–64.

LOCKER, R. H. & HAGYARD, C. J. (1963). A cold shortening effect in beef muscles. *J. Sci. Food Agr.*, **14**, 787–95.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–75.

MARSH, B. B. & LEET, N. G. (1966). Studies on meat tenderness. III. Effect of cold shortening on tenderness. *J. Food Sci.*, **31**, 450–9.

MARSH, B. B., WOODHAMS, P. R. & LEET, N. G. (1968). Studies on meat tenderness. V. The effects on tenderness of carcass cooling and freezing before the completion of rigor mortis. *J. Food Sci.*, **33**, 12–18.

MARTONOSI, A. & FERETOS, R. (1964). Sarcoplasmic reticulum. I. The uptake of Ca^{2+} by sarcoplasmic reticulum fragments. *J. Biol. Chem.*, **239**, 648–58.

MEISSNER, G. & FLEISHER, S. (1971). Characterization of sarcoplasmic reticulum from skeletal muscle. *Biochim. Biophys. Acta*, **241**, 356–78.

MEISSNER, G., CONMER, G. E. & FLEISHER, S. (1973). Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca^{2+}-pump and Ca^{2+}-binding proteins. *Biochim. Biophys. Acta*, **298**, 246–69.

MITCHELL, R. H. & HAWTHORNE, J. N. (1965). The site of diphosphoinositide synthesis in rat liver. *Biochem. Biophys. Res. Commun.*, **21**, 333–8.

MUSCATELLO, U., ANDERSSON-CEDERGREN, E. & AZZONE, G. F. (1962). The mechanism of muscle-fiber relaxation, adenosine triphosphatase and relaxing activity of the sarcotubular system. *Biochim. Biophys. Acta*, **63**, 55–74.

PEACHEY, L. D. (1970). Form of the sarcoplasmic reticulum and T system of striated muscle. In: *The Physiology and Biochemistry of Muscle as a Food*, Vol. 2, pp. 273–310. E. J. Briskey, R. G. Cassens and B. B. Marsh, eds., The University of Wisconsin Press, Madison, Wisc.

PEARSON, A. M., CARSE, W. A., DAVEY, C. L., LOCKER, R. H. & HAGYARD, C. J. (1973). Influence of epinephrine and calcium upon glycolysis, tenderness and shortening of sheep muscle. *J. Food Sci.*, **38**, 1124–7.

SRETER, F. A. (1969). Temperature, pH and seasonal dependence of Ca-uptake and ATPase activity of white and red muscle microsomes. *Arch. Biochem. Biophys.*, **134**, 25–33.

TISDALE, H. D. (1967). Preparation and properties of succinic-cytochrome c reductase (complex II–III). In *Methods in Enzymology, Vol. X*, pp. 213–15. P. W. Estabrook and M. E. Pullman, eds., Academic Press, New York.

WEINER, P. D. & PEARSON, A. M. (1966). Inhibition of rigor mortis by ethylenediamine tetraacetic acid. *Proc. Soc. Expt. Biol. Med.*, **123**, 185–7.

STUDIES ON THE CHEMICAL PROPERTIES OF COWPEA POWDERS SUPPLEMENTED AND DRUM-DRIED WITH DL-METHIONINE

O. ONAYEMI

Department of Food Science and Technology, University of Ife, Ile-Ife, Nigeria

(Received: 15 November, 1977)

ABSTRACT

Food grade methionine was added at 0·2, 0·4 *and* 0·6% *levels, on a solids basis, to dehulled pureed cowpeas* (Vigna sinensis) *for nutritional improvement. The cowpea puree was dried on a laboratory size atmospheric drum drier and comminuted to cowpea powders. The powders, which contained two levels of moisture, were stored in incubators at temperatures up to* 37°C *for a period of* 24 *weeks and evaluated at intervals for chemical changes.*

The drum-drying operation did not appreciably affect the chemical composition of the cowpea powders in terms of protein, thiamin and added methionine. There was good retention of the added methionine in the cowpea powders during storage.

INTRODUCTION

Amino acid supplementation of plant protein foods has been suggested as an effective procedure for producing high quality protein foods which could be used to combat the problem of protein–calorie malnutrition still prevalent in many developing parts of the world (Howe *et al.*, 1965; Hegsted, 1968; Mauron, 1969). Because of their availability and low cost, the use of crystalline amino acids such as lysine and methionine to upgrade the protein quality of foods containing cereals, heat-treated legumes and mixtures has been well established (Bressani & Elias, 1968; Jansen, 1970, 1973; Cremer & Mauron, 1971; Graham, 1971; Zucker, 1973; Bookwalter *et al.*, 1975). Suggestions have been made for the methionine supplementation and processing of common legume foods in order to provide inexpensive sources of protein to many people in Africa and Asia (Anon., 1973).

Cowpea powders supplemented and drum dried with methionine which have good

Fd. Chem. (3) (1978)—
Printed in Great Britain

nutritional and organoleptic properties have been produced (Onayemi & Potter, 1976).

The problems of flavour and possible toxicity at certain levels have been a great limitation to the use of methionine in human foods. Gabby (1973) concluded that the flavour of methionine itself is not good but when small amounts are properly incorporated into human foods it could provide beneficial results. The literature is replete with information on the chemical changes which take place when foods are processed (Baldwin *et al.*, 1951; Harris & Von Loesecke, 1971; Bender, 1972).

With the increased current interest in the nutritional quality of processed foods and the labelling of foods, especially those containing nutrient supplements, it is necessary to provide information on the chemical characteristics of cowpea powders supplemented with methionine. This would enable full use to be made of this material in bakery products such as composite flour and baby foods. This paper describes some of the chemical characteristics of cowpea powders and the factors which influence the stability of the added methionine.

MATERIALS AND METHODS

Source of material

Cowpeas (*Vigna sinensis*) were soaked in water for 18 h, dehulled and pureed. DL-Methionine (Eastman Kodak, Rochester, New York) was added and uniformly dispersed at 0·2, 0·4 and 0·6% levels, on a solids basis, to the puree. Cowpea powders were obtained at the 5·5 and 6·5% levels of moisture by drum drying the cowpea puree on a laboratory size atmospheric drum drier (Onayemi & Potter, 1976). The powders were stored in air-tight bottles in incubators at temperatures of 23, 30 and 37 °C for a period of up to 24 weeks.

Analytical methods

The proximate chemical compositions of the raw cowpeas and the cowpea powders were determined using the Official Methods of Analysis of the AOAC (1970). Thiamin contents of the raw cowpeas, freshly made cowpea powders and stored cowpea powders were determined by the thiochrome method described by the Association of Vitamin Chemists (1966). The recovery and retention of the added methionine in the cowpea powders were estimated using the method of Herrick *et al.* (1972). For methionine determination, performic acid oxidation of the methionine was adopted, followed by acid hydrolysis and thin layer chromatography. The amounts of methionine present in the samples were quantified by reference to a methionine sulphone standard using a spectrodensitometer Model SD 3000 (Shoeffel Instrument Corp. Westwood, New Jersey) at a wavelength of 410 nm. All determinations were made in duplicate.

RESULTS AND DISCUSSION

Proximate chemical composition

The composition of the raw cowpeas and the cowpea powders independent of the level of methionine supplementation is shown in Table 1. From this data it appears that the drum-drying operation did not affect the protein or thiamin content of the cowpea powders when compared with the raw cowpeas. However, the low fibre content of the cowpea powders indicates that they would have high digestibility. The removal of the hulls prior to pureeing the soaked cowpeas suggests that the cowpea product would not cause flatulence, which has been attributed to the presence of hulls in most common legume foods (Hellendoorn, 1973).

TABLE 1
PROXIMATE CHEMICAL COMPOSITION OF THE RAW COWPEAS AND METHIONINE-SUPPLEMENTED COWPEA POWDERS

Determinations	*Raw cowpeas*	*Cowpea powders*[a]
Moisture (% WWB)	8·52	6·50
Dry matter (% WWB)	91·48	93·50
Crude protein (% DWB)	23·90	22·50
Ether extract (% DWB)	1·5	1·25
Crude fibre (% DWB)	6·5	1·0
Ash (% DWB)	0·50	0·5
Nitrogen-free extract (% DWB)	6·0	67·25
Thiamin (mg/100 g)	0·92	0·82

[a] Values are independent of the level of the added methionine.
WWB = Wet Weight Basis.
DWB = Dry Weight Basis.

The levels of thiamin in the cowpea powders after storage are shown in Table 2. It is apparent that there were very slight changes, due to the processing and storage conditions, in the levels of thiamin in the cowpea powders compared with the raw cowpeas. Changes in the thiamin levels have generally been used as indicators of the severity of heat processing treatment and storage conditions (Farrer, 1955). It is reported that the drum-drying process significantly reduced the trypsin inhibitor activities in the cowpea powders (Onayemi & Potter, 1976). The high stability of the

TABLE 2
THIAMIN CONTENT[a] OF COWPEA POWDERS STORED FOR 24 WEEKS

Temperature (°C)	23			30			37		
Levels of added methionine (% DWB)	0·2	0·4	0·6	0·2	0·4	0·6	0·2	0·4	0·6
Powder with 5·5% moisture	0·78	0·75	0·77	0·75	0·76	0·76	0·77	0·75	0·74
Powder with 6·5% moisture	0·78	0·79	0·79	0·76	0·78	0·78	0·74	0·76	0·78

[a] In mg/100 g cowpea powder.
DWB = Dry Weight Basis.

thiamin levels in the cowpea powders indicates that other B-vitamins and functional properties of the cowpea powders such as solubility would not be reduced. Kon *et al.* (1974) reported that slight reductions in the B-vitamin occurred with drum-dried Californian small white beans.

The raw cowpeas had a methionine content of 150 mg per gramme of nitrogen. This value is within the range reported in the literature for cowpea varieties and was used as a basis for supplementing the cowpea puree with methionine prior to the drum-drying process. The recovery of the added methionine was of the order of 95%, thus confirming that methionine is heat stable.

Table 3 shows the retention of the added methionine in the cowpea powders after storage for 24 weeks. The data are the average for two determinations in each case

TABLE 3
PERCENT RETENTION OF ADDED METHIONINE AFTER 24 WEEKS OF STORAGE

Temperature (°C)	23			30			37		
Level of added methionine (% DWB)[a]	0·2	0·4	0·6	0·2	0·4	0·6	0·2	0·4	0·6
Powder with 5·5% moisture	93	93	92	90	91	91	90	89	89
Powder with 6·5% moisture	92	93	91	90	90	89	85	87	88

[a] DWB = Dry Weight Basis.

and are expressed as a percentage of the amounts originally added. The data indicate that for practical purposes the residual methionine would be stable in the cowpea powders under tropical conditions for at least a 24-week period. Similar high retention data have been reported for canned soy-milk and animal feed supplemented with methionine (Dunlop *et al.*, 1974). Rohdenburg & Rosenberg (1956) reported that methionine supplemented to animal feed decreased slightly after a year when the feed was stored at temperatures above 50 °C. It is possible that at this temperature slight decomposition changes occurred in the methionine which produced off-flavour effects due to the presence of such compounds as mercaptans and sulphydryl groups.

In this work no objectionable flavour was produced at any of the levels at which the methionine was added to the cowpea powders. This may be attributed to the heat stable nature of methionine itself, or to the protective effect of other nutrient constituents such as carbohydrates and proteins in the powders as well as the short time–high temperature exposure of the methionine during the drum-drying process.

The data on the stability of methionine and thiamin confirm the observation that, if the conditions of the dehydration process are well controlled, there is no detrimental effect on the quality of the dried product (Thomas & Calloway, 1961; De Groot, 1963; Hollingsworth and Martin, 1972).

The good storage stability data on the chemical properties of the methionine supplemented cowpea powders may also be due to the moisture levels at which the

powders were obtained. These moisture levels are within the range consistent with the stability of many dehydrated products (Salwin, 1963).

This study indicates that the drum-drying process and storage conditions did not significantly affect the chemical characteristics of methionine supplemented cowpea powders. Its low fibre content, high protein content and low levels of methionine should permit this powder to find applications in bakery products and as a supplement to carbohydrate-based baby foods.

REFERENCES

ANON. (1973). *Protein Advisory Group Statement No. 22 on Upgrading Human Nutrition Through the Improvement of Food Legumes*, Bulletin III 2 (1), 24.

AOAC (1970). *Official methods of analysis* (11th edition). Association of Official Analytical Chemists, Washington, DC, 1970.

ASSOCIATION OF VITAMIN CHEMISTS (1966). *Methods of vitamin assay* (3rd ed.). Interscience Publishers, New York, p. 120.

BALDWIN, R. R., LOWRY, J. R. & THIESSEN, R., JR. (1951). Some effects of processing on the nutritive properties of proteins. *Food Res.*, **16**, 107.

BENDER, A. E. (1972). *Processing damage to protein foods—A review. J. Food Technol.*, **7**, 239.

BOOKWALTER, G. N., WARNER, K., ANDERSON, R. A., MUSTAKAS, G. C. & GRIFFIN, E. L. (1975). *Fortification of dry soybean-based foods with* DL-*methionine. J. Food Sci.*, **40**(2), 266.

BRESSANI, R. & ELIAS, L. G. (1968). Processed vegetable protein mixtures for human consumption in developing countries. *Adv. in Fd. Res.*, **16**, 1.

CREMER, H. D. & MAURON, J. (1971). Amino acid fortification. *Archieves Latin Americanos de Nutricion*, **21**(2), 103–22.

DE GROOT, A. P. (1963). The influence of dehydration of foods on the digestibility and the biological value of the protein. *J. Food Technol.*, **17**, 103.

DUNLAP, C. J., GUADANGNI, D. G., MIERS, J. C. & WAGNER, J. R. (1974). Methionine supplement alters flavor, PER of pinto beans canned in tomato sauce. *Food Prod. Develop.*, **8**(1), 88.

FARRER, K. T. H. (1955). The thermal destruction of vitamin B in foods. *Adv. Fd. Res.*, **6**, 257.

GABBY, J. L. (1973). *A Note on the Problems of Food Technology and Acceptance of Methionine in Foods.* PAG Bulletin III No. 3, 32.

GRAHAM, G. C. (1971). Methionine or lysine fortification of dietary protein for infants and small children. In: *Amino acid fortification of protein foods.* (Scrimshaw, N. S. & Altschul, A. M. (Eds.)), MIT Press, Cambridge, Mass., USA.

HARRIS, R. S. & VON LOESECKE, H. (1971). *Nutritional evaluation of food processing.* Avi Publishing Co. Westport, Conn., USA.

HEGSTED, D. M. (1968). Amino acid fortification and the protein problem. *Am. J. Clin. Nutr.*, **21**, 688.

HELLENDOORN, E. W. (1973). Digestibility and flatulence activity of beans. Paper presented at a Meeting on the Nutritional Aspects of Common Beans and other Legume Seeds as Animal and Human Foods. Ribeirao Preto, Brazil, November 6–9.

HERRICK, H. E., LAWRENCE, J. M. & COAHRAN, D. R. (1972). Simple methods for determination of methionine and cystine in legume seeds. *Analytical Biochem.*, **48**, 353.

HOLLINGSWORTH, D. F. & MARTIN, D. F. (1972). Some aspects of the effects of different methods of production and processing on the nutritional value of foods. *World Review of Nutr. and Dietetics*, **15**, 1–34.

HOWE, E. E., GILFILLAN, E. W. & MILNER, M. (1965). Amino acid supplementation of protein concentrates as related to the world protein supply. *Amer. J. Clin. Nutr.*, **16**, 321.

JANSEN, G. R. (1970). Amino acid supplementation and the world food problem. In: *Evaluation of novel protein products* (Bender, A. E. (Ed.)), Pergamon Press, Oxford.

JANSEN, G. R. (1973). Amino acid supplementation of common beans and other legumes. Paper presented at a Meeting on the Nutritional Aspects of Common Beans and other Legume Seeds as Animal and Human Foods. Ribeirao Preto, Brazil, November 6–9.

KON, S., BOOTH, A. N. & WAGNER, J. R. (1974). Legume powders: Preparation and some nutritional and physico-chemical properties. *J. Food Sci.*, **39**, 897.

MAURON, J. (1969). Amino acid imbalance and its bearing on the fortification of food. *Bibl. Nutrio. et Dieta*, **11**, 57.

ONAYEMI, O. & POTTER, N. N. (1976). Cowpea powders dried with methionine: Preparation, storage stability, organoleptic properties, nutritional quality. *J. Food Sci.*, **41**, 48.

ROHDENBURG, E. L. & ROSENBERG, H. R. (1956). Estimation and stability of added DL-Methionine in mixed feeds. *J. Agr. Food Chem.*, **4**(10), 852.

SALWIN, H. (1963). Moisture levels required for stability in dehydrated foods. *J. Food Technol.*, **67**, 34.

THOMAS, M. & CALLOWAY, D. (1961). Nutritional value of dehydrated foods. *J. Amer. Diet. Ass.*, **39**, 105.

ZUCKER, H. (1973). The value of amino acid supplementation. In: *Proteins in human nutrition* (Porter, J. W. G. & Rolls, B. A. (Eds.)), Academic Press, New York.

CHEMICAL COMPOSITION OF SOME LESS COMMONLY USED LEGUMES IN GHANA

J. D. WATSON

Department of Nutrition and Food Science, University of Ghana, P.O. Box 134, *Legon, Ghana*

(Received: 15 August, 1976)

ABSTRACT

Three legumes, viz. mung bean, winged bean (seeds, pods and roots) and the yam bean have been analysed with respect to their moisture, protein, fat, soluble carbohydrate, fibre, mineral and energy content. The seeds provide a valuable source of protein, the winged bean having 29 %, *the mung bean* 23 % *and the yam bean* 19 %. *They are also high in energy content, the winged bean having* 400 *kcals/*100 *g, the mung bean* 310 *kcals/*100 *g and the yam bean* 327 *kcals/*100 *g. In addition to having the highest protein content, the winged bean seeds provide fair amounts of fat* (17·7 %) *and calcium* (204 *mg/*100 *g). The winged bean is significant in that most parts of the plant are edible such as the young leaves, pods and the roots. The roots contain significant amounts of protein and carbohydrate. The production and increased consumption of these legumes has been stressed.*

INTRODUCTION

The increased production and consumption of legumes offers a partial solution to increasing the available protein supplies to any given population. There are however some setbacks in the utilisation of legumes as sources of food and consequently of protein. There is the problem of storage because many legumes are easily infested with various kinds of pests which attack them. With regard to their use as foods there are factors such as palatability, flatulence effect, cooking time, etc., which militate against their increased consumption.

Although wide varieties of legumes are grown in many parts of the world only a few are commonly consumed by man. In recent years attempts have been made to bring about a better utilisation of legumes in the diet and in particular to try and introduce some of the less-used varieties. In Ghana the commonly used legumes

Fd. Chem. (2) (1977)—

include the cowpea, bambara bean, groundnut and lima bean. However, there are several other types of legumes which are already found or could be cultivated. This study was undertaken to provide data on the chemical composition of three different legumes found in Ghana but which are not consumed to any appreciable extent. The legumes studied were the mung bean (two varieties), the winged bean and the yam bean.

MATERIALS AND METHODS

Materials

The samples of mung bean and winged bean were obtained from the University research stations and that of the yam bean from the Volta Region of Ghana where it is commonly found. In addition to the seeds of the winged bean, the pods and the roots also were analysed since they are edible and can serve as sources of food.

Methods

The procedures carried out for the determination of moisture, protein, fat, crude fibre and ash were based on the standard techniques adopted by the Association of Official Agricultural Chemists (1970). The protein value was derived from the nitrogen content by multiplying by the factor 6·25. Of the minerals, calcium was precipitated as calcium oxalate and subsequently determined by titration against permanganate; both phosphorus and iron were determined colorimetrically, the former by the quinol/sodium sulphite method and the latter by the dipyridyl method. The carbohydrate values were computed by difference by subtracting from 100 the sum of the moisture, protein, fat, fibre and ash contents. The energy value was determined with a ballistic bomb calorimeter described by Miller & Payne (1959). The gross energy values thus determined are compared with the physical heats of combustion as computed from the carbohydrate, protein and fat contents using the following values: for carbohydrate 4·15 kcals/g; protein 5·65 kcals/g and fat 9·4 kcals/g. In the computation of the physical heats of combustion of carbohydrate, both the soluble carbohydrate and the fibre content of the foodstuff were taken into account. The metabolisable energy values were derived using the classical Atwater factors 4, 9 and 4 for protein, fat and carbohydrate respectively. However, in the case of contribution of carbohydrate to metabolisable energy, only the soluble carbohydrate content was used in the computation.

RESULTS AND DISCUSSION

The results giving the mean and range values for the various constituents are presented in Table 1.

TABLE 1

CHEMICAL COMPOSITION OF LEGUMES (VALUES IN g/100 g EDIBLE PORTION. EXCEPT MINERALS WHICH ARE EXPRESSED IN mg/100 g)

Foodstuff and description	*Moisture*	*Energy (kcals)*			*Protein*	*Fat*	*Carbo-hydrate*	*Fibre*	*Ash*	*Minerals*		
		Bomb	*Calculated*							*Ca*	*P*	*Fe*
			Physical	*Metab-olisable*								
Mung bean green (4)[a] *Phaseolus aureus*	15·2 (14·6–15·8)	374 (366–382)	379	306	23·0 (22·8–23·1)	1·3 (1·3–1·4)	53·3	3·8 (3·5–4·1)	3·4 (3·3–3·4)	108 (82–130)	430 (430–430)	8·0 (6·0–10·0)
Mung bean black (4) *Phaseolus mungo*	12·0 (11·7–12·2)	415 (385–440)	386	320	22·6 (22·1–22·9)	0·4 (0·3–0·5)	56·5	4·7 (4·4–4·9)	3·8 (3·6–4·0)	135 (127–140)	360 (320–395)	13·5 (11·0–16·5)
Winged bean pods (4) *Psophocarpus tetragonolobus*	86·8 (84·0–90·0)	64 (49–78)	62	43	2·6 (2·0–3·1)	0·6 (0·4–0·7)	6·8	3·2 (2·5–3·9)	0·6 (0·4–0·7)	45 (34–54)	36 (27–43)	15·0 (11·6–18·6)
Winged bean seeds (4)	11·2 (11·0–11·5)	450 (445–455)	490	402	29·1 (29·0–29·2)	17·7 (17·7–17·8)	31·6	6·8 (6·8–6·9)	3·6 (3·6–3·6)	204 (203–205)	376 (375–377)	9·6 (9·5–9·6)
Winged bean roots (4)	65·2 (63·2–67·6)	148 (139–158)	147	129	4·7 (4·4–5·0)	0·1 (0·1–0·15)	27·2	1·5 (1·4–1·6)	0·9 (0·8–0·9)	40 (37–42)	64 (59–67)	3·0 (2·7–3·1)
Yam bean (4) *Sphenostylis stenocarpa*	11·2 (10·8–11·8)	428 (417–446)	390	327	19·1 (18·3–19·9)	0·5 (0·4–0·6)	61·6	5·2 (4·5–6·4)	2·4 (2·3–2·5)	45 (44–46)	Not determined	11·5 (11·0–12·0)

[a] Figure in parentheses denotes number of samples analysed.

Of the legumes examined, the winged bean seeds have the highest protein content, 29 %, followed by the mung bean, 22–23 % and the yam bean, 19 %. The winged bean pods and roots have 2·6 % and 4·7 % protein respectively. All the legumes are low in fat with the exception of the winged bean seeds which contain 17·7 %. The winged bean seeds are also a moderate source of calcium, containing 204 mg/100 g. The results for the mung bean and the yam bean compare favourably with other published data such as those of Platt (1962) and FAO (1968). However, in the case of the winged bean seeds, the protein values obtained in this study are slightly lower (29 %) compared with those reported by other workers (Nicholls *et al.*, 1961, and Cerny *et al.*, 1971) who had protein values of 33 %.

The mung bean has been used extensively in parts of Asia where it is boiled and eaten whole or ground into a flour after the removal of the seed coat, the flour being used in the preparation of various dishes. In recent years it has been grown in Ghana where it has mainly been used as a poultry feed although there is no reason why it cannot be used for human consumption. One of the difficulties of introducing it is its unfamiliarity in this part of the world so that there is a need for education on how it may be used for incorporation into the local dishes.

The yam bean is consumed to a limited extent in Ghana and this is confined mainly to one particular region of the country, namely the Volta Region.

The winged bean is an interesting legume as most parts of the plant are edible. The young pods when tender are sliced and cooked like French beans; the tuberous roots are eaten like potatoes. The unripe seeds are used like peas in soups while the ripe seeds are roasted and eaten like peanuts. The seeds have a pleasant sweet taste even in the raw state although the skin is tough and more difficult to remove than that of the soya bean (Pospisil *et al.*, 1971).

Cerny *et al.* (1971) have shown that the amino acid composition of the protein in winged bean is very similar to that of the soya bean with methionine as the first limiting amino acid. The content of tocopherols, unsaturated fatty acids and that of polyunsaturated essential fatty acids was satisfactory. They showed that an active trypsin inhibitor found in the raw seeds could be destroyed by moist heat and that there was no detectable urease activity. They further demonstrated that the protein efficiency ratio (PER) and net protein utilisation (NPU) of the bean determined with rats were superior to those of groundnuts.

Cerny & Addy (1973) investigated the use of the winged bean as part of a mixed diet (two parts winged bean and three parts maize flour enriched with small amounts of skim milk) in the treatment of children suffering from kwashiorkor. They found that the diets were well accepted and tolerated and the children under study made good clinical progress such that the mixture gave results similar to those of a control diet in which 90 % of the protein content was supplied by skim milk.

The three legumes considered could serve as valuable sources of protein in the Ghanaian diet if their cultivation and consumption can be actively promoted.

ACKNOWLEDGEMENT

The author wishes to thank Miss C. Boakye and Mr F. Torto for technical assistance in carrying out some of the analyses.

REFERENCES

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS (1970). *Methods of Analysis*, 11th Edition.

CERNY, K., KORDYLAS, J. M., POSPISIL, F., SVABENSKY, O. & ZAZIC, B. (1971). The nutritive value of the winged bean, *Brit. J. Nutr.*, **26**, 293–9.

CERNY, K. & ADDY, H. A. (1973). The winged bean in the treatment of kwashiorkor, *Brit. J. Nutr.*, **29**, 105–12.

FOOD AND AGRICULTURE ORGANISATION (1968). *Food composition tables for use in Africa.*

MILLER, D. S. & PAYNE, P. R. (1959). A ballistic bomb calorimeter, *Brit. J. Nutr.*, **13**, 501–8.

NICHOLLS, L., SINCLAIR, H. M. & JELLIFE, D. B. (1961). *Tropical nutrition and dietetics*, 4th Edition, Bailliëre, Tindall and Cox, London.

PLATT, B. S. (1962). Tables of representative values of foods commonly used in tropical countries, *Med. Res. Coun. Spec. Rep. Ser.*, No. 302.

POSPISIL, F., KARIKARI, S. K. & BOAMAH-MENSAH, E. (1971). Investigations on the winged bean in Ghana, *World Crops*, **23**, 250–3.

QUALITY CHANGES OF FROZEN VEGETABLES

P. BAARDSETH

Norwegian Food Research Institute, Postbox 50, *N*-1432 *Aas-NLH, Norway*

(Received: 12 December, 1977)

ABSTRACT

Unblanched and blanched vegetables (carrot, cauliflower, French bean, onion, leek and swede) were stored at $-20\,^{\circ}C$ *and* $-30\,^{\circ}C$ *for up to* 15 *months. Lipid and peroxidase analyses and sensory evaluations were performed every three months.*

Unblanched leek, onion and swede did not change appreciably during the 15 *months' storage, either chemically or organoleptically, and they were scored better than the blanched samples. Carrot, cauliflower and French bean, on the other hand, had to be blanched, but a* 5% *residual activity of peroxidase did not affect the quality during storage.*

INTRODUCTION

It is well known that unblanched vegetables deteriorate in quality when held in frozen storage for extended periods of time. This is mainly due to enzymes present in the living organism; these act in different metabolic processes even at low temperatures. The most important enzymes causing undesirable quality changes in foods are listed in Table 1. According to Svensson (1977) the enzymes can be separated into four groups relating to changes in flavour, colour, texture/consistency and nutritional value. Although the enzymes peroxidase and catalase are often reported to cause off-flavour, the reactions involved have not been conclusively identified (Svensson, 1977).

Peroxidase appears to be the most heat stable enzyme in plants (Lopez *et al.*, 1959; Kyzlink & Chytra, 1959; Reed, 1975). For this reason peroxidase activity is widely used as an index of blanching. It has been generally accepted that if peroxidase is destroyed it is quite unlikely that other enzyme systems will have survived. However, complete inactivation of peroxidase has been shown not to be necessary for quality preservation in frozen vegetables (Bøttcher, 1975*a*,*b*). Lipid oxidation

Fd. Chem. (3) (1978)—
Printed in Great Britain

TABLE 1
FOOD QUALITY-RELATED ENZYMES (SVENSSON, 1977)

	Enzyme	*Catalysed reaction*	*Quality defect*
Flavour	Lipolytic acyl hydrolase (lipase, esterase, etc.)	Hydrolysis of lipids	Hydrolytic rancidity (soapy flavour)
	Lipoxygenase	Oxidation of poly-unsaturated fatty acids	Oxidative rancidity ('green' flavour)
	Peroxidase/catalase	?	'Off-flavour' (?)
	Protease	Hydrolysis of proteins	Bitterness
Colour	Polyphenol oxidase	Oxidation of phenols	Dark colour
Texture/ consistency	Amylase	Hydrolysis of starch	Softness/loss in viscosity
	Pectin methyl-esterase	Hydrolysis of pectin to pectic acid and methanol	Softness/loss in viscosity
	Polygalacturonase	Hydrolysis of α-1,4 glycosidic linkages in pectic acid	Softness/loss in viscosity
Nutritional value	Ascorbic acid oxidase	Oxidation of L-ascorbic acid	Loss in vitamin C content
	Thiaminase	Hydrolysis of thiamine	Loss in vitamin B_1 content

has been claimed to be the main reaction in off-flavour production of frozen vegetables (Lee *et al.*, 1955 and 1956; Lee & Mattick, 1961; Rhee & Watts, 1966; Bengtsson & Bosund, 1966; Chow & Watts, 1969). The oxidation by molecular oxygen of, for example, linoleic and linolenic acids which leads to the formation of strongly odorous compounds is effectively catalysed by metal ions, haematin compounds and the enzyme lipoxygenase in increasing order of activity (Grosch, 1972; Grosch *et al.*, 1974; Eriksson & Svensson, 1974; Gardner, 1975). On heat treatment the lipid oxidation activity of haematin compounds can increase markedly whereas lipoxygenase is inactivated. Lipoxygenase, however, having a far superior catalytic activity, must be considered as a potential deteriorative factor, even at a very low residual activity (Pinsky *et al.*, 1971; Svensson & Eriksson, 1972*a*,*b*, 1974).

When blanching vegetables before freezing, inactivation of the enzymes is dependent upon both time and temperature of the heat treatment (Dietrich *et al.*, 1959, 1960, 1962; Dietrich & Neumann, 1965). Other effects of heating, which may respond differently to time and temperature conditions, include colour changes (Walker, 1964*a*,*b*), softening, gelling of starch and extraction of various soluble components.

Earlier investigations on unblanched and blanched pea, bean, cauliflower, Brussels sprouts, sweet corn, broccoli and spinach (Dietrich *et al.*, 1959, 1960, 1962; Dietrich & Neumann, 1965; Eheart, 1970; Svensson & Eriksson, 1974; Bøttcher, 1975*a*,*b*) show that blanching is necessary in order to avoid quality changes during frozen storage.

The aim of this investigation was to study the quality changes in some other

unblanched and blanched vegetables (carrot, leek, onion and swede). French bean and cauliflower were also investigated to confirm earlier findings. The vegetables were heat treated at different temperatures and for different lengths of time to find the most suitable treatment before freezing.

The storage temperatures, −30 °C and −20 °C, were selected after discussion with the food industry. A temperature of −30 °C represents the storage temperature in a food factory, and −20 °C represents the storage temperature during transportation and handling. Some of the unblanched vegetables were also stored at −85 °C to study any changes taking place at this storage temperature. Lipid and sensory analyses were performed in conjunction with the measurement of lipid oxidation.

MATERIALS AND METHODS

Materials

Carrot (1 cm cubes), cauliflower (florets), French bean (cut 2–3 cm), leek (cut 1 cm) onion (cut $\frac{1}{2}$ cm) and swede (1 cm cubes), one commercial variety of each (Table 2), were water blanched in a food factory.

TABLE 2
VEGETABLE VARIETIES USED IN THE EXPERIMENT

Vegetable	*Botanical name*	*Commercial variety*
Carrot	*Daucus carota*	'Nantes 20'
Cauliflower	*Brassica oleracea* var. *botrytis*	'Pioneer'
French bean	*Phaseolus vulgaris*	'Sabo'
Leek	*Allium porrum*	'Refe'
Onion	*Allium cepa*	'Hegro'
Swede	*Brassica napus* var. *napobrassica*	'Bangholm'

The blancher was a trunnion type, 2·5 m long. The temperature of blanching was measured continuously at three different points in the blancher and two different points in the cooler. The time of blanching was calculated by timing one revolution of the blancher drum. The measured temperatures and times are listed in Table 3. Samples of 20–30 kg were collected from each treatment. One unblanched sample of each vegetable was stored and analysed along with the heat treated samples. Blanched and unblanched samples were put on trays and stored at −40 °C for two days before packing. When packing the samples, triple laminated bags (polyethylene 70 μ, aluminium 9 μ, polyester 12 μ) were used and the samples were finally stored at −20 °C or −30 °C. Lipid analyses and sensory evaluations were carried out every three months up to nine months at −20 °C (except for swede) and

TABLE 3
BLANCHING CONDITIONS FOR CARROT, CAULIFLOWER, FRENCH BEAN, ONION, LEEK AND SWEDE

Vegetable	*Average temperature in the blancher* (°C)	*Average temperature in the cooler* (°C)	*Time* (*sec*)
	82	14	500
	86	29	100
Carrot	92	30	180
	93	12	500
	97	20	100
	88	16	240
Cauliflower	90	15	390
	93	16	195
	81	10	480
French bean	92	22	240
	93	19	480
	96	20	150
Onion	90	20	180
Leek	90	20	180
	80	20	600
	91	19	500
Swede	94	18	200
	95	19	200
	96	19	100

up to 15 months at −30 °C. Unblanched carrot, bean, cauliflower, leek and onion were also stored for 12 months at −85 °C.

Lipid and enzyme analyses

Fresh or frozen homogenised samples were dried in a vacuum oven at 70 °C for 3 h for the determination of moisture content (AOAC, 1975). An extraction method with chloroform, methanol and water described by Hardy & Keay (1972) was used for the total lipid determination. The lipid samples were methylated as described by Luddy *et al.* (1968) and the fatty acid compositions determined by gas liquid chromatography. Peroxidase activity was assayed according to a modified method by Lück (1965). The modification consists of substituting guaiacol (0·5 %) for *p*-phenylendiamine. Horseradish peroxidase (Koch-Light Lab. Ltd, 0·002 mg/ml, 90 units/mg) was used as standard. All unblanched samples were subjected to lipid analyses and peroxidase activity while the heat treated samples were assayed for peroxidase activity only.

