THE JOURNAL OF THE American Oil Chemists' Society

Volume 31 DECEMBER, 1954 No. 12

Comparative Rates of Formation of Fatty Acids in the Soybean Seed During Its Development[1]

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ALTHOUGH the formation of oil in developing soybean seeds has been studied by several workers (5, 6, 10, 11, 12), no attempts were made to follow the individual fatty acids in the soybean at different stages during seed development as was done with flax by Painter (9).

At present two theories exist on the formation of unsaturated fatty acids in developing seeds: a) saturated fatty acids are formed and are then dehydrogenated; b) the individual fatty acids are formed by a separate mechanism (5, 6). Although the interconvertibility of stearic and oleic acids in animals has been demonstrated conclusively (13), no comparable evidence has been reported for a similar mechanism in plant tissue. Present evidence for desaturation of fatty acids in seeds is based on the increase in iodine value of the oil in maturing seeds (5, 6). Grande (4) reported that extracts of certain seeds contained an enzyme system capable of decolorizing a reducible dye in the presence of fatty acids. No change in the iodine value of the substrate was reported.

The purpose of the present study was a) to compare the relative rates of formation of the chief fatty acids in developing soybean seeds and b) to attempt to demonstrate the existence of a fatty acid dehydrogenase. It was hoped that these studies would aid in our understanding of the mechanism of formation of the unsaturated fatty acids.

Experimental

Methods. The Lincoln variety of soybean was grown on Brookston-Crosby complex soil in 1948 and on Floyd-Raub complex soil in 1949. Planting dates were May 20, 1948 and June 1, 1949. The plants were well nodulated each year. The 1949 crop was more uniform than those grown in 1948. To obtain seed of known age, soybean plants were tagged at the beginning of the flowering period. A small tag was attached to the plant, and the number nodes in bloom, approximately two, were noted on the tag. Any pods present on the tagged node were removed. Three days later all flower buds on the tagged area which had not opened were removed, and the next two nodes which had then come into bloom were tagged. In the 1948 season approximately 20,000 blossoms were tagged on the main stems of the plants, and samples were collected from these. In 1949 the work was repeated.

The first seed samples were collected as soon as they had reached a size suitable for analysis, which was approximately three weeks after tagging. Successive samples were collected every 3 or 4 days until about 45 days after tagging. During the latter phase of the growth period samples were collected weekly. A total of nine samples was collected each year.

The fresh pods were stored in a refrigerator at 5°C. until they could be shelled. Small quantities of beans which could be shelled in 15 min. were removed and shelled by hand. The shelled beans were returned to the refrigerator. After shelling, the beans were divided into two equal portions. One portion was dried in an oven at 50°C. for 24 hrs. This procedure reduced the moisture content to about 5%. The dried samples were stored in screw cap glass jars which had been flushed with nitrogen at minus 18°C., until analyzed. The second portion was placed in cellophane bags and stored at minus 18°C. until used for the enzyme phase of the work.

Oil content was determined by a modification of the A.O.C.S. method (7). The samples were ground in a Wiley mill until most of the sample passed through a 20-mesh screen. The coarser particles, consisting mainly of seed coats, were brushed into the ground portion and thoroughly mixed. Duplicate 2-g. samples were weighed into extraction thimbles of double thickness which had been lined with filter paper folded to fit the sides of the thimbles as an aid in retention of fine particles. After extraction for 10 hrs. with redistilled hexane in a Goldfisch extractor, the partially-extracted meal was reground in a mortar and extracted an additional 6 hrs. The clear hexane solutions were reduced in volume on a steam bath and were stripped of solvent under reduced pressure of a water pump at 40°C. for 2 hrs.

For the fatty acid analysis fresh oil was obtained by shaking 10 g. of the ground beans 10 min. with four 50-ml. portions of redistilled hexane. The mixture was centrifuged and the supernatant layer filtered. The combined filtrates were reduced in volume on a steam bath with a stream of nitrogen bubbling through the solutions. The oil was freed of solvent in a vacuum oven at 40°C. After nitrogen was bubbled through the oil samples and the container thoroughly flushed with nitrogen, the samples were stored at —5°C. until analyzed. This procedure gave an oil of peroxide number less than 1.0.

