Vitamin A Activity of Acidulated Soybean Soapstocks in Chicks[1]

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Abstract

Acidulated soybean soapstock increased the vitamin A storage in the livers of young chicks receiving a practical-type diet and improved the survival of chicks reared on vitamin A-deficient diets. The biological activity was mainly associated with one of the carotenoids present in the acidulated soapstock. This pigment is characterized by its red color on the MgO column, its position below lutein but slightly above cryptoxanthin on the same column, and its *single* absorption max at 460 mμ in the visible region. Evidence is presented to show that the pigment is an artefact formed from lutein during the acidulation of raw soapstock and that it is identical with 3′-hydroxy-3,4-dehydro-β-carotene, a dehydration product of lutein.

Introduction

ACIDULATED SOYBEAN soapstock (ASS) is used in Israel as a supplement for poultry feeds. This material is known to improve growth and feed efficiency (3) and has been reported to increase skin pigmentation (4). Other products prepared from soybean soapstock have been patented as pigmenters for poultry (8,10).

Recently it was reported from this laboratory (2) that ASS enhances the liver storage of vitamin A in chicks. The purpose of the present research was to clarify the reason for this effect.

This work was carried out in three stages. The first stage comprised a series of feeding tests designed to evaluate the effect of ASS on the vitamin A storage in the livers of chicks receiving a practical-type diet, as well as the effect of ASS on the growth and survival of vitamin A-deficient chicks. In the second stage, a chromatographic fractionation of the carotenoid pigments of ASS was undertaken and feeding tests were carried out with some of the fractions obtained. This led to the separation of an active pigment. In the third stage, an attempt was made to characterize the biologically active carotenoid and to clarify the mechanism of its formation from inactive precursors present in the crude oil.

Experimental

Animals. All experiments were carried out with New Hampshire x White Leghorn cockerels obtained from a commercial hatchery. Birds were kept in thermostatically-controlled, electrically-heated batteries equipped with raised wire floors. Experimental feeding was preceded by a depletion period of two weeks. The average amt of vitamin A after depletion was 10–30 IU/liver. Chicks were then divided into groups according to their body wt, in a manner to insure that the average starting wt was equal for all groups.

Diets. The basal diets used in the different experiments are presented in Table I. Reference to these diets is made in the tables giving results of the experiments.

Statistical Evaluation. Results of each feeding experiment were evaluated by analysis of variance. Significance of differences between different groups was tested by the studentized range q test.

Chemical Methods. Liver vitamin A was assayed by the procedure of Ames et al. (1).

Tocopherols were determined by the column-chromatographic procedure of Bro-Rasmussen and Hjarde (5), with reduced sizes of samples and column. Readings were made at 520 mμ on a Bausch & Lomb Spectronic colorimeter, pure tocopherols being used for the calibration. Elution peaks of the chromatograms were identified by parallel runs with authentic tocopherols.

Fractionation of the carotenoids from ASS was carried out as follows: 10 g ASS were saponified by refluxing for 30 min with 100 ml ethanol and 10 ml KOH 50% (w/v). After dilution with 200 ml water, the unsaponificable matter was extracted with ether, transferred to methanol and freed from the bulk of sterols by cooling to −15C and filtering. The pigments were then transferred to a few ml hexane and chromatographed on a column of MgO and Hyflo Super-Cel 1:1 10 mm wide and 150 mm high. After elution of the carotenes and other hydrocarbons with 2% acetone in hexane, the xanthophylls were developed with hexane-acetone 85:15. This resulted in the separation of four less strongly adsorbed pigments which could be further purified by rechromatography on calcium hydroxide, using hexane-acetone (95:5) for development. The more strongly retained xanthophylls could be partially separated on MgO and Hyflo Super-Cel by development with hexane-acetone-ethanol (82.5:15:2.5). Good resolution of the resulting fractions could be achieved by rechromatography on zinc carbonate and development with benzene-ether (80:20).

