Since reconstituted soybean oils undergo typical reversion, although completely free of naturally occurring unsaponifiables, the reversion process cannot be attributed wholly to the effect of these materials.

The function of the unsaponifiables, as described here, is minor in soybean oil reversion. Since autoxidation increases unsaponifiable content, the adverse effect noted by previous workers could well have been an effect of materials that had collected in the unsaponifiables resulting from oxidation, not representative of the naturally occurring materials.

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The Effect of Germination Upon the Fat of the Soybean[1]

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Abstract

Soybeans of the Chippewa variety of two crops, 1956 and 1957, were germinated in the dark at 25C and the levels of total dry matter and crude fat of both the seedling axis and cotyledons were determined at various periods up to 12 days. The fatty acid content, neutral fat content of the crude fats of the cotyledons, and the fatty acid composition of neutral fat were determined. The fatty acid composition was measured by the ultraviolet spectrophotometric method.

There was a continuous decrease in the total dry matter and crude fat of the cotyledons and whole seedlings of soybeans during 12 days of germination, contrary to observations of some of the earlier workers. Although there was a preferential utilization of the non-fat dry matter during the first two days of germination, there was a slight but significant loss of fat, which gradually increased with the germination time.

Surprisingly little change in the fatty acid composition of the reserve triglycerides occurred even during their most rapid loss from the cotyledons. However, observed changes were statistically significant. No loss of oleic acid occurred until after the second day of germination and its more rapid loss, compared to the other fatty acids, occurred during the period of most rapid fat loss.

The significance of this observation and its relationship to oleic acid as the key intermediary in fat metabolism in plants is discussed.

Introduction

SINCE HELLRIEGEL'S (6) classic work with sunflower seed, the progress of both the fat and the dry matter of oleaginous seeds during germination in the dark has remained uncertain. Increases (2,3,7,11,14, 18) and decreases (16,17,19,20,23) in both these values have been reported with almost equal frequency. Variations in the fat composition as revealed by the iodine value have also been in dispute. Improved techniques enabled Heumann (7) with pumpkin seeds and Combie and Comber (2) with watermelon seeds to show that only minor changes in the fatty acid composition occurred during germination. The most notable change was an increase in the oleic acid content during the early stages.

The object of the present work was to follow the dry matter and fat level of soybeans during their germination in the dark. The fatty acid composition was examined by a combination of the I.V. and spectrophotometric determinations on the refined (chromatographic) fat.

Materials and Methods

A. *Materials.* Representative samples of the Chippewa beans were manually harvested, at maturity, from experimental plots in 1956 and 1957, at Guelph, Ontario. All beans having wrinkled, discolored, or damaged coats were rejected. The average bean weight for both crop years was 140 mg. To minimize and standardize variations in bean weights, the beans selected had individual weight within the range of twice the standard deviation, i.e., 120 mg to 160 mg. Thus 68% of the original material was selected as suitable for experimentation. These beans were placed in sealed bottles and refrigerated until required.

B. *Sterilization.* Prior to germination, the beans were surface sterilized by a detergent wash (1% solution of Tide) followed by successive treatment with aqueous sodium hypochlorite (3.3%) and sorbic acid (0.2%). There was no change in either the viability or the growth rate of treated beans as compared to untreated controls.

C. *Germination.* Weighed lots of beans were germinated upon wet facial tissue in the dark in water saturated atmosphere at 25C for varying periods of time up to 12 days. At least 6 separate determinations were made for each period.

D. *Analytical Methods.* The seedlings were separated into the axis, cotyledons, and seed coats, the latter being discarded.