Sensory evaluation

The sensory evaluation was performed by seven trained judges. A seven-point scale was used: 7 was the highest score and 1 the lowest (less than 4—not acceptable) for each of the following parameters:

Colour appearance.
Off-odour.
Off-flavour.
Characteristic taste.
Texture.
Total score.

Before the sensory evaluation the samples were put into boiling water and boiled for 1 min (500 g in 500 ml). All unblanched and blanched vegetables were evaluated.

RESULTS AND DISCUSSION

The results of the chemical and peroxidase analyses of fresh vegetables are shown in Table 4.

TABLE 4
THE CONTENT OF MOISTURE, TOTAL LIPIDS, FATTY ACIDS AND PEROXIDASE ACTIVITY OF VARIOUS FRESH VEGETABLES

Vegetable	*Moisture* %	*Total lipids* (% *fresh weight*)	*Fatty acid composition* (% *of total fatty acids*) *Palmitic* $C_{16:0}$	*Stearic* $C_{18:0}$	*Oleic* $C_{18:1}$	*Linoleic* $C_{18:2}$	*Linolenic* $C_{18:3}$	*Peroxidase activity* (*units*/100 *g fresh vegetable*)
Carrot	85	0·4	18	1	6	69	6	1
Cauliflower	91	0·4	20	2	10	16	52	36
French bean	88	0·2	22	4	4	24	46	5
Onion	88	0·1	23	2	23	49	3	0
Leek	91	0·3	18	1	2	32	47	2
Swede	90	0·1	17	2	18	25	38	44

Carrot

During storage unblanched carrot changed significantly ($p < 1\%$) (linear regression) in sensory properties at $-30\,°C$. After nine months of storage the carrots were unacceptable due to the development of off-flavour and off-odour (Fig. 1(a)). A slight bleaching of the colour could also be detected. The moisture content increased significantly ($p < 5\%$) during storage while the total lipid content decreased significantly ($p < 1\%$) (Table 5). Similar changes could also be observed in unblanched carrot, stored at $-85\,°C$ for 12 months. Whole carrot stored in bulk at $0\,°C$ and 86% relative humidity for 20 weeks shows the same changes (Baardseth, 1976). Therefore, the changes are probably of a metabolic nature, independent of storage temperature and form (whole or cut). The sensory properties of the carrot stored at $-85\,°C$ were acceptable with respect to flavour, but the texture was tough

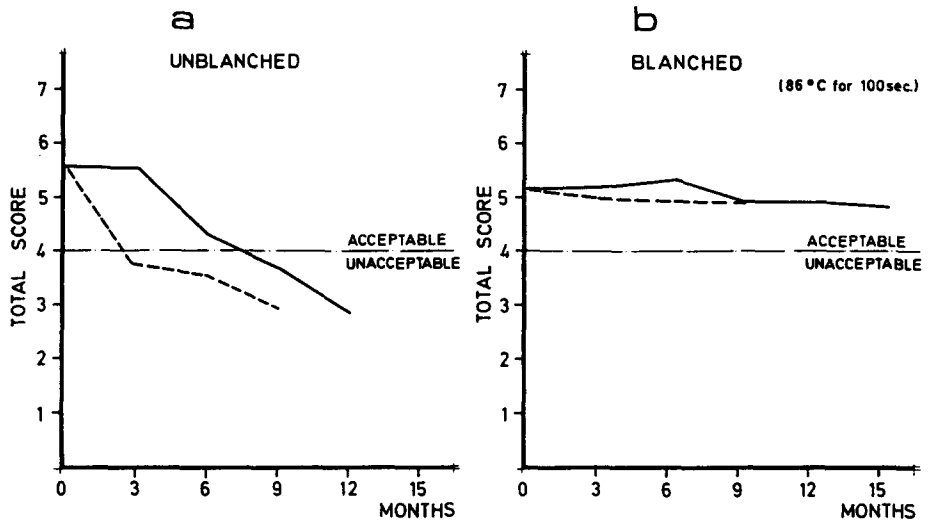


Fig. 1. Sensory evaluation (total score) of carrot stored at −30 °C (——) and −20 °C (-----).

and the typical carrot aroma (odour) had faded. The peroxidase activity and the 'unsaturated ratio'

$$\frac{\text{linoleic + linolenic acid}}{\text{palmitic + stearic acid}}$$

did not change significantly during storage either at −30 °C or at −85 °C. Blanching of carrot resulted in better product stability during storage. The heat treatment

TABLE 5

LIPID AND PEROXIDASE CHANGES IN UNBLANCHED CARROT DURING STORAGE AT −30 °C AND −20 °C

Months of storage	*Lipid and enzyme analyses*	*Moisture (%)*	*Total lipids (% fresh weight)*	*'Unsaturated ratio'* $\frac{C_{18:2} + C_{18:3}}{C_{16:0} + C_{18:0}}$	*Peroxidase activity (units/100 g of fresh vegetable)*
	0	84·6	0·36	4·28	0·2
	3	86·2	0·30	4·03	0·4
at −30 °C	6	85·4	0·30	4·15	0·3
	9	86·4	0·30	4·21	0·3
	12	86·2	0·27	3·44	0·2
	15	86·8	0·26	3·98	0·4
	0	84·6	0·36	4·28	0·2
at −20 °C	3	86·2	0·20	3·96	0·4
	6	85·6	0·27	4·13	0·3
	9	85·8	0·26	3·60	0·3

which gave the best score was 86 °C for 100 sec (Fig. 1(b)). The other treatments showed no differences in flavour or colour but the texture varied from soft to hard.

French bean

Unblanched French bean stored for longer than three months at either −20 °C or −30 °C was found to be unacceptable (Fig. 2(a)). On the other hand, after 12 months' storage at −85 °C the unblanched bean was acceptable in taste and smell, but the colour had become brown–white.

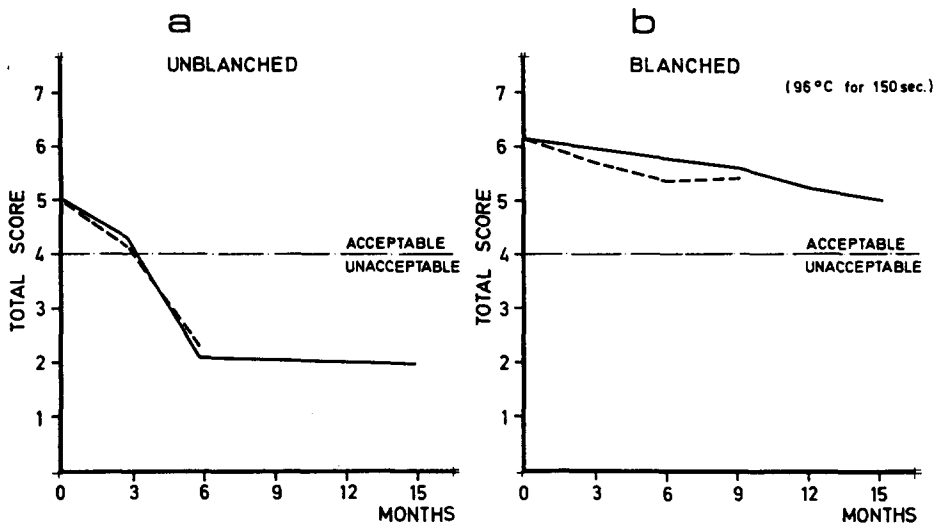


Fig. 2. Sensory evaluation (total score) of French bean stored at −30 °C (——) and −20 °C (- - - -).

Steinbuch (1975) found no difference in sensory properties between blanched bean stored at −70 °C and at −24 °C. Lee *et al.* (1955) tested unblanched bean stored at −18 °C and an off-flavour could be detected after four weeks. Together, these results show an inverse relationship between shelf life and temperature.

No significant differences were found in any of the measured lipid and enzymic parameters.

The heat treatment of 96 °C for 150 sec gave the best storage stability with beans in this investigation (Fig. 2(b)). Extended heat treatment results in a brownish coloured bean. The texture also becomes quite soft.

Cauliflower

Unblanched cauliflower was unacceptable after 3 months' storage at −30 °C (Fig. 3(a)). Off-flavour and off-odour developed and the colour became brownish. The

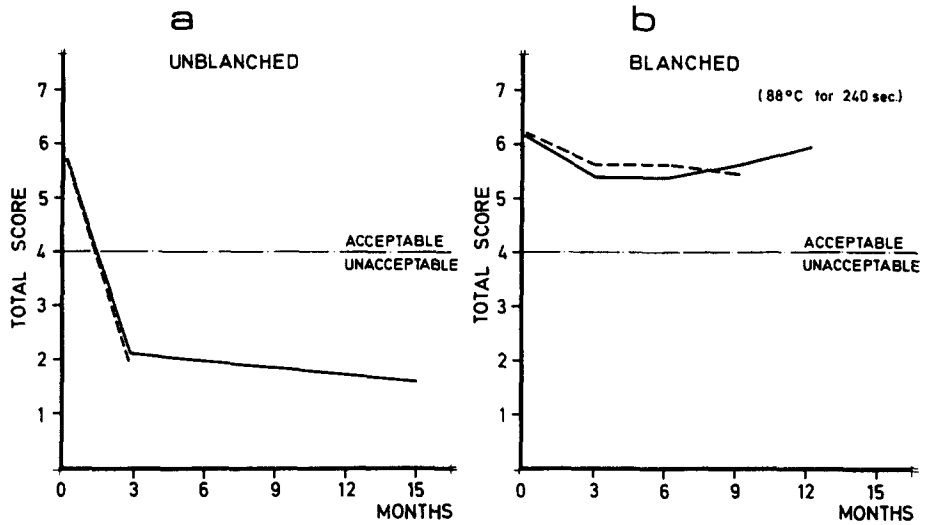


Fig. 3. Sensory evaluation (total score) of cauliflower stored at −30 °C (——) and −20 °C (- - - -).

lipid content decreased significantly during storage ($p < 1\%$) while the unsaturated ratio did not change in a linear manner (Table 6). Similar changes were found in the unblanched sample stored at −85 °C for 12 months.

The blanching condition of 88 °C for 240 sec was found to be the most suitable of the treatments selected (Fig. 3(b)). Shorter heat treatment gave a slight off-flavour after 9 months of storage. The differences between treatment at 88 °C for 240 sec and 90 °C for 390 sec were observed mainly in the texture.

TABLE 6

LIPID AND PEROXIDASE CHANGES IN UNBLANCHED CAULIFLOWER DURING STORAGE AT −30 °C AND −20 °C

Months of storage	*Lipid and enzyme analyses*	*Moisture (%)*	*Total lipids (% fresh weight)*	*'Unsaturated ratio'* $\frac{C_{18:2} + C_{18:3}}{C_{16:0} + C_{18:0}}$	*Peroxidase activity (units/100 g of fresh vegetable)*
	0	91·0	0·37	3·32	36
	3	90·7	0·30	2·05	29
at −30 °C	6	90·5	0·33	2·57	27
	9	90·5	0·31	2·69	41
	12	90·9	0·29	2·38	27
	15	90·6	0·27	2·37	32
	0	91·0	0·37	3·32	36
at −20 °C	3	91·0	0·24	2·10	24
	6	90·7	0·29	2·01	29
	9	90·9	0·24	2·14	45

Leek and onion

Unblanched leek and onion were acceptable even after 15 months' storage at −30 °C (Fig. 4(a)). None of them developed off-flavour or off-odour, but the colour changed from green to yellow–green in the leek and from white to yellow in the onion sample. No lipid changes could be detected by the methods used, but some loss of characteristic taste was found. Unblanched leek and onion stored at −85 °C for 12 months showed similar results.

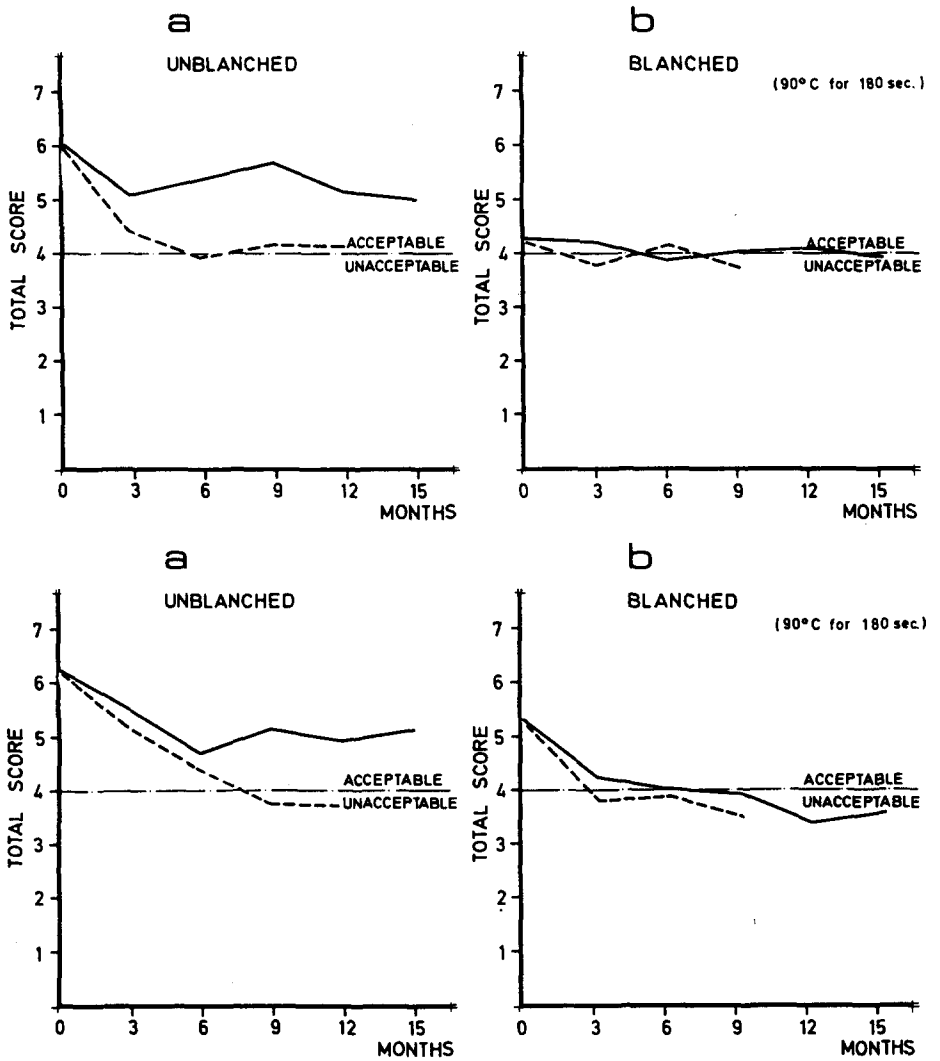


Fig. 4. Sensory evaluation (total score) of onion (top) and leek (bottom) stored at −30 °C (——) and −20 °C (- - - -).

The blanched leek and onion were less acceptable due to loss of characteristic taste (Fig. 4(b)). The leek also became very sticky and caused problems on the processing line. Taste is the most important sensory property for leek and onion and it is therefore important to notice that no off-flavour or off-odour developed during storage at −20 °C or −30 °C.

Swede

Unblanched swede did not develop off-flavour or off-odour during storage at −30 °C (Fig. 5(a)). The colour was only slightly bleached and no significant changes

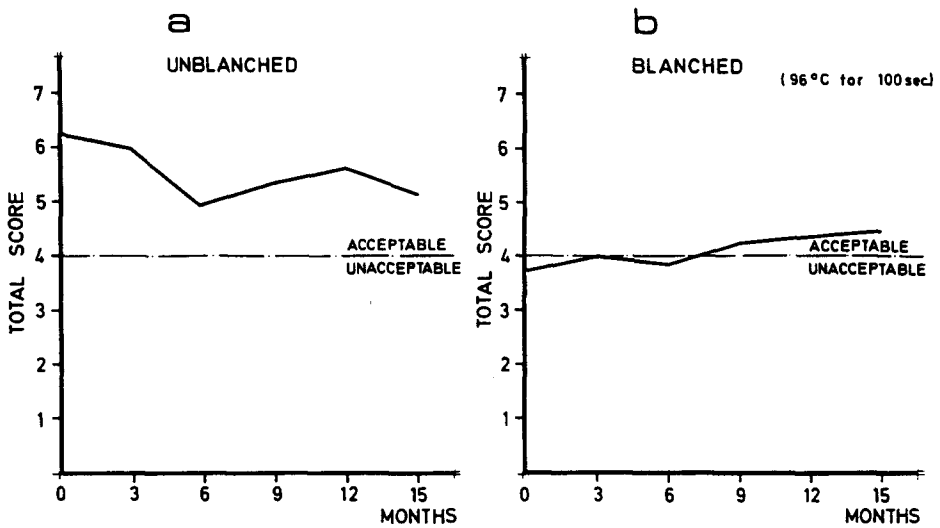


Fig. 5. Sensory evaluation (total score) of swede stored at −30 °C (——).

were found in the chemical parameters analysed. Blanched samples scored lower due to loss of characteristic taste (Fig. 5(b)).

CONCLUSIONS

The peroxidase activity after blanching was less than 5 % of the original activity for all the vegetables and no reactivation was found after storage at either −20 °C or −30 °C. The residual activity did not affect the quality during storage, confirming the results of Bøttcher (1975*a*,*b*).

Generally, the sensory evaluation of the frozen vegetables shows no significant differences between storage at −20 °C and at −30 °C. Unblanched carrot,

cauliflower and French bean had already developed off-flavour and off-odour after nine, three and six months' storage, respectively at −30 °C. In carrot and cauliflower a significant reduction in the total lipid content was found but not in French bean. Pinsky *et al.* (1971) have found a high lipoxygenase activity in cauliflower, a low activity in bean and none in carrot. In this investigation cauliflower was found to have a high peroxidase activity, while bean and carrot had low activities. These findings, together with the observed reduction in the amount of unsaturated fatty acids in stored cauliflower, indicate that a lipid oxidation had taken place. Higher stability of carrot and bean during storage is probably due to the lower enzyme activity.

Unblanched onion, leek and swede did not develop detectable off-flavour or off-odour and no changes in total lipid content were found. In onion no lipoxygenase (Pinsky *et al.*, 1971) or peroxidase have been found. This indicates either the absence of lipid oxidation or low lipid oxidation. In swede the peroxidase activity is high, but the lipoxygenase activity low (Baardseth, 1977). No lipid oxidation could be detected in this experiment, which may be explained by the existence of natural antioxidant.

ACKNOWLEDGEMENTS

I am grateful to Nora-Sunrose Konservesfabrikker A/S, Oslo, Norway for all the assistance and materials given to this project.

REFERENCES

AOAC (1975). Moisture. In *Official Methods of Analysis of the Association of Official Analytical Chemists* (12th edition), Washington, DC, p. 394.

BAARDSETH, P. (1976). *Analysis of lipid and peroxidase activity in carrot during maturation and storage.* NINF Report (in Norwegian). No. 27, pp. 1–7.

BAARDSETH, P. (1977). *Enzyme degradation of plant lipids.* NINF information (in Norwegian). No. 2, pp. 42–4.

BENGTSSON, B. & BOSUND, I. (1966). Lipid hydrolysis in unblanched frozen peas (*Pisum sativum*), *J. Food Sci.*, **31**, 474–81.

BØTTCHER, H. (1975*a*). The enzyme content and the quality of frozen vegetables. I. Residual peroxidase activity, *Nahrung*, **19**, 173–9.

BØTTCHER, H. (1975*b*). The enzyme content and the quality of frozen vegetables. II. Effects on the quality of frozen vegetables, *Nahrung*, **19**, 245–53.

CHOW, L. & WATTS, B. M. (1969). Origin of off-odors in frozen green beans, *Food Technol.*, **23**, 113–14.

DIETRICH, W. C., NUTTING, M. D., OLSON, R. L., LINDQUIST, F. E., BOGGS, M. M., BOHART, G. S., NEUMANN, H. J. & NORRIS, H. J. (1959). Time–temperature tolerance of frozen foods. XVI. Quality retention of frozen green snap beans in retail packages, *Food Technol.*, **13**, 136–45.

DIETRICH, W. C., BOGGS, M. M., NUTTING, M. D. & WEINSTEIN, N. E. (1960). Time–temperature tolerance of frozen foods. XXIII. Quality changes in frozen spinach, *Food Technol.*, **14**, 522–7.

DIETRICH, W. C., NUTTING, M. D., BOGGS, M. M. & WEINSTEIN, N. E. (1962). Time–temperature tolerance of frozen foods. XXIV. Quality changes in cauliflower, *Food Technol.*, **16**, 123–8.

DIETRICH, W. C. & NEUMANN, H. J. (1965). Blanching Brussel sprouts, *Food Technol.*, **19**, 1174–7.

EHEART, M. S. (1970). Effect of storage and other variables on composition of frozen broccoli, *Food Technol.*, **24**, 69–71.

ERIKSSON, C. E. & SVENSSON, S. G. (1974). Activation energy of reaction catalyzed by alcohol dehydrogenase, lipoxygenase, denatured peroxidase and hematin, *Lebensm.-Wiss. u. Technol.*, **7**, 38–41.

GARDNER, H. W. (1975). Decomposition of linoleic acid hydroperoxides. Enzymic reaction compared with nonenzymic, *J. Agric. Food Chem.*, **23**, 129–36.

GROSCH, W. (1972). The enzyme lipoxygenase—Its proper ties and action on foods, *Fette, Seifen, Anstrichmittel*, **74**, 375–81.

GROSCH, W., LASKAWY, G. & FISHER, K. H. (1974). Oxidation of linoleic acid in the presence of hemoglobin, lipoxygenase or by singlet oxygen identification of the volatile carbonyl compounds, *Lebensmittel-Wiss. u. Technol.*, **7**, 335–8.

HARDEY, R. & KEAY, J. N. (1972). Seasonal variations in the chemical composition of Cornish mackerel, *Scomber scombrus* (L), with detailed reference to the lipids, *J. Food Technol.*, **7**, 125–37.

KYZLINK, V. & CHYTRA, M. (1959). Conditions of thermal inactivation of redox enzymes that deteriorate the quality of canned fruit and vegetables. Scientific papers from Institute of Chemical Technology, Prague, pp. 9–42.

LEE, F. A., WAGENKNECHT, A. C. & HENING, J. C. (1955). A chemical study of the progressive development of off-flavor in frozen raw vegetables, *Food Res.*, **20**, 289–97.

LEE, F. A., WAGENKNECHT, A. C. & GRAHAM, R. (1956). Influence of vining on the development of off-flavor in frozen raw peas, *Food Res.*, **21**, 666–70.

LEE, F. A. & MATTICK, L. R. (1961). Fatty acids of the lipids of vegetables. I. Peas (*Pisum sativum*), *J. Food Sci.*, **26**, 273–5.

LOPEZ, A., BOCKLET, M. F. & WOOD, C. B. (1959). Catalase and peroxidase activity in raw and blanched southern peas, *Food Res.*, **24**, 548–51.

LÜCK, H. (1965). Peroxidase. In *Methods of enzymic analysis* (Bergmeyer, H. U. (Ed.)) (2nd edition), Verlag Chemie, pp. 895–96.

LUDDY, F. E., BARFORD, R. A., HERB, S. F. & MAGITMAN, P. (1968). A rapid and quantitative procedure for the preparation of methyl esters of butteroil and other fats, *J. Amer. Oil Chem. Soc.*, **45**, 549–52.

PINSKY, A., GROSSMAN, S. & TROP, M. (1971). Lipoxygenase content and antioxidant activity of some fruits and vegetables, *J. Food Sci.*, **36**, 571–2.

REED, G. (1975). Oxidoreductases. In *Enzymes in food processing*, Academic Press, New York, p. 219.

RHEE, K. S. & WATTS, B. M. (1966). Lipid oxidation in frozen vegetables in relation to flavour change, *J. Food Sci.*, **31**, 675–9.

STEINBUCH, E. (1975). Die Qualität von Tiefgekühlten grünen Bohnen, in Abhängigkeit von Temperatur und Zeit. (DKV) Jahrestagung 1975—Bad Dürkheim (DKV) Tagungsbericht bl 2, 299–306. 2. Jahrgang.

SVENSSON, S. G. (1977). Inactivation of enzymes during thermal processing. In *Physical, chemical and biological changes in food caused by thermal processing*. (Høyem, T. & Kvåle, O. (Eds.)), Applied Science Publishers, Ltd, London, Chapter 12, pp. 202–17.

SVENSSON, S. G. & ERIKSSON, C. E. (1972*a*). Thermal inactivation of lipoxygenase from peas (*Pisum sativum* L): 1. Time–temperature relationships and pH-dependence, *Lebensm.-Wiss. u. Technol.*, **5**, 118–23.

SVENSSON, S. G. & ERIKSSON, C. E. (1972*b*). Thermal inactivation of lipoxygenase from peas. (*Pisum sativum* L): II. Influence of substrate and reaction products, *Lebensm.-Wiss. u. Technol.*, **5**, 124–7.

SVENSSON, S. G. & ERIKSSON, C. E. (1974). Thermal inactivation of lipoxygenase from peas (*Pisum sativum* L): IV. Inactivation in whole peas, *Lebensm.-Wiss. u. Technol.*, **7**, 145–51.

WALKER, G. C. (1964*a*). Colour deterioration in frozen french beans, *J. Food Sci.*, **29**, 383–8.

WALKER, G. C. (1964*b*). Colour deterioration in frozen french beans. 2. Effect of blanching, *J. Food. Sci.*, **29**, 389–92.

CHEMICAL STUDIES ON SOME NIGERIAN CARBONATED AND ALCOHOLIC BEVERAGES

A. O. KETIKU, B. K. OGUNMODEDE, A. O. FALASE & A. O. OMOLOLU

University of Ibadan, Ibadan, Nigeria

(Received: 19 August, 1976)

ABSTRACT

Carbonated beverages and different brands of lager beer bottled in Nigeria were analysed for carbohydrates, proteins, minerals, vitamins and alcohol, in order to provide basic data for the appraisal of their nutritive value. Seven carbonated beverages and five brands of lager beer were so analysed. The carbohydrate content of the carbonated drinks ranged from 9·52–13·93 g/100 g. They all contained sucrose, glucose and fructose. Calcium ranged from 0·97–1·71 mg/100 g; potassium, 0·21–0·27 mEq/100 g; sodium, 1·58–5·20 mEq/100 g. They contained no detectable amount of iron, traces of vitamin C, and negligible amounts of thiamin and riboflavin. The beers contained between 2·56 and 4·17 g carbohydrates/100 g. The main sugar was maltose. Alcohol (by weight) ranged from 3·77 % to 5·20 %. They were poor sources of minerals and vitamins. Some nutritional implications of these data are discussed.

INTRODUCTION

The demand of many Nigerians for carbonated beverages, simply referred to as 'soft drinks', and lager beers, has so greatly increased in recent times that many breweries and bottling companies have had to expand their factories to produce more of these beverages. The Federal Government of Nigeria, too, has been sympathetic enough as to allow the controlled importation of these beverages to discourage hoarding of the locally produced ones and for effective price control. Whilst the literature is replete with data on the nutrient content of such beverages in other countries (Jacobs, 1959; Davidson, 1961; McCance & Widdowson, 1967) no such published data are available on Nigerian beverages. This work was, therefore, embarked upon to estimate the nutrient content of these beverages for the purpose of providing data for the future compilation of Nigeria's Food Composition Tables, for the use of Food Consumption Survey teams, for the possible use of the Nigerian Standards

Fd. Chem. (2) (1977)—
Printed in Great Britain

Organisation and in view of the role of excessive and prolonged intake of alcohol in the aetiology of such ailments as cardiomyopathy (Asokan *et al.*, 1972; Godwin & Oakley, 1972), liver cirrhosis and some nutritional disorders (Davidson *et al.*, 1975).

MATERIALS AND METHODS

Materials

All the beverages analysed were bought from retailers. They included seven brands of carbonated beverages and five types of lager beer.

Methods

Alcohol was estimated using the AOAC methods of analysis (Association of Official Agricultural Chemists, 1970).

Total carbohydrate was determined using the phenol-sulphuric acid reagent of Dubois *et al.* (1951) and a glucose standard curve.

The beverages were qualitatively analysed for individual sugars by paper-chromatography using *n*-butanol, acetic acid and water (4:1:1 volumes) as irrigant. The sugars were identified using silver nitrate in acetone and ethanolic sodium hydroxide (Trevelyan *et al.*, 1950) and naphthoresorcinol and trichloroacetic acid (Partridge, 1948). Sucrose, fructose and glucose were found to be present in the carbonated beverages, with the latter two in greater proportions than sucrose. The beers contained maltose, and two other unidentified spots with Rf values lower than maltose and present in traces.

Protein was estimated by determining nitrogen using the Kjeldahl method and multiplying by 6·25.

Minerals were determined using atomic absorption spectrophotometry. Phosphorus was estimated by the method of Kitson & Mellon (1944).

Vitamins were estimated by the methods of vitamin assay of the Association of Vitamin Chemists (1966).

RESULTS AND DISCUSSION

Tables 1 and 2 below show the chemical composition of the beverages.

Carbonated beverages (Table 1)

The sugar content of the carbonated beverages ranged from 9·52 to 13·93%. Two cola beverages amongst them (C_2 and C_4) contained 12·68% and 11·31% respectively. These figures are slightly higher than those reported for similar drinks (10%–10·5%) by Toulousse (1933) and Jacobs (1959). The figure for a ginger ale (C_6) fell within the conventional range of 9·5–10% (Jacobs, 1959). These carbonated

TABLE 1
CHEMICAL COMPOSITION OF CARBONATED BEVERAGES

Carbonated beverages	*Carbohydrates (as glucose) (g/100 g)*	*Na*	*K*	*Ca*	*Mg*	*Fe*	*P*	*Thiamine*	*Riboflavin*	*Vit. C*
		(mEq/100 g)		*(mg/100 g)*				*(mg/100 g)*		
C_1	10·00	1·80	0·25	1·71	0·39	Nil	3·17	0·004	0·10	0·27
C_2	12·68	1·74	0·21	0·97	0·32	Nil	17·50	0·005	0·09	0·28
C_3	13·93	1·58	0·24	1·31	0·31	Nil	Nil	0·01	0·11	0·18
C_4	11·31	2·32	0·21	1·02	0·33	Nil	1·55	0·01	0·14	0·16
C_5	13·42	4·16	0·27	1·45	0·33	Nil	0·61	0·004	0·07	0·47
C_6	9·52	3·25	0·23	0·98	0·35	Nil	1·20	0·003	0·12	0·22
C_7	11·04	5·20	0·23	1·36	0·31	Nil	Nil	0·005	0·13	0·25

TABLE 2
THE CHEMICAL COMPOSITION OF LAGER BEERS

Beer types	*% Alcohol (by volume)*	*% Alcohol (by weight)*	*Carbohydrates (as glucose) (g/100 g)*	*Na*	*K*	*Ca*	*Mg*	*Fe*	*P*	*Thiamine*	*Riboflavin*	*Vit. C*
				(mEq/100 g)		*(mg/100 g)*				*(mg/100 g)*		
B_1	5·73	4·57	2·90	0·77	7·86	0·30	13·00	Nil	26·46	0·01	0·02	—
B_2	4·73	3·77	2·56	2·36	6·44	1·06	10·67	Nil	21·89	0·01	0·02	—
B_3	6·52	5·20	2·79	3·18	7·74	1·00	12·82	Nil	27·95	0·02	0·02	—
B_4	6·01	4·80	3·93	1·70	9·29	0·61	13·82	Nil	24·96	0·01	0·02	0·01
B_5	6·23	4·97	4·17	1·15	11·48	0·30	16·65	Nil	37·32	0·02	0·02	0·01

beverages are usually sold in bottles of minimum content 300 ml. By drinking 300 ml of these carbonated beverages, between 462 and 672 kJ of energy may be obtained. For school children, who are the major consumers of these beverages as refreshments in schools or at parties, a daily consumption of 300 ml may provide up to 9% of their daily energy requirement of 7·9 MJ (Idusogie, 1971). The vitamins were present in negligible amounts. However, the ascorbic acid content of the lemon-flavoured beverage (C_5) was outstanding, and up to 1·5 mg might be obtained per bottle of beverage. They were all low in minerals and contained no detectable iron. Sodium was, however, more abundant than other elements. Even at these levels (1·58–5·2 mEq/100 g), it is still possible for patients with congestive heart failure, on a daily sodium allowance of 40 mEq (Davidson *et al.*, 1975) per day, to drink any of these beverages.

Lager beers (Table 2)

They were all found to be low in vitamins. There is, therefore, a need for drinkers to eat a wide range of foodstuffs that are rich in vitamins to avoid avitaminosis. Thiamine was present in traces, the riboflavin figures of 15–19 μg/100 g fell below the range of 30–120 μg obtained by Davidson (1961) for some American beers. Though beers are generally regarded as poor sources of potassium (Davidson *et al.*, 1975), consumption of even a bottle of Nigerian beer (600 ml) may substantially contribute to potassium intake (between 3·86–6·89 mEq). They were all low in sodium and might be included in a low salt diet as previously suggested by Olmstead *et al.* (1954, 1955). Nigerian lager beers were found to be relatively high in alcohol (3·77% –5·20% by weight). Their alcohol content exceeded those cited by Watt & Merrill (1963)—3·6% by weight—and Platt (1962)—3% by weight for European beer. The nutritional implications of the relatively high alcohol content of Nigerian beers are that drinkers will derive more energy per unit weight of beer drunk, i.e. between 672 kJ and 916 kJ of energy per bottle; the period of alcohol clearance from the blood will be increased, and the rate of development of certain diseases precipitated by prolonged and excessive intake of alcohol will be faster in such drinkers than in those consuming beers of lower alcohol content. It must be noted that most Nigerian breweries prefer to sell their beer in bottles of 600 ml minimum content rather than in 333 ml cans or bottles.

The carbohydrate content ranged from 2·6% to 4·2%, and these carbohydrates may supply 246–398 kJ of energy per 600 ml bottle of beer.

The crude protein figures obtained were in close agreement with those earlier reported by Watt & Merrill (1963) and Davidson (1961).

CONCLUSION

In the light of the data above, it may be necessary for some Nigerians to modify

accordingly the quantities consumed of these beverages in consonance with their physiological integrity. In particular, the authority responsible for establishing standards in the country should formulate a policy on the level of inclusion of alcohol and carbohydrates in beverages to be consumed in Nigeria, taking into account Nigerians' great flair for parties.

REFERENCES

ASOKAN, S. K., FRANK, M. J. & WITHAM, A. C. (1972). Cardiomyopathy without cardiomegaly in alcoholics, *American Heart Journal*, **84**(1), 13–18.

Association of Official Agricultural Chemists (1970). *Official Methods of Analysis*, 11th Ed. pp. 157–9. Washington DC.

Association of Vitamin Chemists (1966). *Methods of Vitamin Assay*, 3rd Ed., pp. 120–64 and pp. 281–320. Interscience Publishers, London.

DAVIDSON, C. S. (1961). Nutrient content of beers and ales, *New Engl. J. Med.*, **264**, 185–6.

DAVIDSON, S., PASSMORE, R., BROCK, J. F. & TRUSWELL, A. S. (1975). *Human nutrition and dietetics*, 6th Ed. pp. 90–3 and p. 695. Churchill Livingstone, London.

