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Biopotency of Vitamin A in Fortified Flour after Accelerated Storage

The biopotency and availability of the stabilized vitamin A remaining in a fortified flour after storage under accelerated conditions (40 or 45 °C) were studied by estimating relative biopotency by a maleic anhydride procedure and by comparing growth and body storage of vitamin A in rats fed diets containing the stored or fresh flours. The quantity of vitamin A remaining in the stored flour, as determined colorimetrically, had practically the same biopotency as when originally added and was fully available to rats as a source of vitamin A for growth and body storage.

In 1974 the Food and Nutrition Board of the National Academy of Sciences proposed a new fortification policy for cereal-grain products to replace that adopted in 1943 (*Federal Register*, 1943) by adding vitamin A, pyridoxine, folic acid, calcium, magnesium, and zinc and increasing the amount of iron (National Academy of Sciences, 1974). Before a decision could be made on implementation of the proposal it was necessary to prepare and test the fortified products under appropriate conditions of manufacture, storage, and use.

When fortified flours, which contained 0.28 mg of retinol equivalents/100 g along with the other added nutrients, were stored under accelerated conditions at 40 °C, they lost about 30% of the stabilized vitamin A in 3 months (Parrish et al., 1978) and 45% in 6 months. But there was a question whether the remaining vitamin A, as determined colorimetrically (Association of Official Analytical Chemists, 1975), was bioactive and available to meet the vitamin A requirements or whether it had undergone isomerization or degradation so that the analytical values overestimated the actual vitamin A activity (Parrish, 1977).

This is a report on relative biopotency and bioavailability of stabilized vitamin A in stored fortified flour as measured by two different methods: (1) estimation of relative biopotency by the maleic anhydride procedure (Ames and Lehman, 1960) and (2) growth of rats and storage of vitamin A by those rats when fed diets containing the fortified flour. Although there are reports on losses of vitamin A in other stored fortified flours, as determined by standard analytical procedures, nothing was found on the biopotency of the stabilized vitamin A remaining in the flour.

EXPERIMENTAL SECTION

Fortified bread flour, 9.9% protein, was stored for 6 months at 40 °C and then placed in a freezer until used for the feeding trial. Vitamin and mineral additions (Table I), plus amounts naturally in the flour, brought contents of those nutrients to the proposed fortification levels, with overages of 5–20% to provide for normal manufacturing and storage losses (American Bakers Association Inter-Industry Committee, 1976).

Table I. Vitamin-Mineral Additions to Flour (g/100 kg)[a]

thiamin mononitrate	0.565
riboflavin	0.396
niacinamide	4.62
pyridoxine hydrochloride	0.44
folic acid	0.057
vitamin A palmitate, 250-SD	4.4
tricalcium phosphate	0.66
reduced iron	2.52
calcium sulfate	665
magnesium oxide	44
zinc oxide	2.1

[a] All ingredients listed, except the last three, were added as a premix in a starch base, 40% of premix weight.

The original proposed fortification for vitamin A of 0.48 g/100 g was determined to be too high and was changed by the panel to 0.28 mg/100 g of retinol equivalents (Hepburn, 1976); that level was used in this study. Iron fortification was not increased to 8.81 mg/100 g because approval of an increase seemed unlikely (*Federal Register*, 1977). Vitamin A was added as stabilized vitamin A palmitate, 250-SD (Hoffman-LaRoche). Because of overages in the vitamin A product, premix, and flour, vitamin A content by analysis (Association of Official Analytical Chemists, 1975) was about 25% higher than the proposed level. All vitamin A data in this report are based on analytical values.

To provide sufficient vitamin A for a good test of estimated biopotency by the maleic anhydride procedure (Ames and Lehman, 1960), vitamin A palmitate, 250-SD,

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Table II. Diets Fed Rats for 4 Weeks[a]

ingredient	diets[b] std 1 & exptl 1, g/kg	std 2 & exptl 2, g/kg
flour	450	225
casein ("vitamin-free")	123	150
corn starch	180	330
glucose	64	110.5
Wesson oil	48.5	50
cellulose	19	19
sodium chloride	20	20
calcium carbonate	4.5	5.5
monocalcium phosphate (monohydrate)	11	10
vitamin premix[c]	40	40
mineral premix[d]	40	40