Dry weights of the plant parts were obtained by the vacuum oven method, A.O.A.C. Official Methods 13.3. Total crude fat was determined by exhaustive extraction of the finely ground, dried material with n-hexane as described in A.O.C.S. Official Method Ba 3.38. During the evaporation of the solvent, the crude

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TABLE I

Analysis of Seedling Axis, Cotyledons, and Crude Fat of the Cotyledons from Chippewa Soybeans of 1956 Crop germinated in the dark at 25C

Germination time (days)	Dry Weight		Crude Fat				Cotyledon Crude Fat					
	Seedling axis %[b]	Cotyledons %[b]	Seedling axis		Cotyledons		Free fatty acids				Neutral fat %	Neutral non-triglycerides	
			%[c]	mg[a]	%[c]	%[b]	%		mg[a]			%	mg[a]
	(2)	(3)	(4)	(5)	(6)	(7)	(8)		(9)		(10)	(11)	(12)
½	2.5	87.9 ± 1.9	11.9	0.36	23.2	20.6	0.22	± 0.003	0.057	± 0.01	99.2	0.6	0.14
2	2.7	85.3 1.8	11.7	0.37	23.5	20.0	0.35	0.04	0.09	0.02	98.8	0.8	0.23
3	4.2	82.7 3.3	7.1	0.29	.....	.....	.....	.....	.....	.....	.....	.....	.....
4	5.6	79.5 1.3	3.6	0.32	22.8	18.2	0.54	0.003	0.13	0.04	98.0	1.5	0.34
6	7.9	76.9 1.0	3.3	0.33	21.4	16.4	0.93	0.01	0.19	0.03	97.8	1.3	0.39
8	9.6	71.7 0.9	2.8	0.30	18.8	13.5	1.11	0.07	0.20	0.06	96.4	2.5	0.44
10	12.4	67.3 1.8	2.5	0.40	17.2	11.5	1.55	0.25	0.23	0.05	94.2	4.2	0.64
12	15.1	59.0 0.8	1.9	0.39	16.0	9.4	1.91	0.09	0.23	0.07	93.2	4.8	0.58

[a] Weight per plant part (seedling axis or cotyledon pair).
[b] Weight expressed as a percentage of the original, ungerminated, dry bean.
[c] Percentage of the plant part, dry weight.

extract was protected by a nitrogen blanket. A 200 mg sample of the crude fat, dissolved in hot isopropyl alcohol was titrated with 0.01*N* alcoholic NaOH using O-cresol phthalein as the indicator to measure the FFA content. The reproducibility of this method was satisfactory. The standard deviation of the results from several analyses of the same crude soybean oil was ± 0.03%.

The residual fat was refined by passage of the chloroform solution of the crude fat through an activated alumina column. The method was essentially that reported by Hoffpauir (5) but it was modified for scaling down five-fold. The I.V. of the refined residual fat (neutral fat) was determined by the Wijs method. The level of saturated, oleic, linoleic, and linolenic acids in the neutral fat was determined by the 11% glycerol-KOH isomerization methods of Brice *et al.* (1) using the constants and calculations of A.O.C.S. Method L12a-55. Analysis of a standard soybean oil sample gave a reproducibility of ± 0.1 units for the I.V., oleic, linoleic, and linolenic acid contents and 0.4% for the saturated acid level.

Results and Discussion

The dry weight of the seedling axis increased continuously during the germination period studied. While the percent crude fat decreased continuously the total amount of crude fat per seedling axis remained unchanged (column 5, Table I). This finding agrees with the observations of Miller (16) with sunflower seeds, and Crombie and Comber (2) with watermelon seeds.

There was a continuous decrease in the mean dry weight of the cotyledon pairs, but variations of individual determinations, expressed by the standard deviation, indicated overlapping between consecutive periods. These variations were attributed to differences in the growth response of the individual beans. Though the beans were selected so that their weights were within the limits of 120 to 160 mg. some of this variation must be due to the differences in weight. In order to circumvent this variation the dry weights of the cotyledon pairs were expressed as a percentage of the dry weight of that bean sample prior to its germination, and these values, along with the standard deviations, are listed in column 3, Table I. As a result, the incidence of overlapping receded to below the 20% level, and the continuous loss of dry weight from the cotyledons became more definite, especially during the first few days of germination. The increased rate of dry weight depletion after the tenth day of germination was believed due to the onset of senescence.

