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The Lipids of the Wheat Embryo. I. The Fatty Acids

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The earlier work on wheat germ oil consisted for the most part in the measurement of certain physical and chemical characteristics.[2,3,4,5]

There is marked disagreement in the literature concerning the individual fatty acids present in the oil. Thus Powers and Salway[6] state that the saturated fraction is made up of equal parts of palmitic and stearic acids while Jamieson and Baughman[7] found mostly palmitic acid with a relatively small amount of stearic acid and a still lesser proportion of lignoceric acid. Frankforter and Harding[3] found little or no stearic acid in wheat germ oil. Powers and Salway[6] state that the unsaturated fraction of this oil is "practically pure linolic acid" while other investigators[3,5,7] indicate the presence of oleic and linolenic acids. Roller[8] states that ricinoleic acid is present in the unsaturated fraction although he cites no experimental evidence for this assumption.

The phosphatides of wheat germ have been investigated recently by Channon and Foster.[9]

An effort was made toward a more detailed study of the fatty acids of wheat germ oil than has been hitherto conducted. This paper describes the work on the identification of these fatty acids and their glyceride structure.

Experimental

Preparation and Analysis of Starting Material

Fresh wheat germ was obtained from a high protein Marquis spring wheat by means of the usual milling procedure. The product was further purified by flattening between smooth steel rolls and sifting over a No. 18 wire to remove as much bran as possible. The germ analyzed as follows.

	%
Moisture	11.2
Ash	4.29
Lipid (alcohol–ether extract)	13.68
Ether extract	10.81
Acetone extract	11.91
Nitrogen	4.79
Acidity (calcd. as sulfuric acid)	0.092

The lipid obtained by alcohol–ether extraction contained 0.415% nitrogen (micro Kjeldahl) and 1.23% phosphorus (Pregl).

A total of 5.5 kilos of the fresh germ was extracted with 11.5 liters of 95% ethyl alcohol followed by 21 liters of ethyl ether C. P. When the alcohol extract was cooled to −12°, a precipitate formed. This was filtered off and the ether extract added to the filtered alcohol solution when more precipitation of the greenish-white granular material occurred.

Carbohydrate Fraction.—Both precipitates, I, that obtained from cooling the alcohol extract, and II, that obtained on the addition of the ether solution to the filtered alcohol extract, gave a strong Molisch reaction and were found to be a mixture of carbohydrate materials. Both precipitates were dissolved in water and shaken with ether to remove traces of fat. The water solution of Carbo-

(1) Condensed from one section of a thesis presented by B. Sullivan to the Faculty of the Graduate School of the University of Minnesota in partial fulfilment for the degree of Doctor of Philosophy, June, 1935. Paper No. 1395, Journal Series, Minnesota Agricultural Experiment Station.
(2) De Negri, *Chem.-Ztg.*, **22**, 976 (1898).
(3) Frankforter and Harding, THIS JOURNAL, **21**, 758 (1899).
(4) Alpers, *Chem.-Ztg.*, **42**, 37 (1918).
(5) Ball, *Cereal Chem.*, **3**, 19 (1926).
(6) Powers and Salway, *Pharm. J.*, **91**, 117 (1913).
(7) Jamieson and Baughman, *Oil and Soap*, **8**, 138 (1932).
(8) Roller, *J. Phys. Chem.*, **35**, 3286 (1931).
(9) Channon and Foster, *Biochem. J.*, **28**, 853 (1934).