For large-scale preparation of pigment fractions for feeding tests, pigments from 650 g ASS were chromatographed on large (40 x 400 mm) columns

TABLE I

Composition of the Diets Used in the Different Experiments

Ingredient	Diet number				
	1	2	3	4	5
Sorghum	51.7	30.0	35.0	64.8	55.8
Soybean oil meal	35.0	49.0	39.0	25.0	33.0
ASS or oil		6	6		6
Cellulose (Alphacel)		10.2	6.7		
Wheat bran	5	5	5	4	4
Fish meal	5	5	5	3	4
Ground oyster shells	1.4				
Dicalcium phosphate	1.0				
Fortified salt[a]	0.3	Ibid.	Ibid.	Ibid.	Ibid.
Coccidiostat (Bifuron)	0.1				
Antibiotic (Aurofac 20)	0.1				
Vitamin premix[b]	0.4				
Calories metabolic energy, cal./100 g	281	277	277	241	322
MC/P[c]	48.8	48.0	48.0	61.8	59.4

[a] Saline salt—guaranteed to contain Mn 2.6%, I 0.048%, Fe 0.14%, Cu 0.1%, Co 0.008%, Zn 2.0%, Mo 0.044%.

[b] Vitamin premix/kg diet: riboflavin 2.6 mg, Ca panthothenate 15 mg, vitamin B_{12} 0.006 mg, vitamin D_3 750 i.c.u., menadione sodium bisulphite 6 mg.

[c] Calculated according to Combs' definition (metabolizable cal/lb/ per cent protein) and using Frap's metabolizable energy values as given in Titus, "Scientific Feeding of chickens," 1955, 3rd ed., Table 18.

[1] The National and University Institute of Agriculture, Rehovot, Israel. 1963 Series, 648-E.

TABLE II

Effect of ASS and Vitamin E on Liver Storage of Vitamin A from Two Types of Conc

Vitamin A conc	Diet No.	Supplement	Wt at 6 weeks, g	Vitamin A	
				IU/liver	% storage
Gelatin-coated	1	None	654	511	14.4
	3	ASS	674	723	20.3
	1	Vit. E	670	567	15.6
Wax-coated	1	None	676	231	6.8
	3	ASS	658	353	11.1
	1	Vit. E	686	292	8.3

The additives were supplied at the following levels: a) vitamin A, gelatin-coated: 300; and wax-coated: 275 IU/100 g; b) ASS: 6%; c) vitamin E, as d,l-alpha tocopheryl acetate:204 mg/kg.

The diets were kept isocaloric through the appropriate addition of Alphacel.

Each treatment was given to 30 chicks.

Statistical analysis of the data for liver storage of vitamin A shows that the ASS treatment differs to a highly significant extent ($P < 0.01$) from both other treatments, while the effect of vitamin E was not significantly different from the control.

of MgO and Hyflo Super-Cel. A single development with hexane-acetone (85:15) was sufficient for the separation of the fractions needed for the tests. These fractions were removed mechanically from the column and the pigments eluted with ethanol and ether.

Absorption spectra were determined in a Beckman DU spectrophotometer, while pigment concn were estimated in a Bausch & Lomb Spectronic colorimeter.

Vitamin A Activity of ASS in Chicks

When the effect of ASS on the vitamin A storage in chick livers was first reported (2), the hypothesis was advanced that this may have been due to the tocopherols present in ASS. Furthermore, since a wax-coated vitamin A conc was used in the experiment, the ASS supplement might conceivably have increased the absorption of vitamin A from the intestine by its solvent action on the waxy matrix.

An experiment was therefore undertaken to test the influence of added ASS and vitamin E on the vitamin A storage in chicks receiving a gelatin-coated, as well as wax-coated vitamin A preparation. Table II gives the results obtained.