Since Panalaks (20) has shown that the dry weights of the coats of soybeans did not alter during the period of germination used in this study, the sum of the dry weights of the seedling axis and the cotyledon pairs is representative of the dry weight of the whole seedling. Preliminary experiments showed that the fat content of the coats remained constant at about 0.74% during this period. The sums of the dry weights of the cotyledons and the seedling axis decreased as germination progressed (Table I). Thus it was concluded that there was a continuous loss of dry matter from the whole seedling. This loss was not as rapid nor extensive as that which occurs in the cotyledon alone due to increasing dry weight of the seedling axis. Variations of individual determinations from the mean was slightly greater than that observed with the cotyledons alone and, as a result, the incidence of overlapping between consecutive periods was increased. These variations were larger when beans unselected as to size were used and the small differences observed in this work, especially in the earlier stages of germination, were obscured.

Increases in the crude fat content of both the whole seedling and the cotyledon were often observed after the first two days of germination when the results were expressed as % of the final dry weight (column 6, Table I). Comparison of the final fat content with that originally present in the ungerminated bean (column 7, Table I) revealed that such increases were apparent and probably due to a greater utilization of the non-fat solids during this initial stage of germination, agreeing with the observations reported by McKinney *et al.* (15). Therefore, there was a continuous loss of crude fat during the germination period of 12 days, with the rate increasing after the fourth day. Earlier reports concerning increases in the fat content of soybeans and other oleaginous seeds during the earlier stages of germination did not refer to that present in the bean prior to germination; therefore it is difficult to believe that they represent real increases. McKinney *et al.* (15) reported no loss of the crude fat during the first 3 days of germination of Hawkeye soybeans when compared to the ungerminated bean, whereas there was a total loss of dry matter from the whole seedling of 1.5% based on the original bean dry weight. This finding could well result from a difference in the growth rate of the beans used, as compared to beans used in this study. Evidence for this difference in the seedling lengths after 3 days' germination is found [¼ inch to ½ inch (15) as compared to ¾ inch to 1 inch for seedling used here].

The FFA content of crude fat from the cotyledons (column 8, Table I) increased continuously during germination, whereas the FFA of the cotyledon in-

TABLE II

The Iodine Value and Fatty Acid Composition of the Neutral Fat from Cotyledons of Soybeans Germinated in the Dark at 25C

Germination time—days	Iodine value (Wijs)	Percent of Natural Fat			
		Sat'd	Oleic	Linoleic	Linolenic
½	142.3 ± 0.2	16.2	12.6	55.9	10.9
2	142.4 0.1	15.8	13.2	55.9	10.7
4	142.8 0.3	15.5	13.8	55.8	10.7
6	142.8 0.2	15.5	13.4	55.9	10.8
8	143.1 0.4	15.6	12.6	56.8	10.6
10	143.5 0.3	15.5	12.5	56.8	10.8
12	143.7 0.3	17.4	10.9	57.5	10.8

creased continuously for the first 6 days only, and remaining more or less constant thereafter (column 9, Table I). Similar observations have been reported for tung kernels by Johnston and Snell (12) and for cotton seed by Olcott and Fontaine (19). The latter reported that lipase activity followed the FFA level, attaining its maximum at about the same time. McKinney *et al.* (15) reported an initial decrease from 0.44% to 0.14% in the FFA of the crude oil and showed no increase until after the fifth day of germination. Since the fat from beans in this study, prior to germination, exhibited a low FFA content of 0.2% it is not surprising that no decrease was perceived. Also, the more rapid growth rate of the beans in this study could account for this low value and the increase in the FFA prior to the fifth day of germination.

There was a continuous almost linear increase in the components absorbed by alumina from the crude fat of cotyledons during germination. The arithmetical difference between this fraction and the FFA content, termed the neutral non-triglyceride fraction (NNTF), increased continuously (columns 11 and 12, Table I). The NNTF is composed of phospholipids, and to a lesser extent sterols and other unidentified fat-soluble compounds. Thus the decrease in neutral fat was slightly greater than that of crude fat, with the difference becoming greater as germinations progressed (column 10, Table I). Crombie and Comber (2) observed similar increases in a like fraction in watermelon seeds during germination, supporting the findings of Houget (10) who reported a two-fold increase in the phospholipid content of rape seeds, and of MacLachlan (13) who found a two-fold increase in the sterol content of soybeans during germination in the dark.