hydrate I was concentrated *in vacuo* and clarified with 130 cc. of 20% lead acetate. Powdered disodium hydrogen phosphate was added to the filtrate to precipitate the excess lead and the resultant precipitate filtered off. The filtrate was made up to 500 cc. and sugar was determined by the Bertrand method. A 10-cc. aliquot required 6.8 cc. of 0.05 *N* potassium permanganate or 500 cc. contained the equivalent of 0.50 g. dextrose. After inversion with hydrochloric acid, 1 cc. of the solution required 13 cc. of 0.05 *N* permanganate or 500 cc. contained the equivalent of 9.23 g. of sucrose. Although this carbohydrate fraction gave some reduction before inversion, showing the presence of a reducing sugar, a much greater reduction was obtained after inversion, suggesting the presence of sucrose. The osazone from the inverted solution was prepared by adding phenylhydrazine hydrochloride and sodium acetate in the required amounts. Recrystallization from dilute alcohol gave characteristic crystals of glucosazone, m. p. 208°. The filtrate remaining after the sugar determinations was evaporated *in vacuo* to a thick sirup and ground under absolute alcohol. The material was then treated with 250 cc. of pyridine and 130 cc. of acetic anhydride. The mixture was allowed to stand several days at room temperature and was then poured into 1.5 liters of ice water. The precipitate was filtered, dissolved in hot methanol and decolorized with carbon. The filtrate, after concentration, was allowed to stand in the refrigerator several days. The white precipitate was recrystallized once from methanol and twice from ethanol and then dried in a vacuum desiccator. It weighed 1 g. and melted at 228–229°. On ignition, the compound was found to be ash free.

Anal. Found: C, 51.09; H, 5.81; mol. wt. (Rast), 884; $[\alpha]^{27°}D$ −16.7° (in chloroform). The acetyl group was determined by the method of Kunz and Hudson:[10] CH_3CO found, 44.60.

No acetylated sugar could be found in the literature which corresponded to the above analyses. With the exception of α-cellobiose octaacetate, very few of the acetylated disaccharides or trisaccharides which are known have such high melting points. Cellobiose is not known to occur naturally but in view of the fact that the melting point of this compound checked with that of α-cellobiose octaacetate, a mixed melting point with pure α-cellobiose octaacetate was determined. The melting point of the unknown substance (228.5°) was depressed to 210–213° on admixture with α-cellobiose octaacetate. Analysis of the known acetylated disaccharides and trisaccharides requires a carbon content of 49.6% and a hydrogen content of 5.6%. The carbon content of this unknown is over 1% too high. In view of the several recrystallizations and the sharp unchanged melting point, it is not thought that the variance in the analysis obtained could have been due to some impurity. Molecular weight determinations by the Rast camphor method also proved unsatisfactory for identification purposes. However, this is not so surprising because abnormal molecular weight determinations on acetylated sugars have been reported frequently in the literature. Thus raffinose, when acetylated, gives, by the Rast method, the value required by a disaccharide instead of a trisaccharide.[11] The value for molecular weight, 884, is somewhat low for an acetylated trisaccharide.

The amount of material was so small that complete identification was not possible.

Carbohydrate fraction II was also concentrated *in vacuo* to a small volume. The solution was clarified with basic lead acetate and the precipitate filtered off after standing overnight. The excess lead was removed from the filtrate by passing hydrogen sulfide through it until no further precipitation occurred. After filtration, the solution was decolorized with Norit and evaporated *in vacuo* to a thick sirup. The sirup was ground under absolute alcohol whereby a yellowish white powder weighing 12.2 g. was obtained. Sugar was determined by the Bertrand method before and after inversion. The powder was found to contain 36.57% sucrose and 2.56% dextrose.

This material (10.8 g.) was acetylated by adding 166 cc. of pyridine and 92 cc. of acetic anhydride. After standing two weeks at room temperature, solution was complete and the liquid was poured into 1 liter of ice water. The 9 g. of viscous material which separated was dissolved in hot methanol with Norit, filtered and allowed to cool. After several days, white crystals separated which, after recrystallization from ethyl alcohol, melted at 229°. This compound was identical in analysis and melting point with the acetylated sugar obtained from Carbohydrate I.

Colin and Belval[12] state that wheat germ contains only sucrose and raffinose, both of which when acetylated have a dextro rotation and a lower melting point than this acetylated carbohydrate. Unfortunately, after analyses were complete, not enough of the acetylated carbohydrate was left for hydrolysis and reacetylation. Work on this compound is being repeated in order to obtain sufficient material for complete identification.