The slight effect caused by the tocopheryl acetate was not significant, while ASS caused a highly significant increase in storage for both gelatin-coated and wax-coated materials. Although the latter gave a lower storage, compared to the gelatin-coated product, in agreement with previously reported results (2), the relative increase in storage brought about by the ASS was approx the same in both cases.

A subsequent experiment was undertaken in order to find out whether tocopheryl acetate would be more effective if used in a fat-rich diet; whether free

TABLE III

Liver Storage of Vitamin A in Chicks Receiving ASS, the Unsaponifiable Matter from ASS, Vitamin E and Olive Oil

Group	Diet No.	Supplement	Wt at 6 weeks, g	Vitamin A	
				IU/liver	% storage
I	1	None	631	884	25.4
II	1	d,l-α-tocopherol	632	976	26.8
III	1	ASS	622	1218	36.6
IV	3	Unsaponifiable	605	1158	33.2
V	2 (6% olive oil)	None	675	1031	26.5
VI	2 (6% olive oil)	d,l-α-tocopherol	630	892	28.4
VII	2 (6% olive oil)	d,l-α-tocopherol acetate	626	977	28.7
VIII	2 (6% olive oil)	Unsaponifiable	603	1130	32.4

Additives were supplied at the following levels: a) vitamin A (gelatin-coated): 300 IU/100 g; b) ASS: 6%; c) unsaponifiable matter: equivalent to 6% ASS; and vitamin E, as the acetate or the free tocopherol: 115 IU/kg, including the amt supplied by the olive oil.

Each treatment was given to 20 chicks.

Analysis of variance of all data for vitamin A storage in the livers reveals a significant difference ($P < 0.05$) between the basal and ASS treatments. When groups I,II,IV,V,VI and VIII were analyzed as a factorial design, a significant difference ($P < 0.02$) was found between the unsaponifiable and both other treatments.

TABLE IV

Effect of ASS, Vitamin E and Refined Soybean Oil (RO) on the Survival of Chicks Kept on Vitamin A-Deficient Diet No. 4

Supplement	Avg survival time, days	Survivors at 81 days[a]
None	44.8	0/36
RO, 4.5%	44.4	0/36
d,l-α-tocopheryl acetate, 106 mg/kg	50.5	0/36
ASS, 6%	.....	34/36
ASS after depletion[b]	.....	12/32

[a] No. of chicks surviving/No. at start of experiment.

[b] Feeding of ASS started after 14 days depletion.

Statistical analysis shows that the tocopheryl acetate treatment differs to a highly significant extent ($P < 0.01$) from both the negative control and the refined soybean oil treatment.

tocopherol would be more efficient than the ester; and whether the storage-enhancing agent is contained in the unsaponifiable fraction of ASS. The setup of the experiment may be seen from Table III which also shows the results obtained. ASS again significantly improved vitamin A storage when compared with both the control and the tocopherol-supplemented groups. The unsaponifiable matter of ASS was nearly as effective as ASS itself. The reason for the higher body wt in group V is not known. As in the previous experiment, the effect of vitamin E was not significant.

A survival experiment was carried out next, to help to decide whether the increased storage brought about by ASS was the result of improved utilization of preformed dietary vitamin A, or whether ASS itself exhibited provitamin A activity.

In this experiment the diets were not isocaloric, therefore, two control groups were included; one involving the basal diet only and the second group receiving the basal diet plus 4.5% refined oil. This level of refined oil was assumed to be equivalent to 6% ASS in its metabolizable energy content. Table IV gives the results of this survival test.

It is seen that while the refined oil did not produce any effect and α-tocopherol caused a highly significant, but slight increase in survival time, ASS caused a spectacular increase in survival.

ASS was unique in its pronounced vitamin A activity. Some samples of acidulated foots from cottonseed and sunflowerseed oil had slight but significant activities, while acidulated coconut and corn oil soapstocks were not significantly active.