[a] 3140 units vitamin A/kg in diets 1; 1570 units in diets 2. [b] Standard diets 1 and 2 prepared from flour stored without fortification; experimental diets 1 and 2 prepared from stored, fortified flour (see text). [c] Except for vitamins in the fortified experimental flour and vitamin A, the premix contained vitamin additions to meet requirements, plus 2.5 g of methionine/kg diet, in a starch-glucose carrier. [d] Except for the quantities of Fe and Zn in the fortified experimental flour, the premix contained supplemental K, Mg, Mn, Cu, Zn, and Se, to meet requirements, and Fe to meet approximately 90% of the total requirement, in a starch-glucose carrier.

was added to fortified bread and corn flours before they were stored to raise the vitamin A contents to approximately ten times the proposed level. Because even at the higher level the particles of stabilized vitamin A product constituted only about 0.05% of the total flour weight, one would expect similar storage changes in vitamin A at both the lower and higher fortification levels. The bread and corn flours were stored for 3 months at 45 °C; bread flour was stored an additional 6 months at 45 °C and then at room temperature for 3 months.

Male albino rats, Charles River strain, were weaned when 21 days old and given a basal diet without vitamin A but otherwise similar to the experimental diet (Table II) for 5 days. Rats then were selected within a 5-g weight range, distributed at random to four groups of ten each, placed in individual wire-bottom stainless-steel cages in an animal room maintained at about 27 °C, and given the diets (Table II) and water ad lib for 4 weeks. Rats were checked and weighed weekly, and records were kept on feed consumption. Diets were mixed just before the feeding study was begun. They were placed in a freezer immediately after mixing and only enough for 4 or 5 days of feeding was removed at a time.

The diets were designed to meet the requirements of the growing rat (National Academy of Sciences, 1972), except for vitamin A. Vitamin C also was added to the diet at approximately the human requirement adjusted on a weight basis. Water-soluble vitamins and trace minerals, except iron, were added to requirement levels with no allowance for that naturally in the flour. Iron was provided at about 90% of the requirement.

In experimental diets 1 and 2, vitamin A at 3140 and 1575 IU per kg, respectively, was supplied solely by that in stored fortified flour. Earlier studies had indicated that if the vitamin A was available, it should provide enough for growth and adequate serum levels in rats receiving diets 1 and 2 and result in markedly higher vitamin A storage in the livers of rats fed diets 2. If, however, there was a significant decrease in vitamin A availability, it would be reflected in less growth and serum vitamin A in rats fed the experimental diet containing the lower vitamin A level than in rats receiving the same amount of vitamin A from the standard diet. The standard diets 1 and 2, used for comparison with the experimental diets, were prepared from unfortified stored flour to which the vitamins and minerals were added only when diets were prepared. In the standard diets vitamin A was supplied as USP Reference Standard, an oily solution of retinyl acetate, a well-utilized source of vitamin A.

Vitamin A in flours was determined by the AOAC colorimetric method (Association of Official Analytical Chemists, 1975), modified by adding sodium sulfite and ascorbic acid before alkaline hydrolysis, which appeared to increase some analytical values slightly and make the extraction procedure somewhat easier. The biopotency method (Ames and Lehman, 1960) was modified only be extracting with hexane instead of ether. The serum vitamin A method (Kimble, 1939) was modified to use a smaller sample. Liver vitamin A was determined by alkaline hydrolysis, extraction with ether and hexane, and colorimetry similar to the method used for flour.

RESULTS AND DISCUSSION

The weight gains were higher and serum vitamin A contents lower for rats fed diets made with stored, fortified flours (Table III), but those differences were not significant ($P = 0.05$). As expected, much more vitamin A was stored in livers of rats with the higher intakes of vitamin A. At the lower level of vitamin A in the standard and experimental diets, vitamin A contents per liver were in the same general range; they were, however, significantly higher ($P = 0.05$) in rats fed the standard diet. But at the higher dietary levels of vitamin A in the standard and experimental diets, and at both levels, when results were calculated on a per gram of liver basis, the differences were not significant.