DUBOIS, M., GILLES, K., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1951). A colorimetric method for the determination of sugars, *Nature*, **168**, 167.

GODWIN, J. F. & OAKLEY, D. M. (1972). The Cardiomyopathies, *British Heart Journal*, **34**, 545–52.

IDUSOGIE, E. O. (1971). The nutritional requirements of the Nigerian population, *African Journal of Medical Sciences*, **3**(1), 53–6.

JACOBS, M. B. (1959). *Manufacture and Analysis of Carbonated Beverages*, p. 47. Chemical Publishing Co. Inc., New York.

KITSON, R. E. & MELLON, M. G. (1944). The determination of phosphorus in plant material using phospho-vanadomolybdate complex, *Ind. Eng. Chem.* (Anal Ad.), **16**, 397.

MCCANCE, R. A. & WIDDOWSON, E. M. (1967). Alcohol and energy value of common alcoholic beverages, *Med. Res. Coun. Spec. Rep. Ser.*, No. 297, HMSO, London.

OLMSTEAD, E. G., CASSIDY, J. E. & MURPHY, F. D. (1954). Nutritional value of beer with reference to the low salt diet, *Amer. J. Clin. Nutrit.*, **2**, 392–5.

OLMSTEAD, E. G., CASSIDY, J. E. & MURPHY, F. D. (1955). The use of beer in the low salt diet with special reference to renal disease, *Amer. J. Med. Sci.*, **3**, 49–53.

PARTRIDGE, S. M. (1948). Filter-paper chromatography of sugars, *Biochem. J.*, **42**, 238.

PLATT, B. S. (1962). Tables of representative values of foods commonly used in tropical countries, *Med. Res. Counc. Spec. Rep. Ser.*, No. 302, HMSO, London.

TOULOUSSE, J. H. (1933). *Natl Bottlers' Gaz.*, **52**(618), 45 (cited by Jacobs (1959) above).

TREVELYAN, W. C., PROCTOR, D. O. & HARRISON, J. S. (1950). Detection of sugars on paper chromatograms, *Nature*, **166**, 444.

WATT, B. K. & MERRILL, A. L. (1963). Composition of foods—raw, processed, prepared. *US Department of Agriculture Handbook*, No. 8.

SOME FACTORS INFLUENCING CAECAL ENLARGEMENT INDUCED BY RAW POTATO STARCH IN THE RAT

E. A. EL-HARITH & R. WALKER

Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, Great Britain

&

G. G. BIRCH & G. SUKAN

National College of Food Technology, University of Reading, Weybridge, Surrey KT13 0DE, Great Britain

(Received: 25 October, 1976)

ABSTRACT

The phenomenon of caecal enlargement in rats fed raw potato starch as their sole dietary carbohydrate source is examined. The caeca of experimental animals were enlarged 800–1700% *. They contained undigested starch, an increased number of micro-organisms and a lower concentration of ions and were of lower pH* (5·5) *than those of control animals* (*pH* 7·0).

Duodenal contents had a lower specific activity of amylase expressed either as whole duodenal contents or on the basis of duodenal protein which was also depressed. This suggests that the potato starch either: (*a*) *contains an inhibitor of pancreatic amylase,* (*b*) *lacks an amylase activating or stabilising factor or* (*c*) *causes changes in the duodenal secretions.*

The resistance of potato starch to pancreatic amylase, together with the lower level of amylase in the duodenum, results in undigested starch reaching the caecum and there probably acting as a substrate for the caecal microflora. The mechanism whereby these events result in caecal enlargement is still unclear.

INTRODUCTION

Diets containing various types of glucose syrups and dextrins have been found to cause caecal enlargement in the rat (Birch & Etheridge, 1973; Birch *et al.*, 1973; Chalvardian & Stephens, 1970; Etheridge, 1974) and recently gross caecal

Fd. Chem. (2) (1977)—
Printed in Great Britain

enlargement has been reported in rats fed raw potato starch (El-Harith *et al.*, 1975, 1976*a* and *b*). The effect may be controlled by the osmotic value of the caecal contents (Leegwater *et al.*, 1974). Caecal enlargement was so gross as to be lethal in at least 21 % of rats fed raw potato starch at dietary levels of 30 % or more while no deaths occurred in control animals fed raw maize starch or in animals receiving gelatinised potato starch. Death of animals with grossly enlarged caeca appeared to result from respiratory insufficiency due to compression of the diaphragm (El-Harith *et al.*, 1976*a*). The effect is reversible since experimental animals which have maize starch substituted for potato starch in their diet revert to normal after a period of time (El-Harith *et al.*, 1975, 1976*a* and *b*).

Since caecal enlargement is quite commonly observed after feeding particular types of dietary carbohydrate such as lactose (Reussner *et al.*, 1963), analogous polyols such as sorbitol (Morgan & Yudkin, 1957), maltitol (Hosoya, 1972) and polyethylene glycol (Loeschke *et al.*, 1973) the effect deserves more detailed study.

MATERIALS AND METHODS

Animals and protocols

Weanling male Wistar albino rats of an inbred SPF-derived colony, of 40–50 g initial body weight, were used for all experiments. Animals were housed singly in cages with raised screen floors. Environmental conditions were controlled at a temperature of 20 °C ± 2°, a relative humidity of 45 % ± 5 %, and with a 12 h alternate light/dark cycle. Food and water were provided *ad libitum* and food intake and body weight were recorded weekly or more frequently as indicated below.

Materials and methods

Raw maize, potato and rice starches were obtained from BDH Chemicals Ltd (Poole, Dorset, Great Britain), cassava starch from Laing National Ltd (Manchester, Great Britain) and sorghum starch from the Food Processing Centre (Khartoum North, Sudan). Extracted maize and potato starches were obtained by refluxing with dioxan in a Soxhlet apparatus for 4 h. Autoclaved potato starch was prepared by moistening the raw starch with water and heating it in an autoclave at 120 °C for 2 h then drying at 40 °C for 24 h. Glucose syrups (DE 17 or DE 43) were obtained by a reverse osmosis fractionation procedure and freeze-dried (Birch & Kearsley, 1974).

Caecal fluid was obtained by homogenising caecal contents with deionised water (1:1 w/w). Electrolyte concentrations were determined in caecal fluid and blood serum as follows: sodium and potassium were determined in an EEL (Evans Electrosolenium Ltd) flame photometer and chloride by the method of Schales & Schales (1941). Osmolalities were determined with the Halbmicro-Knauer automatic osmometer. Pancreatin (Hopkin and Williams Ltd) was used for the

enzymatic hydrolysis of starches. The enzyme solution was prepared by shaking 250 mg of pancreatin with 50 ml of distilled water. The suspension was centrifuged and the supernatant liquid was used as the source of α-amylase. The starch (250 mg) was suspended in 40 ml of distilled water, phosphate buffer (Sorensen's, M/15, pH 7·0, 2 ml) and enzyme solution (30 iu amylase, 5 ml) were added and the volume made up to 50 ml with distilled water. The mixture was incubated at 37 °C with continuous shaking. Aliquots were withdrawn at intervals (2, 4, 8 and 12 h), centrifuged and the total soluble sugars determined in the supernatant by the method of Dubois *et al.* (1956).

For determination of amylase activity in the duodenum, an appropriate sample of the fluid from the duodenal lumen was collected immediately post-mortem after cervical dislocation and diluted with phosphate buffer (M/15, pH 7·0). Soluble maize or potato starch (BDH Chemicals, Poole, Dorset, Great Britain) made as 2% aqueous solution were then mixed with an equal volume of the diluted duodenal juice and the mixture incubated at 37 °C for 1 h. The reducing sugars (as maltose) were then determined by the 3,5-dinitrosalicylic acid method of Dahlqvist & Borgström (1961). For the determination of protein in the duodenal fluid the method of Lowry *et al.* (1951) was employed.

Experiment 1: Four groups of ten animals were maintained for three weeks on a diet with the following basic composition: carbohydrate 71 %, protein (casein) 16 %, corn oil 5 % (Craigmillar Ltd), vitamin mix 4 % (Cooper's Nutritional Products) and mineral mix 4 % (Cox Ltd). The carbohydrate source was varied between groups as follows: group 1A—raw maize starch; group 2A—extracted maize starch; group 1B—raw potato starch and group 2B—extracted raw potato starch.

At termination the animals were anaesthetised with Nembutal (20–35 mg/kg body weight) and bled from heart punctures and sacrificed by cervical dislocation. The caeca and intestinal sections were examined and weighed as necessary.

For the water balance study, three animals from each of groups 1A and 1B were singly housed in metabowls, on day 15 of the experiment. After a three-day period of adaptation to the metabowls, food intake, water intake, volume of urine and faecal weight were recorded daily for a period of three days (days 18–20, inclusive).

Experiment 2: To investigate whether a low molecular weight carbohydrate is needed for 'priming action' of the amylase activity (and hence to help reduce the phenomenon of caecal enlargement), animals were fed on small amounts of freeze-dried glucose syrup fractions.

Twelve groups of five animals were fed for three weeks on diets with a basic composition similar to the one described above. Six groups of these received maize starch as the main source of carbohydrate (groups $1C^1$, $2C^1$, $3C^1$ and $1D^1$, $2D^1$, $3D^1$). The six test groups ($1C^2$, $2C^2$, $3C^2$ and $1D^2$, $2D^2$, $3D^2$) received potato starch as the main source of carbohydrate.

Freeze-dried glucose syrups (DE 17 and DE 43) were included in the diets and partially replaced the 71 % carbohydrate part of the diet at levels of 5, 10 and 15 %.

The food intake and body weight were recorded weekly and at termination the animals were autopsied and the caeca excised and weighed.

RESULTS AND DISCUSSION

The inclusion of raw potato starch (whether extracted with dioxan or not) in the diet of rats resulted in significantly lower food efficiencies compared with similar diets containing maize starch (Table 1).

TABLE 1
MEAN FOOD EFFICIENCY VALUES OF RATS FED DIETS CONTAINING 71 % STARCH AS SOLE DIETARY CARBOHYDRATE

	1*A* (m)	2*A* (ext. m)	1*B* (p)	2*B* (ext. p)
First week	0·40	0·40	0·33	0·19
Second week	0·41	0·33	0·27	0·28
Third week	0·39	0·20	0·22	0·19
Third week (corrected)	0·37	0·19	0·14	0·09

m = raw maize starch; *p* = raw potato starch, *ext.* = dioxan extracted.
Food efficiency = increase in body weight (g)/food intake (g).
Corrected food efficiency = increase in body weight-weight of caecum (g)/food intake (g).
1A; 2A maize starch fed groups.
1B; 2B potato starch fed groups.

Dioxan treatment of the starches also resulted in a lowering of the food efficiency despite the fact that this treatment *increased* the rate of hydrolysis of maize starch by pancreatic amylase (Fig. 1) and had little effect on the rate of hydrolysis of raw potato starch. This effect of dioxan treatment may reflect a toxic effect of residues of dioxan in the extracted starches since this compound has been reported to be neurotoxic in the rat and to cause morphological changes in the hepatorenal system with an LC_{50} of 37 mg/litre by inhalation (Kulagina, 1960). Goldstein *et al.* (1970) reported that the maximum concentration of dioxan tolerated by rats and mice was 0·2 mg/litre.

Extraction of starches with dioxan removes certain minerals such as phosphorus compounds and polar lipids and, in the case of maize starch, this increased the rate of hydrolysis by amylase (Fig. 1) which is in accordance with the postulate that helical phospholipid/amylose complexes (Priestley, 1974) or phosphates (Bhotiyakornkiat & Birch, 1972; Turvey & Hughes, 1958) stabilise starches to hydrolytic attack by amylases. Potato starch retains more phosphate after dioxan

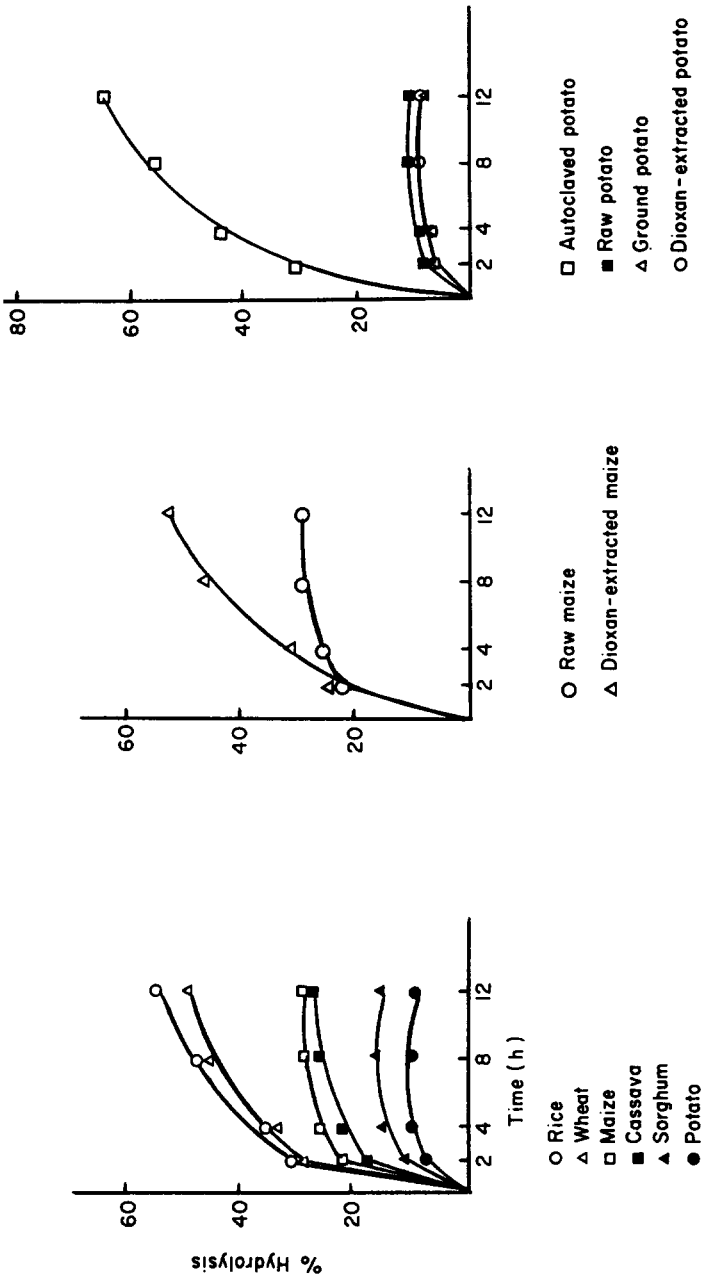


Fig. 1. The rate of hydrolysis of different starches (*in vitro*) by pancreatic α-amylase.

extraction than does maize starch (Bhotiyakornkiat & Birch, 1972) and this is probably involved in cross-linking between amylose molecules conferring a paracrystalline structure to the raw potato starch granule (Banks & Greenwood, 1975). Such a structure is probably responsible for the peculiar resistance of potato starch to amylase which results in the rate of hydrolysis being lower than any of the other starches examined, while destruction of this crystallinity by autoclaving greatly increases the rate of hydrolysis (Fig. 1).

As previously reported (El-Harith *et al.*, 1976), raw potato starch caused a gross enlargement of the caecum (Table 2) relative to maize starch; in addition, dioxan treatment itself caused further significant increases in caecal size. The animals receiving potato starch, raw or extracted, exhibited undigested starch granules in caecal contents and faeces while those receiving maize starch or autoclaved potato starch did not and this favours the view that the paracrystalline nature of the potato starch granule and its peculiar resistance to amylase are responsible for the caecal enlarging propensity. In this context potato starch granules are noted for their high swelling power (perhaps related to their esterified phosphorus content) and ability to absorb minerals, particularly Ca^{2+} and Mg^{2+}.

TABLE 2
ANALYSIS OF CAECAL CONTENTS AND SERUM ELECTROLYTES IN RATS FED RAW AND DIOXAN-EXTRACTED STARCHES

Parameter		1*A* (71% m)	2*A* (71% ext. m)	1*B* (71% p)	2*B* (71% ext. p)
Filled caecum wt g/100 g bd wt		1·23 ± 0·13	2·21^{a} ± 0·36	9·59^{b} ± 0·45	13·18^{b}† ± 1·95
Osmotic value m osml/Kg		228 ± 10	231 ± 14	202 ± 12	188^{a} ± 13
Caecal contents electrolytes					
μeqv./g wet wt	Na^+	107 ± 8	106 ± 8	67^{b} ± 5	83^{a}† ± 4
	K^+	31 ± 2	32 ± 2	24^{a} ± 3	29 ± 1
	Cl^-	20 ± 1	19 ± 3	16 ± 2	15 ± 3
Serum electrolytes m eqv./l	Na^+	125 ± 4	124 ± 4	127 ± 5	121 ± 6
	K^+	4·8 ± 0·2	4·7 ± 0·1	4·6 ± 0·1	4·2 ± 0·3
	Cl^-	109 ± 2	112 ± 3	115 ± 3	112 ± 2

m = raw maize starch, *p* = raw potato starch, *ext.* = dioxane extracted.
Mean values significantly different from those of group 1A are marked $^{a} = P < 0{\cdot}05$, $^{b} = P < 0{\cdot}001$.
The values of group 2B which are significantly different from group 1B values are marked † = $P < 0{\cdot}05$.
1A; 2A maize starch fed groups.
1B; 2B potato starch fed groups.

The ionic concentrations and osmolality of the caecal contents indicated a tendency towards lower values in the animals receiving potato starch (Table 2) and the Na^+/K^+ ratio (2·8–2·9) was lower in the potato starch than the maize starch fed groups (3·3–3·5), but this was without significant effect on serum electrolytes.

The greater caecal enlargement and lowered food efficiency consequent on dioxan extraction may reflect effects of residual dioxan on caecal microflora since some antibiotics also cause caecal enlargement (Savage & Dubos, 1968).

Partial replacement of dietary starch by dried glucose syrups (DE 17 or DE 43) had little effect on food efficiencies (Table 3) and in the case of DE 17 syrup the effect on caecal size was no more than expected on partial replacement of a refractory starch with a readily utilisable carbohydrate. Incorporation of DE 43 syrup in the potato starch-containing diet was without effect on caecal weight. Again, this is not altogether unexpected because DE 43 glucose syrup itself causes some caecal enlargement (Birch *et al.*, 1973).

TABLE 3
EFFECT OF FEEDING GLUCOSE SYRUPS (OF DE17 AND DE43) TO WEANLING RATS. ON THEIR FOOD EFFICIENCY AND CAECAL WEIGHT

Group	*Percent and source of dietary carbohydrate*	*Caecal weight g/100 g bd wt*	*Food efficiency First week*	*Second week*	*Third week*
$1C^1$	5*gs* + 66*m*	1·50 ± 0·05	—	0·34	0·57
$2C^1$	10*gs* + 61*m*	1·61 ± 0·06	—	0·31	0·62
$3C^1$	15*gs* + 56*m*	1·57 ± 0·13	—	0·41	0·66
$1C^2$	5*gs* + 66*p*	9·62 ± 1·38	—	0·39	0·34
$2C^2$	10*gs* + 61*p*	8·77 ± 0·37	—	0·35	0·39
$3C^2$	15*gs* + 56*p*	7·87 ± 0·99	—	0·34	0·35
$1D^1$	5*gs* + 66*m*	1·43 ± 0·07	0·49	0·37	0·32
$2D^1$	10*gs* + 61*m*	1·22 ± 0·06	0·51	0·37	0·32
$3D^1$	15*gs* + 56*m*	1·44 ± 0·10	0·51	0·31	0·37
$1D^2$	5*gs* + 66*p*	7·44 ± 0·78	0·35	0·26	0·24
$2D^2$	10*gs* + 61*p*	7·60 ± 1·05	0·43	0·28	0·21
$3D^2$	15*gs* + 56*p*	8·09 ± 1·03	0·43	0·19	0·27

gs = glucose syrup, *m* = raw maize starch and *p* = raw potato starch.
All the C groups received glucose syrup of DE = 17 while all the D group received glucose syrup with DE = 43.
Values listed are means of five animals/group.

When the amylase activity (against potato and maize starches) and protein content of the duodenal fluid of the animals used in the first experiment were determined *in vitro*, both were found to be lower in the group receiving raw potato starch (1B) than in the maize starch fed group (1A) (Fig. 2). The enzyme activities were, however, lowered more than the proteins. The fluid retention which is characteristic of all alimentary tissue below the stomach (in the raw potato starch fed groups) is underlined by examining the water balance in the two groups 1A and 1B (Table 4). This could account, in some measure, for the lower protein content in the duodenal fluid, but the marked lowering of the enzyme activity may possibly be ascribed to the presence of an inhibitor or the absence of an activator for amylase. Which of these two explanations best fits the facts must await more detailed enzyme study, but the marked caecal enlarging effect of the raw potato starch and the even more marked effect of the dioxan extracted material favours the notion that an activator is being lost. It is possible, for example, that a low molecular weight carbohydrate is needed for 'priming action' (Whelan, 1971) if the amylase is to successfully hydrolyse potato starch, and certain minerals such as Ca^{2+} and Mg^{2+}

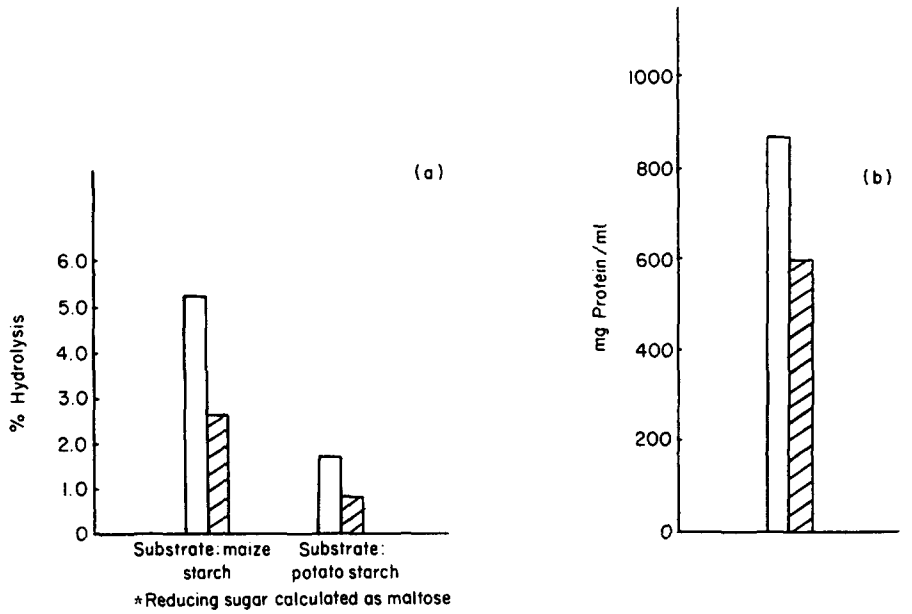


Fig. 2. (a) Amylase activity (reducing sugar calculated as maltose) of duodenal fluid in rats fed maize starch (white columns) or potato starch (hatched columns) diets. (b) Protein content of duodenal fluid in rats fed maize starch (white column) or potato starch (hatched column) diets.

are known stabilisers for amylase (Vallee *et al.*, 1959). The first of these possibilities was ruled out after the feeding experiment (Experiment 2) in which decreasing proportions of the dietary raw potato starch were replaced by DE 17 or DE 43 dried glucose syrup, a substance containing a complete range of possible priming sugars (Birch *et al.*, 1970). The results indicated that the glucose syrup addition conferred no protection against caecal enlargement, except for the proportional differences

TABLE 4

WATER BALANCE† IN YOUNG RATS FED ON RAW MAIZE OR RAW POTATO STARCHES

Parameter (per day per animal)	*1A (71% maize starch)*	*1B (71% potato starch)*
Water intake (g)	17·7 ± 0·5	16·7 ± 0·8
Volume of urine (ml)	8·8 ± 0·7	$5·2^a$ ± 0·6
Wet faecal weight (g)	0·7 ± 0·2	$7·4^b$ ± 1·0
Dry faecal weight (g)	0·6 ± 0·1	$4·5^b$ ± 0·5
Per cent moisture in faeces	23·9 ± 2·9	$36·7^a$ ± 2·5
Water balance	48·7 ± 4·3	51·4 ± 2·1

Values listed are mean daily determinations of three animals on three consecutive days.
Mean values which are significantly different from the values of the control group (1A) are marked $^a = P < 0·01$, $^b = P < 0·001$.

† Water balance calculated as $= \frac{(\text{water intake} - \text{water lost in urine and faeces}) \times 100}{\text{water intake}}$

expected by partial replacement of the potato starch (Table 3). The second possibility, that the divalent ions are absent, might not be anticipated in view of the mineral additions included in our experimental diets. However, pure polysaccharides may chelate metal ions (Angyal & Pickles, 1972*a* and *b*) and indeed the metal complexing properties of carbohydrates are already well known (Rendleman, 1966). Thus the mineral additions may not have been sufficient to compensate for the well known mineral absorbing property of the raw potato starch (Hollo *et al.*, 1962) which in particular absorbs Ca^{2+} and Mg^{2+} ions about twice as much as other starches. The rate of absorption of these ions by potato starch is again much higher than the rate of absorption by other starches.

The caecal microflora (especially lactic anaerobes) count is elevated in group 1B (receiving raw potato starch) and this will be the subject of a further paper from these laboratories. The pH of caecal contents is low (5·5) in group 1B compared with the control group 1A (7·0); this could be due to the apparent changes in the gut microfloral population. It is possible that maltodextrins, produced by limited amylase activity, are poorly absorbed and utilised, and that their humectant properties (Kearsley & Birch, 1975*a* and *b*) result in fluid retention.

The poor utilisation of raw potato starch has been recognised for some time (Booher *et al.*, 1951; Yoshida & Morimoto, 1955); Ketiku & Oyenuga (1973) have illustrated some similarity between yam and potato in this respect. More recently, Whittemore *et al.* (1975*a* and *b*) have studied the poor metabolisable energy associated with whole raw potato diets in pigs and have observed enlarged caeca engorged with particles of raw potato. These authors point out that there is a loss of measurable metabolisable energy when gut microflora convert undigested carbohydrate into volatile acids and intestinal gas, and such considerations may well undermine the significance of food efficiency calculations. Clearly, the phenomenon of caecal enlargement is not confined to rats fed on potato starch diets and it may possibly be a general manifestation of certain types of dietary carbohydrate interacting with the alimentary canal.

The mechanism by which caecal enlargement is effected remains unclear but the postulate that osmolality of the gut contents may play a part is worthy of consideration (Leegwater *et al.*, 1974). Although the osmolality tended to be lower in the enlarged caeca (Table 2) it is remarkable how similar these values are and this suggests that a homeostatic mechanism may be operative, controlling water absorption and osmolality in the caecum. In that case, caecal enlargement may be considered an adaptive rather than a toxic response which only produces pathologic sequelae in extreme cases.

REFERENCES

ANGYAL, S. J. & PICKLES, V. A. (1972*a*). Equilibria between pyranoses and furanoses I, *Aust. J. Chem.*, **25**, 1695.

ANGYAL, S. J. & PICKLES, V. A. (1972*b*). Equilibria between pyranoses and furanoses II. *Aust. J. Chem.*, **25**, 1711.

BANKS, W. & GREENWOOD, C. T. (1975). In: *Starch and its components*, Edinburgh University Press, 242.

BHOTIYAKORNKIAT, V. & BIRCH, G. G. (1972). Phosphate content in glucose syrup conversion, *Process Biochem.*, **7**, 25.

BIRCH, G. G. & ETHERIDGE, I. J. (1973). Chemical and physiological properties of glucose syrup components, *Starke*, **25**, 235.

BIRCH, G. G., ETHERIDGE, I. J. & GREEN, L. F. (1973). Short-term effects of feeding rats with glucose syrup fractions and dextrose, *B. J. Nutr.*, **29**, 87.

BIRCH, G. G., GREEN, L. F. & COULSON, C. B. (1970). *Glucose syrups and related carbohydrates*, Amsterdam. London and New York, Elsevier, 118.

BIRCH, G. G. & KEARSLEY, M. W. (1974). The fractionation of glucose syrups by reverse osmosis, *Starke*, **26**, 220.

BOOHER, L. E., BEHAN, I. & MCMEANS, E. (1951). Biological utilization of unmodified starches, *J. Nutrition*, **45**, 75.

CHALVARDIAN, A. & STEPHENS, S. (1970). Lipolitic effect of dextrin versus sucrose in choline deficient rats, *J. Nutr.*, **100**, 397.

DAHLQVIST, A. & BORGSTROM, B. (1961). Digestion and absorption of disaccharides in man, *Biochem. J.*, **81**, 411.

DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances, *Anal. Chem.*, **28**(3), 350.

EL-HARITH, E. A., WALKER, R. & DICKERSON, J. W. T. (1975). Some gastrointestinal effects of raw and processed potato starch, *J. Sci. Fd. Agric.*, **26**, 538.

EL-HARITH, E. A., DICKERSON, J. W. T. & WALKER, R. (1976*a*). Potato starch and caecal hypertrophy in the rat, *Fd. Cosmet. Toxicol.*, **14**, 115.

EL-HARITH, E. A., DICKERSON, J. W. T. & WALKER, R. (1976*b*). On the nutritive value of various starches for the albino rat, *J. Fd. Sci. Agric.*, **27**, 521.

ETHERIDGE, I. J. (1974). Ph.D. Thesis (Reading Univ.) Nutritional and technological studies on glucose syrups.

GOLDSTEIN, I., DUMITRU, E., DAVID, V. & MELINTE, L. (1970). Toxicity of glycol derivatives, *Igiena*, **19**(4), 209. *Chem. Abst.*, **75**, 33269.

HOLLO, J., HUSZAR, J., SZEJTH, J. & PETLO, M. (1962). Calcium and magnesium absorption in starch, *Starke*, **14**, 343.

HOSAYA, N. (1972). Effect of sugar alcohol on the intestine, *IX Int. Congr. Nutr.*, Mexico City.

KEARSLEY, M. W. & BIRCH, G. G. (1975*a*). Selected physical properties of glucose syrup fractions obtained by reverse osmosis: I—Specific rotation, average molecular weight, solubility rate, *J. Fd. Technol.*, **10**, 625.

KEARSLEY, M. W. & BIRCH, G. G. (1975*b*). Selected physical properties of glucose syrup fractions: II—Hygroscopicity, *J. Fd. Technol.*, **10**, 613.

KETIKU, A. O. & OYENUGA, V. A. (1973). Dehydrated yam and cassava as sources of energy to the laboratory rat. *W.A.J. Biol. Appl. Chem.*, **16**(2), 9.

KULAGINA, N. K. (1960). On the toxic action of dioxan, *Akad. Med. Nauk SSSR*, 142. *Chem. Abst.*, **58**, 869.

LEEGWATER, D. C., DE GROOT, A. P. & VAN KALMTHOUT-KUYPER, M. (1974). The aetiology of caecal enlargement in the rat, *Fd. Cosmet. Toxicol.*, **12**, 687.

LOESCHKE, K., UHLICH, E. & HALBACH, R. (1973). Caecal enlargement combined with sodium transport stimulation in rats fed polyethylene glycol, *Proc. Soc. Exptl. Biol. Med.*, **142**, 96.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, **193**, 265.

MORGAN, T. B. & YUDKIN, J. (1957). The vitamin-sparing action of sorbitol, *Nature (London)*, **180**, 543.

PRIESTLEY, R. J. (1974). Ph.D. Thesis (Reading Univ.)—Physiochemical studies of rice starch.

RENDLEMAN, J. A. (1966). Complexes of alkali metals and alkaline earth metals with carbohydrates. In: *Advances in carbohydrate chemistry*, Vol. 21 (ed. M. L. Wolfrom), London, Academic Press.

REUSSNER, G. (Jr.), ANDROS, J. & THISSEN, R. (Jr.) (1963). Studies on the utilization of various starches and sugars in the rat, *J. Nutr.*, **80**, 291.

SAVAGE, D. C. & DUBOS, R. (1968). Alterations in the mouse caecum and its flora produced by antibacterial drugs, *J. Exp. Med.*, **128**(1), 97.

SCHALES, O. & SCHALES, S. S. (1941). A simple and accurate method for the determination of chloride in biological fluids, *J. Biol. Chem.*, **140**, 879.

TURVEY, G. R. & HUGHES, R. C. (1958). The enzymic degradation of raw starch granules, *Proceedings of the Biochemical Society*, **69:1**, 4.

WHELAN, W. J. (1971). Enzymatic explorations of the structures of starch and glycogen, *Biochem. J.*, **122**, 609.

WHITTEMORE, C. T., TAYLOR, A. G., MOFFAT, I. W. & SCOTT, A. (1975*a*). Nutritive value of raw potato for pigs, *J. Sci. Fd. Agric.*, **26**, 255.

WHITTEMORE, C. T., MOFFAT, I. W. & TAYLOR, A. G. (1975*b*). Influence of cooking upon the nutritive value of potato and maize diets for growing pigs, *J. Sci. Fd. Agric.*, **26**, 1567.

VALLEE, B. L., STEIN, E. A., SUMMERWELL, W. N. & FISCHER, E. H. (1959). Metal content of amylases of various origins, *J. Biol. Chem.*, **234**, 2901.

YOSHIDA, M. & MORIMOTO, H. (1955). Utilization of sweet potato starch by rats and its effect on the digestion of dietary protein, *J. Nutr.*, **57**, 565.

GAS CHROMATOGRAPHIC DETERMINATION OF AVAILABLE LYSINE

BABOO M. NAIR, A. LASER, A. BURVALL & N-G. ASP

Applied Nutrition, Department of Food Science, University of Lund, Chemical Centre, Lund, Sweden

(Received: 28 December, 1977)

ABSTRACT

The available lysine was converted to homoarginine by guanidation. After acid hydrolysis the homoarginine was converted to its trifluoro-acetic-butyl derivative and separated in a mixed phase silicone column.

The method was applied to a series of heat treated skim milk powder samples for determination of available lysine. This method is very well correlated to the protein efficiency ratio of diets where lysine was the limiting amino acid. The method appears to be a satisfactory measure of the available lysine.

INTRODUCTION

The nutritive value of food proteins depends on the presence of the essential amino acids in the required quantities and also on their biological availability. Some of the essential amino acids exist in food materials in a limited quantity. Few of them become unavailable to the digestive enzymes for proteolysis due to their reaction with carbohydrates. Lysine, methionine and tryptophan could be simultaneously limiting and unavailable. An amino acid analysis would give the amount of amino acids in a food sample, but, due to heat treatment during processing and long storage, the amino acids form stable compounds, called Maillard products, especially with reducing sugars. Maillard products are many and form in various ways, the most important being the reaction where the ε-amino group of lysine, being very reactive, acts with glucose, fructose, galactose, lactose, etc., which are also very reactive, to produce Maillard products.

Lysine, whose ε-amino group is engaged in a Maillard component, is not available for enzymic hydrolysis. Amino acid analysis of a food protein sample after

Fd. Chem. (3) (1978)—

traditional acid hydrolysis with 6N HCl for 20 h at 110 °C would give a lysine content, an unknown part of which is nutritionally unavailable.

There are a few methods for the determination of available lysine.

