Plant Lipases in Emulsions of Water in Oil[1]

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An important criterion of the value or "grade" of a vegetable oil is based on the content of free fatty acids therein. The factor responsible for the formation of such free fatty acids (except in rather well-defined special cases) appears to be something connected with the seed itself, and commonly regarded as a lipolytic enzyme. Whether these plant lipases are part of the normal composition of the seed or come from contaminating organisms, their action is to produce free acids and thus decrease the value of the oil.

It is generally recognized that seeds rich in fats should contain a lipase; otherwise it would be difficult to explain the synthesis of glycerides in the plant and their subsequent utilization as food for the germinating seed. Investigations on the seeds of a large variety of plants have demonstrated the presence of a phytolipase [cf. Bamann and Ulmann (2)] but usually not in the quantities that might be expected from the behavior of the oil on occasion. However our knowledge of the action of lipases and our ability to demonstrate their presence by adequate laboratory techniques are both far from perfect at present. This paper reports the first results of a study being undertaken to clarify the role of plant lipases in normal seeds and in the behavior of the oils made from them. The present work has been carried out with the lipase of the castor bean *(Ricinus communis)* because it is one of the few common plants from which a large quantity of lipase may be obtained. Even with this enzyme, which when properly prepared is very active, it is apparent that laboratory handling may cause great and frequently total inactivation, often for unexplained reasons. It seems quite probable that other plant lipases are not necessarily less active, but only less stable. Ricinus lipase preparations may be classified into two types: a "lipase milk" as first described by Hoyer (3) and later concentrated to a "lipase cream" by centrifuging, as described by Willstätter and Waldschmidt-Leitz (11), and a lipase powder, obtained by defatting shelled or unshelled castor beans. Of the large number of lipase powder preparations that have been described it is probable that those of Longenecker and Haley (7) were the most active. The lipase powders possess the advantage of stability on prolonged storage and the disadvantage that they contain the highly toxic material, ricin (5).[2]

It is already apparent that most studies on plant lipase [though not all, see (7)] have been conducted with emulsions of oil in water whereas the reverse condition, that of an emulsion of water in oil, usually obtains under natural conditions. It has now been observed that under the latter conditions even small quantities of active lipase are capable of producing a spectacular development of free fatty acid. Provided time is allowed, the extent of hydrolysis depends more upon the water available in the emulsion than upon the amount of the enzyme itself. Neutralization of the free acids formed seems to be unnecessary for the continued action of the enzyme. On the other hand, certain substances of the phospholipide class were found to be very good substrates (as might be expected), but others of the same class proved to be powerful inhibitors.

Method of Assay. Measurement of lipolytic activity in an emulsion of a little water and much oil, instead of the reverse, required a procedure differing greatly from that usually employed. A study of the course of lipolysis of cottonseed oil is described later and furnishes the basis for the method of assay used throughout this paper. The lipase cream (250 mg.) is stirred with 200 mg. of water, and this mixture is then stirred with 25.0 g. of oil. For the results reported in this paper, hand stirring with a glass rod was invariably used for the initial mixing of the cream and oil. The cream emulsifies readily with the oil, and subsequent stirring is of little value when the amount of water added to 250 mg. of cream does not exceed 0.5 ml.[3] No buffer is required, and it is unnecessary to neutralize the liberated acid for the action of the enzyme to continue. The mixture of enzyme cream, water, and oil is allowed to stand for 60 minutes at about 25°. A weighed portion (1 to 5 grams) of the reaction mixture is then dissolved in 20 to 25 ml. of a previously neutralized mixture of alcohol and ether (1:1), containing 5 drops of the mixed indicator of Kleinzeller and Trim (4), and titrated with standard alcoholic potassium hydroxide. Unless otherwise stated, all values for lipolytic activity are expressed as percentage of free acids produced in the reaction mixture. Figure 1 (25°) may be used to correct an activity value determined at some time varying from 45 to 90 minutes, to the standard reaction period of 1 hour. The foregoing method is extremely simple and sufficiently accurate for the present purpose. Replicate results that check within a margin of 5% have always been consistently obtained.