Estimated biopotencies of vitamin A in stored flours were all 95% or higher (Table IV), indicating practically no changes in biopotency. Since all data, except for the one difference noted above, indicated good utilization of the stabilized vitamin A in fortified flours stored for 6 months under accelerated conditions (40–45 °C) and the bioavailability, as estimated chemically, was not changed by storage, stabilized vitamin A in flour stored over a period of time under normal, less rigorous conditions should be highly available to meet dietary requirements. Thus the principal concern would be gradual loss of vitamin A during storage (Parrish et al., 1978), not loss of

Table III. Results of 4-Week Rat Feeding Trial

	diet			
	std 1	exptl 1	std 2	exptl 2
weight gain, g	182 ± 18[a]	194 ± 17	191 ± 9	194 ± 16
food eaten, g	397 ± 45	434 ± 31	424 ± 26	428 ± 16
feed efficiency	0.458	0.447	0.450	0.453
serum vitamin A, μg/100 mL	43.5 ± 7.8	37.6 ± 7.6	44.9 ± 4.6	40.8 ± 6.1
liver vitamin A, μg/liver	139 ± 25	135 ± 25	32 ± 4[b]	26 ± 4[b]
liver vitamin A, μg/g of liver	12.5 ± 2.02	12.6 ± 2.43	2.65 ± 0.47	2.35 ± 0.53

[a] Averages and standard deviation. [b] Significant difference ($P = 0.05$) for same level vitamin A in the diet.

Table IV. Estimated Biopotency of Vitamin A in Stored, Fortified Flours

storage conditions	corn flour	white bread flour
3 months, 40 °C	99	98
3 months, room temp.	98	99
3 months, 45 °C	97	95
12 months[a]		95

[a] Nine months at 45 °C, 3 months at room temperature.

bioactivity of vitamin A remaining in the flour.

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Vacuum Thermolysis of 1-Deoxy-1-sarcosino-D-fructose

Thermolysis of the title compound at 140 °C in vacuo yields products that support the earlier suggestion of a preferred decomposition pathway for Amadori compounds substituted with a secondary amino acid. The degradation route proceeds largely through 2,3-enolization of the ketose, with loss of the amino acid, to form a methyl α-dicarbonyl intermediate. The mechanism differs from those of Amadori compounds derived from a secondary amine or primary amino acid in the decomposition of the first-formed intermediate from the 2,3-enolization process. One of the hexose dehydration products derived from this intermediate, 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one, comprises 63% of the isolated distillate. Sarcosine derivatives, a piperazine (31% of the distillate), substituted pyrroles, 2-furfurol, pyrones, and a furanone are other classes of the volatile compounds identified. The findings again demonstrate that the described pyrone and 2,3-dimethyl-4-hydroxy-3(*H*)-furanone are important compounds for the development of browning aromas from sugar–amino acid reaction.

Thermally initiated browning of 1-amino-2-ketoses (Amadori compounds) and the subsequent set of complex reactions that follow characterize in part the genesis of volatile compounds important to food acceptance (Hodge, 1967; Mills et al., 1969, 1970; Reynolds, 1970; Hodge et al., 1972; Mills and Hodge, 1976). These nonvolatile intermediates can be formed by reaction of free and bound amines, amino acids, peptides, or proteins with reducing sugars. The results of earlier model studies, that the decomposition of Amadori compounds had shown a significant difference in decomposition of the 1-substituted 2-ketose when the substituent was changed from a secondary amine to an amino acid (Mills et al., 1970; Mills and Hodge, 1976), are now confirmed with recent reports of the thermolysis of other model 1-amino-1-deoxy-2-ketoses, fructoses containing a 1-valine, -proline, -alanine, or a -4-aminobutyric acid moiety (Shigematsu, 1976; Shigematsu et al., 1977). Because of the later work, the sarcosino-D-fructose study is abbreviated and the conclusions are presented as further support to the initial investigations (Mills et al., 1970; Mills and Hodge, 1976). Our findings demonstrate that the current hexose degradation path agrees with the earlier results (Mills and Hodge, 1976), supporting the concept that 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one, its dehydrogenation product, **13**, and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone are indicators of the browning process in heated or cooked foods (Ledl et al., 1976).

The title Amadori compound was thermally decomposed and the resulting distillate was fractionated by gas–liquid chromatography. The products identified (Table I) were isolated from this distillate except for sarcosine, which was found in the pyrolysis residue, and structural identifications were made spectrometrically. Most assignments were verified by comparison of either gas–liquid chromatography (GLC), infrared (IR), or mass spectral (MS) data, or all of these techniques, with those from synthetic or authentic compounds. As in the past, the low-temperature thermolysis (140 °C) produced fewer types of degradation and rearrangement products compared to those formed at the higher temperatures (Shigematsu, 1976), but these are still important to flavor and aroma development.

Nitrogenous products **1**, **2**, **3**, and **8** are related by class to compounds found in the thermolysis of prolino-D-fructose; their genesis has been described (Mills and Hodge, 1976). Of the nitrogen-containing components, compound **4** represents the only five-carbon hexose fragment, and similar products were not observed in the