Chromatography and Biological Testing of Carotenoid Fractions from ASS

The unsaponifiable constituent responsible for the vitamin A activity of ASS was likely to be found among the carotenoids in which ASS is particularly rich. Total carotenoid values ranging from 120–250 μg/g were found by us in samples of ASS supplied by different producers and at different times. Carotene could be detected in traces only. This is in con-

TABLE V

Some Carotenoids from a Sample of ASS, in Order of Increasing Retention on a MgO Column

Pigment	Color on column	Approx amt[a] μg/g	Extinction peaks in hexane	
			maxima mμ	minima mμ
F[b]	.....	1	.....	.....
A-1	Yellow-orange	12	424, 446, 476	430, 464
A-2	Yellow	39	417, 438, 466	420, 456
A-3[c]	Yellow		422, 446, 475	428, 462
A-4	Red	14	460	.....
X[d]	.....	96	.....	.....

[a] Measured at the wavelength of max absorption and expressed as β-carotene.

[b] Forerun, including small amt carotenes and fluorescent pigments.

[c] Different from cryptoxanthin which is similarly located on the column, but exhibits two extinction maxima at 451 and 477 mμ.

[d] Xanthophylls, resolvable into 23 different pigments, including lutein.

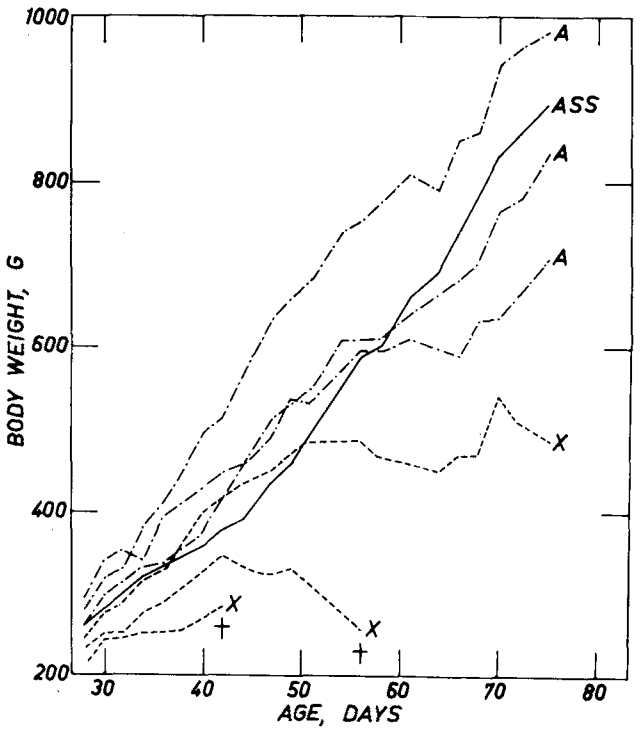

FIG. 1. Individual growth curves of six chicks, three of which received the "A" fraction and the remaining three the "X" fraction of ASS. The fractions were dissolved in an amt of refined soybean oil equal to the wt of ASS from which they had been prepared and added to the diet at a level equivalent to 6% ASS. The average increase in wt of 30 chicks receiving 6% ASS is also shown. All chicks were kept on the vitamin A-deficient diet 4 for 14 days before receiving the supplements.

trast with crude soybean oil which was found to contain 19–30 μg carotenoids, including 0.5–1.3 μg β-carotene/g. Tocopherol levels in ASS were similar to, or slightly lower, than those of crude soybean oil, confirming our conclusion that tocopherol plays no significant role in the increase of vitamin A storage caused by ASS.

Table V presents the position, approximate amt and spectrophotometric characteristics of some of the pigments from ASS. Following a small forerun which included fluorescent pigments and traces of carotenes, four essentially epiphasic (hexane and 90% MeOH) pigments were recognized on the MgO column. They remained mainly unchanged upon rechromatography on calcium hydroxide and are designated A-1 through A-4. Four xanthophyll bands ("X") appeared in the upper part of the MgO column. These could be resolved into a large number of pigments upon rechromatography on zinc carbonate.