Lipids

The combined alcohol–ether extract of the fat was filtered, dried with anhydrous sodium sulfate and again filtered. The solvent was partly distilled off *in vacuo* in a current of nitrogen at room temperature and made up to a definite volume (5 liters). For the determination of all the characteristics, small amounts of the stock solution were made up to 250 cc. with dry ether or chloroform and a definite number of cc. pipetted into the reaction flask. At the same time, an aliquot or multiple of the volume taken for analysis was pipetted into a platinum dish and weighed after removal of the solvent *in vacuo* at 70°. This procedure was especially advantageous for micro determinations where direct weighing and transferring of the small amount of fat used would have been inaccurate.

The characteristics obtained on the wheat germ lipids are recorded in Table I together with those found by two other investigators. These figures are the average of at least three and, in many cases, several individual determinations. Methods used for the determination of these characteristics were the Rosenmund and Kuhnhenn method[13] and the Yasuda micro adaptation,[14] both of which use pyridine sulfate dibromide, were found preferable to the older methods, such as the Hanus and Wijs, for

(10) Kunz and Hudson, THIS JOURNAL, **48**, 1978 (1926).

(11) Haworth, Hirst and Ant-Waorinen, *J. Chem. Soc.*, 2368 (1928).

(12) Colin and Belval, *Bull. soc. chim. biol.*, **16**, 424 (1934).

(13) Rosenmund and Kuhnhenn, *Z. Nahrs. U. Genussm.*, **46**, 154 (1923).

(14) Yasuda, *J. Biol. Chem.*, **94**, 401 (1931).

TABLE I

PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE WHEAT GERM LIPIDS

	Present investigation Alcohol–ether extraction (1935)	Jamieson and Baughman ether extraction (1932)	Ball ether extraction (1926)
Specific gravity	0.9326 (26°/26°)	0.9268 (25°/25°)	0.9249 (25°/1°)
Refractive index	1.4800 (20°)	1.4762 (20°)	1.4686 (17.5°)
Acid value	6.95	7.6	21.48
Saponification value	184.0	186.5	184.13
Iodine number	125.0 (Rosenmund)	125.6 (Hanus)	123.64 (Wijs)
Thiocyanogen number	84.7	79.7	......
Hexabromide number	2.28	Trace	......
Acetyl value	16.7	9.9	......
Reichert–Meissl value	0.77	0.2	0.475
Polenske number	0.44	0.35	0.25
Hehner number	89.0	......	93.7
Soluble acids as butyric, %	1.44	......	......
Ester number	177.05	......	162.65
Unsaponifiable matter, %	4.00	4.70	3.59
Iodine number of unsaponifiable matter	......	97.3	......
Thiocyanogen number of unsaponifiable matter	......	62.0	......
Saturated acids (Twitchell), %	16.00	......	......
Saturated acids (Bertram), %	17.87	......	......
Saturated acids (lead salt–ether), %	......	13.3 (corr.)	15.83 (I no. 27.4)
Unsaturated acids	84.00[a]	75.3 (corr.)	84.17[b]
Iodine number of total fatty acids	129.9	......	128.11
Thiocyanogen number of total fatty acids	79.3	......	......
Iodine number of unsaturated fatty acids	153.0	160.7	145.97
Mean mol. wt. of total fatty acids	278.0	......	272.72
Mean mol. wt. of saturated fatty acids	262.2	......	245.08
Mean mol. wt. of unsaturated fatty acids	281.0	......	276.85

[a] % of total fatty acids determined. [b] % of total fatty acids by difference.

the determination of iodine numbers. The Hanus method gave consistently lower results than the Rosenmund or Yasuda methods on wheat germ oil and its fractions. Thiocyanogen numbers were determined by the procedure of Kaufmann and Keller.[15] Other determinations, such as acid number, saponification number and unsaponifiable matter, were conducted as outlined by Grün[16] or by Jamieson.[17] Alcohol used for saponifications or as a solvent for titrations was made aldehyde-free by the method of Dunlap.[18] Wherever pure anhydrous ether was necessary, it was prepared as follows: C. P. ethyl ether (so-called anhydrous) was washed three times with one-quarter of its volume of 3% neutral potassium permanganate. It was then washed twice with a saturated solution of calcium chloride and allowed to stand over crystalline calcium chloride. The ether was then filtered and stored over sodium.