Sangers reaction with FDNB to ε-dinitrophenyl-lysine (ε-DNP-lysine) is the basic principle for the so far, widely used method for the determination of available lysine (Carpenter, 1960). After the reaction and acid hydrolysis the yellow coloured ε-DNP-lysine is separated from the remainder by ether extraction and the absorption is then measured spectrophotometrically. This method is useful for comparatively pure protein samples which contain relatively small amounts of carbohydrates. ε-DNP-lysine is not completely stable on acid hydrolysis and corrections using a standard substance are necessary.

Mauron & Bujard (1963) suggested guanidation of the free ε-amino group of lysine to produce homoarginine. The available lysine is converted to homoarginine which is stable during acid hydrolysis and the homoarginine is eluted as in the amino acid analysis by ion exchange chromatography.

This paper reports on a method for the determination of available lysine by converting it to homoarginine followed by acid hydrolysis. Homoarginine is determined by gas–liquid chromatography after converting it to its trifluoroacetic-butyl derivative. Simultaneously, the relevance of the method has been checked through animal experiments with varying lysine deficient diets.

EXPERIMENTAL

Reagents

Lysine and arginine were obtained from BDH Chemicals, Poole, England. Homoarginine was obtained from Calbiochem, San Diego, California 92112, USA. Butyl-stearate in *n*-butanol was obtained from Regis Chemical Co., Chicago, Illinois, USA. Butanol and methylene chloride were obtained from Merck, Darmstadt, West Germany and redistilled. Trifluoroacetic anhydride and orthomethyl-isoureahydrogensulphate were obtained from Eastman Kodak products. β-lactoglobulin was obtained from Sigma Chemical Co., St Louis, USA while all other chemical reagents were of analytical grade.

Apparatus

A Pye-Unicam gas chromatograph, model 104, with a flame ionisation detector and 1600 mm × 4 mm id glass column containing a mixed phase packing of 2·0 w/w % OV-17 and 1·0 w/w % OV-210 on 100/120 mesh Supelcoport, obtained from Regis Chemical Co., Chicago, Illinois, USA, was used in the experiments.

Samples

The milk sample was standardised cow's milk. For heat treatment spray-dried skim milk powder was used.

Experimental animals

Male rats, 20–23 days old, bought from Anticimex, Stockholm, Sweden, were used as the experimental animals.

METHODS

Guanidation was carried out by the method described by Mauron & Bujard (1963). After guanidation the protein was precipitated with trichloracetic acid and centrifuged. The precipitate was hydrolysed with 6N HCl at 110 °C for 20 h. An aliquot of the hydrolysate was evaporated and cleaned through an ion exchange column.

The eluate was evaporated and then derivatised as described by Kaiser *et al* (1974). The experiments for the quantitative evaluation of derivatisation and chromatography were carried out with pure standard samples of lysine, arginine and homoarginine using butyl stearate as internal standard. Evaluation of the chromatogram was by triangulation at a column temperature of 190 °C. The method was applied to β-lactoglobulin and milk samples in order to study the guanidation step and various samples of heat-treated skim milk powder to evaluate the entire method.

In the animal experiments to determine protein efficiency ratios ten rats in individual cases were used and their increase in weight was followed for a period of 28 days (Eggum, 1973).

Each sample of heat treated skim milk powder was incorporated into a diet having 10 % protein (equal parts from milk protein and gluten).

Room temperature was 26 °C and humidity 58 % and there was a free supply of water.

RESULTS AND DISCUSSION

Derivatisation and gas chromatography of homoarginine, arginine and lysine

As shown in Fig. 1, the derivatives of homoarginine, arginine and lysine are well separated from each other and from butyl stearate under the chromatographic conditions used. Table 1 shows the results of six repeated injections of the equimolar mixture of derivatives of HArg, Arg and Lys and the relative responses. The relative standard deviation is 4–6 %. Table 2 shows the data from ten separate derivatisations and chromatography. The relative response for all the amino acids studied shows a relative standard deviation of 10–12 %. This includes derivatisation, gas chromatography and evaluation of chromatograms by triangulation.

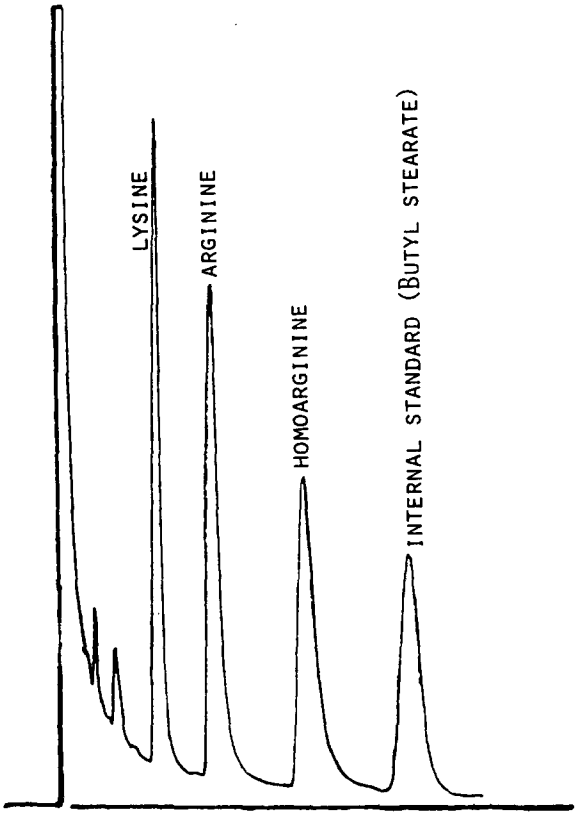


Fig. 1. Separation of a standard mixture of lysine, arginine, homoarginine and butyl stearate from a glass column (1600 mm × 4 mm id) containing a mixed phase of 2·0 w/w % OV 17 and 1·0 w/w % OV 210 on 100/120 mesh Supelcoport at 190 °C.

TABLE 1

RELATIVE RESPONSE OF A SINGLE STANDARD SAMPLE OF THE DERIVATIVES OF LYSINE, ARGININE AND HOMOARGININE SHOWING CHROMATOGRAPHIC PRECISION ON REPEATED INJECTION

	Aaa/Ais		
	HArg.	*Arg.*	*Lys.*
1	1·18	1·16	0·97
2	1·16	1·14	0·90
3	1·13	1·06	0·87
4	1·08	1·06	1·00
5	1·07	1·06	0·89
6	1·13	1·10	0·92
MV	1·13	1·09	0·93
σ	0·04	0·04	0·05
% Rel σ	3·54	3·66	5·37

TABLE 2

PRECISION OF DERIVATISATION AND CHROMATOGRAPHY OF A STANDARD MIXTURE OF LYSINE, ARGININE AND HOMOARGININE

	Aaa/Ais		
	HArg.	*Arg.*	*Lys.*
1	1·15	1·20	1·17
2	1·50	1·66	1·53
3	1·64	1·67	1·22
4	1·38	1·46	1·33
5	1·62	1·72	1·54
6	1·45	1·52	1·36
7	1·73	1·80	1·21
8	1·54	1·63	1·43
9	1·45	1·39	1·32
10	1·41	1·41	1·36
MV	1·49	1·55	1·35
σ	0·16	0·18	0·13
% Rel σ	10·73	11·61	9·62

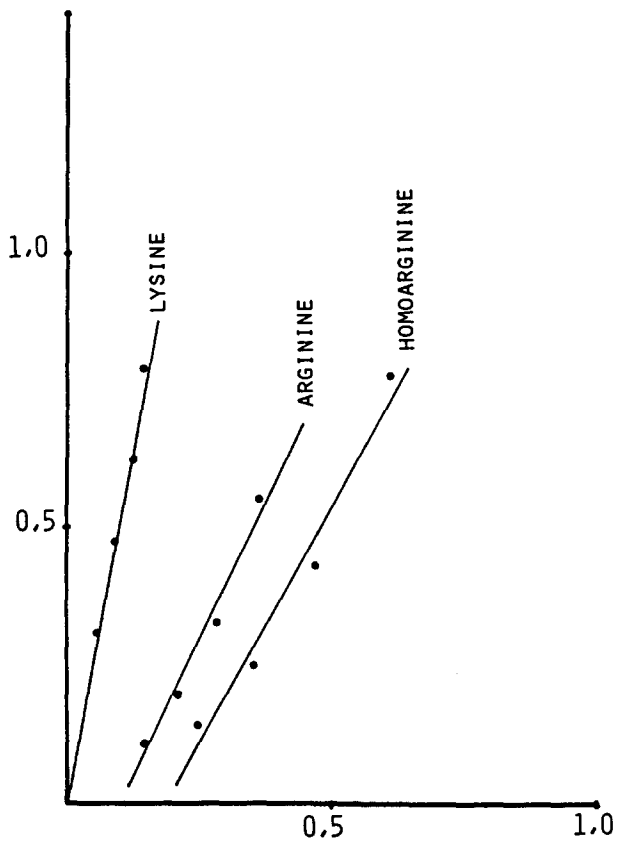


Fig. 2. Response linearity for the derivatives of lysine, arginine and homoarginine. Ordinate: Aaa/Ais. Abscissa: μmol injected.

Response linearity

Figure 2 shows the response linearity graphs for homoarginine, arginine and lysine. An aliquot from a standard solution of the amino acids was derivatised and various dilutions were made with methylene chloride. Some 5 ml of the derivatives were injected. It is clear from the figure that there is a linear relationship between the amount of amino acid injected and its peak area when the same sample with internal standard is injected after dilution. There exists a perfect linearity for lysine throughout the range from zero upwards. For homoarginine and arginine the

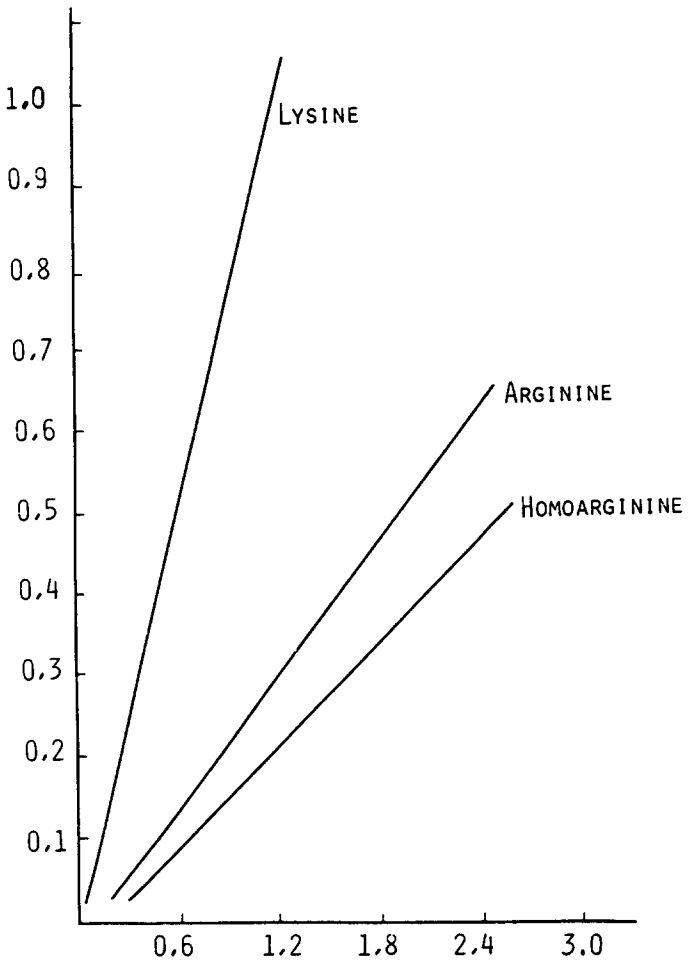


Fig. 3. Calibration curves for lysine, arginine and homoarginine. Ordinate: Aaa/Ais. Abscissa: μmol injected.

linearity curve could not be drawn through the zero point. Therefore, a calibration curve was necessary for homoarginine and arginine. As we had to inject very low amounts of homoarginine we used a calibration curve as shown in Fig. 3.

Guanidation, acid hydrolysis and derivatisation and gas chromatography were carried out with a sample of milk powder and internal standard to see if there is any substance which would interfere with the procedure. The chromatogram in Fig. 4

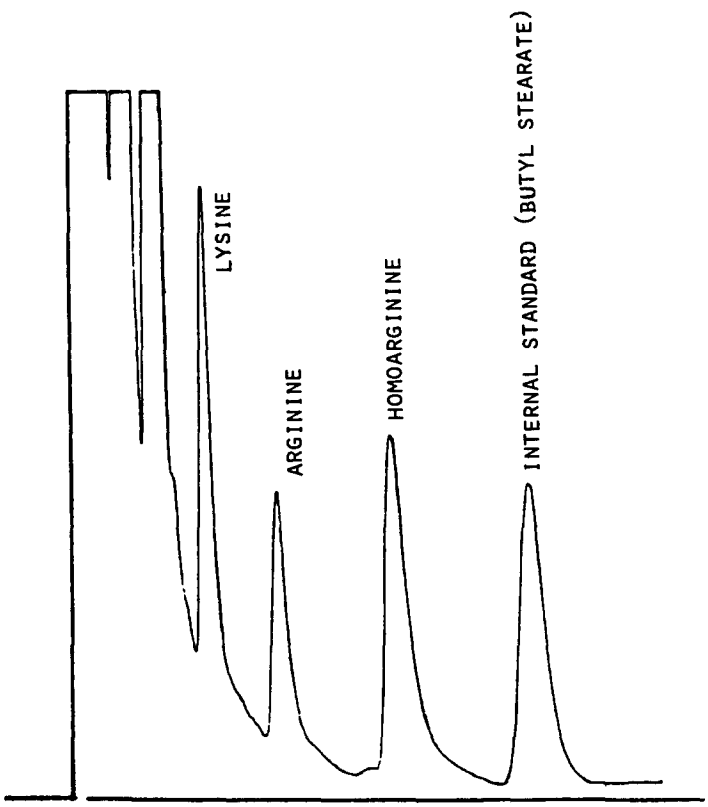


Fig. 4. Separation of lysine, arginine, homoarginine and butyl stearate after guanidation, hydrolysis and derivatisation of a sample of milk.

shows the excellent separation of all the relevant amino acids without any interfering peak. In order to check the reproducibility of the entire procedure seven samples of skim milk were carried through the various steps of the method. The results are presented in Table 3. The relative standard deviations for the quantities of homoarginine/arginine and homoarginine/lysine were 6% and 5%, respectively.

TABLE 3
PRECISION OF GUANIDATION, HYDROLYSIS, DERIVATISATION AND GAS CHROMATOGRAPHY OF SKIM MILK PROTEIN SAMPLES

	HArg.	*Aaa/Ais Arg.*	*Lys.*
1	1·16	0·58	0·70
2	1·10	0·48	0·68
3	1·25	0·53	0·74
4	1·28	0·56	0·72
5	0·92	0·42	0·49
6	1·01	0·44	0·60
7	1·03	0·43	0·47
MV	1·10	0·49	0·63
σ	0·13	0·07	0·11
% Rel σ	11·81	13·26	17·46

Application of the method for evaluating the effect of heat treatment in comparison with determination of the protein efficiency ratio by animal experiments

The experiments were carried out as described above. Skim milk powder samples were subjected to various degrees of heat treatment to decrease the availability of lysine due to Maillard reaction. Results of determinations of total lysine and available lysine are presented in Table 4. The total lysine content of the non heat-treated skim milk powder used as a control sample was 7·3% of the protein. Guanidation had converted 87% of the total lysine to homoarginine in this sample. On analysing β-lactoglobulin and a sample of lyophilised skim milk 90% of the lysine was determined as homoarginine. This shows that the skim milk powder was, in fact, manufactured under a mild heat treatment procedure. Such a procedure does not seem to destroy arginine to any considerable degree.

The results of animal experiments are shown in Table 5. The protein efficiency ratio values show that a very light browning would cause considerable reduction in nutritive value. The protein efficiency ratio of the diets, including the heat treated

TABLE 4
CONTENT OF AVAILABLE LYSINE AND TOTAL LYSINE IN SOME HEAT TREATED SAMPLES OF SKIM MILK POWDER

Sample	*Available lysine* (g/16 g N)	*Total lysine* (g/16 g N)	*Available lysine % of non heat treated sample*	*Available lysine % of total lysine in non heat treated sample*
Non heat treated	6·32	7·23	100	87
1/2	4·92	5·90	78	68
I	3·78	4·86	60	52
II	3·70	5·06	59	51
III	3·70	5·32	59	51

TABLE 5

PROTEIN EFFICIENCY RATIO OF CERTAIN SAMPLES OF HEAT TREATED SKIM MILK POWDER

Sample No.	*Duration of heat treatment (100°) (h)*	*PER*	PER_{Corr}
0	0	3·18	2·51
1/2	12	1·88	1·48
I	24	1·39	1·10
II	48	0·96	0·76
III	96	0·44	0·35

$$PER_{Corr} = PER_{Sample} \times \frac{2{\cdot}50}{PER_{Casein}}$$

skim milk powder, was found to correlate well with lysine contents determined by the method described here. The three samples which were mostly heat treated contain similar quantities of available lysine. This could mean that the lysine is made non available only to a certain extent by Maillard reaction, while the browning continues further.

REFERENCES

CARPENTER, K. J., *Biochem. J.*, 77 (1960) p. 604.
EGGUM, B. O., *Beretning fra försøgslaboratoriet* (1973) p. 406.
KAISER, F. E., GEHRKE, C. W., ZUMWALT, R. W. & KUO, K. C., *J. Chromatogr.*, 94 (1974) p. 113.
MAURON, J. & BUJARD, E. Proc. Int. Congr. Nutr. (Edinburgh) (1963) p. 167.

ACCELERATED SHELF-LIFE TESTING FOR OXIDATIVE RANCIDITY IN FOODS—A REVIEW

J. O. RAGNARSSON & T. P. LABUZA

Department of Food Science and Nutrition, University of Minnesota, St Paul, Minnesota 55108, *USA*

(Received: 28 November, 1976)

ABSTRACT

Accelerated shelf-life test (ASLT) methods for processed foods are receiving greater attention. In this paper, current ASLT methodology for fatty foods is reviewed with particular emphasis on the testing of antioxidant effectiveness.

In all the classical ASLT methods temperature is the dominant acceleration factor used. Its effect on the rate of lipid oxidation is best analysed in terms of the overall activation energy, E_A for lipid oxidation. It is an inherent assumption in these tests that the E_A is the same in both the absence and the presence of antioxidants. An analysis of the rate equations for the uninhibited versus the inhibited oxidation indicates, however, that the E_A may be considerably higher in the latter case. ASLT data collected at 60–65 °C bear this out and show that such tests lead to sizeable, but predictable, underestimation of the shelf-life extension by antioxidants for room temperature. In comparison, data collected at 98–100 °C are much less predictable. At this higher temperature E_A-variations are generally smaller and both under- and overestimation of shelf-life is found. In addition, the use of such high temperatures for complex foods is ruled out because of secondary reactions of other food components.

Other acceleration parameters for shelf-life used are the oxygen pressure, reactant contact and the addition of catalysts. The effect of these factors, although much less important than that of temperature, is discussed.

INTRODUCTION

Rancidity of edible oils and fatty foods due to lipid oxidation is a serious problem in some sectors of the food industry. Factors which have contributed to this problem in recent years are the increased emphasis on polyunsaturated dietary lipids and the fortification of certain foods with iron. Because of the unfortunate consequences of

Fd. Chem. (2) (1977)—
Printed in Great Britain

lipid oxidation in foods it is critical that information about the oxidative stability of susceptible food items be obtained before they are marketed.

The food manufacturer would like to employ methods which can give a reasonably accurate indication of the product shelf-life in a relatively short period of time. Many accelerated shelf-life tests (ASLT) are available. Since the rate of a reaction increases exponentially with the absolute temperature, this parameter is usually singled out to speed up the oxidation in such tests (for explanation of symbols and abbreviations used see Table 1).

The first step in a typical ASLT study is to select a suitable method for testing the food product under consideration. Next, a sample is placed under the conditions of the test and the induction period θ_s is measured, i.e. the time, usually in hours, required to reach a specified end-point. The last and the most difficult step is to translate the value for the induction period obtained into actual product shelf-life in months of storage. Usually this is done with some arbitrary factor based on prior experience. As most highly oxidisable foods contain added antioxidants these methods are often used to evaluate the effectiveness of antioxidants.

There are several reasons why a review of the ASLT methodology is presented here. Originally these methods were designed to be used for homogeneous lipids such as animal fats and vegetable oils. Unfortunately, only a few studies have been carried out to evaluate these methods critically. At least two such studies (Pohle *et al.*, 1964; Paul & Roylance, 1962) raise serious doubts about the usefulness of these tests even when applied to fats and oils. Pohle *et al.* (1964) concluded from their data that in order to obtain any useful information from the current ASLT methods, each test must be calibrated for each individual fat formulation.

Another serious drawback of the currently-used ASLT methods is the fact that, with the exception of the Active Oxygen Method, they are not standard methods. Different workers use different versions and modifications, which greatly complicates matters.

Finally, there is a growing interest in applying ASLT methods to formulated foods. The feasibility of using existing methods for this purpose should be evaluated.

Basically, four parameters are manipulated in ASLT procedures to speed up the oxidation and development of rancidity in foods or oils. These are listed in Table 2. Increased temperature is the most common and effective means of accelerating the oxidation. The rate of the reaction is exponentially related to the temperature; thus shelf-life should decrease logarithmically with increasing T. In *single-component* lipid systems oxidation can be represented by the following equation:

$$RH + O_2 \xrightarrow{K} ROOH \rightarrow \text{Secondary Products} = \text{rancidity or end of shelf-life}$$

where: RH = polyunsaturated lipid substrate; O_2 = oxygen; ROOH = lipid hydroperoxide (primary product) and K = overall rate constant for ROOH production.

TABLE 1
ABBREVIATIONS AND SYMBOLS USED

Symbol	*Meaning*
a	Inhibition, reaction (10)
A	Pre-exponential factor
A·	Antioxidant radical
AH	Primary antioxidant
AOM	Active Oxygen Method
ASLT	Accelerated Shelf-Life Test
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
c	Chain transfer, reactions (6) and (−10)
d	Dimerisation, reaction (9)
D	Bond dissociation energy, kcal/mole
E_a, E_i, E_p, E_t	Activation energies for elementary steps
E_A	Overall activation energy
EDTA	Ethylene diaminetetraacetic acid
f	Reaction (8)
HVO	Hydrogenated vegetable oil
i	Initiation, reaction (2)
k	Rate constant for elementary step
K	Overall reaction rate constant
K_A	Overall inhibited rate constant
K_M	Overall uninhibited rate constant
M	Metal ion
M^{n+}	Metal ion, lower oxidation state
$M^{(n+1)+}$	Metal ion, upper oxidation state
o	Propagation, reaction (4)
OAM	Oxygen Absorption Method
OBM	Oxygen Bomb Method
p	Propagation, reaction (5)
PG	Propyl gallate
psia	lb/in^2 absolute pressure
PUFA	Polyunsaturated fatty acid
PV	Peroxide value, meq ROOH/kg lipid
Q_{10}	Temperature coefficient, k_{T+10}/k_T
R	Universal gas constant, 1·987 cal/K-mole
R·	Lipid radical
RH	Lipid substrate
RO·	Oxyl radical
ROO·	Peroxyl radical
ROOH	Hydroperoxide
SOT	Schaal Oven Test
t	Termination, reaction (7)
T	Temperature in °C or K
TBHQ	Tertiary butylhydroquinone
θ	Time
θ_s	Induction period (elevated T) or shelf-life (room temperature)

On a kinetic basis $K = A\,e^{-E_A/RT}$ where A, the pre-exponential factor, and E_A, the activation energy, stay approximately constant as long as the mechanism does not change. Some workers, in analysing storage studies, use Q_{10}-values to determine the

TABLE 2
COMMON ACCELERATION PARAMETERS

Parameter	*Normal range*
Temperature (°C)	60–140
Oxygen pressure (psia)	3–165
Added metals (ppm in lipid)	25–100
Reactant contact	variable

accelerating effect instead of activation energies. These quantities are related through the following equation:

$$\log Q_{10} = (2{\cdot}189E_A)/(T + 10)T = \log\left[\frac{\text{shelf-life at } T + 10}{\text{shelf-life at } T}\right]$$

where: Q_{10} = increase in rate or decrease in shelf-life for a 10 °C increase in T; T = temperature in K and E_A = energy of activation in cal/mole.
Unlike the activation energy, the Q_{10}-value is a strong function of temperature and thus a poor criterion of the temperature sensitivity of the rate constants. As can be seen, the higher the E_A, the larger will be the increase in rate resulting from an increase in temperature. If the Q_{10} for the control is known it is usually applied to the test system containing the antioxidant in question. Obviously if E_A changes this Q_{10} factor is meaningless, as will be discussed.

Lipid oxidation can be broken down into a number of elementary step reactions where each step has its own activation energy. The controlling overall activation energy E_A can thus change as a result of a temperature elevation alone. Also, the addition of antioxidants, change in the oxygen pressure and other factors can alter the mechanism and hence the activation energy. These E_A changes, therefore, could cause erroneous prediction of the shelf-life at room temperature on the basis of data collected at a higher temperature, when the shelf-life of a control system is compared with that of one with added antioxidants.

In multi-component formulated food systems the effect of temperature can be very complex. A number of changes, both physical and chemical, may occur as the temperature is increased as listed in Table 3. Some of these changes could drastically affect the chemical reactivity through their effect on the distribution of reactants, metal binding properties, viscosity, metal prooxidant effectiveness and other variables. The water activity will increase as the temperature is increased and water can evaporate from the food. The fat—if solid at room temperature—can undergo crystal structure changes and eventually melt, contact new catalytic surfaces or drip out of the food. Once melted, oxidation will no longer be limited to the surface of the fat. Some end-products of the browning reaction between carbohydrates and proteins have antioxidative properties (Kirigaya *et al.*, 1968). Various proteins and

TABLE 3
EFFECTS OF 'HIGH' TEMPERATURES ON FOODS

Component	*Process*	*Critical T-Zone*
Water	Transfer	Above ambient
	Evaporation	Above 60 °C
Fat	Crystal changes	Close to melting point
	Melting	Above melting point
	Transfer	Above melting point
Starch	Gelatinisation	Above 60 °C
Carbohydrates	Non-enzymatic browning	Above 50 °C
	Caramelisation	Above 100 °C
	Charring	Above 100 °C
Proteins	Denaturation	Above 40 °C

enzymes will be denatured including lipoxigenases, peroxidases and other heme-proteins, with subsequent alteration in their pro-oxidative potential (Ericksson *et al.*, 1971). Heating can expose SH-groups which can act as antioxidants. Heme-bound metals may adopt a low-spin configuration, resulting in a loss of catalytic activity (Love & Pearson, 1974). Thus, the probability of a change in the mechanism occurring as a result of an increase in temperature is much greater in complex foods than in simple lipid systems. Some of the reactions listed in Table 3 can become significant at temperatures as low as 40 to 50 °C. This temperature range should therefore not be exceeded in ASLT studies for foods. The question remains as to whether such a moderate temperature elevation alone provides a sufficient acceleration in the oxidation rates for evaluation of shelf-life.

Other parameters besides the temperature which may accelerate the oxidation are listed in Table 2. At *oxygen pressures* close to atmospheric and higher, little effect of the oxygen pressure on the rate is obtained. However, at higher temperatures the effect of oxygen can become considerable (Bateman, 1954), especially for fats which are high in polyunsaturated fatty acids. Similarly, stirring, air bubbling and the use of 'inert' carriers or other means of promoting the *reactant contact* should have little effect provided the lipid is completely melted, the temperature is relatively low and no trace contaminants are introduced in the process. At the high temperatures of most ASLT methods, however, increasing the contact between oxygen and substrate can have an effect on the rate. Finally, by *adding pro-oxidants* such as transition metals one can accelerate the oxidation. This parameter is seldom used as it may cause changes in the mechanism of lipid oxidation. In conclusion, the temperature is generally by far the most important acceleration parameter used in ASLT methods.

THE EFFECT OF TEMPERATURE ON LIPID OXIDATION RATES

Lipid oxidation is caused by a free-radical chain reaction occurring through a series of steps. Each one has associated with it a rate constant. The most important steps in

the mechanism of the oxidation of polyunsaturated fatty acid esters are shown in Table 4. As indicated, the reaction can be divided into four phases: Initiation, propagation, termination and inhibition.

TABLE 4
MECHANISM OF LIPID OXIDATION

	$k_{30°C}$	E_a
Initiation		
(1) $RH + M^{(n+1)+} \longrightarrow R\cdot + H^+ + M^{n+}$	f (metal)	f (metal)
(2) $ROOH + M^{(n+1)+} \xrightarrow{k_2} ROO\cdot + H^+ + M^{n+}$	f (metal)	f (metal)
(3) $ROOH + M^{n+} \longrightarrow RO\cdot + OH^- + M^{(n+1)+}$	f (metal)	f (metal)
Propagation		
(4) $R\cdot + O_2 \xrightarrow{k_p} ROO\cdot$	10^7–10^9	0
(5) $ROO\cdot + RH \xrightarrow{k_p} ROOH + R\cdot$	10^2	5–7
(6) $A\cdot + RH \xrightarrow{k_c} AH + R\cdot$	slow	5–10
Termination		
(7) $ROO\cdot + ROO\cdot \xrightarrow{k_t} ROOOOR$	10^7	0–3
(8) $ROO\cdot + A\cdot \xrightarrow{k_f} ROOA$	fast	0–3
(9) $A\cdot + A\cdot \xrightarrow{k_d} A_2$	slow	0–3
Inhibition		
(10) $ROO\cdot + AH \underset{k_a}{\overset{k_a}{\rightleftharpoons}} ROOH + A\cdot$	10^4	0·8

RH = lipid substrate = methyl linoleate or linolenate
R· = substrate radical (di- or tri-allyl)
RO· = lipid oxyl radical
ROO· = lipid peroxyl radical
ROOH = lipid hydroperoxide
AH = primary antioxidant = BHT
A· = antioxidant radical (phenoxy)
$k_{30°C}$ = rate constant at 30 °C, litres/mole-sec
E_a = energy of activation, kcal/mole

Uri (1961*a*); Ingold (1973); Howard (1973); Howard & Furimsky (1973); Bateman (1954); Korcek *et al.* (1972); Ingold (1968).

The initiation can be either metal-, light- or thermally-catalysed. Singlet oxygen initiation has been implicated in the early initiation process (Rawls & van Stanten, 1970). When the lipid contains suitable sensitisers such as chlorophyll or polycyclic hydrocarbons, singlet oxygen initiation may dominate throughout in the presence of ultraviolet or visible light (Labuza, 1971; Grosch, 1975). Normally, however, as soon as hydroperoxides have formed, reactions (2) and (3) predominate. Waters (1971) does not believe that reaction (2) is important in metal-catalysed lipid oxidation. Kochi (1973), in contrast, has suggested that reaction (2) does occur and is rate-limiting in non-polar and poorly co-ordinating solvents such as lipids, especially in the presence of transition metals which are powerful oxidising agents such as Co and Mn.

The propagation occurs through steps (4) and (5). At ambient conditions the concentrations of oxygen and methyl linoleate in purified methyl linoleate are of the order of 10^{-3} molar and 1 molar respectively; thus reaction (5) is rate-limiting and the overall rate is approximately independent of the oxygen pressure. As the energy of activation for reaction (5), E_p is much higher than E_0 (Koreck *et al.*, 1972)—the same is not true at 100 °C. If weak, non-hindered antioxidants are present the A· radical can also act as a chain carrier through reactions (6) and (− 10), especially at high temperatures and at high antioxidant concentrations.

The termination is dominated by reaction (7) at atmospheric pressure. In the presence of primary antioxidants termination steps (8) and (9) also occur. The former is normally 'fast' whereas reaction (9) is very slow for 2,4,6-tri-substituted phenols (Ingold, 1973).

The inhibition takes place via reaction (10). Howard & Furimsky (1973) measured the E_A for a number of amines and phenolic antioxidants. For highly hindered phenols and amines the E_A was approximately 0–1 kcal/mole whereas the corresponding value for phenol and aniline was about 5 kcal/mole (Chenier *et al.*, 1974).

Mahoney (1969) has pointed out that for hydroquinones and sterically hindered phenols (hereafter referred to as ideal inhibitors) reactions (8) and (9) are much faster than reactions (6) and (− 10), whereas, for non-hindered phenolic antioxidants (i.e. non-ideal antioxidants), the four reactions can proceed at comparable rates. Commercial primary food antioxidants generally belong to the former category.

The low value of E_A obtained for hindered phenols and aromatic amines has been explained in terms of the formation of a hydrogen-bonded free-radical complex prior to the transfer of hydrogen (Howard & Furimsky, 1973). The activation energy for the back-reaction, E_{-A} may be roughly estimated by subtracting the bond dissociation energy of AH, D_{AH} from that for ROOH, D_{ROOH}. Generally the latter is about 88 (Howard, 1973) while the former is about 80 for hindered phenols, e.g. 81 kcal/mole for 2,6-di-tert-butylphenol (Chenier *et al.*, 1974). Therefore E_{-A} in this case is close to 7 kcal/mole. Hence, at high temperatures and once significant hydroperoxide has built up, the back-reaction can become important, giving the antioxidant some pro-oxidant properties. Reaction (6) also has a relatively high activation energy and would be expected to dominate the chain transfer process at low hydroperoxide concentration.

In the absence of primary antioxidants the limiting equation at 'high' oxygen pressures has been shown to be (Labuza, 1971):

$$[\mathrm{ROOH}]^{1\,2} = (k_i[\mathrm{M}]/k_t)^{1\,2} k_p[\mathrm{RH}]\theta/2 = K_M\theta/2 \tag{11}$$

thus

$$E_A = E_p + 0{\cdot}5(E_i - E_t) \approx 6 + 0{\cdot}5(E_i - 0) \tag{12}$$

where: θ = time and K_M = overall uninhibited reaction rate constant.

It should be noted that the rate does not depend on the oxygen pressure. At 'low' oxygen pressures, however, an oxygen-dependent rate-expression with a lower E_A is found.

Because of the change in termination with the presence of ideal primary inhibitors the following first order rate-expression is obtained (Labuza, 1971):

$$\ln [ROOH] = \ln [ROOH]_0 + k_i[M]k_p[RH]\theta/K_A[AH] = \ln [ROOH]_0 + K_A\theta/[AH] \quad (13)$$

thus:

$$E_A = E_i + E_p - E_a \approx E_i - E_a + 6 \quad (14)$$

where: $[ROOH]_0$ = initial hydroperoxide concentration and K_A = overall inhibited reaction rate constant.

In this case the rate is also oxygen-independent, even at quite low oxygen pressures. As shown, the rate of lipid oxidation is inversely proportional to the antioxidant concentration.

A comparison of eqns. (12) and (14) shows that the E_A will be considerably higher in the presence of a primary antioxidant than for the corresponding control if E_i is the same. This is, of course, quite logical and indicates that these inhibitors lower the rate of the oxidation at least partly by increasing the overall energy of activation. It must be realised, however, that eqn. (13)—and hence eqn. (14)—applies only for ideal primary inhibitors. Especially for relatively non-hindered phenolic antioxidants at high antioxidant concentrations, much more complex rate expressions with lower E_A-values can be expected. The same is probably true for the ideal inhibitors. At high temperatures chain transfer reactions become more important and eqns. (13) and (14) no longer apply.