None of the better known provitamins A seemed to be present in significant amounts. Therefore, a survival experiment was undertaken in which the pigments of ASS were grouped into the two main fractions comprising the "A" and "X" pigments, respectively. Each of these fractions was dissolved in an amount of refined soybean oil equal to the wt of ASS from which they had been obtained, the oils were added to the vitamin A-free diet at the level of 6% and fed to groups of three chicks each. Growth curves show in Figure 1. It is clear from these data that the chicks receiving the A fraction grew well and survived, while the X pigments were ineffective in supporting growth and preventing mortality.

In order to decide which of the four A pigments carried the vitamin A activity, these pigments were separated and fed individually, together with the forerun and the combined X fraction. The results of this experiment show in Table VI. It is clear from these data that the vitamin A activity is associated with pigment A-4. Slight but significant activities were exhibited by the X fraction and the crude oil.

TABLE VI

Survival of Chicks Receiving Vitamin A-Deficient Basal Diet No. 5 Supplemented with ASS, Crude Soybean Oil (CO), Refined Soybean Oil (RO), Carotenoid Fractions from ASS, and Various Levels of Vitamin A

Supplement	Avg survival time, days	Survival[a] at 49 days	Survival[a] at 70 days
RO	35.2	0/20	.....
CO	44.5	0/20	.....
ASS	.....	18/20	15/20
RO + F	33.5	0/10	.....
RO + A-1	35.4	0/10	.....
RO + A-2	38.0	0/10	.....
RO + A-3	37.0	0/10	.....
RO + A-4	.....	8/10	.....
RO + X	43.6	0/10	.....
RO + 25 IU/100 g	.....	18/20	9/20
RO + 50 IU/100 g	.....	19/20	.....
RO + 75 IU/100 g	.....	20/20	.....
RO + 300 IU/100 g	.....	20/20	20/20

[a] No. of chicks surviving /No. at start of experiment.

Oils were given at the level of 6%. Pigment fractions (symbols as in Table V) were dissolved in an amount of RO equal to the weight of ASS from which they were prepared, and the solutions were mixed with the diets at the level of 6%.

All chicks were depleted during the first 14 days of life by receiving the basal diet only.

Groups receiving A-4 and two vitamin A supplements (50 and 75 IU/100 g) were discontinued on the 49th day, for technical reasons, while the feeding of ASS and the remaining two vitamin A supplements (25 and 300 IU/100 g) was continued until the 70th day.

Statistical analysis shows that the difference between the negative control (refined oil only) and the treatments was significant ($P < 0.05$) for the X fraction, and highly significant ($P < 0.01$) for the crude oil.

The average growth rates (not shown here) confirm the picture obtained from survival times. A rough calculation based on growth and A-4 content of ASS shows that ASS contains about 5 IU vitamin A activity/g.

The Origin of the Biologically Active A-4 Pigment

At this point the question arose whether the pigment A-4 was already present in the crude oil and accumulated in the soapstock together with other carotenoids during the refining process, or whether it was formed during some processing stage. None of the A pigments could be detected in the crude, and it seemed likely that they were artefacts.

Two steps are involved in the production of ASS from crude, degummed soybean oil: 1) alkali neutralization of the crude, yielding the raw soapstock; and 2) boiling of the raw soapstock with an excess of aqueous H_2SO_4 until clear separation of the oily layer is achieved. The relatively mild alkaline treatment seemed unlikely to alter the structure of the carotenoids; attention was therefore directed toward the effect of acid.

An attempt was made to induce formation of A-4 in crude soybean oil by boiling the latter with dilute H_2SO_4 under conditions roughly approximating those used in industry for the acidulation of raw soapstock. This attempt proved to be successful and the results show in Table VII. It is seen that A-4 appears in the oil thus treated, but its concn soon reaches a plateau. The combined A pigments increase steadily.