Saponification of the fat was achieved by heating for eight hours on a steam-bath with 5% alcoholic potassium hydroxide (made from aldehyde-free ethyl alcohol) in an atmosphere of nitrogen. After removal of the unsaponifiable fraction with ethyl ether, the acids were recovered in the usual manner by acidification with dilute hydrochloric acid and subsequent extraction with ether. The ether solution was dried with anhydrous sodium sulfate, filtered, made up to 1 liter and aliquots taken for all determinations.

The method of Twitchell[19] for the separation of the saturated fatty acids (which depends on the relative solubility of the lead salts in 95% ethanol) was found to be preferable in every respect to the lead salt–ether method. Curiously enough, the Twitchell method, which was developed in this country, has been widely used in Europe and but seldom by fat investigators in the United States, most of whom have employed the lead salt–ether method. The objection to the latter procedure is due to the fact that the saturated acids are always contaminated with some unsaturated acids for which a correction needs to be made. In the case of wheat germ fat, the saturated acids obtained by the Twitchell method gave an iodine number of less than 1.5 before any recrystallization. It was found important in the Twitchell method, as has been also observed by Hilditch,[20] that the temperature must not fall below 15° and that the fatty acid to alcohol ratio be maintained at approximately 1:10. The lead salts of the saturated acids lower than myristic are, of course, increasingly soluble in 95% ethyl alcohol and would be found in the unsaturated fraction.

The more recent method of Bertram[21] for the separation of the saturated acids was also found to be highly sat-

(15) Kaufmann and Keller, *Z. angew. Chem.*, **42**, 20 and 73 (1929).

(16) Adolf Grün, "Analyse der Fette und Wachse," Erster Band, Julius Springer, Berlin, 1925.

(17) George Jamieson, "Vegetable Fats and Oils," Chemical Catalog Company, New York, N. Y., 1932.

(18) Dunlap, THIS JOURNAL, **28**, 395 (1906).

(19) Twitchell, *J. Ind. Eng. Chem.*, **13**, 806 (1921).

(20) Hilditch and Priestman, *Analyst*, **56**, 354 (1931).

(21) Bertram, *Z. Unters. Lebensm.*, **55**, 179 (1928).

isfactory. This procedure depends on the oxidation of the unsaturated glycerides in the soap solution by permanganate after removal of the unsaponifiable matter. The Bertram method always gave 1–2% higher values for the saturated acids from wheat germ than the Twitchell procedure. Since both methods were conducted several times, this difference was due to no accidental variation but might indicate the presence of a slight amount of hydroxy acids in the saturated fraction as weighed or a small amount of myristic acid, more of which is obtained by the Bertram method than by the Twitchell method.

Identification of the Saturated Fatty Acids.—The mixed saturated fatty acids (23 g.) separated by the Twitchell method were esterified in ether solution with diazomethane. The latter was prepared by flowing an excess of nitrosomethylurethan from a separatory funnel on a concentrated solution of sodium ethylate in a distilling flask and the diazomethane formed was distilled directly into a solution of the mixed acids in dry ether. The mixed methyl esters were distilled twice at 4 mm. pressure from a Ladenburg distilling flask. Three fractions were collected (one of which remained in the flask) which weighed as follows

Fraction 1 (145–155°)	16.93 g.
Fraction 2 (155–170°)	4.39 g.
Fraction 3 (residue)	1.71 g.