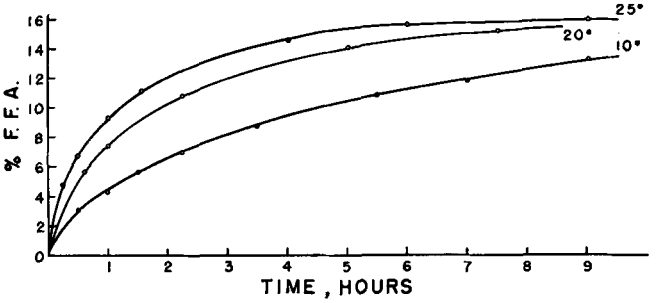

FIG. 1. Rate of splitting of cottonseed oil by lipase cream.

[1] Enzyme Research Division Contribution No. 129. Presented at the Spring Meeting, American Oil Chemists' Society, Atlanta, Ga., May 1-3, 1950.

[2] Longenecker (6) found it necessary to conduct the operations incident to the preparation of the enzyme and also the weighing of the sample for use, in an especially constructed box. The preparation and use of lipase cream does not require these precautions since only oily or moist materials are handled.

[3] Stirring this mixture with a blender for 1 minute at low speed results in total inactivation of the enzyme, but stirring with a magnetic stirrer has no harmful effect. When using lipase powder preparations under the conditions described for the lipase cream, a definite advantage is gained by stirring with a magnetic stirrer for a period of 5 minutes, as better and more reproducible emulsions are thereby obtained.

Preparation of Enzyme Material. When preparations of lipase cream were made by the method of Willstätter and Waldschmidt-Leitz (11, 12), it was observed that many of them were inactive. The less active preparations were frequently capable of activation by the addition of an electrolyte, as shown in Table 1A. The solution of electrolyte was 0.5 *M* and

TABLE IA

Effect of Salts on Lipase Activity

Time, hrs.	Control	NaOAC	$(NH_4)_2SO_4$	NaCl	KH_2PO_4
1	0.9	1.9	1.8	1.6	2.1
3.5	2.0	4.0	4.6	8.0	6.0
19	6.7	7.7	9.4	9.2	9.6
27	7.7	8.6	13.9	8.8	12.5
43	8.0	9.1	14.7	9.6	14.4
50	....	9.1	....	9.4	....
69	8.5	9.5	14.5	9.2	13.8

replaced the water used in the standard assay procedure, i.e., the reaction mixture consisted of 25.0 g. oil, 0.25 g. lipase cream, and 0.20 ml. of 0.5 *M* salt solution. However the addition of various electrolytes had no activating effect on creams which were somewhat more active, Table 1B. For reasons that

TABLE IB

Effect of Salts on Lipase Activity

Time, hrs.	Control	NaCl	Na_2SO_4[a]	Na_2SO_4[b]	$(NH_4)_2HPO_4$	KH_4PO_4	$(NH_4)_2SO_4$
1	1.4	1.4	1.4	1.4	0.6	1.6	1.4
3	2.7	2.7	2.7	2.6	0.6	3.1	2.6
4	6.6	6.2	6.3	6.2	0.7	7.5	6.1
114	10.9	9.9	9.8	9.4	0.5	12.0	8.5

cannot be explained, totally inactive preparations were also obtained—latterly with great frequency, perhaps owing to the increased age of the beans. Various treatments, such as soaking the beans in water, allowing the "milk" to stand in the cold, adding magnesium, manganese, or sodium salts to the castor bean milk or attempting partially to digest the castor bean proteins in the "milk" with trypsin or papain resulted in little or no improvement in the quality of the cream prepared subsequently.

It was found however that adjustment of the pH of the suspension of beans ground with water to pH 4.2-4.8 with acetic or hydrochloric acids invariably produced a very active cream.[4] Table II shows the yield and activity of creams prepared at various pH's. The reported yield of cream represents the amount obtained from 10 grams of shelled beans. The following method for the preparation of lipase cream from castor beans was therfore adopted.