TABLE VII

Effect of Boiling Crude Degummed Soybean Oil with an Equal Volume of 10% (v/v) Sulfuric Acid on the Composition of Carotenoids. Symbols as in Table V

Duration of treatment, min	Carotenoids, μg/g				
	Total	β-carotene	A-(1-3)	A-4	X
0	30.3	1.1	0	0	28.4
2	30.5	1.2	—— 2.2 ——		27.2
5	27.3	1.3	4.5	3.1	18.3
15	27.6	1.2	8.7	2.7	15.0
30	22.6	0.8	12.6	3.6	4.4

On the other hand, the xanthophylls decrease constantly during the treatment, while β-carotene is relatively little affected under these conditions. Since lutein is the main pigment present in the crude, the data provide some evidence that lutein might be the precursor of A-4.

More definite proof for the role of lutein in the formation of A-4 was provided by acid treatment of chromatographically pure lutein prepared from soybean oil and from dehydrated alfalfa meal. These tests were carried out with *p*-toluenesulfonic acid in benzene as described below, since better yields were obtained under these conditions. Lutein was converted to A-4 to the extent of 24%, while pure zeaxanthin yielded 6% A-4. Moreover, fair yields of the provitamin could be obtained by applying the same treatment directly to crude soybean oil and dehydrated alfalfa meal. These yielded 6 and 83 μg provitamin/g starting material, respectively. In all these cases, the provitamin could be readily localized on the MgO column by its red color, and was identified by its characteristic single absorption peak at 460 mμ in both hexane and ethanol and by its mixed chromatogram with A-4 obtained from ASS. The conditions used in these treatments and subsequent separation of A-4 were essentially similar to those used for preparing the pigment from alfalfa lipids (6). Details are given below:

Lutein and Zeaxanthin. 0.5–1 mg carotenoids was dissolved in 100 ml analytical grade benzene, 0.15 mg *p*-toluenesulfonic acid ("Practical," Matheson, Coleman and Bell) was added and the mixture refluxed for 30 min. After cooling, the benzene solution was washed free of acid, dried and freed of solvent under a stream of CO_2 and reduced pressure. The residue was dissolved in a few ml hexane and chromatographed as usual.

Crude Soybean Oil. 10 g oil were treated with 0.1 g *p*-toluenesulfonic acid as described above. After washing, drying and removal of the solvent, the oil was assayed for A-4 by saponification and chromatography of the unsaponifiable matter, as usual.

Dehydrated Alfalfa Meal. 10 g dehydrated alfalfa meal were treated as described above, except for the use of a Dean-Stark moisture trap connected to the reaction flask and the reflux condenser. The reflux with acid was followed by an additional 10 min reflux with barium hydroxide (2 g) to neutralize the acid and destroy chlorophyll. After cooling, the benzene solution was sucked through a layer of Hyflo Super-Cel, the solvent was removed and the chromatography carried out as usual.

The role of acid in the induction of vitamin A activity was further demonstrated by an experiment in which acid-treated crude soybean oil was fed to vitamin A-depleted chicks and its effect compared to that produced by the non-treated crude oil. The oils were included in diet No. 5 at the 6% level and fed to groups of 20 chicks. Refined soybean oil served as a control. The average survival times for the groups receiving refined oil, crude oil and acid-treated crude oil were, 52.9, 49.1 and 66.7 days, respectively, the acid-treated oil producing a highly significant ($P < 0.01$) increase in survival time.

Discussion

The vitamin A activity of ASS represents an interesting example of "induced" activity, a provitamin being formed from an inactive precursor, i.e., lutein, as the result of the action of mineral acid during the acidulation of raw soapstock.