The total weight was 23.03 g. Each fraction was saponified with 5% alcoholic potassium hydroxide and the acids recovered in the usual way. Each fraction was recrystallized once from ethanol. Neutralization equivalents were as follows

Fraction 1, 0.3500 g. required 13.5 cc. 0.1 *N* KOH = 259
Fraction 2, 0.2764 g. required 9.67 cc. 0.1 *N* KOH = 286
Fraction 3, 0.1963 g. required 5.34 cc. 0.1 *N* KOH = 367.6

Palmitic acid has a molecular weight of 256.25. The *p*-bromophenacyl ester of this first fraction melted at 85.5°. Hann, Reid and Jamieson[22] report a melting point of 86° for this ester of palmitic acid. A mixed melting point with the ester prepared from pure palmitic showed no depression. Fraction 1, which made up 73.51% of the saturated fraction, was, therefore, palmitic acid. Stearic acid has a molecular weight of 284.28. The molecular weight found (286) as well as the phenacyl ester of this fraction (m. p. 69°) shows that fraction 2, which amounted to 19.06% of the mixed esters, was stearic acid. Fraction 3, which comprised 7.43% of the total methyl esters, was lignoceric acid as proved by its neutralization equivalent of 367.6 (theoretical, 368) and the melting point of its phenacyl derivative (87–88°).

The following saturated acids occur in wheat germ: palmitic, stearic and lignoceric. This confirms the findings of Jamieson and Baughman,[7] although the proportion of palmitic acid found by them was larger (91% of the saturated fraction) and of stearic and lignoceric acids smaller than in the present investigation.

The Unsaturated Fatty Acids

Calculation of the Unsaturated Fatty Acids from the Iodine and Thiocyanogen Values.—The total fatty acids recovered after removal of the unsaponifiable fraction of the wheat germ lipids had a neutralization value of 202, an iodine number of 130, and a thiocyanogen number of 79.3. The iodine number of 130 is the average of replicate determinations (ranging from 127–131) on several samples of the fatty acids prepared during the course of this investigation. The thiocyanogen number of 79.3 is the average of five determinations on various preparations of the total fatty acids, which were 80.0, 77.6, 79.4, 77.6 and 81.7, respectively. Kaufmann and Keller[15] have shown that thiocyanogen adds to the double bonds in a different way than bromine or iodine. Thus, while bromine and iodine will add to all the double bonds in the fatty acid series, thiocyanate adds to only one bond of the two present in linolic acid, to only two of the three double bonds in linolenic acid, but adds quantitatively to the one double linkage in oleic acid. As a result of this peculiarity of thiocyanogen, it is possible (assuming the correctness of the above assumption with all mixtures) to calculate from the iodine and thiocyanogen values and the percentage of saturated acids the proportion of the various unsaturated acids present in a mixture of fatty acids according to the following equations:

$$O = (100 - G) - 1.104 \text{ (Iodine number } - \text{ Thiocyanogen number)}$$
$$L = (100 - G) - 1.104 \text{ (2 Thiocyanogen number } - \text{ Iodine number)}$$
$$Ln = -(100 - G) - 1.104 \text{ (Thiocyanogen number)}$$

where G is the per cent. of saturated acids, O, oleic acid, L, linolic acid and Ln, linolenic acid. The validity of the method when oleic, linolic and linolenic acids are present depends, among other things, on the assumption by Kaufmann that the thiocyanogen value of linolenic acid is 183. Smith and Chibnall[23] do not believe that the Kaufmann procedure gives accurate values when applied to all mixtures of fatty acids in which β-linolic and β-linolenic acids are present. It is of utmost importance in these determinations that all reagents and glassware are dry. A large excess of potassium iodide (exactly 20 cc. of 15% solution) gave the most uniform results. In the majority of cases where a mixture of saturated acids, oleic, linolic and linolenic acids are present, it is undoubtedly true that the use of the Kaufmann method gives a very valuable index of the unsaturated fatty acids which are present. In this case, using the figures 129.9 iodine number, 79.3 thiocyanogen number and 16.00% saturated acids, the percentages of the unsaturated acids in the wheat embryo were calculated according to the equations given above with the following results