A slight but continuous increase in the I.V. (from 142.3 to 143.7) of the neutral fat was observed during the 12-day germination period. The overall increase of 1.4 I. V. was found to be significant at the 1% level, as was a 0.5 unit increase between the one-half and fourth days. The I.V.'s reported by earlier workers were determined on the crude fatty extract of the whole seedling which contained increasing amounts of non-triglyceride matter as germination progressed, and could not be representative of the residual triglycerides. MacLachlan (13) observed that the I.V. of the crude fatty matter from the seedling axis of germinating soybeans was at least 30 I.V. units below that of the cotyledons, and since the proportion increased in the crude fat of the whole seedling, as germination progressed, the resultant I.V. would be lower than that of the fat from the cotyledons alone.

The oleic acid content of the neutral fat increased from 12.6% to 13.8% by the end of 4 days' germination, decreasing continuously thereafter to 10.9% after 12 days. Heumann (7), using the thiocyanogen-iodine value method, observed a similar initial increase in the oleic acid content of the crude fat of pumpkin seedlings. Crombie and Comber (2) using

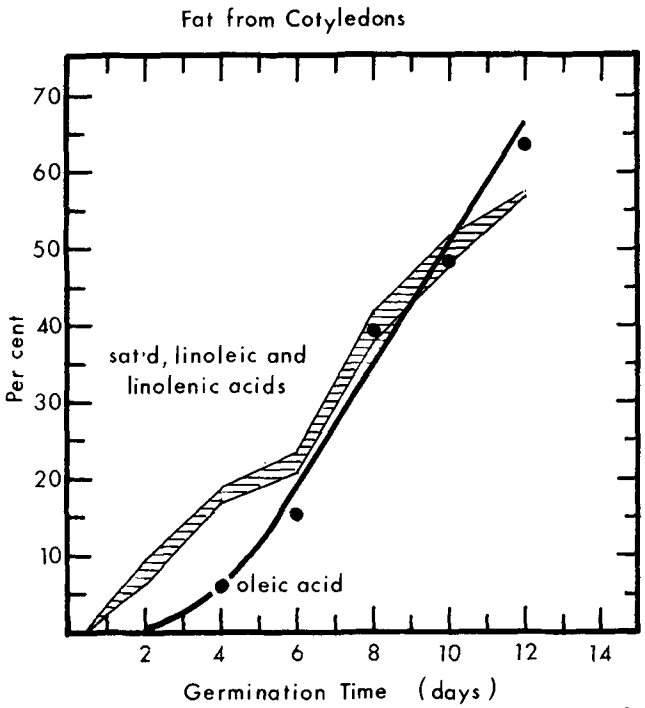

FIG. 1. The loss of the fatty acids of the neutral fat of the cotyledons from Chippewa soybeans of the 1957 crop, germinated in the dark at 25C. (The values are calculated as a percentage of that present in the cotyledons after the period of imbibition of water.)

a quantitative, reverse phase, chromatographic technique were able to measure directly the composition of the triglyceride bound fatty acids of the watermelon seed during germination, and reported a slight but significant increase in both the oleic and palmitic acids during first 3 to 4 days of germination. The loss of oleic acid from the cotyledons, expressed as percentage of that originally present, is plotted against germination time in Figure 1. There was no loss of oleic acid during the first 2 days, which is coincident with the period of the slowest loss of fat. The rate of loss of oleic acid increased continuously thereafter until it surpassed that of the other fatty acids in latter stages of germination.

The linoleic acid content of the neutral fat remained constant at 55.8% until after the fourth day, increasing to 57.5% by the end of the twelfth day. In contrast, Holman (9) reported a continuous decrease in both the linoleic acid and the linolenic acid content of the crude residual fat of whole soybean seedlings during 6 days' germination in the dark. It is possible that Holman's measurements may have been affected by the increasing proportions of non-triglyceride matter in crude fat from the whole seedling. In this study the increase in linoleic acid coincided with a more rapid loss of oleic acid from the neutral fat. Thus, the slight increase could well be due to more rapid utilization of oleic acid.