Shelled castor beans are ground in a mortar to a greasy paste. Distilled water equivalent to twice the weight of the beans is then added in small portions with frequent grinding during the course of one hour. The originally smooth paste assumes a somewhat granular appearance during this operation although the presence of granular particles cannot be detected by probing with the pestle. At the end of the hour the material is transferred to a beaker, with the aid of an additional quantity of water equal to about half that added during the grinding, and the

[4] Hoyer (3) in 1907 obtained active preparations by grinding shelled castor beans with water, and allowing them to stand for several days, using chloral hydrate as a preservative. He noted that the mixture became acid, and identified lactic and carbonic acids as two of the acidic products. Hoyer also found that addition of an acid would produce an active lipase milk at once.

TABLE II

Effect of pH of Castor Bean Milk on Yield and Activity of Lipase Cream

Milk	pH	Yield, g.	Activity
B	3.5	2.7	3.2
B	4.0	2.6	5.1
A	4.0	3.1	5.8
C[a]	4.0	6.0	1.6
B	4.25	3.5	5.8
C	4.25	6.2	5.4
B	4.50	2.1	4.7
B[b]	4.50	3.9	4.8
C	4.50	7.7	6.6
A	4.50	3.5	6.8
C	4.75	7.3	6.2
B	5.0	2.2	2.6
C	5.0	7.3	5.9
A	5.0	0.7	2.4
A	5.5	7.3	0.2
A	6.0	5.5	0.2
A[c]	6.7	3.5	0.1

[a] Cream C was prepared from milk to which 0.2 *M* phthalate buffer, pH 4.25 was added before adjustment of pH.
[b] Phthalate buffer, pH 4.50, used to wash cream, instead of usual water wash.
[c] Natural pH of castor bean milk.

lipase milk is stored overnight in a covered beaker at 2° to 4°. Phthalate buffer (0.2 *M*, pH 4.25) is then added. The amount added has been varied from one-half the volume of the milk to a volume equal to that of the milk, without affecting the yield or activity of the cream. The pH of the buffered milk is then adjusted to 4.5, and the milk is centrifuged. The cream is removed with the aid of a small glass spoon.[5] The cream is transferred to another centrifuge tube containing distilled water, washed by agitation, and centrifuged. It is then given a second similar washing, transferred to a glass-stoppered weighing bottle, and stored in a refrigerator until used. A typical yield was 19.9 grams of twice-washed cream from 25 grams of shelled beans.

Creams have also been prepared from unshelled beans, by the above procedure. The yield and activity of the cream so obtained is not greatly different from that prepared from shelled beans, but it is dark in color. Several attempts to prepare lipase cream by grinding castor beans with water in a ball mill resulted in inactive preparations.

The behavior of the cream on storage is somewhat peculiar. A decrease in activity of 8 to 10% during the first 24 hours is not unusual. It has sometimes been observed that the next 8 to 10 days' storage at 2° to 4° results in an increase of activity of 25 to 30% above the original activity value. After 43 days' storage at 2° to 4° a lipase cream with an original activity of 6.5 decreased to 4.0. Mold growth in the cream was observed at that time.

Action of Lipase Cream on Cottonseed Oil. The rate of lipase action on cottonseed oil at several temperatures is given in Figure 1. The rather small temperature coefficient of ricinus lipase is noteworthy. It will be observed that an increase in temperature from 10° to 20° results in an activity increase of about 50% instead of the more general doubling of activity with a 10° temperature increase. This behavior is similar to that of the pig pancreas lipase, which Balls and Tucker (1) found to be surprisingly active at temperatures as low as −30°.

We have also made comparisons of enzyme activity at temperatures of 2° to 4° and −16° to −18°. In

[5] The glass spoon was made by cutting a test tube of suitable size, about 5-6 mm., from the closed end and sealing that to a glass rod about 3 mm. in diameter. The rod is sealed to the edge of the rounded shallow cup from the test tube, in a direction parallel to the length of the tube.

these mixtures 19.8 grams of lipase cream was mixed with 19.8 grams of water and then with 1,500 grams of cottonseed oil. After mixing, 30- to 40-gram portions were placed in 50-ml. flasks, which were stoppered and placed in storage in an electric refrigerator at 2° to 4°, or in a cold storage room maintained at —16° to —18°. The data so obtained is given in Table III.[6]