	% of total fatty acids
Oleic acid	28.14
Linolic acid	52.31
Linolenic acid	3.55

A somewhat similar type of calculation can be used for the iodine and thiocyanogen numbers determined on the fat itself as was done by Jamieson and Baughman.[7] This is not so accurate since the per cent. of the unsaponifiable matter as well as of the saturated acids must be used. The use of the fat itself instead of the fatty acids introduces the error of substitution as well as addition of the

(22) Hann, Reid and Jamieson, THIS JOURNAL, 52, 818 (1930).

(23) Smith and Chibnall, *Biochem. J.*, 26, 1345 (1932).

halogens to the sterols and other unsaturated compounds present in the unsaponifiable matter. Jamieson and Baughman found on the ether extracted fat from wheat germ an iodine number (Hanus) of 125.6, a thiocyanogen number of 79.7 with an analysis of 13.3% saturated acids (lead-salt-ether method) and 4.7% unsaponifiable matter. They calculated the percentage of unsaturated acids as follows as acids in the original oil: oleic acid 26.6%; linolic acid, 39.1%; and linolenic acid, 9.60%.

Bromination of the Unsaturated Fatty Acids.—The unsaturated acids (5.325 g. separated by the Twitchell process) were brominated in dry ether (55 cc.) with an ether solution of bromine at -5 to $-10°$ according to the conventional method of Eibner and Muggenthaler.[24] The bromine used had previously been dried by shaking with concentrated sulfuric acid. After standing for three hours at $-5°$, the precipitate was filtered through a sintered glass crucible (10 G 3) and washed with four portions (10 cc. each) of ice-cold dry ethyl ether. The precipitate weighed 0.3165 g. after drying at 95°, which is equivalent to 5.94% linolenic hexabromide or 2.18% α-linolenic acid.[25] The precipitate dissolved completely in boiling benzene, indicating the absence of any octabromides. After one recrystallization, the precipitate melted at 176°. The melting point of linolenic hexabromide is given variously from 175–181°. The melting point depends somewhat on the temperature at which the precipitate is dried.

Anal. Calcd. for $C_{18}H_{30}O_2Br_6$: Br, 63.32. Found: Br, 62.84.

The ether was evaporated from the filtrate *in vacuo* and the residue dissolved in petroleum ether (b. p. 30–60°), refluxed on a steam-bath, cooled and placed in an ice chest overnight. The heavy precipitate was filtered, washed with ice-cold petroleum ether and dried at 100°. It weighed 2.9132 g. The petroleum ether filtrate was concentrated to about one-third its original volume. When cooled, an additional 0.1152 g. crystallized, giving a total weight of 3.0284 g. of linolic tetrabromide. This is equivalent to 56.87% of linolic tetrabromide or 26.57% of α-linolic acid. The precipitate was decolorized with Norit and recrystallized from ligroin. Beautiful white needles were obtained, m. p. 113.5°.

Anal. Calcd. for $C_{18}H_{32}O_2Br_4$: Br, 53.33. Found: Br, 52.95, 53.39.