Iodine values were determined by the Wijs 30-min. method (7). Individual fatty acids were determined by the spectrophotometric method as modified by Brice, Swain, Schaffer, and Ault (2). Methyl alcohol was redistilled over sodium hydroxide and zinc before use as the solvent for the isomerized soaps.

[1]Journal Paper No. 717 of the Purdue Agricultural Experiment Station, Lafayette, Ind.

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The method used for the detection of fatty acid dehydrogenases was based on that reported by Grande (4). The method consisted of extracting 25 g. of frozen soybeans with 100 ml. of KH_2PO_4 buffers, pH 5 to 8, in a Waring blendor for 10 min. The slurry was shaken with 50 ml. of ether and the emulsion centrifuged. The aqueous layer was removed and divided into two equal portions. One portion was added to a 200-ml. flask containing an equal volume of .02 M. lipid substrate, 10 mg. ATP, and 2 ml. 1.01% methylene blue. The following lipid substrates were used: 0.02 M. potassium stearate, myristate and oleate, tripalmitin, monostearin, and monopalmitin. The other portion was added to a flask containing all the above constituents except that an equal volume of the buffer used for extraction was substituted for the lipid substrate. The flasks were flushed 10 times with nitrogen and incubated at 35°C. until decolorization of the dye occurred. After decolorization more dye was added and its decolorization time noted.

In one variation of this procedure the aqueous extracts were precipitated with ammonium sulfate at half-saturated and saturated concentration. These precipitates were redissolved and used in place of the crude extracts in the above procedure. In the trials where the decolorization of methylene blue was accelrated by the presence of the lipid material, the solutions were extracted with ether and iodine values of the extract determined (7). The difference in iodine values of the extracts with and without added lipid was taken as a measure of enzymic dehydrogenation. The neutralization equivalent of the substrate was determined by the method outlined by Marcali and Reiman (8).

Results. Oil percentage of the beans increased about four-fold during the period from 24 to 41 days after blossoming (Tables I and II). Although only about one-half of the total oil had been deposited at this time, subsequent changes in oil percentage were minor. The iodine value of the oil was highest in the young beans and decreased to a constant level before one-half of the oil was deposited (Tables III and IV).

TABLE I

Changes in Field-Grown Lincoln Soybeans During Maturation (1948)

Days after blossoming	Moisture	Oil	Av. weight in mg. per bean				
			Oil	Saturated acid	Oleic acid	Linoleic acid	Linolenic acid
	%	%					
24	86.7	5.4	0.3	0.04	0.06	0.1	0.07
27	75.0	9.5	1.5	0.3	0.2	0.7	0.3
32	71.6	13.2	5.9	1.1	1.0	2.9	0.6
37	69.0	18.4	14.4	2.7	2.8	7.1	1.2
41	62.5	20.3	22.0	4.0	4.2	11.4	1.6
45	63.0	20.9	23.9	4.8	3.5	12.9	1.8
48	62.5	20.3	26.5	5.2	4.9	14.4	1.7
54	58.0	20.3	33.0	6.5	4.3	18.7	2.1
62	16.0	21.4	40.5	7.3	6.7	22.2	4.7

Individual as well as total fatty acids per bean generally increased with each successive sample. In the few cases in which decreases were observed, the magnitude was not enough to account for the increase of another fatty acid during the same period. Since oleic and saturated fatty acids were determined indirectly, these small apparent decreases could have been due to inaccuracy of the methods (1).