TABLE III

Lipase Activity at Low Temperature

2° to 4° C.		—16° to —18° C.	
Time, hrs.	% f.f.a.	Time, hrs.	% f.f.a.
0.5	7.1	47	8.2
2	8.8	119	9.1
4	9.7	405	12.1
8	10.5	764	13.8
23	12.6	1292	15.0
47	13.6	1820	15.0
119	15.6	2516	17.1
405	18.9		
764	21.6		
1292	23.5		
1820	24.0		
2492	25.5		

It should be noted that approximately one hour was required to attain the storage temperature and free fatty acids were being formed much more rapidly during this time than when the samples had cooled to the storage temperature. There were probably some differences between the individual subsamples especially at —18°, as is evidenced by the 15% free fatty acids in the samples stored for 1,292 and 1,820 hours whereas the 2,516-hour sample had shown an increase to 17.1%. The marked difference in the activity of lipase at 2° and —18° was doubtless due to the fact that both fat and water are solid at the lower temperature. The activity of lipase at temperatures at which both the fat and water are solid was discussed by Balls and Tucker (1).

The reaction did not appear to be either a first or second order reaction but did appear to follow the Schütz rule $\sqrt{\text{time}}/\text{extent of hydrolysis} = k$) during the early part of the reaction. Indeed at a temperature of 10° the Schütz rule was obeyed during the 9 hours required to attain an acid concentration of 13.3%. At 20° deviation from the square root relationship was noted after 2.25 hours, when 10.8% free acid had been formed, while at 25° deviation from this law was observed after 1.6 hours, when 11.1% free acids were present. These data are shown in Table IV.

[6] The subdivision of the large sample into 20- to 30-grams subsamples was necessitated by the impossibility of obtaining uniform samples from the cold mixtures. This procedure also helped to attain the storage temperatures more rapidly. At the end of the storage time the samples were heated to about 80° to inactivate the lipase.

Lipase cream, prepared as described, contains from 45 to 60% of water; stoichiometrically, 250 mg. of the cream can furnish sufficient water to hydrolyze 25 to 30% of the 25 grams of cottonseed oil used in the test, but lipase action stops when one-half to two-thirds of this water has been used. Addition of water to a lipase-oil system that has become static due to lack of water results in further splitting of the fat, but at a much slower rate than the original. This slower rate is probably due to the fact that the water thus added is not emulsified as well as that present in the cream.

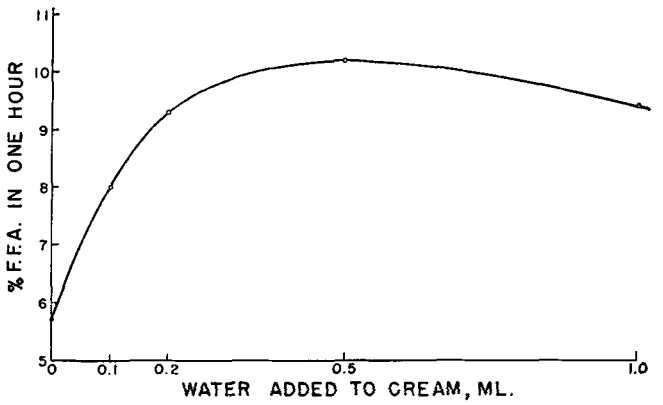

FIG. 2. Effect of addition of water to 0.25 g. lipase cream before mixing the cream with 25.0 g. of oil.

The addition of water to the cream before mixing it with cottonseed oil increases the rate of splitting, up to about 2% of water, based on the weight of oil to be employed. This effect is shown in Figure 2. It will be noted that the value obtained by the assay procedure described above is well below the limit imposed by the available water. Figure 3 shows the

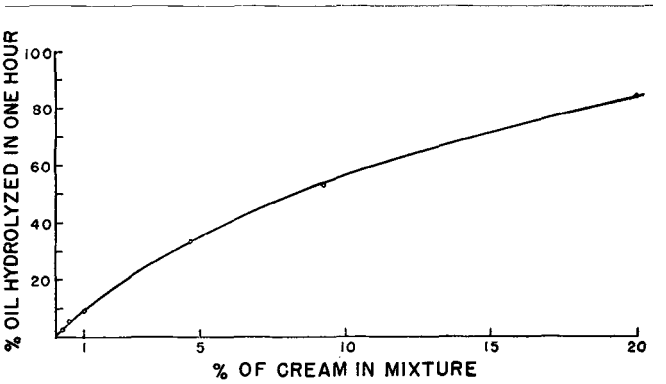

FIG. 3. Effect of increasing concentration of lipase cream.