The petroleum ether filtrate from the linolic tetrabromide was evaporated to dryness and taken up in dry ethyl ether. The ether solution was washed with sodium sulfite to remove the excess of bromine and then evaporated. The residue weighed 7.2445 g. The bromine content of the oily residue as determined by duplicate Carius combustions was 45.37 and 45.23%, respectively. Since the theoretical bromine content of oleic dibromide is 36.18%, the higher bromine percentage found shows that some β-linolic tetrabromide and possibly some β-linolenic hexabromide was present. The residue (5.7 g.) which contained 45.3% bromine was debrominated by the method of Rollet.[26] After saponification of the ester, the acid was recovered on acidification and taken up in petroleum ether. The petroleum ether solution was thoroughly washed until free from acid, dried with sodium sulfate, filtered and evaporated to a small volume. The mixed acids were again brominated when additional 0.2 g. of linolic tetrabromide (m. p. 112°) was obtained. The filtrate was again debrominated according to Rollet's method and the acid recovered. The iodine value was found to be 108, whereas the theoretical iodine value for oleic acid is 89.9. This shows that although oleic acid is present, pure 9,10-dibromostearic acid was not obtained. The *p*-phenylphenacyl ester of this fraction was prepared. After several recrystallizations from aqueous alcohol, the correct melting point could be obtained. Drake and Bronitsky[27] report a melting point of 60° for this ester of oleic acid. As a result of this bromination, the following compounds were isolated and identified: linolenic hexabromide, linolic tetrabromide and oleic dibromide. The per cent. of α-linolenic acid found was 2.18% of the unsaturated fraction or 1.83% of the total fatty acids. The per cent. of β-linolenic acid can be calculated from the amount of total linolenic acid as measured from the iodine-thiocyanogen calculations. The amount of β-linolenic acid found by this means was 1.72% of the total fatty acids (3.55–1.83). The per cent. of α-linolic acid formed on the first bromination was 26.57% of the unsaturated fraction or 22.32% of the total fatty acids. The amount of β-linolic acid can be calculated from the bromine content of the mixed linolic tetrabromide-oleic dibromide fraction if the absence of the liquid hexabromide is assumed. Since β-linolenic acid is present in small amounts, the calculation of β-linolic acid from the bromine content of the mixed residue and of oleic acid by difference, are only very rough approximations. The β-linolic acid was, therefore, calculated as the difference between the total linolic acid found by the Kaufmann procedure and the amount of the α-form isolated on bromination or 29.99% β-linolic acid (52.31–22.32). Oleic acid was then determined by difference and was found to be 28.14%. The unsaturated fatty acids of wheat germ oil were, therefore, found to be present in the following amounts.

	%
Total unsaturated fatty acids	84.00
α-Linolenic acid	1.83
β-Linolenic acid	1.72
α-Linolic acid	22.32
β-Linolic acid	29.99
Oleic acid (by difference)	28.14

No acid of greater unsaturation than three double bonds was present.

No other complete bromination on the wheat germ lipids has been reported. Ball[6] reported a compound melting at 162° obtained on brominating the unsaturated acids from wheat germ but did not purify or identify this compound. He carried the bromination no further. Jamieson and Baughman[7] found only a trace of the ether-insoluble hexabromide on brominating the fatty acids from wheat

(24) Eibner and Muggenthaler, *Farben-Ztg.*, **18**, 131 (1912).

(25) There is no definite configurational meaning in the terms α and β as they are commonly used for the double and triple bond acids. The α designation refers to those acids forming insoluble bromo addition compounds, while the β refers to the isomers forming soluble bromo addition compounds with the solvents used.

(26) Rollet, *Z. physiol. Chem.*, **62**, 410 (1909).

(27) Drake and Bronitsky, THIS JOURNAL, **52**, 3715 (1930).

oil. Since they found 9.6% linolenic acid by calculation from the iodine and thiocyanogen numbers of the fat, they conclude that most of the linolenic acid is of the form which gives the ether-soluble hexabromide. The ether-insoluble form was found to predominate in this investigation.

It is well known that bromination of unsaturated fatty acids and the separation of the bromides by means of their different solubilities in such solvents as ethyl ether and ligroin does not give a quantitative measure of the total amounts of linolic or linolenic acids present in a mixture owing to the presence of geometrical isomers. This subject has been investigated by Rollet.[28] Erdmann has established the pre-existence of the so-called liquid or β-linolic acid. Debromination of the soluble bromides from linolic and linolenic acids results in isomerization of some of the β-form to the α-form. The yields are variable and dependent on the nature of the fatty acids present in the mixture. It is thought that the best procedure available at the present time for the quantitative estimation of mixtures of linolenic, linolic and oleic acids is the measurement of the per cent. of the α- or insoluble forms of linolic acid and linolenic acid by bromination and the measurement of the β- or so-called soluble forms by subtraction of the amount of the α modification from the total amount of each of these acids as determined by the iodine–thiocyanogen calculation.