The first successful attempt to convert inactive xanthophylls to vitamin A-active products was carried out by Von Euler et al. in 1934 (7). Treatment of lutein and zeaxanthin with PBr_3 in benzene yielded unidentified amorphous products which supported growth in vitamin A-deficient rats. Free hydrobromic and phosphorous acids were liberated in the course of the reaction, therefore it is possible that the active product resulted from the action of these acids on lutein and zeaxanthin.

A few years later, Quackenbush et al. (9) investigated the reaction between acids and lutein and described several reaction products, among them a carotenoid having adsorption and spectrophotometric properties similar to those of the A-4 pigment of ASS. The vitamin A activity of this pigment was not tested, although other reaction products were examined and found to be inactive. Quackenbush et al. showed that these products also appeared in certain types of silage prepared with acids.

In 1943, Zechmeister and Sease (12) obtained three crystalline products by melting a mixture of lutein, naphtalene and boric acid (or tetraboric acid or boric anhydride). Because the compounds were believed to be reduction products of lutein, they were named "desoxyluteins." They appeared to be inactive by rat growth test. However, 13 years later, Zechmeister and Petracek (11) reinvestigated the same three compounds and reported that one of them ("deoxylutein I") supported rat growth to the extent of 10% of the activity of β-carotene. The structure assigned to this carotenoid was 3′-hydroxy-3,4-dehydro-β-carotene, i.e. that of a dehydration product of lutein in which the 3-hydroxy-α-ionone moiety had been converted to a 3,4-dehydro-β-ionone ring.

The provitamin A found by us in ASS and designated A-4 is undoubtedly identical with "deoxylutein I": it has the same single absorption peak at 460 mμ and is similarly located on the chromatographic column. Furthermore, it is formed from lutein by reaction with acids, together with two inactive pigments—A-1 and A-3—whose properties correspond to those of the inactive deoxyluteins II and III. Finally, we have recently found (6) that the acid-induced conversion product of lutein (which is identical with the A-4 pigment of ASS) is a provitamin A_2 for the chick. This lends further support to the structure incorporating a 3,4-dehydro-β-ionone ring, assigned to the active carotenoid by Zechmeister and Petracek (11).

Although our experiments were not designed for an accurate determination of the vitamin A activity of ASS, an approx estimate of 5 IU/g was obtained from growth data. The level of provitamin in various batches of ASS ranged from 9–14 μg/g, but exact evaluation of the vitamin A activity of this carotenoid must await further work.

The relatively high xanthophyll content of ASS is noteworthy: it reflects the selective transfer of xanthophylls to the raw soapstock during neutralization of the crude oil. Obviously, this favorably affects the amount of provitamin subsequently formed. It is not known to what extent this and other acid-induced conversion products of lutein contribute to the pigmenting power of ASS for skin and yolk.

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REFERENCES

1. Ames, S. R., H. A. Risley and P. L. Harris, Anal. Chem. *26*, 1378 (1954).
2. Ascarelli, I., and M. Senger, J. Sci. Food Agr. *13*, 332 (1962).
3. Bornstein, S., and B. Lipstein, World's Poultry Sci. J. *17*, 167 (1961).
4. Bornstein, S., and B. Lipstein, *Ibid.* *19*, 172 (1963).
5. Bro-Rasmussen, F., and W. Hjarde, Acta Chim. Scand. *11*, 44 (1957).
6. Budowski, P., I. Ascarelli, J. Gross and I. Nir, Science *142*, 969 (1963).
7. von Euler, H., P. Karrer and A. Zubrys, Helv. Chim. Acta *17*, 24 (1934).
8. Kruse, N. F., and W. W. Cravens, U.S. Pat. 2,924,525 (1960).
9. Quackenbush, F. W., H. Steenbock and W. H. Peterson, J. Am. Chem. Soc. *60*, 2937 (1938).
10. Wittee, N. H., and E. Sipos, U.S. Pat. 2,929,714 (1960).
11. Zechmeister, L., and F. J. Petracek, Arch. Biochem. Biophys. *61*, 243 (1956).
12. Zechmeister, L., and J. W. Sease, J. Am. Chem. Soc. *65*, 1951 (1943).