To illustrate the significance of eqns. (12) and (14) one can consider a reaction, in this case lipid oxidation ($RH + O_2 \xrightarrow{K} ROOH$), which can occur via two different routes, path 1 and path 2, where $K_1 = A_1 e^{-E_1/RT}$ and $K_2 = A_2 e^{-E_2/RT}$. If path 1 represents lipid oxidation in the absence of primary antioxidants and path 2 in the presence of primary antioxidants then $E_2 > E_1$. The Arrhenius plots corresponding to $A_1 = A_2$ are shown in Fig. 1(a). As shown, the degree of protection by the primary antioxidant increases as the temperature is decreased.

In order to apply these principles to ASLT methodology one must first assume that the overall rate constant (K_1 or K_2) is proportional to the reciprocal of the 'induction period', θ_s, where the induction period (or the shelf-life at room temperature) is defined as the time to reach a constant percent oxidation of the substrate or end of shelf-life. Hence one can plot 'either' ln K or ln θ_s versus $1/T$ K (or T°C, if the temperature interval is small) and obtain linear plots as shown in Figs. 1(a) and (b). These plots both show that the effectiveness increases as T decreases. Thus the overall protection predicted at high temperatures for an antioxidant will usually be less than that found at lower temperatures. On the other hand, if the E_A

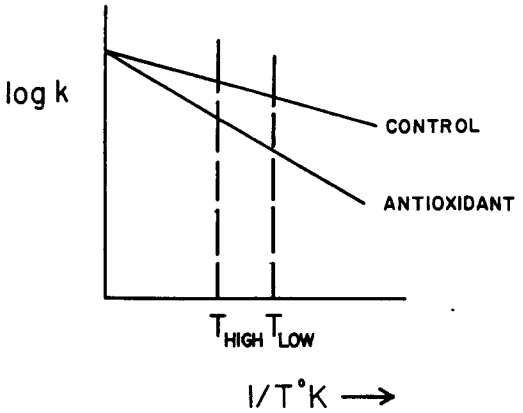


Fig. 1(a). Arrhenius plots for two parallel reactions.

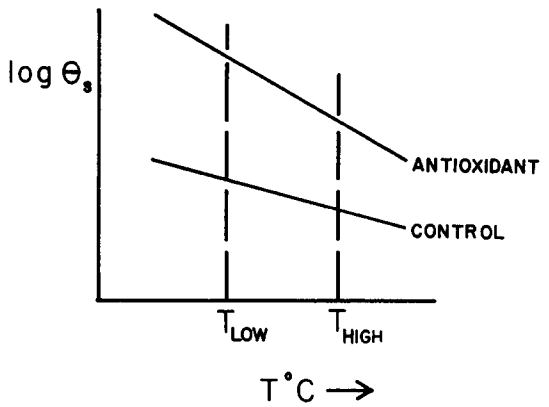


Fig. 1(b). ASLT plots for two parallel reactions.

decreases when the antioxidant is added, the degree of protection projected from high temperatures would overestimate the true shelf-life.

Bolland (1949) assumed that $E_i = 30$ kcal/mole for all purified olefins. In foods the E_i is much lower. Labuza (1972) found that in the range 37–52 °C for lipid oxidation in a chicken/cellulose/glycerol food system, E_A was about 10 in the absence of added antioxidants, indicating that E_i may have been as low as 8 kcal/mole. In the presence of added EDTA the E_A rose to about 17, presumably because the metal catalysts were inactivated, allowing an increase in E_i (to about 22 kcal/mole).

These considerations have important implications for high temperature testing of antioxidants at a single temperature. When fats such as animal fats, which contain

very low levels of primary antioxidants (*cf.* eqn. (12)), are tested against the same fat containing ideal antioxidants (*cf.* eqn. (14)) the temperature coefficients are quite different. Thus, any quantitative prediction for lower temperatures is impossible unless several temperatures are used. Besides, the order of effectiveness (ranking) at the higher temperature may not be the same at a lower temperature, since E_A may vary, depending on the structure of the antioxidant. The same is true for the evaluation of chelating agents since the E_i will change as compared with the control. However, if the only objective is to qualitatively rank antioxidants of similar structure (e.g. hindered phenols) at a single temperature, the test is probably useful.

Some other important considerations include: (1) Especially for weak, non-hindered antioxidants, very complex rate equations have been obtained, indicating that different mechanisms can occur (Scott, 1965, Ingold, 1970). (2) Volatility of antioxidants can become important at high temperatures, e.g. for BHT (Klaui, 1971). (3) If two phases, such as water and fat, are present the solubility in each phase and pH may become important, especially for the low-molecular weight gallates (Cornell *et al.*, 1970).

Lea (1960) prepared purified tocopherol-free cottonseed, linseed and cod liver oil esters which respectively were high in linoleate, linolenate and more unsaturated fatty acid esters. From the induction period (time to reach PV = 100 meq/kg), the E_A-values for the control oils respectively were about, 20, 11 and 13 kcal/mole. For samples containing various hindered phenols the corresponding E_A ranges were 35–40, 27–35 and 13–20. Agreement with eqns. (12) and (14) thus seems to have been excellent for cottonseed oil if E_i is taken as 30 kcal/mole. In this case the gallates and hydroquinones behaved very similarly to the phenols. Agreement could be considered fair for the other substrates if one assumes lower values for the E_i. However, in the linseed oil the gallates and hydroquinones did not show the same behaviour as did the phenolic antioxidants and thus the E_A-value was somewhat different.

ASLT METHODS FOR PREDICTING OXIDATIVE STABILITY

(a) *The Schaal Oven Test (SOT)*

This method was developed in the baking industry in the 1920s. No published reference by its originator exists (Dugan, 1955). Joyner & McIntyre (1938) recommended that 50 g samples be held in 250 ml beakers with watch-glass on the top and maintained at about 63 °C. The samples were smelled daily until the rancid point was reached. Lea (1962) advocated the use of peroxide values to monitor the oxidation and the use of much smaller samples (0·2 ml) which were kept in small glass cups, the oil forming about a 2 mm layer on the bottom.

The temperature called for in this method is much lower than in most other ASLT procedures. This method can therefore be recommended as the one having the fewest possible problems. Hartman *et al.* (1975) feel that this method gives a better

correlation with an actual shelf-life test than does the Active Oxygen Method. However, especially for complex foods, a temperature of 60 °C is too high. The end-points used—either a rancid odour or a peroxide value of 70–120—are appropriate for correlating with the shelf-life at lower temperature.

(b) *Oxygen Absorption Methods (OAM)*

Many versions of the Oxygen Absorption Method are available. The most commonly used procedures are those of Sylvester–Martin and Eckey. In the former method (Sylvester *et al.* (1942) as modified by Martin (1961)), 100 to 1000 mg samples of lipid are kept in 30 ml flasks connected to mercury manometers. These are connected to a pressure recorder. The sample is kept at atmospheric pressure in oxygen at 100 °C. The end-point was taken as the time when a marked drop in pressure occurred. If the sample absorbed oxygen only gradually throughout, the end-point was taken at the organoleptic rancid point. In order to get a sharp end-point with vegetable oil, Sylvester found it necessary to replace the air with oxygen.

Eckey (1946) proposed a somewhat similar design in which 1 g of lipid was suspended in 12·5 g of 'pure silica sand' in a 50 ml flask. The temperature was maintained at 80 °C. The end-point was taken as the time for the sample to absorb 3 ml of oxygen as calculated at 0 °C, 760 mm. The author used air as the surrounding atmosphere, but suggested a modified design that could be used with pure oxygen.

The temperature used in these methods is considerably higher than that used in the Schaal Oven Test. This is a serious disadvantage even for simple lipids. Of the two methods considered the Eckey method involves a somewhat lower temperature, but this method has two additional disadvantages. Firstly, the sand is bound to introduce trace pro-oxidants. Generally the use of the sand increased the rate 1·4 to 6 times. The least acceleration occurred with a sample containing phosphoric acid. This acid can act as a metal-chelating agent, indicating that the acceleration was probably due to metal contaminants. The other problem is the end-point chosen. The significance of the induction period obtained depends on the degree of unsaturation of the oil. If the lipid to be tested contains 10 % polyunsaturated fatty acids (PUFA), 1 g of lipid contains about 0·3 mmoles PUFA. Three millilitres of O_2 at 1 atm and 0 °C correspond to about 0·13 mmoles O_2. Thus, at the end-point, the sample would be about 40 % oxidised. In contrast, rancidity in foods usually occurs when the lipids are 0·1 to 3 % oxidised (Labuza, 1971).

A considerable disadvantage of both methods is that at the relatively low oxygen pressures used (3 and 15 psia in the Eckey and Sylvester–Martin methods, respectively) the rate can easily become dependent on the oxygen pressure and the rate of oxygen dissolution. There are three reasons why this can occur. At the high temperatures used: (a) a shift in the mechanism occurs so that now higher oxygen pressures are needed to remove the oxygen dependence of the reaction rate (Bateman, 1954); (b) the solubility of oxygen in the lipid decreases (Bateman *et al.*, 1951) and (c) the rate of oxidation increases dramatically, decreasing the oxygen

concentration in the lipid more and more as the reaction proceeds (Bateman & Gee, 1951). The dependence on oxygen will be expected to increase with the degree of unsaturation of the lipid.

Sylvester *et al.* (1942) found that for palm kernel oil the induction period was almost cut in half when the air was replaced with pure oxygen. Pohle *et al.* (1962), using a considerably higher oxygen pressure, found less than a 10% increase in rate as the oxygen pressure was increased from 65 to 115 psia. At higher temperatures the dependence was found to be even greater, as expected (Bennett, 1964).

(c) *The Active Oxygen Method (AOM)*

The Active Oxygen Method, or the Swift Stability Test, as it is sometimes called, was originally proposed by King *et al.* (1933) and later modified slightly by Riemenschneider *et al.* (1943). Twenty millilitre samples of lipid are kept in 1 in × 8 in glass tubes and clean dry air at 2·33 cm^3/sec is bubbled through. The temperature is maintained at 97·8 °C. Periodically about 0·2 ml samples are withdrawn and the peroxide value (PV) is determined until it reaches 120 meq/kg. Unlike most other ASLT methods, this one has been rigorously standardised.

The main problem with this method is the high temperature used. Generally, an arbitrary multiplying factor is used, based on previous experience, to give an estimate of the shelf-life at room temperature. The method obviously cannot be used with formulated foods.

Another problem is the arbitrary air bubbling rate. Since one is trying to compare the data with room temperature conditions where the rate is indeed oxygen-independent, ideally the rate in the AOM test should be oxygen-independent also. The use of bubbling does, of course, speed up the rate of dissolution. Air at atmospheric pressure, however, will not suffice to achieve oxygen independence even if one assumes that the oxygen concentration in the lipid equals the oxygen solubility under the conditions used. As oxygen solubility depends almost linearly on the oxygen concentration in the head space, the only way to maintain oxygen independence is to use higher oxygen pressures. The final problem is that antioxidants such as BHT which are relatively volatile can evaporate from the sample (Klaui, 1971).

Luckman *et al.* (1953) used specially prepared and cleaned iron tubes instead of glass tubes to shorten the induction period. A two- to six-fold increase in the rate was obtained with hydrogenated vegetable oil. In contrast, only a 20% increase was obtained when the fat contained 0·08% added isopropyl citrate. This modification has not been generally accepted. The easiest and most accurate way of increasing the level of pro-oxidants is to add the metal directly in the form of a salt.

(d) *The ASTM Oxygen Bomb Method (OBM)*

This method has long been used (ASTM Committee D-2, ASTM Standards on

Petroleum Products and Lubricants, pp. 254–7, 1955) to determine the resistance of petrols to gum formation (Scott, 1965). Gearhart *et al.* (1957) were the first to use this method for food lipids. The authors added 15–30 g of lipid to a glass container which was fitted into the bomb. The oxygen pressure used was either 65 or 115 psia and the temperature 99 °C. The induction period was taken as the time to reach the mid-point of the first hour during which a pressure drop of at least 2 psia/h was obtained. The reproducibility has been shown to be excellent (Pohle *et al.*, 1962). Stuckey *et al.* (1958) modified the method by using a smaller sample which was spread on tissue paper in order to increase the contact between the lipid and the oxygen. For lard a five- to eight-fold increase in the rate was obtained in this way.

Three problems occur with this method. Firstly, the temperature is too high. Secondly, as in the Eckey method, the degree of oxidation of the sample at the end-point depends on the % PUFA in the lipid. When a 6 g sample of 10 % PUFA-lipid is to be tested in a 200 ml bomb the sample will be about 40 % oxidised at the end of the induction period, if one assumes a total pressure drop of 2 psia. Finally, unwanted contaminants can be introduced from the tissue paper.

Pohle *et al.* (1963) proposed a modification whereby a fat soluble copper-salt was added to the oil at a level of 25 or 100 ppm. Approximately a ten-fold increase in the rate was obtained in this manner. This method is not generally accepted.

(e) *Other methods*

A number of other methods have been suggested for specific applications. The Weight-Gain Technique (Sherwin, 1968) is based on the increase in weight of the lipid as it continues to absorb oxygen. This method is not recommended. Unless the weight-gain is calibrated against oxygen uptake data the method is meaningless.

A variety of methods have been proposed specifically to evaluate antioxidant effectiveness. Cort *et al.* (1975) used a modification of the Schaal Oven Test (Lea's version) which is carried out at 45 °C and which they term the Thin Layer Test. This method is strongly recommended, but it will be slow when potent antioxidants are tested.

Because of the problems experienced with the high-temperature ASLT methods, alternative means of acceleration have received more interest recently. Most of these tests use some form of metal-containing pro-oxidants. According to Betts & Uri (1968) metal ions, when they act as pro-oxidants, usually increase the rate in proportion to their concentration to the one-half or first power. Hence the addition of such pro-oxidants can be used to give a powerful catalytic effect.

Uri (1961*b*) has pointed out that since the temperature coefficients of antioxidant efficiency vary with the nature of the antioxidant, high-temperature ASLT studies are open to criticism. Furthermore, since lipid oxidation is generally trace-metal catalysed, adding metallic pro-oxidants may be a more meaningful method of acceleration. The author used ferrous phthalocyanine as a catalyst for linoleic acid

oxidation in an ethyl benzoate solvent at 25 °C. He compared the effectiveness of five antioxidants (propyl gallate and four flavonoids) at a concentration close to 50 ppm. The order of effectiveness was found to be similar to that obtained using a control containing no added pro-oxidant. It should be pointed out that the advantage of using complexed iron is that it is equally effective in the presence of metal chelating agents.

Berner *et al.* (1974) used hemin catalyst which they added to a lipid emulsion at 45 °C, pH 7·2 and measured the oxygen uptake using an oxygen analyser. The method was used for lard containing 0·02 % of one of several antioxidants. The AOM method was used for comparison. As expected, the two methods ranked the antioxidants quite differently. In particular the more water-soluble antioxidants (PG and TBHQ) were much less effective in the former method, probably in part because of their higher partition into the water phase.

The use of metallic pro-oxidants in ASLT studies, perhaps in conjunction with a moderate temperature elevation, should be given careful consideration. One of the problems involved is that some foods contain higher levels of endogenous metals than others. Therefore, different amounts must be added to different foods to give the same percent acceleration. If metal salts are used, problems will be encountered when metal-chelating compounds are present. By using metal complexes such as phthalocyanins or hematin-catalysts, this may be avoided. However, the possibility of a change in the mechanism can become much greater, e.g. when hematins are used (Kendrick & Watts, 1969). In selecting a suitable catalyst, the emphasis should be on using one which is already present in the product and which is likely to be the dominating catalyst for lipid oxidation in that particular food. One would question, therefore, the use of heme-catalysts for accelerating the oxidation of vegetable oils.

ASLT STUDY RESULT ANALYSIS

Unfortunately, ASLT studies where the overall rate constants K_A and K_M are calculated are almost non-existent. In calculating E_A-values for such studies one must rely on data for the induction period obtained. The use of different end-points and the concurrent presence of other acceleration parameters makes the calculated values inaccurate at best. An added obstacle, when it comes to interpreting the data, is the loose characterisation of the substrates given. Neither trace metal content nor the fatty acid distribution is generally known. Obscure terms such as 'stabilised lard,' 'shortening A' and 'hydrogenated vegetable oil' are often the only information given.

Because of the different temperatures used for different methods it is convenient to divide this survey into two parts depending on the T-zone under consideration.

(a) *Ambient to* 65 °C

In this range the Shelf-Life Study carried out at or close to room temperature and the Schaal Oven Test are the most commonly used methods. The most interesting data available are those of Pohle *et al.* (1964) who compared various different ASLT methods. When comparing Shelf-Life Test data collected at 29·5 °C with data obtained using the Schaal Oven Test at 60 °C an E_A-value of about 14 kcal/mole can be calculated for both lard and tallow. When 0·01 % BHA was added, the value increased to 18–20 kcal/mole. In contrast, the E_A for hydrogenated vegetable oil (HVO) was about 18–20 and did not change when BHA was added. These results are in line with what would be predicted on the basis of eqns. (12) and (14) and the fact that most vegetable oils already contain close to an optimum concentration of primary antioxidants (tocopherols).

(b) *Ambient to* 100 °C

The most important ASLT methods carried out in the vicinity of 100 °C are the AOM, OBM and the two oxygen absorption methods. Of these methods only the OBM, especially if used with a dispersant, will be close to giving oxygen-independent kinetics. The dispersant, however, is bound to be a source of pro-oxidative contaminants. Of the other methods the AOM is intermediate and the absorption methods are the most severely oxygen-dependent.

Pohle *et al.* (1962) found the Eckey OAM at 100 °C to be twice as fast as the AOM. Stuckey *et al.* (1958) found the OBM *without* a dispersant to be 1·4 times faster than the AOM when lard was the substrate. *With* a dispersant the OBM was three to six times faster still. Based on these considerations, the OBM seems to be the best of the high-temperature ASLT methods.

Pohle *et al.* (1964) made extensive comparisons between different high-temperature ASLT methods and concluded that the OBM was the most precise and gave the best correlation with product stability. When comparing their shelf-life test data at 29·5 °C with AOM data, the calculated E_A for lard is 20 and for tallow 14 kcal/mole. In the presence of 0·01 % BHA these values dropped to 16 and 11 kcal/mole, respectively instead of increasing as expected. In this case, predictions about antioxidant effectiveness, based on the control, would cause the degree of protection afforded by the antioxidant under normal storage conditions to be overestimated. Why the E_A dropped is unexplainable.

When the OBM-data for these same fats are compared to the Shelf-Life Test data the calculated E_A becomes about 19 kcal/mole for lard and 10 kcal for tallow. The corresponding values in the presence of 0·01 % BHA are 19 and 13 kcal/mole. Similarly, the value of HVO is 16 kcal/mole both with and without added BHA.

Thompson & Sherwin (1966) collected data at 43·3 °C (storage studies) and 98·9 °C (AOM) for safflower oil. Their data show that E_A can vary from 13–24 kcal/mole, depending on the antioxidant used. Generally, however, the value

increased from 17 for the control to 19–20 when antioxidants were added. In this case predictions would lead to underestimation of protection at room temperature.

Paul & Roylance (1962) collected data at 10–20 °C (Shelf-Life Test) and 98·4 °C (AOM) for two batches of peanut oil: (1) factory oil, refined in iron vessels and (2) further purified oil, using batch 1 as starting material. The E_A for the batch 1 control was about 9 and dropped to 7·5 kcal/mole in the presence of some primary antioxidants (0·01 % level). This difference may seem slight, but in this case indicated a 60–70 % increase in the AOM-induction period whereas no corresponding increase in shelf-life occurred. For batch 2 the E_A for the control was about 17 kcal/mole, indicating that the purification procedure (Crossley *et al.*, 1962) removed active pro-oxidants, possibly trace metals. In the presence of primary antioxidants (0·01 or 0·02 % level) the E_A appears to have been considerably lower.

The data of Paul & Roylance (1962) show that the AOM is unreliable, not only for testing antioxidants but also for testing batches of oil processed in different ways. The shelf-life test data collected indicated that the purification procedure increased the shelf-life of the control—in some cases by over 100 %—while the AOM data showed a dramatic drop in the induction period and thus a shorter shelf-life.

These results illustrate that temperature-accelerated test procedures for rancidity are generally more accurate for vegetable oils and stabilised animal fats. However, even for such lipids, high temperature test procedures can give very misleading results. In some cases the protection at 100 °C is greater than at room temperature, whereas in other cases the reverse is true. The Schaal Oven Test seems to yield much more predictable results although more data are needed to prove this. It seems quite possible that in this test one may be able to correct for E_A-differences between samples with and without antioxidants using eqns. (12) and (14).

The erratic behaviour at high temperatures is undoubtedly caused at least in part by the increased importance of pro-oxidative side-reactions of the antioxidants, when the antioxidant radical, A·, becomes an effective chain carrier as discussed above, causing a change in mechanism of antioxidation and possibly a drop in the activation energy. It seems that in the studies reviewed here, these reactions are not sufficiently important at 60 °C to greatly affect the results. Shelf-life studies for both model systems and foods are currently being carried out in our laboratories using oxygen absorption in the range 25–45 °C. Results will be presented in the future.

ACKNOWLEDGEMENT

This project was supported in part by project 18-72 of the University of Minnesota Agricultural Experiment Station, contract NAS-9-12560, Lyndon Johnson Space Center, Houston, Texas and a grant-in-aid from the Pillsbury Co., Minneapolis, Minnesota. This paper is scientific paper No. 9583 of the University of Minnesota Experiment Station.

REFERENCES

BATEMAN, L., BOLLAND, J. L. & GEE, G. (1951). Determination of absolute rate constants for olefinic oxidations by measurement of photochemical pre- and after-effects. Part II—At 'low' oxygen pressures. *Trans. Faraday Soc.*, **47**, 274–85.

BATEMAN, L. & GEE, G. (1951). Determination of absolute rate constants for olefinic oxidations by measurement of photochemical pre- and after-effects. Part I—At 'high' oxygen pressures. *Trans. Faraday Soc.*, **47**, 155–64.

BATEMAN, L. (1954). Olefin oxidation. *Quart. Reviews*, **8**, 147–67.

BENNETT, J. E. (1964). Studies of the oxygen bomb method for determining shortening stabilities, *J.A.O.C.S.*, **41**, 505–7.

BERNER, D. L., CONTE, J. A. & JACOBSON, G. A. (1974). Rapid method for determining antioxidant activity and fat stability, *J.A.O.C.S.*, **51**, 292–6.

BETTS, A. T. & URI, N. (1968). Catalyst-inhibitor conversion in autoxidation reactions, *Adv. Chem. Series*, (75) (Americ. Chem. Soc.), 160–81.

CHENIER, J. H. B., FURIMSKY, E. & HOWARD, J. A. (1974). Arrhenius parameters for reaction of tertbutylperoxy and 2-ethyl-2-propylperoxy radicals with some nonhindered phenols, aromatic amines and thiophenols, *Can. J. Chem.*, **52**, 3682.

CORNELL, D. G., DEVILBISS, E. D. & PALLANSCH, M. J. (1970). Partition coefficients of some antioxidants in butteroil-water model systems, *J. Dairy Sci.*, **53**, 529.

CORT, W. M., SCOTT, J. W. & HARLEY, J. H. (1975). Proposed antioxidant exhibits useful properties, *Food Technol.*, **29**, 46.

CROSSLEY, A., DAVIES, A. C. & PIERCE, J. H. (1962). Keeping properties of edible oils. Part III. Refining by treatment with albumin, *J.A.O.C.S.*, **39**, 150–65.

DUGAN, L. (1955). Stability and rancidity, *J.A.O.C.S.*, **32**, 605–9.

ECKEY, E. W. (1946). An oxygen absorption method for examination of fat, *Oil and Soap*, **23**, 38–45.

ERICKSSON, C. E., OLSSON, P. A. & SVENSSON, S. G. (1971). Denatured hemoproteins as catalysts in lipid oxidation, *J.A.O.C.S.*, **48**, 442.

GEARHART, W. M., STUCKEY, B. N. & AUSTIN, J. J. (1957). Comparison of methods for testing the stability of fats and oils, and of foods containing them, *J.A.O.C.S.*, **34**, 427–30.

GROSCH, W. (1975). Auflauf und Analytik des oxydativen Fettverderbs, *Z. Lebensm. Unters.-Forsch.*, **157**, 70–83.

HARTMAN, L., ANTUNES, A. J., GARRUTI, R. S. & CHAIB, M. A. (1975). The effect of free fatty acids on the taste, induction periods and smoke points of edible oils and fats, *Lebensm.-Wiss. u.-Technol.*, **8**, 114–18.

HOWARD, J. A. (1973). Homogeneous liquid-phase autoxidation. In: *Free radicals*, ed. J. K. Kochi. New York, Interscience, Vol. II, 4–61.

HOWARD, J. A. & FURIMSKY, E. (1973). Arrhenius parameters for reaction of tert-butylperoxy radicals with some hindered phenols and aromatic amines, *Can. J. Chem.*, **51**, 3738–45.

INGOLD, K. U. (1968). Inhibition, *Adv. Chem. Series* (75) (Amer. Chem. Soc.), 296–305.

INGOLD, K. U. (1970). The inhibition of autoxidation by phenols. In: *Essays in free-radical chemistry*, Spec. Publ. 24 (Chem. Soc.), 285–93.

INGOLD, K. U. (1973). Rate constants for free radical reactions in solution. In: *Free radicals*, ed. J. K. Kochi, New York, Interscience, Vol. I, 36–113.

JOYNER, N. T. & MCINTYRE, J. E. (1938). The oven test as an index of keeping quality, *Oil and Soap*, **15**, 184–6.

KENDRICK, J. & WATTS, B. M. (1969). Acceleration and inhibition of lipid oxidation by heme compounds, *Lipids*, **4**, 454–8.

KING, A. E., ROSCHEN, H. L. & IRWIN, W. H. (1933). An accelerated stability test using the peroxide value as an index. *Oil and Soap*, **10**, 105–9.

KIRIGAYA, N., KATO, H. & FUJIMAKI, M. (1968). Studies on antioxidant activity of nonenzymic browning reaction products. Part I. Relations of color intensity and reductones with antioxidant activity of browning reaction products, *Agr. Biol. Chem.*, **32**, 287–90.

KLAUI, H. (1971). The functional (technical) uses of vitamins. In: *Vitamins*, University of Nottingham Seminar, ed. M. Stein, London, Churchill Livingstone, 110–41.

KOCHI, J. K. (1973). Oxidation-reduction reactions of free radicals and metal complexes. In: *Free radicals*, ed. J. K. Kochi, New York, Interscience, Vol. I, 591–683.

KORCEK, S., CHENIER, J. H. B. & INGOLD, K. U. (1972). Absolute rate constants for autoxidation. XXI. Activation energies for propagation and correlation of propagation rate constants with carbon-hydrogen bond strengths, *Can. J. Chem.*, **50**, 2285–97.

LABUZA, T. P. (1971). Kinetics of lipid oxidation in foods, *CRC Crit. Rev. Food Technol.*, **2**, 355–404.

LABUZA, T. P. (1972). Mechanisms of deterioration of intermediate moisture foods, NASA, *Contractor Report*, NASA CR-114861.

LEA, C. H. (1960). Antioxidants in dry fat systems: Influence of the fatty acid composition of the substrate. *J. Sci. Food Agr.*, **11**, 143–50.

LEA, C. H. (1962). The oxidative deterioration of food lipids. In: *Lipids and their oxidation*, ed. H. W. Schultz, the AVI Publishing Co., Inc., 3–28.

LOVE, J. D. & PEARSON, A. M. (1974). Metmyoglobin and nonheme iron as pro-oxidants in cooked meat, *Agr. Food Chem.*, **22**, 1032–4.

LUCKMANN, F. H., MELNICK, D. & MILLER, J. D. (1953). A new accelerated holding test involving aeration of oils in iron tubes, *J.A.O.C.S.*, **30**, 602–6.

MAHONEY, L. R. (1969). Antioxidants, *Angew. Chem.* Internat. Edition. **8**, 547–55.

MARTIN, H. F. (1961). A modified recording oxygen absorption apparatus for the determination of oxidative stability of lipid material, *Chem. and Ind.*, (10) 364–7.

PAUL, S. & ROYLANCE, A. (1962). Keeping properties of edible oils.—Part I. The use of accelerated tests for assessment of keeping properties of oils and the value of antioxidants, *J.A.O.C.S.*, **39**, 163–5.

PIERCE, J. H., CROSSLEY, A. & DAVIES, A. C. (1962). Keeping properties of edible oils. Part II Refining by treatment with alumina, *J.A.O.C.S.*, **39**, 165–8.

POHLE, W. D., GREGORY, R. L. & TAYLOR, J. R. (1962). A comparison of several analytical techniques for prediction of relative stability of fats and oils to oxidation, *J.A.O.C.S.*, **39**, 226–9.

POHLE, W. D., GREGORY, R. L. & VAN GIESSEN, B. (1963). A rapid oxygen bomb method for evaluating the stability of fats and shortenings, *J.A.O.C.S.*, **40**, 603–5.

POHLE, W. D., GREGORY, R. L., WEISS, T. J., VAN GIESSEN, B., TAYLOR, J. R. & AHERN, J. J. (1964). A study of methods for evaluation of the stability of fats and shortenings, *J.A.O.C.S.*, **41**, 795–8.

RAWLS, H. R. & VAN SANTEN, P. J. (1970). Singlet oxygen: A possible source of the original hydroperoxides in fatty acids, *Ann. N.Y. Acad. Sci.*, **171**, 135–7.

RIEMENSCHNEIDER, R. W., TURER, J. & SPECK, R. M. (1943). Modifications of the Swift Stability Test, *Oil and Soap*, **20**, 169.

SCOTT, G. (1965). Atmospheric oxidation and antioxidants. Amsterdam, Elsevier Publishing Co.

SHERWIN, E. R. (1968). Methods for stability and antioxidant measurement. *J.A.O.C.S.*, **45**, 632A–49A.

STUCKEY, B. N., SHERWIN, E. R. & HANNAH, F. D. (1958). Improved techniques for testing fats and oils by the oxygen bomb method, *J.A.O.C.S.*, **35**, 581–4.

SYLVESTER, N. D., LAMPITT, L. H. & AINSWORTH, A. N. (1942). Determination of the stability of oils and fats, *J. Soc. Chem. Ind.*, **61**, 165–9.

THOMPSON, W. & SHERWIN, E. R. (1966). Investigation of antioxidants for polyunsaturated edible oils, *J.A.O.C.S.*, **43**, 683–6.

URI, N. (1961*a*). Physico-chemical aspects of autoxidation. In: *Autoxidation and antioxidants*, ed. W. O. Lundberg, Vol. X, New York, Interscience, 55–106.

URI, N. (1961*b*). Mechanism of antioxidation. In: *Autoxidation and antioxidants*, ed. W. O. Lundberg, Vol. I, New York, Interscience, 133–69.

WATERS, W. A. (1971). The kinetics and mechanism of metal-catalyzed autoxidation, *J.A.O.C.S.*, **48**, 427–33.

GELATINISATION OF STARCH AND WHEAT FLOUR STARCH—A REVIEW

JUHANI OLKKU†

Department of Food and Agricultural Engineering, University of Massachusetts, Amherst, Massachusetts 01002, *USA*

&

CHOKYUN RHA

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, *USA*

(Received: 27 February, 1978)

ABSTRACT

Gelatinisation of wheat starch is reviewed, making comparisons with other starches.

Starch in wheat kernel endosperm is briefly described. Various aspects of gelatinisation of starch in water are considered, including swelling and interaction with water before the onset of gelatinisation, increase in consistency during gelatinisation, gelatinisation temperature, types of gelatinised system and gel and paste formation upon cooling. In addition, the traditional rationale of gelatinisation and gelatinisation from the viewpoint of water mobility are discussed.

Experimental methods used to study gelatinisation are also reviewed, as are the effects of starch concentration, protein, pentosans, surface active agents, fats, salt and sugars on gelatinisation and shear rate on consistency.

INTRODUCTION

Gelatinisation of starch is a basis for many types of food production. Processes such as the baking of bread, the gelling of pie fillings, the production of pasta products, the fabrication of starch-based snack foods and the thickening of sauces are all dependent on proper gelatinisation of starch to produce a desirable texture or consistency of the end product.

† Present address: Technical Research Centre of Finland, Food Research Laboratory, 02150 Espo 15, Finland.

Fd. Chem. (3) (1978)—
Printed in Great Britain

Food industries make use of several types of starch. Tuber starches, such as potato and tapioca, and grain starches, such as corn and wheat, are the most commonly used. Since the availability and cost of starches vary from district to district, as does their practical utility, a great deal of research has been aimed at differentiating various starches and their gelatinisation behaviour. However, the basic phenomenon of gelatinisation itself seems to require a more thorough explanation. Miller *et al.* (1973) give an explanation for the rise in consistency during gelatinisation. Studies by Jaska (1971) suggest further considerations for research in this area.

The main emphasis of this review is on the gelatinisation phenomenon of wheat starch and on the influence of the amount of water and various other ingredients, as well as constituents of wheat kernel, on gelatinisation of this starch. Other starches will only be referred to by way of comparison, or to aid in explaining the basic phenomenon.

Starches are often chemically modified for special purposes. Although modified starches are gaining importance, they have not been included in this study.

THE WHEAT STARCH IN THE WHEAT KERNEL ENDOSPERM

Starch granules in the wheat grain

The wheat grain can be roughly divided into three parts: (1) germ, 2%; (2) endosperm, 85% and (3) husk, 13%. Modern methods of milling are designed to produce flour from the endosperm which is free of germ and husk. The wheat endosperm has a cellular structure and each cell is filled with starch granules, contained within thin cellulose walls and varying in diameter from 1 to 40 microns. Between these starch granules is a proteinaceous material which contains, in addition to proteins, the minerals, colouring matter and enzyme of the endosperm (Knight, 1965). In an electron microscope study by Barlow *et al.* (1973), the entire area between starch granules in the endosperm was found to be filled with material that stained as protein. Water-soluble proteins are confined to locations immediately surrounding the granules and this area is capable of rapid swelling upon hydration. There is evidence of residues of the original amyloplast membranes, rich in lipids separating the starch and protein, as well as those of endoplasmic reticulum, existing around the starch granule.

The soluble proteins associated with starch granules form an electrophoretically complex group. Associated with them are carbohydrates which give rise to glucose on hydrolysis. The total water-soluble material appears to play the role of a cementing substance between starch granules and storage protein. Part of the endosperm carbohydrates are pentosans (Jelaca & Hlynka, 1971). Wheat flour contains about 0·5 to 0·8% of water-soluble pentosans and 2 to 3% in total of water-soluble and insoluble pentosans.

Wheat starch contains about 30% amylose and 70% amylopectin. Commercially available wheat starches usually have about 12% moisture and 0·2% protein (Knight, 1965).

Starch granules

Starch granules vary in size and shape and every starch can be classified as to origin by means of microscopic inspection. Wheat starch is unusual in that the size of the granules can vary greatly. The smallest granules are about 2 microns and the largest about 35 microns in diameter. By comparison, corn starch has more uniform granules about 15 microns in diameter and potato starch consists mainly of large granules about 15 to 100 microns in diameter.