TABLE IV

Ratio of Square Root of Time of Reaction to Fatty Acid Liberated

10°			20°			25°		
% f.f.a.	$\sqrt{t}$	$\frac{\sqrt{t}}{\%\text{ f.f.a.}}$	% f.f.a.	$\sqrt{t}$	$\frac{\sqrt{t}}{\%\text{ f.f.a.}}$	% f.f.a.	$\sqrt{t}$	$\frac{\sqrt{t}}{\%\text{ f.f.a.}}$
3.1	0.71	0.228	5.6	0.77	0.138	4.8	0.50	0.104
4.3	1.00	0.232	7.4	1.00	0.135	6.8	0.71	0.104
5.6	1.22	0.218	10.8	1.50	0.139	9.3	1.00	0.107
7.0	1.50	0.214	14.1	2.24	0.159	11.1	1.25	0.112
8.8	1.87	0.213	15.3	2.74	0.182	14.0	2.00	0.143
10.8	2.34	0.217	17.2	3.00	0.174	15.7	2.45	0.156
11.9	2.64	0.222				16.1	3.00	0.186
13.3	3.00	0.225						

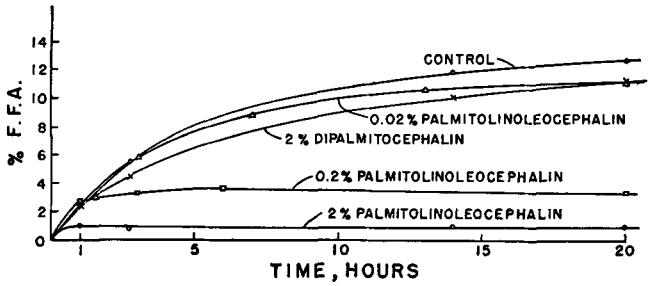

FIG. 4. Effect of synthetic cephalins on lipase cream of moderate activity.

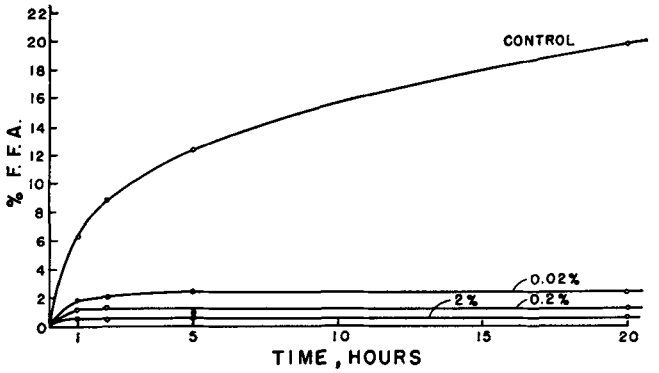

FIG. 5. Effect of palmitolinoleocephalin on cream of high activity.

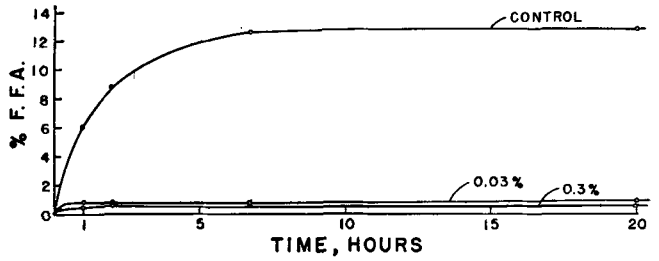

FIG. 6. Inhibition of lipase cream by egg phosphatide.

extent of splitting of cottonseed oil when varying amounts of cream are added. In all cases the mixtures contained 0.20 ml. of water, which is included in the reaction mixture for the purpose of calculating the percentage of cream in the reaction mixture. All titrations are corrected for the acid in the cream used although this is only significant when the concentration of the cream exceeds 1%. For these data the percentage of free fatty acids in the mixture has been calculated to the percentage of cottonseed oil hydrolyzed.