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Determination of the Glyceride Structure of Fats: Distribution of Individual Saturated and Unsaturated Acids[1]

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Abstract

A method has been devised which gives the distribution of saturated and unsaturated fatty acids. It involves fractionation of the triglycerides into groups on the basis of total unsaturation by employing chromatography on a silicic acid-silver nitrate column. The glyceride composition of each fraction is then determined by gas-liquid chromatography (GLC) of the oxidized glycerides. Using this method, the glyceride composition of lard and cocoa butter was determined to give quantitative amt of 24 and 18 glycerides, respectively. Duplicate analyses agreed to within ±0.5%. The fatty acid composition calculated from the glyceride composition agreed to within ±1.5% with that of the original fat. This approach provides a new basis for the evaluation of the glyceride tyes in natural fats and for the first time permits the quantitative determination of all the chemically different glycerides of myristic, palmitic, stearic, oleic, linoleic and linolenic acids in a fat.

Introduction

IN A RECENT publication from this laboratory (16), we have reported a method for the determination of glyceride composition of natural fats which involves oxidation of the fat by permanganate-periodate and subsequent GLC of the oxidized esterified glycerides. Although this method gives the distribution of the individual saturated fatty acids in the glycerol moiety, the unsaturated acids are estimated together as azelaoglycerides. In order to obtain the distribution of the unsaturated acids as well, it is necessary to first quantitatively separate the fat into groups differing in unsaturation. de Vries (3) has recently described such a method. Using a column of silicic acid impregnated with silver nitrate, and varying proportions of benzene in petroleum ether as eluting solvent, he obtained clear cut separation of tristearin, oleodipalmitin, stearodiolein and triolein. In this paper we describe a method of glyceride analysis based on fractionation of a fat into groups differing in the degree of unsaturation followed by GLC analysis of oxidized, esterified glyceride fractions thus obtained. The method has been applied to the study of glyceride composition of lard and cocoa butter.

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Experimental

Materials

Benzene was purified as outlined by Vogel (14), by shaking it with coned H_2SO_4, washing, drying and distilling using a fractionating column. The firt and the last 50 ml were rejected and the middle fraction was collected.

Mallinckrodt silicic acid, 100 mesh, analytical grade was used.

Samples of synthetic triglycerides were obtained from Canada Packers, Toronto, and purified by silicic acid chromatography (6).

Lard and cocoa butter were commercial samples and had iodine values of 66 and 39 respectively.

Methods

Column Chromatography. A mixture of tristearin, oleodistearin, palmitodiolein and triolein was separated on a silver nitrate impregnated silicic acid column as described by de Vries (3). Subsequently, lard was fractionated into 5 fractions as follows: 20 g silver nitrate impregnated silicic acid (3) and 10 g celite were mixed together in a mortar and pestle and packed on to a column (18 mm diam) with 30%, by volume, of benzene in Skellysolve F (a hydrocarbon fraction with a br of 35–58C) to give a column length of 28 cm. The column was covered with black paper during operation. Lard (146 mg) in 3 ml 30% benzene in Skelly F was added at the top of the column. Elution was commenced with 40% benzene in Skelly F. The rate of elution was 0.5 ml/min and fractions of 20 ml were collected. A change in the eluting solvent was made only after a peak started coming down. Thus 55, 80 and 100% benzene solutions were used to elute fractions 2,3 and 4, respectively. Fraction 5 was eluted with ether. Solvent systems for effecting separations were arrived at on the basis of preliminary runs. Cocoa butter was fractionated into 4 fractions using the same general procedure. Fractions belonging to each peak were pooled together. Thin layer chromatography (TLC) of these fractions by the method of Padley (1) gave single spots. The wt of each fraction was then determined.

GLC Analysis. A portion of each fraction was in-