The structure of the starch granule is usually taken to be amorphous near the surface of the granule and the amylopectin and amylose macromolecules are thought to be of a crystalline nature inside the granule. The granule is then totally or partially covered with protein, as described above, which explains why starch always contains some protein when manufactured industrially.

GELATINISATION OF STARCH IN WATER

Swelling and interaction with water before the onset of gelatinisation

Wheat starch is generally considered to be insoluble in water (Knight, 1965). This may be correct for practical purposes but interaction of starch and water does occur before the initial temperature of gelatinisation is reached, as detected by the disappearance of polarising crosses under a microscope, or by Amylograph rise in consistency.

Gough & Pybus (1971) treated undamaged wheat starch granules with 50 °C water for 72 h. The gelatinisation temperature increased and gelatinisation occurred more suddenly than in the control material, indicating modification of the internal structure of the granules.

Kerr (1950) distinguishes various phases in the breakdown of the starch molecule structures. In the first phase, which occurs before the onset of gelatinisation, water is slowly and reversibly taken up. This has been confirmed by Jaska (1971) in his study using Proton Magnetic Resonance (PMR). At this stage the mobility of water decreases as the temperature is increased from 20 to 60 °C and it is thought that water is being reversibly complexed with the starch molecules in the granule. Although this change is reversible at temperatures below the onset of gelatinisation, continued exposure of the starch to water can cause changes in the granule itself (Gough & Pybus, 1971).

Gelatinisation phenomena

Traditional theoretical rationale of gelatinisation: Glicksman (1969) gives a traditional explanation of starch gelatinisation. The constituent molecules in the

starch granules are held together by hydrogen bondings. When aqueous suspensions of starch granules are heated, a temperature is reached at which hydrogen bonding forces are weakened to the point where water can be absorbed by the granules. At this temperature, called the 'initial gelatinisation temperature', the granules swell tangentially and simultaneously lose their birefringence. These phenomena start at the hilum or botanical centre of the granules and spread rapidly to the periphery. Gelatinisation begins in the intercellular areas where the hydrogen bondings are weakest. It occurs in different temperature ranges for different starches. Values for wheat starch are: initial gelatinisation temperature, 58 °C; midpoint, 61 °C and end point, 64 °C.

As the temperature of the aqueous suspension is increased above the gelatinisation range, hydrogen bondings continue to be disrupted, water molecules become attached to hydroxyl groups and the granules continue to swell. As a direct result of granule swelling, there is an increase in starch solubility, paste consistency and paste clarity. With the continued swelling of granules, starch particles that have become fully hydrated separate themselves from the intricate micellar network and diffuse into the aqueous medium.

In a concentrated starch paste, the individual granules gelatinise and swell freely until all the available water has been imbibed. As they swell, the swollen starch granules become increasingly susceptible to shear disintegration. The bonding forces in the granule also become weaker as heating is continued and the susceptibility of the granule to mechanical and thermal breakdown increases. When the granules have swollen to occupy the entire volume, some of the starch solubles, which had earlier diffused into the surrounding aqueous region, may now diffuse back into the highly swollen granules. The system becomes a gel-like mass held together by associative bondings. The hot starch pastes may be viewed as a mixture of swollen starch granules and granule fragments, together with colloidally and molecularly dispersed starch molecules.

Upon cooling, the starch polymers tend to associate or retrogradiate to give a substantial increase in consistency. This phenomenon is governed by the concentration of amylose, the length of amylose chains and the state of dispersion of amylose chains.

In summary, the gelatinisation of starch takes place as follows:

(1) Granules hydrate and swell to several times their original size.
(2) Granules lose their birefringence.
(3) Clarity of the mixture increases.
(4) Marked, rapid increase in consistency occurs and reaches a peak.
(5) Linear molecules dissolve and diffuse from ruptured granules.
(6) Mixture retrogrades to a paste-like mass or gel.

This behaviour has been described more succinctly by Bechtel *et al.* (1964). Wheat starch swells in two stages:

(1) A small amount of swelling between 60 and 70 °C, involving disruption of weakly bound or readily accessible amorphous sites.
(2) Subsequent rapid swelling at 80 to 90 °C, involving disruption of more strongly bound or less accessible sites.

Upon continued heating, the swollen granules disrupt into fragments. This fragmentation is distinct from the stages discussed above.

Gelatinisation and water mobility: Jaska (1971) studied gelatinisation of starch with the PMR technique to complete the traditional theory, but he also contradicts it regarding the order of hydration of starch molecules and their physical changes inside the granule. The PMR method gives information on the mobilisation of starch and water molecules during the gelatinisation process.

When the temperature of a starch suspension is increased but remains below the gelatinisation range, water mobility is reversibly decreased, indicating the adsorption of water to starch molecules. At the gelatinisation stage the mobilisation of water increases, indicating rapid transfer of soluble starch into solution. Later there is a slight increase in mobilisation of water and starch. It is also concluded that the structure of the water–starch complex may extend well beyond the water molecules which are directly bonded to starch.

When 40 % potato starch was slowly heated, a rapid drop in PMR line width was observed at 58 °C, which is thought to be the onset of gelatinisation and the transfer of soluble starch into solution. In a 20 % starch solution, the mobility of water is similar to that of free water until, at the onset of gelatinisation, the 20 % sample shows a radical decrease in water mobilisation. This is attributed to a sudeen increase in hydration together with a decrease in water mobilisation. At temperatures above gelatinisation, the mobility of water slowly increases. This slow increase in water mobility is probably due to a decrease in the microviscosity of starch granules with increasing temperature. The average water mobility will depend upon the ratio of water molecules in close association with starch to total water. It will also depend on the mobility of the starch molecules with which the water molecules are associated.

Jaska (1971) concludes that part of the starch is in solution inside the granule before it escapes out of the granule. Most of the soluble starch solubilises over a relatively narrow temperature range. Only part of it escapes the granule to be measured in the first stage of solubilisation. As the temperature is increased, the insoluble granule breaks down, releasing the solution inside the granule at the second stage of solubilisation.

Increase in consistency during starch gelatinisation

The increase in consistency that occurs when starch suspensions are heated was traditionally attributed to granules imbibing more and more water as they swell. They thus increase the chances of starch granules coming into contact with each

other (Miller *et al.*, 1973). Theoretically this is a plausible explanation for the increase in consistency, since increase in consistency is a measure of hydrodynamic volume and of the work required to move granules past each other as they continue to expand (Rha, 1975).

However, Miller *et al.* (1973) have studied the increase in consistency of heated wheat starch water suspensions, using light micrographs and scanning electron microscopy (SEM) of freeze dried samples, taken at different stages of heating. It was found that the maximum consistency of wheat starch suspensions occurs after most of the granule swelling ceases. The increase in consistency is due mainly to the exudate, which appeared as a filamentous network when a fully heated starch suspension was freeze dried. When a network was not observed, there was no increase in consistency. Thus, consistency of a wheat starch paste is not due to increased size of granules but mainly to the exudate network in the fluid. These same results appeared in the limited tests with corn, waxy maize and potato starch.

Starch granules swell equally in the presence or absence of sodium steryl fumarate up to 70 °C. At 95 °C the granules are much larger in diameter in the presence of this agent. Without sodium steryl fumarate, granules break down to about one-third of their original size. However, the consistency of the mixture with sodium steryl fumarate is much less than that without it. This is further evidence that the sharp increase in consistency of a cooked starch is not a result of the swelling and increase in starch particle size but rather of a network formed by the exudate.

If a large amount of water is present, the exudate is released in amounts sufficient to make consistency a function of temperature alone. The exudate is released in relatively large amounts as the temperature approaches 90 °C (Longley & Miller, 1971). It forms a continuous and complex filamentous network throughout the suspension upon freeze drying. The extent of the contribution of swollen granules to the consistency remains to be determined.

Types of gelatinised system and gel and paste formation upon cooling

The behaviour of starch is governed to a large extent by the affinity of hydroxyl groups in one molecule for those in another (Glicksman, 1969). This is particularly true when starch pastes cool. When starch granules cool, randomly oriented starch molecules tend to aggregate and crystallise out of the solution. In the linear starch amylose the straight chains can orient themselves in parallel fashion with large numbers of hydroxyl groups in one chain in close proximity to those in adjacent chains. The chains are bound together with hydrogen bondings to form aggregates of low solubility. If solutions are sufficiently concentrated, gels are formed. This process is known as retrogradation, which is basically a crystallisation phenomenon and an important factor in the use of starch.

The amylose fraction is considered to be primarily responsible for gel formation. It is able to form gels at a concentration as low as 1·5%. Using corn starch, the amount of amylose needed for gels of equivalent strength is about three times

greater in the absence of amylopectin than in its presence. The results of Ott & Hester (1965) confirm the dual role of amylose in gel formation: (1) as the chief material for forming gel networks to entrap unabsorbed water and (2) as a binding material linking intact starch granules or fragments of granules. The swelling pattern of starch granules appears to determine which role of the linear fraction is more significant.

Gelatinisation is believed to result from the formation of a three-dimensional network which binds the swollen granules. This network is assumed to be crystalline in nature and is thought to consist of segments of linear and branched molecules forming a continuous structure (Miller *et al.*, 1973). Parts of one molecule may be present in several crystalline regions.

Ott & Hester (1965) specify four types of gel system (Fig. 1). Figure 1(A) shows a gel system containing amylose with water trapped or bound in a three-dimensional network. If granular starch is present to provide some structure, a smaller amount of amylose is needed to form a gel.

Figure 1(B) shows a gel with highly swollen and fragmented starch granules. A high proportion of water is held by the fragments. Amylose is necessary to join the fragments into a continuous network. Since the number of fragments is large, a high proportion of amylose is required.

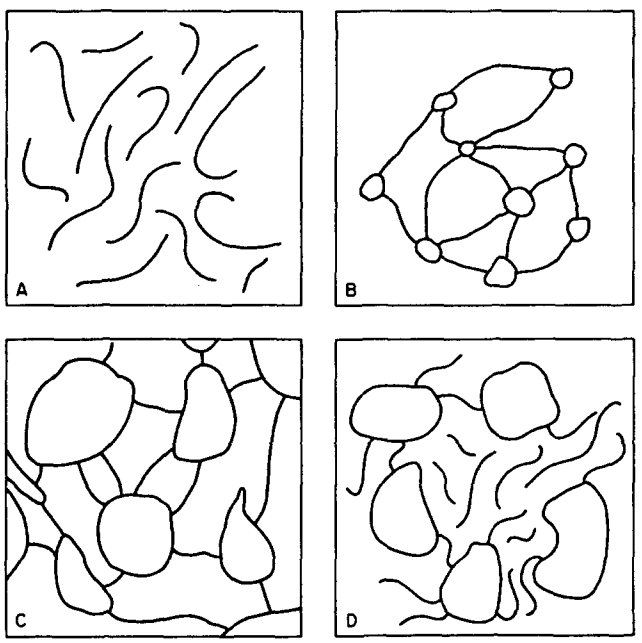


Fig. 1. Different types of starch gels. (Adapted from Ott & Hester (1965).)

Figure 1(C) shows a gel with highly swollen and intact granules. A high proportion of water is held by the granules. Only a limited amount of amylose is needed to join the large particules into a continuous network.

Figure 1(D) shows a gel with intact starch granules which are not highly swollen. Amylose is necessary to bind or entrap free water and to join granules in a continuous network.

Thus, the factors involved in the formation and determining characteristics of starch gels are: the type and size of starch granules, as well as their age and previous treatment; the paste concentration; cooking time and temperature; agitation during cooking; time and temperature of storage after cooking and types and amounts of added ingredients.

The changes in gel structure brought about by increased crystallisation or retrogradation of starch molecules may be viewed as a continuation of those changes converting the viscous paste into a gel. In food processing these are generally undesirable, such as skin formation on puddings and weeping in gravy and pie fillings.

Study of starch gelatinisation

Several methods have been used to study starch gelatinisation. Kofler hot-state microscopy has been used in a method described by Watson (1964). Other microscopic measurements have been reported by Seidemann (1963) and by Kainuma *et al.* (1968), who used photopastegraphs. Miller *et al.* (1973) also used photomicrographs which are photographs, taken through a microscope, of the samples taken during gelatinisation in an Amylograph. Morgan (1940) has used photometric measurements to determine gelatinisation temperatures.

Scanning electron microscopy (SEM) of freeze dried Amylograph samples was studied by Miller *et al.* (1973). Although SEM can demonstrate in detail the arrangement of structure in the various stages of gelatinisation, the preparation of the sample for the microscopy may alter it so that it is no longer representative of the actual starch gel.

As mentioned earlier, Jaska (1971) used PMR to study water mobilisation during gelatinisation. This technique has a great potential for studying phenomena during the gelatinisation process.

The most widely used methods for studying the gelatinisation process have been those based on the changes in consistency during gelatinisation. Examples of commercially available instruments are the Corn Industries Viscograph, used by Bean & Osman (1959) and the MacMichael instrument, used by Dahle (1971). Most commonly used is the Brabender Viscoamylograph or Amylograph.

A Viscoamylograph records the force induced by a starch slurry on a five spindle sensing head. The slurry is heated at the rate of 1·5 °C a minute in a cylindrical container with several spindles having a capacity of about 500 ccm. In more recent

models the rotation speed of the sample holder can be varied from 30 to 150 rpm. The usual means of recording the force is by means of a spring-loaded cartridge. Voisey & Nunes (1968) have converted the instrument to electronic recording, thus improving its accuracy and expanding the range of measurement.

The Brabender Viscoamylograph has one disadvantage as an instrument for measuring the rheological behaviour of starch suspensions. The instrumental design is such that analysis of the stress fields—and of the complex property consisting of shear, compression and elongation—is not possible. Interpretation of Brabender curves is discussed by Mazurs *et al.* (1957), Elder & Schoch (1959), Schoch (1961), Greenwood (1964), Sandstedt & Abbott (1964) and Smith (1967), among others.

According to Glicksman (1969), the method of Schoch & Maywald (1956) is widely used in industry and gives a great deal of information on each Brabender curve. Some typical Brabender curves are given in Fig. 2. A curve for wheat starch has been superimposed (Knight, 1965).

The Brabender curves show five critical regions, A, B, C, D and E, as illustrated in Fig. 2 and described below.

(A) The 'peak consistency', or the highest consistency, irrespective of temperature, encountered during preparation of a paste.
(B) The ease of cooking the starch, which is shown by the consistency of the paste at 95 °C in relation to the peak consistency.
(C) The paste stability or resistance to breakdown, which is shown by consistency after cooking 1 h at 95 °C.
(D) A measure of setback, or gelling ability, which is shown by the consistency of the cooked paste after cooling to 50 °C.
(E) Stability of the cooked starch paste, which is shown by the final consistency after stirring 1 h at 50 °C.

Crossland & Favor (1948) used a technique employing CMC solutions instead of pure water in order to understand the two-phase swelling of starch, i.e. the swelling of amorphous areas at lower temperatures and the subsequent swelling at higher temperatures as discussed above in the section on 'Gelatinisation and water mobility'. The method has been refined by Sandstedt & Abbott (1964). These methods have been used by Medcalf & Gilles (1965) and by Medcalf *et al.* (1968). The method makes a change of slope visible in the curve after the first phase of gelatinisation.

Goto & Yokoo (1969) used a Brabender Plastograph to measure the gelatinisation properties of highly concentrated starch suspensions, a purpose for which the Amylograph is not suitable. The increase in consistency takes place at a lower temperature in the Plastograph. Goto (1969) also found that the Plastograph consistency had nearly reached maximum at the temperature where the Amylograph consistency began to rise. With the method of Goto & Yokoo (1969) wheat starch

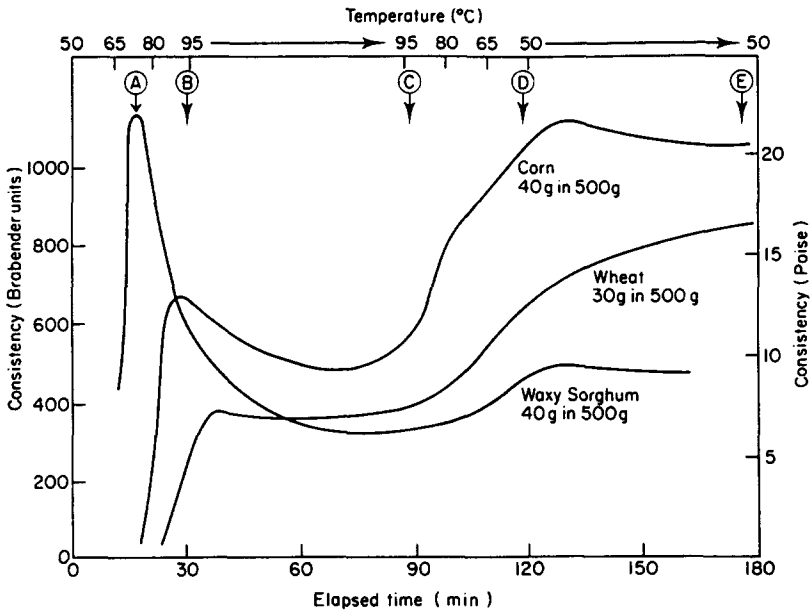


Fig. 2. Brabender curves of some starches. (Adapted from Glicksman (1969) and Knight (1965).)

gives the lowest gelatinisation temperature and highest peak consistency of cereal starches. All cereal starches show a sudden decrease in consistency at about 97 °C. This does not occur with tuber starches. Goto (1969) defines the following parameters from plastograms:

(1) Initial gelatinisation temperature: temperature at which consistency reached 50 BU.
(2) Terminal gelatinisation temperature: temperature at which the curve reaches its peak.
(3) Critical gelatinisation temperature: temperature at which initial consistency of plastogram reaches a minimum or first levels off. This temperature is 42 °C for wheat starch or about 10 °C below the initial gelatinisation temperature.

When examining the gelatinisation temperature, it should be taken into consideration that the microscopic/hot stage gives the gelatinisation temperature which is largely independent of concentration and heating rate but as a function of the granular structure whereas the viscographic determination is dependent on concentration, heating rate and shearing condition. The latter provides more information regarding the subsequent swelling behaviour which has a more practical implication in actual processing.

TABLE 1
GELATINISATION TEMPERATURE OF STARCH[a]

Goto (1969) Temperature (°C)		*Seidemann* (1963) Temperature (°C)		*Schoch & Maywald* (1956) Temperature (°C)	
Initial	*Terminal*	*Initial*	*Terminal*	*Initial*	*Terminal*
54	67	54·5	65·0	52	63
		58·5	65·5		

[a] Adapted from Goto (1969).

Gelatinisation temperatures of wheat starch

Glicksman (1969) gives wheat starch the following range of gelatinisation temperatures: initial, 58 °C; midpoint, 61 °C; endpoint, 64 °C. Some examples of the initial and terminal gelatinisation temperatures are given in Table 1.

Table 2 gives the temperatures of the onset of gelatinisation for 50 % wheat starch suspensions based on the PMR studies of Jaska (1971). He considers the difference

TABLE 2
TEMPERATURE FOR ONSET OF GELATINISATION FOR 50 % WHEAT STARCH SUSPENSION DETERMINED BY PMR[a]

Heating rate	*Temperature for onset of gelatinisation* (°C)
10 °C/5 min (2 °C/min)	61
5 °C/5 min (1 °C/min)	56

[a] Adapted from Jaska (1971).

between his values and those of others to be due to a difference in the definition of gelatinisation. However, his lower heating rate value is in agreement with that of Seidemann (1963) and close to others. Higher onset temperature at higher rate of heating (2 °C/min) (Jaska, 1971) indicates that under the experimental conditions in this concentrated starch suspension the time required to reach equilibrium state for gelatinisation lags behind heating rate.

EFFECT OF CONCENTRATION, SHEAR RATE AND CHEMICAL COMPOUNDS ON GELATINISATION

Effect of starch concentration on gelatinisation

In the past, studies of starch gelatinisation have been concerned mainly with systems of low starch concentrations. However, in the food industries concentrations up to more than 90 % starch are used in a variety of products.

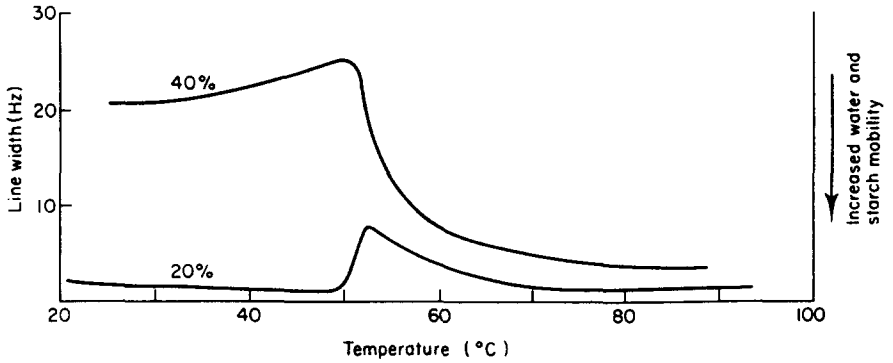


Fig. 3. Schematic diagram PMR for 40% and 20% potato starches. (Adapted from Jaska (1971).)

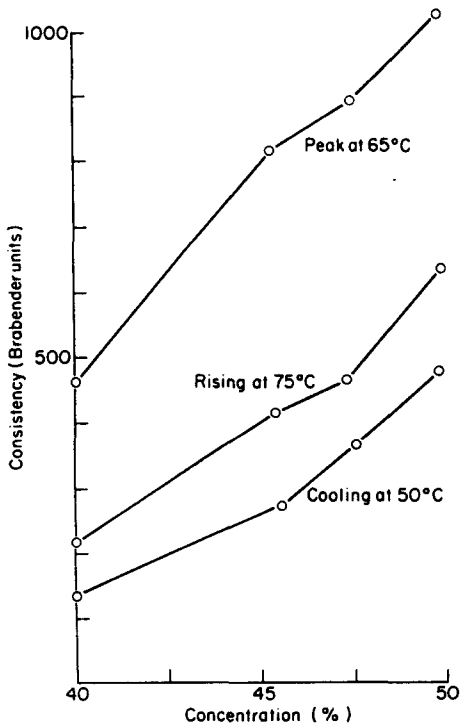


Fig. 4. Consistency of wheat starch as a function of starch concentration in a plastograph. (Adapted from Goto & Yokoo (1969).)

At temperatures below gelatinisation, Jaska (1971) noticed that in 20% starch solutions the water mobility change, as reflected by the PMR line width, followed that of pure water. On the other hand, in the same temperature range in 40% solution, a decrease in water mobility was apparent due to absorption of water by starch. This difference is due to the dominance of free water in the 20% sample. At the onset of gelatinisation, which occurs at the same temperature for both solutions, the 40% sample shows a rapid increase in molecule mobility. This is indicated by a large drop in the water line width and is associated with a rapid increase in hydration of starch macromolecules. The 20% sample shows a decrease in average water mobility which is associated with rapid hydration of starch at this concentration. The average water mobility is thus affected by the ratio of water molecules in association with starch molecules to total water. It will also depend on the mobility of the associated starch molecules. Therefore, it appears that the starch to water ratio is the dominant mechanism for regulating water mobility upon gelatinisation.

After the onset of gelatinisation in both 20% and 40% solutions, the average water mobility slowly increases. Curves for 20% and 40% potato starch solutions are given in Fig. 3.

Goto & Yokoo (1969) studied 40·0, 45·5, 47·6 and 50·0% starch suspensions. The higher the concentration, the lower the temperature of rise in the plastograph curve. Higher concentrations thus lowered the gelatinisation temperature and led to a higher peak consistency, as one would expect (Fig. 4).

The consistency of the paste is nearly proportional to the concentration of the starch. In Fig. 4 only wheat-starch is shown, but corn and potato starches show a similar behaviour. The temperatures at which peak consistency was reached were almost the same for each concentration.

Effect of shear rate on consistency

As previously mentioned, swollen starch particles become vulnerable to shear. Although granules are found to decrease in size after an initial increase (Miller *et al.*, 1973), the quantitative analysis of starch granule size is scarce in the literature.

Goto & Yokoo (1969) studied the effect of rotational speed on consistency of highly concentrated potato starch suspensions (Fig. 5). Temperature of gelatinisation and temperature of peak consistency were the same at all rotational speeds. It was found that increase in rotational speed or shear rate resulted in higher consistencies. The consistency at a given temperature was found to be proportional to the speed of rotation, even within this limited range. Therefore, it is pertinent that the rate of shear be kept constant in comparative studies of starch suspensions. This study did not consider the effect of shear on starch suspension after gelatinisation, which would also be of interest. The effect of shear should be considered when a complex ingredient, such as flour, is used instead of pure starch, since other components such as protein would interact differently with starch.

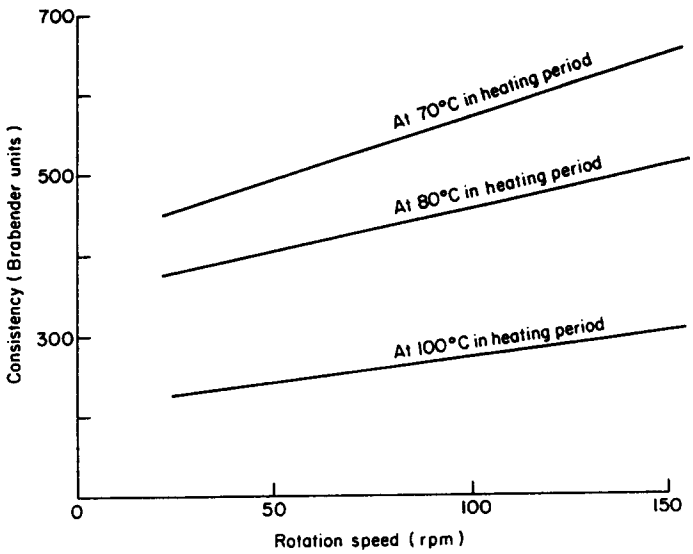


Fig. 5. Effect of rotational speed on consistency of 47·6% potato starch slurry in a plastograph. (Adapted from Goto & Yokoo (1969).)

Effect of protein on starch gelatinisation

Every industrially produced wheat starch contains some protein. Knight (1965) gives a figure of 0·2% as a typical protein concentration of wheat starch. The gelatinisation is more complex when starch in wheat flour is present with the natural protein. While studying the effect of sucrose on the properties of some starches and flours, Hester *et al.* (1956) came to the conclusion that the protein content of flours offers the most obvious explanation for the differences between flours and their starches, although the possible role of other constituents of flour should not be overlooked.

Protein and starch form complexes in the flour while being gelatinised. According to Takeuchi (1969), the starch protein interaction is due to the attraction of opposite charges. Dahle (1971) has studied the starch-binding effects of wheat flour protein from this standpoint and found that association of gelatinised wheat starch and wheat protein occurs at acid and neutral pH but is diminished at alkaline pH. Modification of the wheat protein by heat denaturation results in loss of the starch-binding properties of the wheat protein. The consistency of the wheat protein–starch systems is altered by heat denaturation of the protein. Table 3 shows the effect of the protein content and protein denaturation on the consistency of gelatinised starch.

It appears that protein forms complexes with starch molecules on the granule surface, preventing escape of exudate from the granule and therefore interfering with the increase in consistency. Dahle (1971) concludes from the ratio of extracted

TABLE 3
EFFECT OF PROTEIN CONCENTRATION AND DENATURATION ON CONSISTENCY OF GELATINISED STARCH[a]

Ratio of starch and protein components		*Consistency*	*MacMichael*
Starch solution	*Protein extract*	*Unheated protein*	*Heated protein*
5	15	5	5
10	10	7	12
15	5	12	18

[a] Adapted from Dahle (1971).

flour protein to total protein in a flour of normal protein content (11·15 %) that the amount of protein present in flour is adequate for forming complexes with starch. This does not include the non-extractable protein which might also be able to react with starch.

From the theory of oppositely charged colloids it can be reasoned that at alkaline pH both starch and protein bear negative charges and complexing does not occur. At acidic pH wheat protein bears a positive charge and complexes can be formed.

Hwang (1963) observed that a strong secondary maximum seemed to appear when starch suspensions in milk, not containing predenaturated proteins, were heated. It was confirmed by Stalder (1964) that the secondary maximum was due to milk proteins that were not denaturated before the starch milk suspension was heated. Thus, the role of protein seems to depend on the state of protein before heat is applied. This same phenomenon was observable when heating took place in sugar solutions (Grant, 1968). It seems reasonable to assume that the starch protein complex retards or inhibits the release of starch exudate into the aqueous medium.

Berry & White (1966) have shown that the gluten of wheat flour increases the gelatinisation temperature of wheat starch. Proteins and lipids present in flour also complex with each other and starch, thus complicating the overall picture (Olcott & Mecham, 1947).

Effect of pentosans on starch gelatinisation

It has already been mentioned that 'the possible role of other constituents of flour should not be overlooked' in gelatinisation studies of wheat flour (Hester *et al.*, 1956). According to Jelaca & Hlynka (1971), wheat flour contains about 2 % to 3 % of water-soluble and water-insoluble pentosans, of which the water-soluble fraction represents only about 0·5 % to 0·8 % of the total weight of the flour. It has been estimated that pentosans absorb about a third of the total water in a normal dough, which shows their extremely high affinity for water and the general importance of these minor components.

Water-soluble pentosans have the property of dissolving in water to give an extremely viscous solution whereas water-insoluble pentosans seem able to become highly hydrated in water without actually going into solution. The water-soluble

pentosans absorb 9·2 times, and the water-insoluble pentosans 8 times, their weight in water. The water-insoluble pentosans absorb 4·8 times their weight in water in a gluten–water system, 6·9 times in a starch–water system and 6·5 times in a reconstituted gluten–prime starch dough.

The absorption curve of water for pentosans is dependent on the rate of shear used in mixing. Water-insoluble pentosans show a sudden increase in water absorbed at 56 rpm and the water-insoluble pentosans show a similar jump at 105 rpm. Therefore, with their high affinity for water, the pentosans may well interfere with the water absorption of starches during gelatinisation.

Effect of surface active agents and fats on starch gelatinisation

Dahle & Muenchow (1968) studied the effects of solvent extraction on the cooking characteristics of spaghetti, a typical pasta product made by gelatinising the starch in wheat flour. It had already been observed by Brokaw *et al.* (1964) that certain lipids, such as monoglycerides, enhance the quality of cooked spaghetti. The effect was attributed to the ability of the monoglycerides to complex amylose. Lipid–protein binding phenomena which occur when flour is mixed with water have been studied (Olcott & Mecham, 1947). Dahle & Muenchow (1968) found that removal of lipid or protein resulted in increased amylose in cooking water. Removal of lipids led primarily to greater stickiness. The lipids supplement protein in being essential structural components of spaghetti and other pasta products.

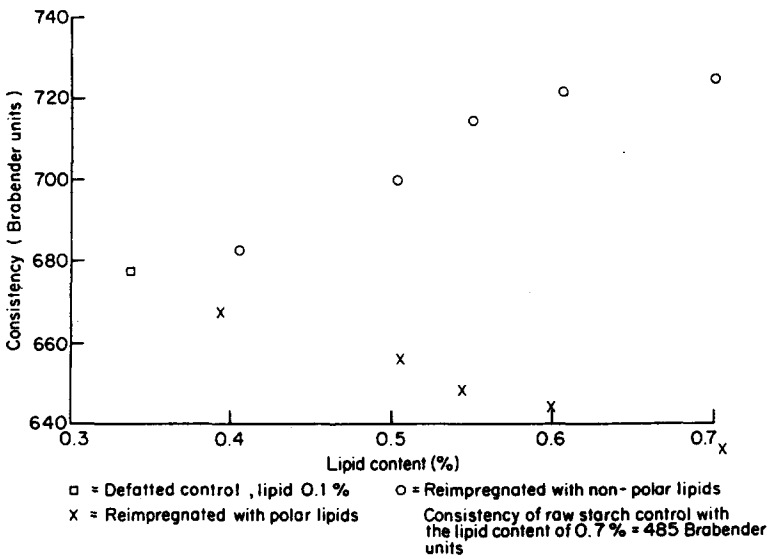


Fig. 6. Effect of concentration of polar and non-polar lipids on consistency of wheat starch suspensions at peak height. (Adapted from Medcalf *et al.* (1968).)

These observations are further evidence of the complexing of starch with lipids. Wheat starches contain about 0·6 % to 0·8 % lipid materials, according to Medcalf *et al.* (1968). These lipid materials are distributed throughout the granule. The polar lipids form a complex with the amylose, or linear starch fraction (Leach, 1965). Studies of lipids added to starch suspensions are reported by Gray & Schoch (1962), Osman & Dix (1960) and Yasumatsu & Moritaka (1964).

Medcalf *et al.* (1968) studied the effect of the polar and non-polar lipid fractions of wheat flour on the pasting characteristics of wheat starch. It was found that the polar and non-polar lipids exhibit different effects on the pasting property of starch. The pasting properties of defatted starch reimpregnated with polar lipids showed reduced consistency in both steps of the CMC technique pasting curve, following the method of Crossland & Favor (1948). Defatted starch reimpregnated with non-polar lipids showed relatively little change in the first step of the pasting curve but a significant increase in maximum peak height. This is illustrated in Figs. 6 and 7.

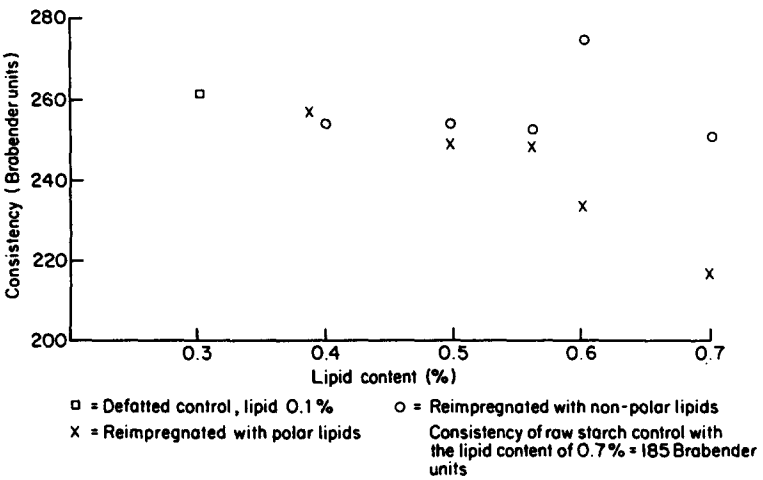


Fig. 7. Effect of concentration of polar and non-polar lipids on consistency of wheat starch suspensions at 76 °C. (Adapted from Medcalf *et al.* (1968).)

The initial step in the pasting curve is attributed to hydration of the amorphous, more accessible regions of the granule (Bechtel *et al.*, 1964). The polar fractions may complex easily with molecules of starch in this region, since they are not closely packed. This complex would tend to retard or reduce the rate of hydration of starch molecules in this region of the granule. Thus, addition of polar lipids should result in a decrease in consistency in this region of the first step of the pasting curve. The reduction in hydration caused by the presence of the polar lipids would also tend to

lower the maximum peak height. Peak height depends on the negative ease of penetration of lipids to the more crystalline, or micellar, regions of the starch granule (Leach *et al.*, 1959). During pasting, consistency continues to increase until water penetrates these micellar regions. Once the micellar regions are hydrated, the granules lose their integrity. Since the polar lipids interfere with the release of exudate from the starch granules and also destroy the micellar arrangement, the consistency of the gelatinised starch decreases. Non-polar lipids would not be expected to produce this result.