Inhibition of Lipase by Phosphatides. Phosphatides are normal constituents of crude vegetable oils, and it was therefore deemed to be of interest to observe the effect of synthetic dipalmitocephalin (8) and of palmitolinoleocephalin (9) on lipase activity. When the latter was found to inactivate lipase, the effect of natural mixed phosphatides as represented by soybean phosphatide, egg phosphatide, and salmon egg phosphatides was also studied. The results are shown in Figures 4 to 8. The phosphatide was dissolved in cottonseed oil to make a 2% solution, heating to 80° to 90°, when necessary, to effect solution. Aliquots of this were used directly, after cooling to room temperature, and were also diluted to make the concentrations of 0.2 and 0.02% phosphatide in the

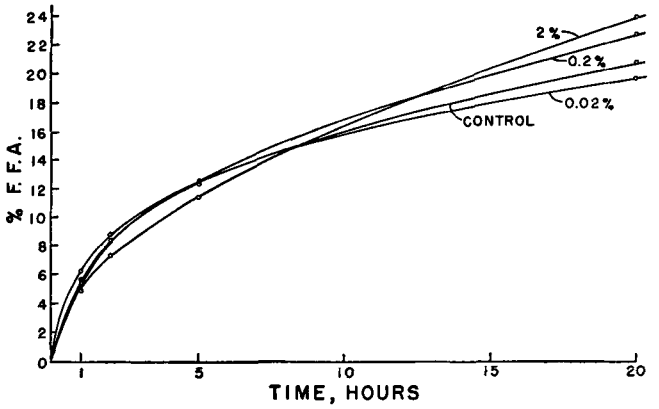

FIG. 7. Effect of soybean phosphatide on lipase.

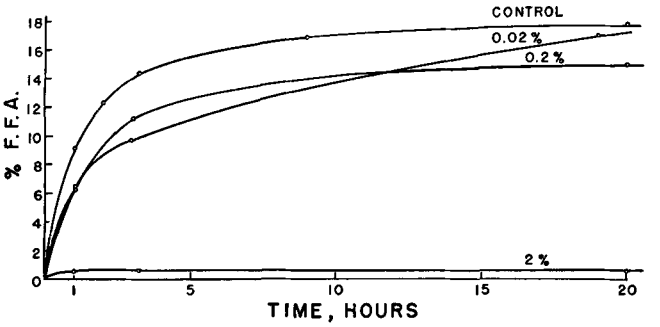

FIG. 8. Effect of salmon egg phosphatide on lipase cream.

oil. These solutions of phosphatide in oil were then added to a mixture of lipase cream and water in the usual way. Corrections were applied to account for the acidity of the phosphatide. The observation that dipalmitocephalin is practically without effect is doubtless due to the insolubility of this substance in cottonseed oil at room temperature. Soybean phosphatide is practically without effect; the increased splitting after 7 or 8 hours is probably due to the improved emulsions obtained in the presence of this substance. The inhibitory phosphatides appear to cause a precipitate in the reaction mixture and probably inactivate the lipase by irreversible combination of the phosphatide at the active centers of the lipase.

Soybean phosphatide doubtless contains cephalins, and we are unable to explain why it is not inhibitory. It may be due to structural differences in the arrangement of the groups attached to the glycerol or to other differences, such as optical activity. In this connection it is of interest to note that synthetic cephalin is unable to serve as substrate for the enzyme in cabbage that splits ethanolamine or serine from cephalin or phosphatidyl serine, but soybean phosphatides are a good substrate for this enzyme system (10).

Summary

A procedure for preparing an active lipase cream, of reproducible activity, from castor beans is described. Shelled castor beans are ground in a mortar to a greasy paste. Water is incorporated in this paste by addition of small quantities, with frequent grinding. This suspension of ground castor beans in water is stored overnight at 2° to 4°, phthalate buffer is then added, and the pH adjusted to 4.5. This suspension is centrifuged and the cream washed twice with water. This lipase cream emulsifies with oils and

reacts rapidly with them to liberate free fatty acids, without neutralization of the fatty acids liberated, or the addition of buffers, or any other substance although the rate of fat splitting is enhanced if about 2% of water is added. The rate of splitting of cottonseed oil at 10° and 20° by this lipase cream indicates that the temperature coefficient of this enzyme is about half of the usual temperature coefficient. The lipase is inhibited by a soluble synthetic cephalin, egg phosphatide, and salmon egg phosphatide, but not by soybean phosphatide.