There was no significant change in the linolenic acid content of the neutral fat during germination, which remained about 10.8 ± 0.1%. Although there appeared to be a decrease of 0.7% in the saturated acids content during the first day, it was not considered significant because of the nature of its derivation. The saturated acids level remained constant at about 15.8% up to the tenth day and showed a marginally significant increase to 17.4% at the end of 12 days. For clarity, the individual values for the loss of linoleic, linolenic, and saturated acids are

not shown individually in Figure 1, but all fall into the bounds of the shaded area.

This initial increase in the oleic acid was not the result of a selective lipolysis for plant lipases have been found to be non-selective (21 and 22). Hydrogenation or dehydrogenation of either the bound or free fatty acids is possible by a fatty acid dehydrogenase system such as has been reported as present in soybeans during germination (4). Although only free long chain acids were reported as precursors for this system, the acceptability of acids bound in triglycerides as precursors should not be neglected. With this last assumption, the accumulation of oleic acid in the residual triglycerides (neutral fat) would not necessitate an active re-esterification process for the incorporation of the newly formed oleic acid into the neutral fat. Such re-esterification would be possible by the lipases as reported by Hilditch (8) or by esterases.

The conversion of other fatty acids into oleic acid as an initial stage in the breakdown of fatty acids appears to be a reversal of that observed in the developing bean. Simmons and Quackenbush (24) concluded from their studies on soybean cuttings that oleic acid was the first formed, and acted as a precursor for the others, which would be possible in the presence of the dehydrogenase system reported by Funicha (4).

With oleic acid as the precursor necessary for further fatty acid breakdown, its later more rapid disappearance during the period of most rapid fat loss could be due to the demand for it exceeding that present in the FFA as a result of the lipase and dehydrogenase systems, and as a result more must be hydrolyzed from the triglycerides. This hydrolysis being non-selective, the other FFA formed must re-esterify and so become prominent in the neutral fat.

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Analysis of the Geometric Isomers of Methyl Linoleate by Gas Chromatography[1,2]

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Abstract

The four geometric isomers of methyl linoleate have been quantitatively determined by gas chromatogrophy on Apiezon L and DEGS polyester capillary columns. Three peaks were eluted from the Apiezon L column: (a) the 9-*cis*,12-*cis* isomer; (b) the 9-*cis*,12-*trans* isomer; and (c) the 9-*trans*,12-*cis* and 9-*trans*,12-*trans* isomers combined. The DEGS polyester column also resolved three peaks: (a) the 9-*trans*,12-*trans* isomer; (b) the 9-*cis*,12-*cis* and 9-*cis*,12-*trans* isomers combined; and (c) the 9-*trans*,12-*cis* isomer. Since the separation of isomers was different on each column, the content of each of the four isomers could be determined from the combined results. Quantitative results agreed closely with the per cent *trans* bonds as determined by infrared analysis.

Introduction

DURING current investigations on the *cis-trans* isomerization of natural fats, it became necessary to determine the precise fatty acid composition of fats produced by such a process. According to Blekkingh, Janssen, and Keppler (1) and Kass, Nichols, and Burr (2), *cis-trans* isomerization with such catalysts as Se, NO_2, or SO_2 produces only geometric isomerization without involving appreciable movement of the double bond along the carbon chain. However, a small amount of conjugation does occur. In the absence of positional isomers, our problem was to find suitable analyses for the geometric isomers of the three common unsaturated fatty acids: oleic, linoleic, and linolenic. This report describes a procedure for the quantitative determination of the four geometric isomers of methyl linoleate by gas chromatography.

There are four possible geometric isomers of methyl 9,12-linoleate: *cis-cis, cis-trans, trans-cis,* and *trans-trans.* Very few analytical methods are available for determining the content of these four isomers in a mixture of fatty acids. Infrared analysis at 10.36 microns (3, 4, 5) indicates only the total percent of *trans* bonds present in a mixture. Jackson, Paschke, Tolberg, Boyd, and Wheeler (6) suggested an analytical method based on the different rates of alkali conjugation of the various isomers. Their procedure yielded only approximate results, and their calibration curves were based on only three of the four possible isomers. McGee (7) has described an enzymatic technique for determining the amount of methyl 9-*cis*, 12-*cis* linoleate in a mixture of its geometric isomers. His method is based on the specificity of lipoxidase in

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