Addition of non-polar lipids resulted in greater maximum consistency. The primary effect of this fraction appears to be prevention of hydration of the micellar regions of the granules. This is in contrast to the polar fraction which has prevention of hydration of amorphous regions as its primary effect.

The polar lipids seem to decrease the peak height, as well as the first micellar region peak height, indicating an inhibition of exudate release at both stages. In contrast, non-polar lipids seem to be non-effective or non-reactant in the first phase of hydration and to enhance the release of exudate at higher temperatures or at peak height of the pastegram. It seems, however, that the proper explanation involves prevention or enhancement of exudate release, rather than prevention of hydration (Miller *et al.*, 1973).

Miller *et al.* (1973) showed that surface active agents can be used to elaborate this point further. Surface active agents such as sodium steryl fumarate reduced the consistency of the heated suspension, after the first peak in a CMC-technique plastogram. The granules continued to swell, to sizes larger than in the water–starch system, but consistency remained at a very low level due to the fact that exudate was not released to the surrounding solution. Thus, an agent like sodium steryl fumarate seems to enter the granule after the first slight swelling, preventing release of the exudate. As a result, the consistency does not increase. Again, this is in agreement with Jaska (1971) that part of the starch is in suspension within the granule itself. The chemical agent could form a complex within the granule, enhancing its internal strength, thus preventing the exudate from escaping into the surrounding solution. In that case, since exudate is not available to form a network in the aqueous phase, peak consistency does not increase.

Effect of salt on the gelatinisation of starch

Ganz (1965) suggests that salts may be used to control swelling of starch. When 2·5 % NaCl was added to wheat starch suspensions which were subsequently heated, peak consistency was markedly increased. This increase was associated with enhancement of 'granule integrity', i.e. the granule remains intact or experiences greater swelling before fragmentation occurs. This view is supported by photomicrographs and also by the delay in reaching the maximum consistency in the presence of NaCl. When NaCl is added at 70 °C, the peak consistency is not

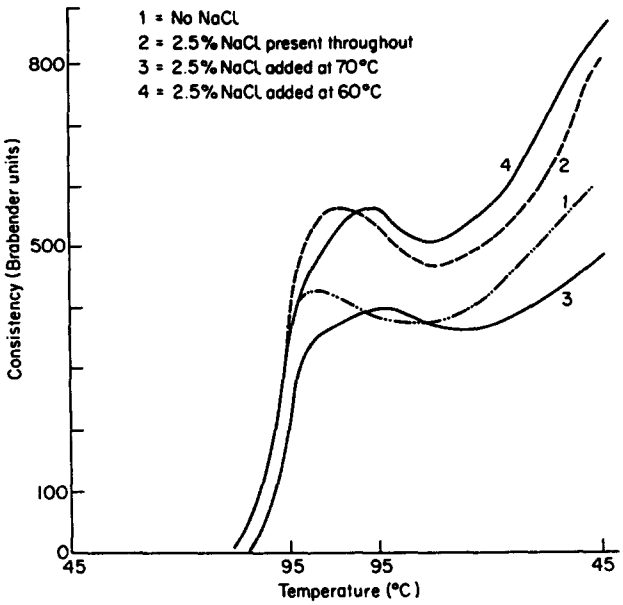


Fig. 8. Amylograph consistency of wheat starch pastes containing NaCl. (Adapted from Ganz (1965).)

enhanced even though it takes more time to reach it. When NaCl is added at 60 °C, peak consistency is enhanced, with more time needed to reach it. The effect of salt on the gelatinisation of starch is shown in Fig. 8.

Ganz (1965) associates the effect of salt with the presence of crystalline regions in the starch granule which have binding forces of varying strengths or different accessibilities. In the 60 °C to 70 °C range, NaCl seemed primarily to affect the weak forces or readily accessible regions involved in swelling. Ganz (1965) assumed that NaCl inhibits the 'opening' of these regions. NaCl also appears to have the secondary effect of either delaying swelling at higher temperatures or altering the course of fragmentation.

Effect of sugars on starch gelatinisation

Glicksman (1969) summarises the process of gelatinisation in the presence of sugar as follows. Sugar inhibits the swelling of starch in a water system and thus retards the gelatinisation of starch. Because of high water solubility, sucrose ties up water molecules, making them unavailable for starch. Greater sugar concentrations increasingly inhibit normal swelling of starch granules and high concentrations cause a marked increase in the temperature at which birefringence disappears. A similar effect is shown in Fig. 9, which is adapted from a plot of starch gelatinisation

temperature versus sugar concentration (Knoch, 1972). The plot in Fig. 9 can be well approximated by two straight lines. If y is the concentration of sugar solution and x the temperature of starch gelatinisation, the straight lines in Fig. 9 can be represented by:

$$y = 1{\cdot}16x - 78 \quad \text{for } x = (70\,^{\circ}\text{C} \sim 105\,^{\circ}\text{C}) \text{ and } y = (5\,\% \sim 45\,\%)$$
$$y = 1{\cdot}66x - 131 \quad \text{for } x = (105\,^{\circ}\text{C} \sim 130\,^{\circ}\text{C}) \text{ and } y = (45\,\% \sim 85\,\%)$$

The strength of gels formed also decreases with increasing sugar content (Bean & Osman, 1959). In jelly confections having 10% starch, 70% sugar and 20% water, the starch cannot be completely gelatinised. When cooked at atmospheric pressure, the starch is often heated in excess water, which is later evaporated, in order to obtain better gelatinisation. In pressure cooking at higher temperatures, starch

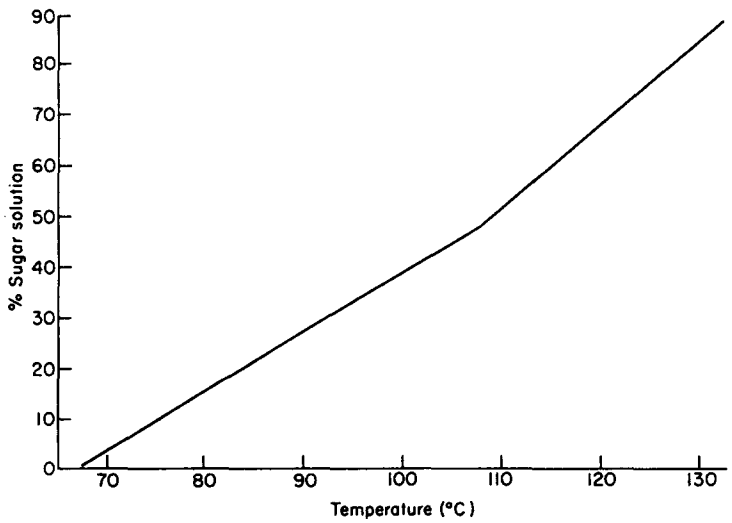


Fig. 9. Gelatinisation temperature of starch in sugar solutions. (Freely adapted from Knoch (1972).)

swells even in the presence of large amounts of sugar. The minimum amount of water needed to obtain conditions of sufficient swelling without high pressures is one part of water to one part of flour (starch) (Knoch, 1972). Starch gelatinised in the presence of small amounts of water under pressure yields a more compact paste of high consistency (Knoch, 1972).

The hydration of starch granules was also inhibited in the presence of sucrose (Hester *et al.*, 1956). The maximum consistency of starch paste was not reached at temperatures below 95 °C, indicating less than complete swelling of starch granules

due to insufficient release of exudate, although the starch mixture was not exposed at the temperature for a sufficient period. The effect is similar to the increase in consistency when NaCl is present (Ganz, 1965). The disintegration of granules was less, and either granule size or extent of folding of the granules was decreased. Less soluble material was diffused from starch granules (Miller *et al.*, 1973). The starch gels were less rigid. At higher sucrose concentrations gels did not form, but instead the result was a paste.

Table 4 shows the effect of sucrose concentration on the gelatinisation of wheat flour. It has been adapted from the work of Hester *et al.* (1956) with a Brabender Viscoamylograph.

The pastes of wheat flour heated in the presence of sucrose had markedly higher

TABLE 4
THE EFFECT OF SUCROSE ON THE GELATINISATION OF WHEAT FLOUR IN AN AMYLOGRAPH†

Grammes of sucrose in 100 g H_2O	*Onset temperature*[a] *(°C)*	*Maximum peak BU/°C*	*BU at 95°C*	*Trend at 95°C*
SSW				
0·00	40	NIR	ab 90	R
15·9	38	NIR	ab 110	R
23·7	38	NIR	ab 110	R
31·6	38	NIR	ab 90	R
39·5	40	NIR	ab 70	R
SHW				
0·00	42	NIR	ab 80	R
15·9	41	NIR	ab 90	R
23·7	41	NIR	ab 50	R
31·6	38	NIR	ab 50	R
39·5	40	NIR	ab 50	R
FSW				
0·00	26	350/90	250	D
15·9	26	660/94	650	D
23·7	28	NIR	700	R
31·6	28	NIR	550	R
39·5	30	NIR	320	R
FHW				
0·00	22	120/85	80	D
15·9	26	310/88	160	D
23·7	26	380/92	320	D
31·6	28	450/93	430	D
39·5	30	NIR	520	R

SSW = Starch from soft wheat flour.
SHW = Starch from hard wheat flour.
FSW = Soft wheat flour.
FHW = Hard wheat flour.
NIR = Not in range of the experiment.
R = Rising trend.
D = Decreasing trend.
[a] Temperature at which consistency starts to increase.
† Adapted from Hester *et al.* (1956).

maximum and terminal consistencies and gels tended to be firmer than those obtained using starch alone. Comparable pastes of wheat starch showed no maximum consistency, little or no change in terminal consistency in the presence of sucrose and weaker gels. Apparently, sucrose had an effect on constituents of flour other than starch, which influenced the paste and gel properties. The syneresis of flour gels was observed to be practically non-existent compared with that of the starch gels.

Campbell & Briant (1957) studied the effect of citric acid and sucrose on wheat starch pastes and gels, using wheat starch of several granule sizes. They found that the amount of amylose in solution increases with increased concentrations of citric acid and decreases with increased concentrations of sugar. Citric acid hastens the granule fragmentation and sucrose delays it. The amylograms showed that wheat starch slurries start swelling at approximately the same temperature, but with different rates of increase in consistency (Campbell & Briant, 1957). Peak consistency—and the time required to reach peak consistency—increased with increasing sugar concentration up to 20 g sucrose per 100 ml water.

There seems to be a change in trend when the sugar concentration per 100 ml of water is increased from 20 g to 40 g. The reasons for this need further study.

Bean & Osman (1959) studied the effects of different sugars on the consistency and gel strength of starch pastes. They used sucrose, dextrose, fructose, maltose, lactose, invert syrup and three corn syrups. The starch used was corn starch, which was more suitable than wheat starch under the conditions of the experiment. Using sucrose and other sugars, maximum consistencies were obtained with 20 % solutions. When sugar concentrations exceed this value, the temperature of the onset of gelatinisation starts to increase and is markedly higher for 50 % sugar concentration. At high concentrations of sugar, the rate of consistency increase is slower than at low concentrations and the maximum value attained is lower. The pastes also show a smaller decrease in consistency after reaching the maximum.

Most of the sugars delayed gelatinisation. The effect of the monosaccharides was not as great as that of the disaccharides. Maximum consistency was increased by monosaccharides and decreased by disaccharides. Corn syrups gave results which can be approximated by their composition. The molar concentrations of the hydroxyl groups available for hydrogen bonding of water are not sufficient to explain water binding of sugar in competition with starch. The differences in gel strength appear at first to reflect the differences in hot paste consistencies, but in practice they are not directly related.

Bean & Osman (1959) conclude that sucrose interferes with starch gelatinisation by inhibiting the swelling of starch granules, increasing the temperature of the onset of gelatinisation, lowering maximum consistency, reducing the thinning of the paste after completion of gelatinisation and decreasing the gel strength upon cooling. However, this conclusion seems only partially true for wheat starch and a more complicated situation arises when wheat flour is used.

DISCUSSION

Wheat starch, wheat protein, pentosans and commonly used ingredients such as salt and sugar interact and contribute to the gelatinisation process of starch. Studies have been carried out to evaluate these factors separately and as part of flour. Experiments were performed mainly at low concentrations of starch due to limits of available instrumentation.

Increase in consistency in the gelatinisation of starch has largely been attributed to granule size and swelling of the granules. However, work by Miller *et al.* (1973) gave an explanation involving the release of exudate, which may have been referred to in some earlier studies as amylose release. The higher consistency of the starch paste is attributed to an extensive swelling of the starch granules prior to disintegration and to the existence of partially intact granular fragments after disintegration. In this context, the effects of other food ingredients and processing parameters on the consistency of the paste can be explained.

Combined effects of variations in ingredient and processing parameters have not been widely studied, especially for industrial operation. However, understanding of the gelatinisation mechanism of starch will be facilitated by PMR studies of water mobility, by SEM studies of structure and by the development of controlled shear-temperature equipment for a wider range and other consistency and texture measuring apparatus.

SUMMARY

Swelling of starch, or reversible uptake of water, occurs before the onset of gelatinisation at temperatures below 60 °C and is followed by further slow swelling at 60 °C to 70 °C, rapid swelling at 80 °C to 90 °C and eventual fragmentation of the starch granules. Part of the starch is solubilised inside the granule before it escapes out of the granule and most of the starch is solubilised over a relatively narrow temperature range. As the temperature is increased the insoluble granule breaks down, releasing the solution inside. This exudate released from granules is mainly responsible for the increase in consistency of starch during gelatinisation.

The type and size of starch granules, their age and previous treatment, cooking time and temperature, time and temperature of storage after cooking and types and amounts of added ingredients all affect the formation of the starch gel and its characteristics. Protein complexes with starch molecules on the granule surface and prevents the escape of exudate from the granule, thus interfering with increase in consistency of starch paste. Pentosans may compete with starch for water and oil and surface active agents may interfere with the release of starch exudate and decrease peak consistency. Salt increases the consistency of wheat starch by inducing greater swelling before fragmentation. Hydration and gelatinisation of starch are retarded by the presence of sucrose.

REFERENCES

BARLOW, K. K., BUTTROSE, M. S., SIMMONDS, D. H. & VESK, M. (1973). The nature of the starch–protein interface in wheat endosperm, *Cereal Chem.*, **50**, 443–54.

BEAN, M. L. & OSMAN, E. M. (1959). Behavior of starch during food preparation. II. Effects of different sugars on the viscosity and gel strength of starch pastes, *Food Research*, **24**, 665–71.

BECHTEL, W. G., GEDDES, W. F. & GILLES, K. A. (1964). Carbohydrates. In *Wheat: Chemistry and technology* (Hlynka, I. (Ed.)), American Association of Cereal Chemists, St. Paul, Minn. Chapter 7, 277–352.

BERRY, G. K. & WHITE, G. W. (1966). An objective method for the measurement of starch gelatinization temperatures, *J. Food Technol.*, **1**, 249–56.

BROKAW, G. Y., LEE, L. J. & NEU, G. D. (1964). Eliminate pasty quality of many starch based foods, *Food Processing*, **29**, 198–202.

CAMPBELL, A. M. & BRIANT, A. M. (1957). Wheat starch pastes and gels containing citric acid and sucrose, *Food Research*, **22**, 358–66.

CROSSLAND, L. B. & FAVOR, H. H. (1948). Starch gelatinization studies. II. A method for showing the stages in swelling of starch during heating in the amylograph, *Cereal Chem.*, **25**, 213–20.

DAHLE, L. K. (1971). Wheat protein–starch interaction. I. Some starch binding effects of wheat-flour proteins, *Cereal Chem.*, **48**, 706–14.

DAHLE, L. K. & MUENCHOW, H. L. (1968). Some effects of solvent extraction on cooking characteristics of spaghetti, *Cereal Chem.*, **45**, 464–8.

ELDER, A. L. & SCHOCH, T. J. (1959). Measuring the useful properties of starch, *Cereal Sci. Today*, **4**(7), 202–8.

GANZ, A. J. (1965). Effect of sodium chloride on the pasting of wheat starch granules, *Cer. Chem.*, **42**, 429–31.

GLICKSMAN, M. (1969). *Gum technology in the food industry*, Academic Press, New York. 554 pp.

GOTO, F. (1969). Determination of gelatinization properties of highly concentrated starch suspensions by Brabender Plastograph. II. Examination of gelatinization curve obtained by Plastograph. *Staerke*, **21**, 267–70.

GOTO, F. & YOKOO, Y. (1969). Determination of gelatinization property of highly concentrated starch suspensions by Brabender Plastograph. I. Examination of test conditions, *Staerke*, **21**(5), 128–32.

GOUGH, B. M. & PYBUS, J. N. (1971). Effect on the gelatinization temperature of wheat starch granules of prolonged treatment with water at 50 °C, *Staerke*, **23**(6), 210–12.

GRANT, V. M. (1968). The effect of heat treatment of milk on selected food systems, M.S. thesis, University of Iowa, Iowa City, Iowa.

GRAY, V. M. & SCHOCH, T. J. (1962). Effects of surfactants and fatty adjuncts on the swelling and solubilization of granular starches, *Staerke*, **14**, 239–46.

GREENWOOD, C. T. (1964). Structure, properties and amylolytic degradation of starch, *Food Technol.*, **18**(5), 138–44.

HESTER, E. E., BRIANT, A. M. & PERSONIUS, C. J. (1956). The effect of sucrose on the properties of some starches and flours, *Cereal Chem.*, **33**, 91–101.

HWANG, Q. S. (1963). A comparison of some characteristics of milk samples with their action on starch-milk pastes, M.S. thesis, University of Illinois, Urbana, Illinois.

JASKA, E. (1971). Starch gelatinization as detected by proton magnetic resonance, *Cereal Chem.*, **48**, 437–44.

JELACA, S. L. & HLYNKA, I. (1971). Water-binding capacity of wheat flour crude pentosans and their relation to mixing characteristics of dough, *Cereal Chem.*, **48**, 211–22.

KAINUMA, K. T., ODA, T. & SUZUKI, S. (1968). Observation of changes of starch granules during gelatinization by a novel photopastegraphy method. I. Design of the photopastegraph, *S.J. Technol. Soc. Starch (Tokyo)*, **16**, 51–4.

KERR, R. W. (1950). Evaluation of modified starches in practice. In *Chemistry and industry of starch*, Academic Press, New York, p. 143.

KNIGHT, J. W. (1965). *The chemistry of wheat starch and gluten*, Leonard Hill, London, 149 pp.

KNOCH, H. (1972). Problems and progress in the making of licorice products, *Manufr. Confectioner*, **52**(10), 42–8, 72–3.

LEACH, H. W., MCCOWEN, L. D. & SCHOCH, T. J. (1959). Structure of the starch granule. I. Swelling and solubility patterns of various starches, *Cereal Chem.*, **36**, 534–44.

LEACH, H. W. (1965). Gelatinization of starch. In *Starch: Chemistry and technology*, Vol. I (Whistler, R. L. & Paschall, E. F. (Eds.)), Academic Press, New York, p. 297.

LONGLEY, R. W. & MILLER, B. S. (1971). Note on the relative effects of monoglycerides on the gelatinization of wheat starch, *Cereal Chem.*, **48**, 81–5.

MAZURS, E. G., SCHOCH, T. J. & KITE, F. E. (1957). Graphical analysis of the Brabender viscosity curves of various starches, *Cereal Chem.*, **34**, 141–52.

MEDCALF, D. G. & GILLES, K. A. (1965). Wheat starches. I. Comparison of physicochemical properties, *Cereal Chem.*, **42**, 558–68.

MEDCALF, D. G., YOUNGS, V. L. & GILLES, K. A. (1968). Wheat starches. II. Effect of polar and nonpolar lipid fractions on pasting characteristics, *Cereal Chem.*, **45**, 88–95.

MILLER, B. S., DERBY, R. I. & TRIMBO, H. B. (1973). A pictorial explanation for the increase in viscosity of a heated wheat starchwater suspension, *Cereal Chem.*, **50**, 271–80.

MORGAN, W. L. (1940). Pasting and identification of starches, *Ind. Eng. Chem. Anal. Ed.*, **12**, 313–17.

OLCOTT, H. S. & MECHAM, D. K. (1947). Characterization of wheat gluten. I. Protein–lipid complex formation during doughing of flours. Lipoprotein nature of the glutenin fraction, *Cereal Chem.*, **24**, 407–14.

OSMAN, E. M. & DIX, M. R. (1960). Effects of fats and nonionic surface-active agents on starch pastes, *Cereal Chem.*, **37**, 464–75.

OTT, M. & HESTER, E. E. (1965). Gel formation as related to concentration of amylose and degree of starch swelling, *Cereal Chem.*, **42**, 476–84.

RHA, CK. (1975). Theories and principles of viscosity. In *Theory, determination and control of physical properties of food materials*. (Rha ChoKyun (Ed.)), D. Reidel Publishing Co., Dordrecht-Holland, pp. 7–24.

SANDSTEDT, R. M. & ABBOTT, R. C. (1964). A comparison of methods for studying the course of starch gelatinization, *Cereal Sci. Today*, **9**(1), 13–18, 26.

SCHOCH, T. J. & MAYWALD, E. C. (1956). Microscopic examination of modified starches, *Anal. Chem.*, **28**(3), 382–7.

SCHOCH, T. J. (1961). Starches and amylases, *Proc. Am. Soc. Brewing Chemists*, 83–92.

SEIDEMANN, J. (1963). Identification of different kinds of starches by microscopic determination of the swelling range, *Staerke*, **15**, 291.

SMITH, R. J. (1967). Characterization and analysis of starches. In *Starch: Chemistry and Technology. Vol. II. Industrial aspects* (Whistler, R. L. & Paschall, E. F. (Eds.)), Academic Press, New York, pp. 569–635.

STALDER, M. J. (1964). The amylograph viscosity of starch-milk pastes: Effects of source and preheating of milk, M.S. thesis, University of Illinois, Urbana, Illinois.

TAKEUCHI, I. (1969). Interaction between protein and starch, *Cereal Chem.*, **46**, 570–9.

VOISEY, P. W. & NUNES, A. (1968). An electronic recording amylograph, *J. Can. Inst. Food Technol.*, **1**, 128–32.

WATSON, S. A. (1964). Determination of starch gelatinization temperature. In *Methods in carbohydrate chemistry, Vol. IV, Starch* (Whistler, R. L. (Ed.)), Academic Press, New York, pp. 240–2.

YASUMATSU, K. & MORITAKA, S. (1964). Changes of characteristics of starch during gelatinization in the presence or absence of fatty acid, *J. Food Sci.*, **29**, 198–202.

EFFECTS OF HYPOCHLORITE TREATMENTS ON A METHIONYL PEPTIDE

J. L. CUQ, C. AYMARD & C. CHEFTEL

Laboratoire de Biochimie et Technologie Alimentaires, Université des Sciences et Techniques du Languedoc, 34060 Montpellier, France

(Received: 1 November, 1976)

ABSTRACT

Treatment of glycyl-L-methionyl-glycine with up to 0·4% (w/v) sodium hypochlorite causes oxidation of methionine residues into methionine sulphoxide and methionine sulphone, and probable deamination of free α amino groups.

INTRODUCTION

Hypochlorite can be used to destroy contaminating aflatoxins of protein meals and concentrates (Natarajan *et al.*, 1975*a*). However, the detoxifying treatment of peanut protein isolates with 0·3% sodium hypochlorite at pH 8 to 9 was found to bring a 50% reduction in tryptophan and tyrosine contents, probably through oxidation (Natarajan *et al.*, 1975*b*).

A more systematic study of the effects of hypochlorite on specific amino acid residues of proteins is needed.

In the present study, using the tripeptide glycyl-L-methionyl-glycine as a substrate, it is shown that 0·01 to 0·1% sodium hypochlorite causes methionine oxidation into methionine sulphoxide. Higher hypochlorite concentrations, longer reaction times, an acid pH and a temperature of 80°C appear to provoke the deamination of free α amino groups and the formation of methionine sulphone.

The molarity (5 mM) of glycyl-L-methionyl-glycine selected for hypochlorite treatments approximately corresponds to that of methionine residues in the experiments with peanut protein isolates (Natarajan *et al.*, 1975*b*).

MATERIALS AND METHODS

5 mM glycyl-L-methionyl-glycine (purchased from Interchim, Montluçon, France) in a 0·1 M, pH 8·0 phosphate buffer is made 0·1% (w/v) in sodium

Fd. Chem. (2) (1977)—
Printed in Great Britain

hypochlorite and held at 60 °C for 30 min. Similar incubations are also performed in other conditions of hypochlorite concentration, pH, temperature and duration, as indicated. In all cases, the incubation medium also contains free norleucine (at a 4·5 mM concentration).

The incubation medium is then frozen in liquid nitrogen and immediately freeze-dried (no remaining hypochlorite can be detected at this stage by iodometric assays). The dried residue is dissolved in distilled water. A part of this solution is analysed by ion-exchange chromatography (see below). The remaining part is made 3N in NaOH (1·31 mg of tripeptide per 2 ml final volume) and hydrolysed for 16 h at 110 °C under nitrogen (a technique known to permit the direct analysis of methionine sulphoxide and methionine sulphone, Neumann, 1967; Cuq *et al.*, 1973). The hydrolysate is brought to pH 2 with 2N HCl; a sample of less than 100 μl is then assayed on a Technicon NC1 analyser. A rapid amino acid separation and analysis (Fig. 1) is obtained as follows: the column (25 cm × 0·6 cm) of Chromobeads C_2 resin is maintained at 60 °C. It is first cleaned with 0·2N NaOH for 5 min, then equilibrated with pH 3·10 citrate buffer for 15 min. The amino acid sample is next introduced into the top of the column and then eluted with pH 3·8 citrate buffer at a rate of

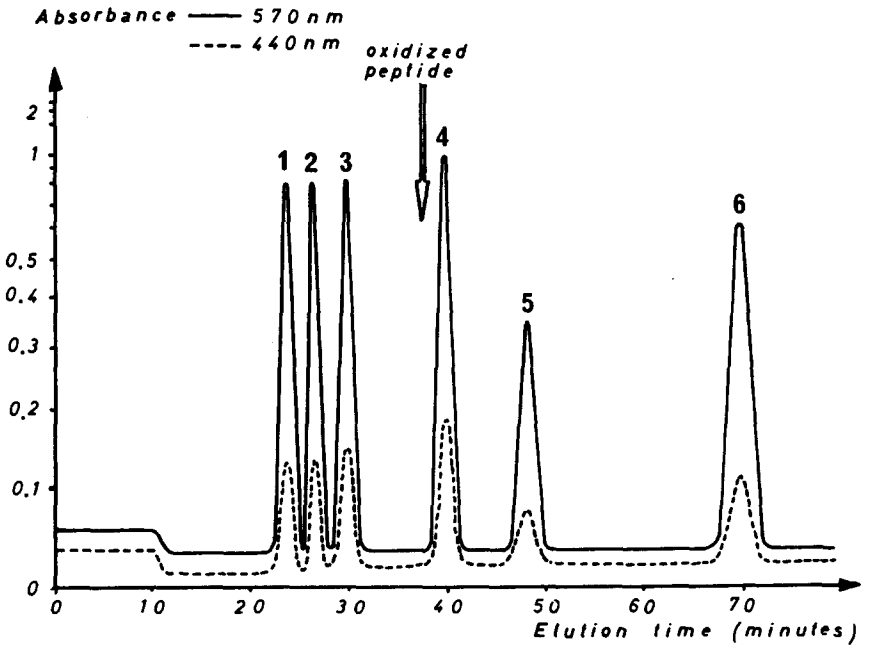


Fig. 1. Chromatogram of glycyl-L-methionyl-glycine and of various amino acids. 125 nmoles methionine sulphoxide (1), 125 nmoles methionine sulphone (2), 100 nmoles glycine (3), 250 nmoles methionine (4), 125 nmoles norleucine (5) and 250 nmoles glycyl-L-methionyl-glycine (6). Chromobeads C_2 resin. 25 cm × 0·6 cm column. 60 °C. NC1 Technicon amino acid analyser.

0·8 ml/min. Both buffers contain per litre, 14·71 g sodium citrate, $2H_2O$; 25 ml of 2N NaOH; 5 ml of thiodiglycol; 10 ml of a saturated solution of Brij 35, and enough 6N HCl for pH adjustment.

Amino acid contents are given as moles of individual amino acid per mole of glycyl-L-methionyl-glycine.

RESULTS AND DISCUSSION

Chromatography on Chromobeads C_2 resin of glycyl-L-methionyl-glycine submitted to 0 to 0·1% (w/v) hypochlorite treatments indicates a decrease in the tripeptide level and the corresponding formation of a derivative (elution time 37 min, Fig. 1) which may well be glycyl-L-methionyl-sulphoxide-glycine. Treatment with 0·2% hypochlorite causes the complete disappearance of glycyl-L-methionyl-glycine and a decrease in free norleucine (forty per cent of the initial concentration), while the level of oxidised peptide remains constant (Fig. 2). Treatment with 0·4% hypochlorite causes the total disappearance of the peptide, of the oxidised peptide and of free norleucine. This is probably due to deamination of N terminal amino groups.

Alkaline hydrolysis of non hypochlorite-treated glycyl-L-methionyl-glycine is

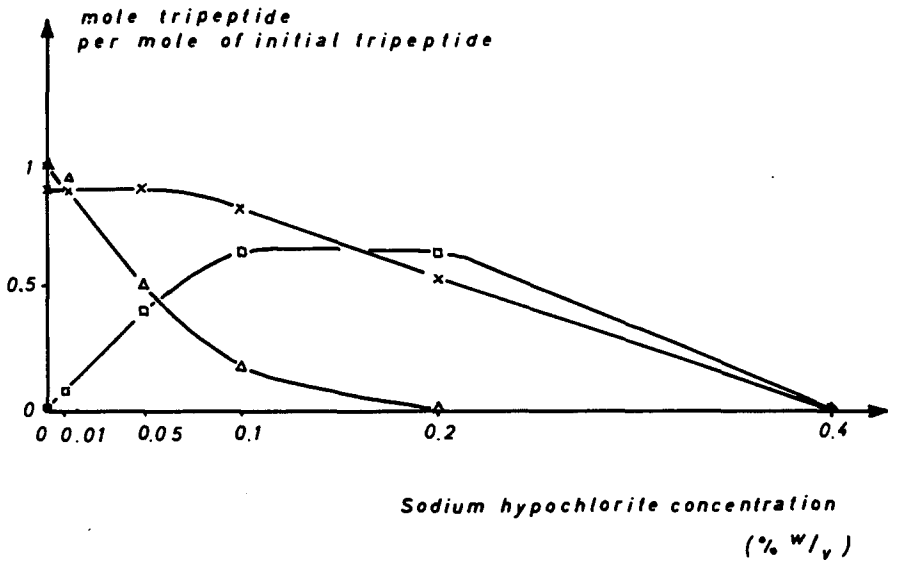


Fig. 2. Effects of sodium hypochlorite treatments on glycyl-L-methionyl-glycine. Influence of sodium hypochlorite concentration (60 °C, 30 min, pH 8·0). △—△ peptide, □—□ oxidised peptide, (× — × norleucine).

complete: no residual peptide can be detected, and the release of glycine is close to the theoretical maximum of 2 moles (Fig. 3). There is, however, a 10% loss in the recovery of methionine, a loss probably due to partial destruction during alkaline hydrolysis.

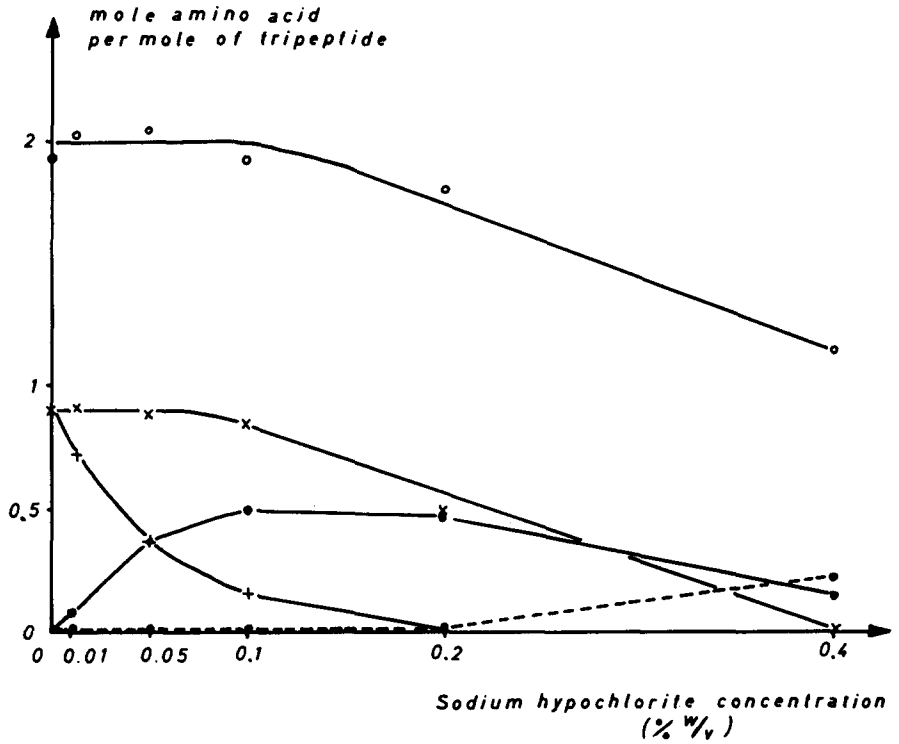


Fig. 3. Effects of sodium hypochlorite treatments on the amino acid composition of glycyl-L-methionyl-glycine. Influence of sodium hypochlorite concentration (60 °C, 30 min, pH 8·0). Amino acid analysis after alkaline hydrolysis. O—O glycine, + — + methionine, ●—● methionine sulphoxide, ●---● methionine sulphone (× — × norleucine).

Treatments with 0·01 to 0·1% hypochlorite cause significant decreases in the methionine content and the formation of methionine sulphoxide (Fig. 3). The latter does not account totally for the decrease in methionine. This effect of hypochlorite differs from that of 0·1 M hydrogen peroxide, which can quantitatively oxidise the methionyl residues of a protein (Cuq *et al.*, 1973). It can be mentioned that Natarajan *et al.* (1975*b*) did not investigate the formation of methionine sulphoxide in hypochlorite-treated peanut proteins.

Treatment with 0·2% hypochlorite provokes an almost complete destruction of methionine, while the content of methionine sulphoxide remains constant (fifty per cent of initial methionyl residues). Of interest is the concomitant decrease in the

glycine and norleucine contents, probably due to oxidative deamination reactions (Fig. 3). No new ninhydrin-positive peak can be detected on the amino acid chromatogram.

Treatment with 0·4% hypochlorite brings about the total disappearance of norleucine and the destruction of one mole of glycine out of the initial two (Fig. 3). It is likely that deamination affects the N terminal glycine residue. It would be of interest to check if hypochlorite can provoke the deamination of free α and ε-amino groups of proteins.