While the amount of all fatty acids increased throughout the period of seed development they increased at different rates (Figure 1). Linolenic acid reached a constant percentage before the beans were 30 days old and remained constant throughout the period of oil deposition. At the 30th day about 30% of the total oil and linoleic acid and about 50% of the saturated and oleic acids had been formed. Oleic and saturated acids reached a constant level about 40

TABLE II

Changes in Field-Grown Lincoln Soybeans During Maturation (1949)

Days after blossoming	Moisture	Oil	Av. weight in mg. per bean				
			Oil	Saturated acid	Oleic acid	Linoleic acid	Linolenic acid
	%	%					
23	84	4.0	0.15	0.02	0.05	0.05	0.03
27	79	6.9	0.5	0.1	0.1	0.2	0.1
31	75	9.9	1.7	1.3	0.3	0.8	0.2
34	72	14.0	3.9	0.4	1.0	1.9	0.4
39	71	19.5	11.5	1.5	3.3	5.3	0.9
46	65	20.7	19.8	3.9	3.7	10.0	1.3
52	58	22.0	29.5	4.8	5.8	15.5	2.0
60	48	22.1	30.0	5.4	4.8	16.4	2.1
67	9	22.0	31.5	5.2	5.6	17.1	2.7

TABLE III

Changes in Composition of Soybean Oil in Developing Beans (1948 Crop)

Age of beans[a]	Iodine number	Fatty acids			
		Linolenic	Linoleic	Oleic	Saturated
		%	%	%	%
24	158.7	23.4	39.0	20.8	12.5
27	145.4	17.5	46.4	15.0	16.8
32	133.1	10.5	48.9	17.3	19.0
37	129.4	8.2	49.3	19.3	18.9
41	130.1	7.2	51.6	18.9	18.0
45	130.8	7.3	53.7	14.7	19.9
48	130.1	6.5	54.6	14.8	19.8
54	131.9	6.4	56.6	12.9	19.8
62	131.3	6.2	54.9	16.5	18.1

[a] Days after blossoming date.

TABLE IV

Changes in Composition of Soybean Oil in Developing Beans (1949 Crop)

Age of beans[a]	Iodine number	Fatty acids			
		Linolenic	Linoleic	Oleic	Saturated
		%	%	%	%
23	142.8	19.9	32.2	33.0	10.5
27	136.0	14.6	42.0	22.2	18.0
31	138.7	14.2	45.4	19.4	15.7
34	140.3	10.3	47.5	26.6	11.4
39	128.0	7.4	46.2	28.9	13.2
46	127.2	6.8	50.6	18.6	19.7
52	132.3	6.9	52.6	19.7	16.4
60	132.9	6.9	54.6	16.3	17.9
67	134.1	7.2	54.3	17.8	16.4

[a] Days after blossoming date.

TABLE V

Decolorization of Methylene Blue by Aqueous Soybean Extracts Plus ATP[a]

Age of beans	Extract volume	pH	Methylene blue	Substrate	Decolorizing time with substrate	Decolorizing time without added substrate
(days)	*(ml.)*		*(ml.)*		*(min.)*	*(min.)*
Mature	25	5.8	2	K-stearate	360+	360+
54	50	5.8	6	K-stearate	175	510+
54	75	8	1	K-stearate	360+	360+
48	30	7	1	K-stearate	240	90
48	30	7	3	K-myristate	250	300+
34	30	6	1	Monostearin[b]	105	85
34	30	6	1	Monopalmitin[c]	85	85
34	30	6	2	K-oleate	240+	240+

[a] Supplied by Armour and Company, Chicago, Ill., as the dibarium salt. The barium was removed by ammonium sulfate precipitation.
[b] Synthesized by the method of Daubert, Longenecker, and Fricke (3).
[c] Supplied by Armour and Company, Chicago, Ill.