Acknowledgments

The author wishes to express his appreciation to A. K. Balls, for suggesting this problem and for his assistance and advice throughout the course of this work. He also wishes to thank G. M. Severson, of the University of Nebraska, for two lots of castor beans; John H. Lathe of the Glidden Company for two samples of soybean phosphatides; G. Ivor Jones of the U. S. Bureau of Fisheries for the salmon egg phosphatides; and Leo Kline of the Western Regional Research Laboratory for the egg phosphatide. He is also indebted to Walter Mann Jr. for assistance in some of the experimental work.

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[Received June 12, 1950]

Sesame Oil. VI. Determination of Sesamin

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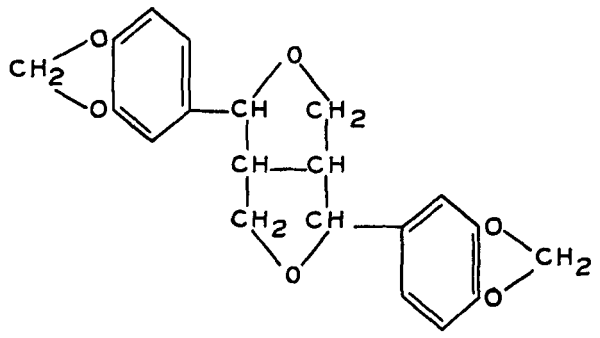
SESAMIN, one of the minor constituents of sesame oil, was first isolated from the acetic acid extract of sesame oil by Tocher (20) in 1890. Villavecchia and Fabris (21) in 1892 obtained sesamin from the unsaponifiable matter of sesame oil. These authors described some of its properties, but no further significant work on sesamin was reported until 1928 when Adriani (1), and Boeseken and Cohen (3), independently, indicated that the correct empirical formula for sesamin was $C_{20}H_{18}O_6$. The structure and properties of sesamin were extensively investigated by Bertram *et al.* (2), Boeseken and Cohen (3), and Cohen (8). The relationship between sesamin and other naturally occurring compounds, especially asarinin, was investigated and discussed by Erdtman (10), Kaku *et al.* (17), Huang-Minlon (14), and Bruchhausen and Gerhard (4). The last mentioned authors proposed a complete structure for sesamin as shown in the accompanying formula.



In 1937 Eagleson (9) found that the insecticidal activity of pyrethrum insecticides was markedly enhanced by the addition of sesame oil. The oil alone was inactive as an insecticide, and of 42 animal and vegetable oils tested only sesame oil acted as a synergist for the pyrethrins. Haller and co-workers (13) fractionated sesame oil by molecular distillation in an effort to isolate the active principle. From the active fractions a crystalline compound was obtained, which was shown to be sesamin. Insecticidal tests with pure sesamin showed that this compound possessed marked synergistic activity with pyrethrins.

Although other unidentified minor constituents of sesame oil have been reported to exhibit synergistic effects (19), sesamin is the only component which has been shown definitely to act as a synergist with pyrethrins; therefore its quantitative determination in this oil is of practical importance.

A colorimetric method for the estimation of sesamin was described in 1944 by Jacobson, Acree, and Haller (16). Since this method has certain disadvantages which will be discussed below, an improved method appears to be desirable. Such a method has been developed and is described in the present report. It is based on the measurement of the ultraviolet absorption of sesame oil (11, 15, 18) following the removal of sesamol by treatment with alkali (6) and correction for the absorption resulting from the presence of sesamolin.

Experimental

Sesame oil contains, besides sesamin, other minor constituents, the absorption characteristics of which are not known. Therefore, the following compounds were prepared: sesamin and sesamolin, by extraction from sesame oil as described elsewhere (5); and sesamol, by synthesis (5). The absorption spectra of these compounds in the ultraviolet region are shown in Figures 1 and 2 together with the absorption spectrum of a crude sesame oil.

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