The 0·4% hypochlorite treatment also causes a decrease in the content of methionine sulphoxide (fifteen per cent of initial methionine residues) and the marked formation of methionine sulphone (twenty-five per cent of the initial methionine residues: Fig. 3). No methionine can be detected on the chromatogram, and therefore 60% of the initial methionine residues cannot be accounted for. The partial N-chlorination of peptide bonds is not ruled out; it could modify the stability of methionine residues or of their oxidation derivatives during alkaline hydrolysis.

It is usually assumed that interaction of hypochlorite with proteins results in oxidative reactions at neutral and alkaline pH while acid pH favours chlorination

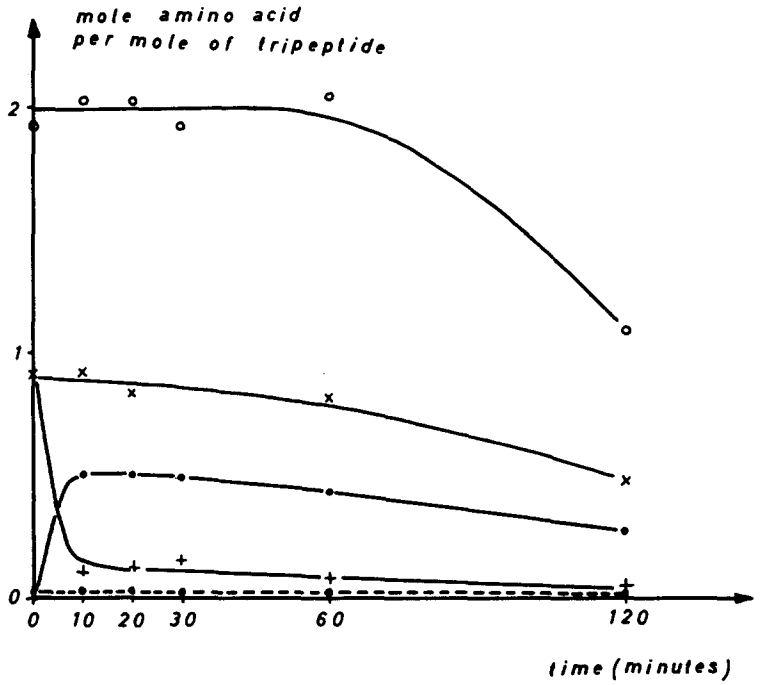


Fig. 4. Effects of sodium hypochlorite treatments on the amino acid composition of glycyl-L-methionyl-glycine. Influence of incubation time (60 °C, pH 8·0, 0·1% w/v sodium hypochlorite). Amino acid analysis after alkaline hydrolysis, O—O glycine, + — + methionine, ●—● methionine sulphoxide, ●---● methionine sulphone (× — × norleucine).

reactions (Natarajan *et al.*, 1975*b*). Such pH effects have not been confirmed here, when glycyl-L-methionyl-glycine was treated at 60 °C for 30 min with 0·1 % (w/v) sodium hypochlorite at pH 3·8, 6·5, 8·2, 10·5 and 11·9. Results obtained in the range of pH 6·5 to 11·9 are similar to those of Fig. 3 (for the same sodium hypochlorite concentration). However, at pH 3·8, the content of methionine sulphoxide decreases by half, while methionine sulphone is formed (corresponding to 13 % of the initial residues). At pH 3·8, also, the content of norleucine and glycine is slightly reduced. It therefore appears that an acid pH favours the oxidation of methionine sulphoxide, and possibly also the deamination of free α-amino groups.

Changes in the temperature of incubation from 0 to 80 °C (for a 30 min, 0·1 % (w/v) sodium hypochlorite treatment at pH 8·0) do not modify the amino acid contents except for a significant formation of methionine sulphone at 80 °C (seventeen per cent of the initial methionine residues).

Experiments have also been performed (with 0·1 % (w/v) sodium hypochlorite at pH 8·0 and 60 °C) for different incubation times (Fig. 4). The decrease in methionine and the formation of methionine sulphoxide are already maximal after 10 min. An incubation time of 2 h brings marked decreases in the contents of glycine, methionine sulphoxide (without significant formation of methionine sulphone) and, to a lesser extent, norleucine.

The effects of hypochlorite treatments on the amino acid composition of tryptophanyl and lysyl peptides are presently under investigation in this laboratory.

ACKNOWLEDGEMENTS

This work has benefited in part from the financial help of the Centre National de la Recherche Scientifique, Paris (ERA No. 614: Modifications biochimiques et nutritionnelles de protéines alimentaires).

REFERENCES

CUQ, J. L., PROVANSAL, M., GUILLEUX, F. & CHEFTEL, C. (1973). Oxidation of methionine residues of casein by hydrogen peroxide. Effects on *in vitro* digestibility, *J. Food Sci.*, **38**, 11–13.

NATARAJAN, K. R., RHEE, K. C., CATER, C. M. & MATTIL, K. F. (1975*a*). Destruction of aflatoxins in peanut protein isolates by sodium hypochlorite, *J. Amer. Oil Chem. Soc.*, **52**, 160–3.

NATARAJAN, K. R., RHEE, K. C., CATER, C. M. & MATTIL, K. F. (1975*b*). Effect of sodium hypochlorite on peanut protein isolates, *J. Food Sci.*, **40**, 1193–8.

NEUMANN, N. P. (1967). Analysis for methionine sulphoxides. In: *Methods in enzymology*, ed. C. H. W. Hirs, S. P. Colowick and N. O. Kaplan, Vol. XI. New York and London, Academic Press. 487–90.

LESSER KNOWN SOURCES OF PROTEIN IN SOME NIGERIAN PEASANT DIETS

I. B. UMOH & O. BASSIR

Department of Biochemistry, University of Ibadan, Ibadan, Nigeria

(Received: 3 November, 1976)

ABSTRACT

The proximate mineral composition and indispensable amino acid pattern were determined for samples of Snail (Vivapara quadrata), *periwinkle* (Littorina littorea), *crayfish* (Palamonetes varians) *and some locally smoked fresh water fish* (Pisces *spp.*).

The crude protein contents of smoked fish, crayfish, snail and periwinkle were 75·31, 74·84, 65·29 *and* 60·93 % *dry matter, respectively. These values were* 56·9, 54·9, 35·2 *and* 26·1 %, *respectively higher than that of whole hen's egg. All the samples, except snail, also had higher ash values. Whole hen's egg, however, contained more crude fat* (40·23 %) *and gross energy* (34·74 *Kj/g*) *than the test samples.*

The minerals calcium and phosphorus were higher in the test samples than in whole hen's egg. Samples analysed contained higher amounts of tryptophan than whole hen's egg and, with the exception of snail, the samples also contained more lysine. The test samples have high chemical scores (A/E ratios). Comparison has also been made of the indispensable amino acid patterns of these samples with those of cow's milk, human milk and the FAO (1957) *provisional pattern.*

INTRODUCTION

There has been, for a long time, an awareness of the persistent shortage of animal protein in the diet of the Nigerian peasant. Evidence from reviews of the literature has indicated clearly that among the factors limiting animal protein supply (particularly via livestock and its products) to these Nigerians have been the prohibitive prices and unavailability (Idusogie, 1971, 1973; Bassir, 1962; Ekpo, 1974). There is, therefore, a natural urge to bridge the wide gap existing between the physiological requirement for protein and the supply from the available

Fd. Chem. (2) (1977)—
Printed in Great Britain

conventional sources in the diets. While the peasants in the Northern States of Nigeria consume cereals, cereal products, fermented milk and milk products and some legumes, in the Southern States—and especially in the riverine areas—they resort to small terrestrial game, fish and other aquatic protein foodstuffs. In general, the Nigerian peasants from the Southern States derive their protein from such animal sources as tortoises, turtles, snails, lizards, snakes and other reptiles, caterpillars, locusts, grasshoppers, crickets, termites, beetles and similar insects, crayfish, fish, crabs, periwinkle, small game and low flying birds. In some areas of Nigeria our survey results (Bassir & Umoh, 1973) have shown that these foods are the only sources of protein in the peasant diets.

It is, however, now being appreciated that, more than anything else, lack of nutritional information on these protein sources is the major problem, rather than the actual shortage of the sources of protein, as was, until now, widely believed. There is a dearth of information on nutrient composition (particularly protein content), indispensable amino acid pattern and mineral composition of these foodstuffs consumed by the Nigerian peasants. The availability of such information would lead to more judicious use of these materials. This paper is a report of a series of analytical results aimed at elucidating the nutrient composition of some of these protein sources. The results are compared with freeze-dried hen's egg.

MATERIALS AND METHODS

Sampling and preparation of the material for analysis

Live samples of edible snails (*Vivapara quadrata*), periwinkle (*Littorina littorea*) and the locally preserved dry forms of both crayfish (*Palamonetes varians*) and smoked fish (*Pisces* spp.) were bought from the local market. Hen's eggs were also bought in the same market.

Snail

The hard shells were broken and the edible portion removed. This was then washed with lime juice to remove the slime, sliced to pieces and then freeze-dried. The dried material was then milled and stored until needed for analysis.

Periwinkle

These were first steeped in hot water for 20 min. The edible portion was removed from the shell with a sharp needle. A sufficient quantity of the material was washed once with water to remove any particles of the shell, then freeze-dried, milled and stored.

Smoked fish

Medium-sized smoked fish of moderate cost, as revealed by our previous survey

(Bassir & Umoh, 1973), was bought. Since the natives usually consume these whole (bones, head and flesh), there was no separation of the bones from the dried flesh. The sample was then milled and stored for analysis.

Crayfish

Samples of the dry crayfish were milled as bought from the market.

Whole hen's egg

Fresh eggs bought from the market were de-shelled. The egg white and the yolk were mixed and freeze-dried. The dried samples were then milled.

ANALYTICAL PROCEDURES

Proximate chemical composition

Analyses of the samples for moisture, crude protein (sample—NX6.25), ether extract, and ash were carried out using the method of the AOAC (1970). The nitrogen-free extract was obtained by difference. Analyses were carried out in triplicate on three separate batches of the samples.

Mineral composition

Calcium and iron were determined by the dry ashing method of the AOAC (1970). Phosphorus was determined from aliquots of the mineral solution by the method of Gomori (1942). The other minerals were estimated from the wet-digested samples (AOAC, 1970), using the Perkin-Elmer Atomic Absorption Spectrophotometer 290. The calorific values of the specimens were determined using a ballistic bomb calorimeter. The results were expressed on a dry matter basis.

Amino acid analysis

The quantitative estimation of the amino acids was carried out according to a procedure based on the report of Spackman *et al.* (1956). Tryptophan was, however, estimated chemically (Miller, 1967) using *p*-dimethylaminobenzaldehyde (DMAB) and sodium nitrite solutions.

RESULTS AND DISCUSSION

Table 1 shows the proximate nutrient composition of snail (*Vivapara quadrata*), periwinkle (*Littorina littorea*), crayfish (*Palamonetes varians*), locally smoked fresh water fish (*Pisces* spp.) and whole hen's egg for comparison purposes. These lesser known foodstuffs were quite rich in crude protein. The ash contents of all the samples except snail were higher than that of whole hen's egg. Whole hen's egg, on

TABLE 1

PROXIMATE NUTRIENT COMPOSITION OF SOME PROTEIN SOURCES AND WHOLE HEN'S EGG (g/100 g DRY MATTER) (Mean value ± SD)

Nutrient	*Smoked fish*	*Periwinkle* (Littorina littorea)	*Crayfish* (Palamonetes varians)	*Snail* (Vivapara quadrata)	*Whole hen's egg*
Dry matter	98·55 ± 4·06	20·12 ± 3·71	97·98 ± 6·34	23·58 ± 4·34	25·34 ± 1·34
Crude protein	75·31 ± 2·82	60·93 ± 2·85	74·84 ± 5·01	65·29 ± 6·51	48·31 ± 0·93
Ash	13·34 ± 1·12	8·40 ± 1·11	8·80 ± 1·88	4·10 ± 0·95	4·63 ± 0·54
Ether extract	11·01 ± 4·33	2·34 ± 0·90	5·53 ± 1·75	3·10 ± 0·33	40·23 ± 0·54
Nitrogen-free extract	—	28·33 ± 3·31	10·83 ± 1·51	27·50 ± 3·18	6·83 ± 1·02
Energy value (Kj/g)	26·39 ± 2·01	19·52 ± 2·15	19·14 ± 2·00	20·13 ± 3·25	34·74 ± 1·98

TABLE 2

MINERAL COMPOSITION OF SOME PROTEIN SOURCES AND WHOLE HEN'S EGG (g/100 g DRY MATTER) (Mean value ± SD)

Mineral	*Smoked fish*	*Periwinkle* (Littorina littorea)	*Crayfish* (Palamonetes varians)	*Snail* (V. quadrata)	*Whole hen's egg*
Calcium	15·31 ± 3·51	5·13 ± 0·35	6·50 ± 0·98	2·31 ± 0·15	0·81 ± 0·02
Phosphorus	2·61 ± 0·55	0·39 ± 0·01	1·40 ± 0·03	0·53 ± 0·09	0·79 ± 0·01
Iron	0·03 ± 0·00	0·04 ± 0·00	0·16 ± 0·01	0·04 ± 0·01	0·02 ± 0·00
Magnesium	0·14 ± 0·01	2·77 ± 0·15	0·45 ± 0·03	0·24 ± 0·01	0·06 ± 0·01
Chloride	0·81 ± 0·04	0·57 ± 0·02	1·65 ± 0·10	0·82 ± 0·11	0·91 ± 0·13
Sodium	0·53 ± 0·01	0·28 ± 0·05	1·22 ± 0·19	0·64 ± 0·08	0·06 ± 0·02
Potassium	1·09 ± 0·11	0·50 ± 0·80	1·20 ± 0·20	0·51 ± 0·01	0·58 ± 0·11
Manganese (mg/100 g)	2·80 ± 0·06	3·22 ± 0·17	5·10 ± 0·18	1·61 ± 0·22	0·95 ± 0·04
Copper (mg/100 g)	1·20 ± 0·10	33·15 ± 2·11	4·10 ± 0·55	2·48 ± 0·18	0·98 ± 0·14
Calcium/Phosphorus ratio	5·90	13·20	4·64	4·36	1·03

the other hand, contained more ether extractable material which may be attributable to the presence of the yolk with its high content of carotene. The gross energy value of 34·74 Kj for whole hen's egg was also higher than for the test samples. Of all these lesser known protein sources, smoked fish contains higher crude protein, ash, ether extractable material and energy than the others.

The mineral composition of the samples and whole hen's egg is shown in Table 2. The calcium contents of the samples were quite high, being 15·31, 5·13, 6·50 and 2·31% dry matter, respectively. Considering these lesser known peasant foods individually, periwinkle seems to be the best source of the trace elements copper, manganese and magnesium. Crayfish is richer in iron, chloride, sodium and potassium than the other test samples and whole hen's egg. In all cases, these lesser known foodstuffs contain higher quantities of iron and copper than whole hen's egg. The importance of these trace elements in the nutrition of the peasant cannot be overemphasised, especially in the villages where there is abundant evidence of anaemia arising from gastroenteritis, hookworm and similar ailments which precipitate iron and copper deficiences.

In Table 3, the indispensable amino acid pattern of the protein sources and whole hen's egg is shown. While whole hen's egg contains more isoleucine (390 mg/gN) and valine (420 mg/gN), all the test samples are better sources of tryptophan. With the exception of *Vivapara quadrata*, the samples are also richer in lysine. Periwinkle (*Littorina littorea*) also contains more leucine, threonine and phenylalanine than whole hen's egg. The protein in these lesser known foodstuffs is also of considerably high quality with chemical scores of 70·89, 75·95, 61·96 and 62·50 for smoked fish, periwinkle, crayfish and snail, respectively. The most limiting amino acids were valine in locally smoked fresh water fish, isoleucine in *Littorina littorea*, threonine in *Palamonetes varians* and methionine in *Vivapara quadrata*.

In Table 4, the indispensable amino acid patterns of the lesser known protein sources are compared with cow's milk, human milk and the FAO (1957) provisional patterns reported by Lunven *et al.* (1973). Periwinkle looks quite outstanding in its indispensable amino acid pattern. Its isoleucine content is 122·6% of that in the FAO (1957) provisional pattern, 112·2% of that in cow's milk and 130·3% of that in human milk. It also contains 224·8% leucine, 220·0% lysine, 211·7% threonine, 153·3% tryptophan, 136·7% valine and 210·6% total aromatic amino acids when compared with the FAO (1957) provisional pattern. It is also richer than cow's milk and human milk in all the indispensable amino acids except the total sulphur amino acids which were not determined. On the other hand *Vivapara quadrata*, *Palamonetes varians* and *Pisces* spp. contained less isoleucine than the FAO (1957) provisional pattern, cow's milk and human milk.

These results certainly indicate that these foodstuffs could provide, on a dry weight basis, much more dietary protein than whole hen's egg. The protein is also of good quality judging from the high chemical scores. Such foodstuffs, like the periwinkle (*Littorina littorea*), are even richer in indispensable amino acids than

TABLE 3

INDISPENSABLE AMINO ACID PATTERN OF SOME PROTEIN SOURCES AND WHOLE HEN'S EGG (mg/gN)

Amino acid	*Smoked fish*	*Periwinkle* (Littorina littorea)	*Crayfish* (Palamonetes varians)	*Snail* (V. quadrata)	*Whole hen's egg*
Isoleucine	244	331	238	238	390
Leucine	439	688	476	469	550
Lysine	750	594	742	439	538
Threonine	231	381	181	249	320
Tryptophan	236	138	219	156	102
Valine	262	369	250	297	420
Methionine	119	129	119	65	133
Phenylalanine	193	384	237	196	244
Total indispensable amino acids	2374	3014	2462	2109	2697
Chemical score	70·89	75·95	61·96	62·50	—

TABLE 4

COMPARISON OF INDISPENSABLE AMINO ACID PATTERN OF SOME PROTEIN SOURCES WITH FAO (1957) PROVISIONAL PATTERN,† COW'S MILK† AND HUMAN MILK† (mg/gN)

Amino acid	*Smoked fish*	*Periwinkle* (Littorina littorea)	*Crayfish* (P. varians)	*Snail* (V. quadrata)	*FAO provisional pattern*† (*FAO*, 1957)	*Cow's milk*† (*FAO*, 1970)	*Human milk*† (*FAO*, 1970)
Isoleucine	244	331	238	238	270	295	254
Leucine	439	688	476	469	306	596	548
Lysine	750	594	742	439	270	487	428
Threonine	231	381	181	249	180	278	280
Tryptophan	136	138	219	156	90	88	105
Valine	262	369	250	297	270	362	284
Total S-amino acids	—	—	—	—	270	208	185
Total aromatic amino acids	331	758	446	340	360	633	421

† Lunven *et al.* (1973).
(—) Not determined.

cow's milk and human milk and contain more than the FAO (1957) recommended quantity of indispensable amino acids. Except in the case of snail, methionine was not limiting. With their high content of lysine, these foods could effectively supplement the cereals and legumes which are widely consumed in many villages in Nigeria. These foodstuffs can also provide mineral elements, especially the trace elements which act at enzyme levels during metabolism. It may be added that despite the high chemical scores of the protein and the high nutrient contents of these lesser known foods, their physiological availability to the consumer is still in doubt. Work is, however, in progress to evaluate their nutritional values by *in vivo* studies with rats.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the assistance of Mr J. Onuntuei in the collection of the samples and to thank Mr O. Hassan for the determination of the gross energy of the samples.

REFERENCES

AOAC (1970). *Methods of analysis of the Association of Official Analytical Chemists* (11th ed.), Washington, D.C., 123–37.

BASSIR, O. (1962). In: *Biochemical aspects of human malnutrition in the Tropics*, ed. A. Junk Den Hang, The Netherlands, 10–15.

BASSIR, O. & UMOH, I. B. (1973). The nutritive adequacy of some peasant Nigerian diets, *Ecology of Food and Nutr.*, **2**, 297–306.

EKPO, E. U. (1974). Field problems in nutrition among peasant communities and suggested remedial measures in relation to developmental programmes with special reference to Nigeria, *Proceedings of the 1st African Nutrition Congress*, University of Ibadan, Ibadan, Nigeria, 1975 (in press).

FAO (1957). The FAO Committee on Protein Requirements. FAO Nutritional Studies, No. 16. FAO. Rome.

GOMORI, G. (1942). A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter, *J. Lab. Clin. Med.*, **27**, 955.

IDUSOGIE, E. O. (1973). Centuries of changing food consumption patterns in African communities. Paper presented at the Conference on Factors Affecting Food Utilization in the Semi-Arid Tropics. Zaria, Nigeria, ABU.

IDUSOGIE, E. O. (1971). The relation of population and nutritional health problems in African communities. Paper presented at the African Population Conference. Accra, Ghana.

LUNVEN, P. & DE ST. MAREQ, E. LEC. (1973). Amino acid composition of hen's egg. *Brit. J. Nutr.*, **30**, 189–94.

MILLER, E. L. (1967). Determination of the tryptophan content of feedingstuffs with particular reference to cereals, *J. Sci. Food Agric.*, **18**, 381.

SPACKMANN, D. H., STEIN, W. H. & MOORE, S. (1956). Automatic recording apparatus for use in chromatography of amino acids, *Fed. Proc.*, **15**, 358.

UMOH, I. B. (1973). Changes in the nutritive values of some Nigerian diets after cooking by certain South Eastern Nigerian traditional methods. Ph.D. Thesis. University of Ibadan, Ibadan, Nigeria.

Technical Note

Pyrazine Compounds in Canned Sweet Corn Flavour

ABSTRACT

Dimethyl pyrazine and dimethylethyl pyrazine were identified in canned sweet corn as important aroma compounds formed during thermal processing.

INTRODUCTION

Canned sweet corn has a characteristic flavour that differs from other canned vegetables. Cooked sweet corn has a smooth, cooked and appetising odour that fresh corn does not have. Several reports have been published on the flavour of field corn, but there are few on sweet corn flavour. Hougen *et al.* (1971) proposed gas–liquid chromatographic (GLC) analysis of headspace vapour of corn in order to classify the chemotaxonomic characteristics of the seed. His group (McKeag & Hougen, 1977) isolated aroma essences from fresh corn by steam distillation and compared varietal differences by the use of GLC peaks. Dimethyl sulphide is the compound that has been studied the most in both fresh and processed sweet corn (Self *et al.*, 1963; Bills & Keenan, 1968; Williams & Nelson, 1973; Flora & Wiley, 1974). Walradt *et al.* (1970) identified thirty-six volatile compounds from popcorn and proposed that pyrazines, furans, pyrrols, carbonyls and substituted phenols are important components of popcorn flavour. This paper reports characteristic aroma compounds isolated from canned sweet corn.

MATERIALS AND METHODS

Two varieties of sweet corn (Style-Pack and Jubilee), grown in upstate New York and processed both at commercial plants and at the pilot plant of the Department of

Fd. Chem. (3) (1978)—
Printed in Great Britain

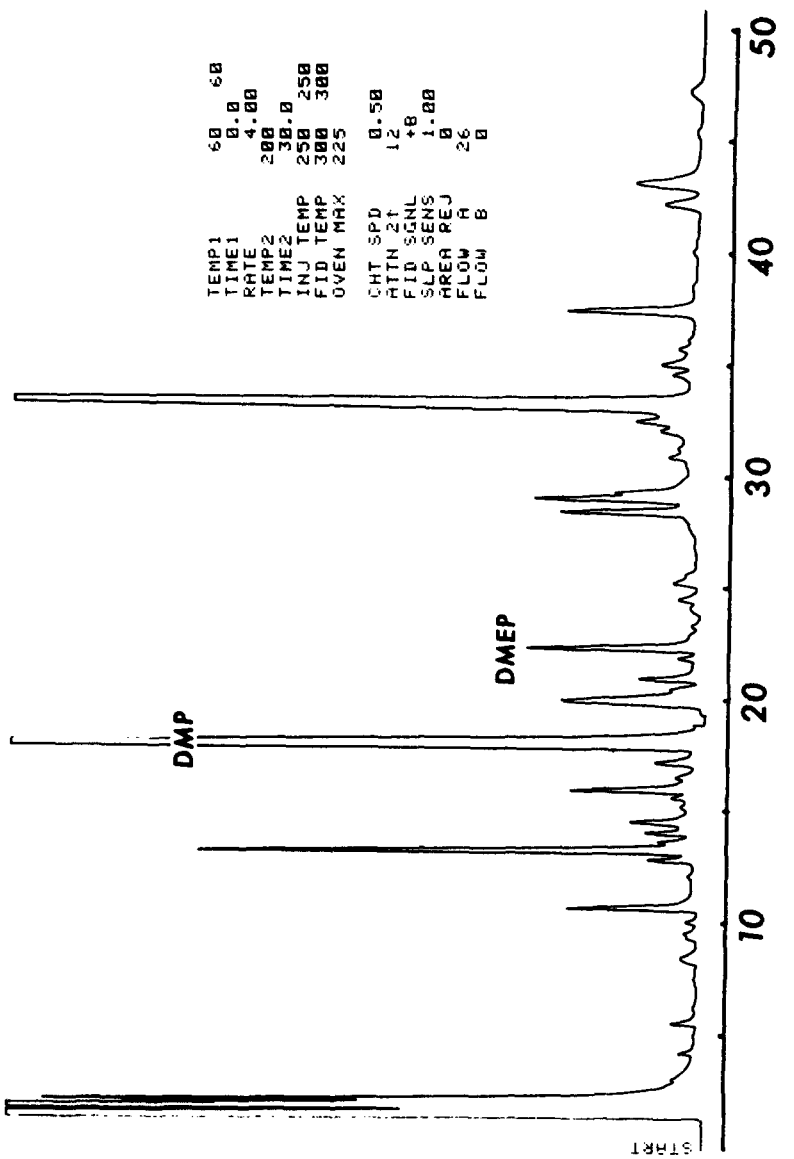


Fig. 1. A typical gas chromatogram of canned sweet corn volatiles on an SP-1000 column using a flame ionisation detector. DMP: 2,5 dimethyl pyrazine, DMEP: 3,6-dimethyl-2-ethyl pyrazine.

Food Science and Technology, NY State Agricultural Experiment Station, were used for this study. Whole contents of three cans (No. 303) of corn were homogenised in a blender with 500 ml H_2O. The slurry was placed in a 5 litre pot attached to the modified Likens and Nickerson distillation apparatus (Flath & Forrey, 1977). A solvent flask containing hexane was attached to the left-hand side of the apparatus. The mixture was boiled for between five and six hours at atmospheric pressure while nitrogen gas was passing through the corn slurry. This extraction procedure was repeated five times and finally 0·4 g of volatile concentrate having a pronounced canned corn aroma was obtained after removal of the hexane solvent. The concentrate was analysed directly by GLC (HP-5830A) using a 2 m × 0·2 cm id stainless steel column packed with 80–100 mesh SP-1000 with N_2 gas as a carrier gas. The GLC conditions used are given in Fig. 1. In order to determine the odour characteristic of each chromatographic peak, a sniffing device (Acree *et al.*, 1976) was used on a Packard gas chromatograph Model 800 equipped with the same column material. Identification of compounds was accomplished by using a Varian 1400 gas chromatograph interfaced to a Bendix 12 Time of Flight mass spectrometer.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram of volatiles from canned sweet corn. The characteristic odour of canned sweet corn (for both varieties) was located in the peaks having retention times of 18·4 and 23·0 min. Most other peaks possessed an undesirable odour usually described as 'harsh', 'green' or 'oxidised oil'. Therefore, no attempt was made to identify these compounds. The first peak was identified as 2,5-dimethyl pyrazine (DMP) whose mass spectrum (molecular ion 108, other important ions at 42, 39, 40, 81, 109 and 91) and GLC retention time were consistent with that of an authentic sample. The second peak was identified as 3,6-dimethyl-2-ethyl pyrazine (DMEP) with a mass spectrum (molecular ion 136, other major ions at 135, 42, 39, 108, 56 and 91) and GLC retention time which coincided with that of the authentic compound. These and other pyrazine compounds have been found in many other processed food products (Maga & Sizer, 1973), including potatoes (Deck & Chang, 1965; Buttery *et al.*, 1971) and roasted peanuts (Mason *et al.*, 1966). Koehler *et al.* (1969) have shown that the amino acid–glucose reaction in model systems produces a large amount of DMP and DMEP. It is generally conceded that pyrazine compounds are generated during the thermal processing of foods. The essence extracted from fresh sweet corn in this study did not show any detectable amounts of DMP and DMEP. Since sweet corn contains an appreciable amount of free amino acids and sugars, it is concluded that the two important pyrazine compounds identified in canned sweet corn were produced by the reaction of these substances during the course of canning.

REFERENCES

ACREE, T. E., BUTTS, R. M., NELSON, R. R. & LEE, C. Y. (1976). Sniffer to determine the odor of gas chromatographic effluents, *Anal. Chem.*, **48**, 1821.

BILLS, D. C. & KEENAN, T. W. (1968). Dimethyl sulfide and its precursor in sweet corn, *J. Agric. Food Chem.*, **16**, 643–5.

BUTTERY, R. G., SEIFERT, R. M., GUADAGNI, D. G. & LING, L. C. (1971). Characterization of volatile pyrazine and pyridine compounds of potato chips, *J. Agric. Food Chem.*, **19**, 969–71.

DECK, R. Z. & CHANG, S. S. (1965). Identification of 2,5-dimethyl-pyrazine in the volatile flavor compounds of potato chips, *Chem. & Indust. (London)*, 1343–44.

DRAVNIEKS, A., REILICH, H. G., WHITFIELD, J. & WATSON, C. A. (1973). Classification of corn odor by statistical analysis of gas chromatographic patterns of headspace volatiles, *J. Food Sci.*, **38**, 34–9.

FLATH, R. A. & FORREY, R. R. (1977). Volatile components of papaya (*Carica papaya* L. solo variety), *J. Agric. Food Chem.*, **25**, 103–9.

FLORA, L. F. & WILEY, R. C. (1974). Influence of cultivar, process, maturity, and planting data on the dimethyl sulfide and hydrogen sulfide levels in sweet corn, *J. Agric. Food Chem.*, **22**, 816–19.

HOUGEN, F. W., QUILLIAM, M. A. & CURRAN, W. A. (1971). Headspace vapors from cereal grains, *J. Agric. Food Chem.*, **19**, 181–3.

KOEHLER, P. E., MASON, M. E. & NEWELL, J. A. (1969). Formation of pyrazine compounds in sugar-amino model systems, *J. Agric. Food Chem.*, **17**, 393–6.

MAGA, J. A. & SIZER, C. E. (1973). Pyrazines in foods. A review, *J. Agric. Food Chem.*, **21**, 22–30.

MASON, M. E., JOHNSON, B. & HAMMING, M. (1966). Flavor components of roasted peanuts. Some low molecular weight pyrazines and a pyrrole, *J. Agric. Food Chem.*, **14**, 454–60.

MCKEAG, R. C. & HOUGEN, F. W. (1977). Varietal differences in aroma essence from maize kernels (*Zea mays* L.), *J. Agric. Food Chem.*, **25**, 417–18.

SELF, R., CASEY, J. C. & SWAIN, T. (1963). The low-boiling volatiles of cooked foods, *Chem. & Indust. (London)*, 863–4.

WALRADT, J. P., LINDSAY, R. C. & LIBBEY, L. M. (1970). Popcorn flavor: Identification of volatile compounds, *J. Agric. Food Chem.*, **18**, 926–8.

WILLIAMS, M. P. & NELSON, P. E. (1973). Effect of hybrids and processing on the dimethyl sulfide potential of sweet corn, *J. Food Sci.*, **38**, 1136–8.

C. Y. LEE,
Department of Food Science and Technology,
New York State Agricultural Experiment Station,
Cornell University,
Geneva, NY 14456, *USA*

BOOK REVIEW

Amine und Nitrosamine. By Ahmed Asker. Vorkommen, Bedeutung, Stoffwechsel und Bestimmung.

Quite apart from their probable contributions to the flavour, aroma and acceptability of items of the diet, amines have become important trace components of foods and drinks as potential precursors to N-nitroso compounds and as possible toxic agents where medication with monoamine oxidase inhibitors is involved or in initiating migraine attacks.

A feature of this book is its list of amines which occur in foods and drinks, including milk and cheese, meat and sausages, fish, wine, beer, etc. The contents of tyramine, which has been found to precipitate attacks of migraine, in food sources are particularly well represented, along with the simpler primary, secondary and tertiary alkylamines. Further sections of this book are concerned with the importance of amines in relation to aroma, non-enzymic browning, quality control, etc., as well as the functions of amines *in vivo* and as diagnostic aids in clinical chemistry. The pathways of the biosynthesis and metabolism of many amines are dealt with in detail and presented conveniently in a tabulated form together with some of the physiological actions of amines.

Finally, so far as amines are concerned, the various methods available for their detection and determination are reviewed in a further section, including their separation and concentration and such methods of detection as paper, thin layer and gas chromatography, mass spectrometry, etc. In fact, this book provides a comprehensive but concise treatise of many of the various aspects of the chemistry and biochemistry of amines. Its treatment of nitrosamines, the other topic of its title, is very cursory in comparison, being restricted to abbreviated accounts of their synthesis, actions and occurrence, and that of their precursors in foods. For those researchers interested in amines *per se*, however, this book is well worth while, but it would have been an advantage to provide an adequate binding to prevent its early collapse, in spite of gentle treatment by the reader.

C. WALTERS

Fd. Chem. (2) (1977)—
Printed in Great Britain

INDEX TO VOLUME 3

(Figures in parentheses are issue numbers)

Fd. Chem. (3) (1978)—
Printed in Great Britain

INDEX TO VOLUME 2

(Figures in parentheses are issue numbers)

Fd. Chem. (2) (1977)—
Printed in Great Britain

FOOD CHEMISTRY

An International Journal

This journal is concerned mainly with the chemistry and biochemistry of foods and chemical and biochemical changes occurring in foods. It is also concerned with sensory and nutritional properties of food within a chemical or biochemical framework. The journal provides a vehicle for the widest possible dissemination of knowledge in food chemistry, mainly as research papers and reviews.

Research papers must be factual and original, and must not have been published elsewhere, though papers whose biological or technological aspects have already been published may be accepted if previously unpublished chemical information and conclusions are now included. The following subjects are among those open for discussion: pure and applied food chemistry; structural changes in molecules during the processing and storage of foods; chemical aspects of nutrition, physiology and health; chemical quality control in food chemical engineering and technology; direct effects on food of the use of agrochemicals; chemistry relating to sensory aspects; chemical additives; chemical toxins; chemical analysis of food; food biochemistry; and chemistry relating to microbiological aspects.

Type of contributions

Research papers
Review articles
Book reviews; Conferences and meetings
Short communications; Letters to the Editor

Submission of papers

Papers should be sent to the Editors (Dr G. G. Birch and Mr L. F. Green, c/o National College of Food Technology, St. Georges Avenue, Weybridge, Surrey, England), to members of the Editorial Board for submission to the Editors, or to the publishers.

Subscriptions

Four quarterly issues per volume (16.5 × 24 cm). Subscription orders should be addressed to:

APPLIED SCIENCE PUBLISHERS LTD, Ripple Road, Barking, Essex, England

Note: The publishers of *Food Chemistry* regret that claims for missing copies can only be entertained if made within six months from the date of despatch.

LIST OF MEMBERS OF EDITORIAL BOARD