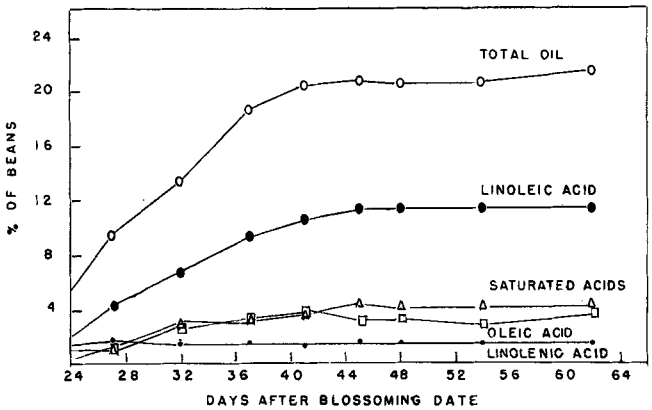

FIG. 1. Increases in oil and its constituent fatty acids with maturity of Lincoln soybeans (1948).

days after the blossoming date. Linoleic acid and total oil reached a constant level at approximately 45 days after the beans were tagged. The ratios of the different acids remained essentially constant after 50 days from the tagging date.

The results obtained from the dehydrogenation experiments were erratic. Some sample data are shown in Table V. In the cases in which added lipid substrate increased the rate of decolorization of methylene blue, the decolorized solution was acidified and extracted with ether and iodine adsorption values obtained on the extract. One of the potassium stearate substrates and one of the monostearin so treated showed increases in iodine values from 0.6 to 3.3 and 0.3 to 3.2, respectively. However, since no increase in iodine value was noted in 11 other trials with potassium stearate and three with monostearin, these small changes were considered insignificant. The possibility of dehydrogenation with subsequent cleavage was investigated by determining the change in neutralization equivalent of the substrate by the method of Marcali and Reiman (8). No change was noted in stearic or myristic acid neutralization equivalent.

Summary

Lincoln soybeans harvested at successive stages of maturity showed continuous increases in amounts present of each of the fatty acids: saturated, oleic, linoleic, and linolenic.

The iodine value and linolenic acid percentage of the oil decreased somewhat during the early stages of seed development. The linoleic acid and total oil percentage in the bean increased continuously until the 50th day then remained constant. Oleic and saturated acids fluctuated.

No evidence for dehydrogenation of saturated fatty acids was obtained either in the oil analyses or in tests of soybean tissues for dehydrogenase activity.

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[Received February 9, 1954]

The Use of Thiobarbituric Acid as a Measure of Fat Oxidation[1,2]

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OBJECTIVE tests for food product deterioration are highly desirable to facilitate research investigations on stability and to aid in specifying product quality. Recently 2-thiobarbituric acid has been proposed as a reagent with potentialities of partially fulfilling these requirements (4, 6, 13). During oxidation of fats, compounds are formed which can be reacted with 2-thiobarbituric acid (TBA) to give red-colored products. Patton and Kurtz (13) investigated the reaction of TBA with milk fat, and Dunkley and Jennings (6) published a procedure for oxidized fluid milk. Biggs and Bryant (4) modified the method for milk and extended its application to milk powder, cheese, and butter. Inasmuch as these investigators confined the use of TBA to milk fat, it was thought that its application to other fats would be of interest and value. Modifications of the TBA tests were developed to meet the objectives of this investigation.

Fats stored at —20, 0, 72, and 100°F.(—29, —18, 22, and 38°C.) and fats oxidized by the active oxygen method (A.O.M.) (14) or by ultraviolet irradiation were examined. Higher TBA values were obtained for soybean oil than for cottonseed oil at comparable peroxide values. This is of interest because of the greater tendency of soybean oil to develop oxidized flavors. The volatile products of oxidation of lard, cottonseed, and soybean oil were examined also, and at comparable peroxide values soybean oil volatiles developed the greatest intensity of color.

Experimental

When oxidized fat samples were dissolved in an organic solvent such as carbon tetrachloride, chloroform, or benzene, reactive material could be extracted

[1] Presented at the 27th Fall Meeting of the American Oil Chemists' Society, Chicago, Ill., Nov. 2-5, 1953.
[2] This paper represents research undertaken by the Quartermaster Food and Container Institute for the Armed Forces and has been assigned No. 450 in the series of papers approved for application. The views and conclusions contained in this paper are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.
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