Oxidation of the Unsaturated Fatty Acids.—The method used for the oxidation was a combination of those described by Rollet,[26,28] Hazura[29] and Lapworth and Mottram.[30]

A 7.565-g. portion of the unsaturated acids separated from the wheat germ lipids by the Twitchell method was taken for oxidation. Sodium hydroxide (8 g.) in 800 cc. of distilled water was added and the solution heated for one hour on a steam-bath. After cooling, 3.5 liters of ice water was added; then 650 cc. of 1% potassium permanganate was poured into the soap solution in a thin stream and with constant shaking at 10°. The solution was allowed to stand for eight minutes and then was decolorized with gaseous sulfur dioxide, fifteen minutes being required for the decolorization. Concentrated hydrochloric acid (160 cc.) was added and the liquid allowed to stand for forty-four hours at room temperature. The white precipitate which settled was filtered by suction and washed with a small amount of petroleum ether (b. p. 60–80°) to remove any unsaturated acids which might have escaped oxidation. The precipitate weighed 9.8 g. after drying in a vacuum desiccator over sulfuric acid. The precipitate was then extracted by refluxing with dry ethyl ether for twenty hours. The ether solution was partly evaporated and 0.47 g. of crystals which separated were filtered, taken up in boiling 95% ethyl alcohol and allowed to crystallize. The precipitate was then recrystallized twice from 95% alcohol. The melting point on the first recrystallization was 129° and on the second, 131–132°. A final recrystallization gave beautiful white crystals melting sharply at 131°. Dihydroxystearic acid melts at 131°.

Anal. Calcd. for $C_{18}H_{36}O_4$: C, 68.35; H, 11.49. Found: C, 67.87; H, 11.40.

(28) Rollet, *Z. physiol. Chem.*, **62**, 422 (1909).
(29) Hazura, *Monatsh.*, **8**, 147 (1887).
(30) Lapworth and Mottram, *J. Chem. Soc.*, 1628 (1925).

The compound is, therefore, dihydroxystearic acid resulting from the oxidation of oleic acid.

α- and β-Sativic Acid (Tetrahydroxystearic Acid).—The residue of the hydroxy acids left after the ether extraction weighed 4.68 g. and had a melting point range of 156–165°. This material was boiled with 1 liter of water and 1.6 cc. of concentrated hydrochloric acid according to the procedure of Nicolet and Cox.[31] The process of recrystallization from this solution was repeated several times with the following results

	Ppt. obtained, g.	M. p., °C.
1st Recrystallization	0.35	157
2nd Recrystallization	.17	155
3rd Recrystallization	.28	155–157
4th Recrystallization	.20	157
5th Recrystallization	.13	157
6th Recrystallization	.31	157
7th Recrystallization	.35	159–161

In order to avoid too much loss, the mother liquor from the first recrystallization was used for the third recrystallization and the mother liquor from the second recrystallization for the fourth treatment. The first six fractions weighed 1.44 g. The combined first six fractions (A), again recrystallized from 700 cc. of water and 1.1 cc. of concentrated hydrochloric acid, had a melting point of 156–157°. The same treatment was repeated and a melting point of 155–156° obtained. The material was then recrystallized from glacial acetic acid, giving a melting point of 155–158°. The precipitate was then subjected to another series of recrystallizations from 1 liter of water and 1.6 cc. of hydrochloric acid.

	M. p., °C.
11th Recrystallization	156–157.5
12th Recrystallization	155–155.5
13th Recrystallization	155–155.5

The substance as finally obtained weighed 0.1 g. and had a sharp melting point (155–155.5°).

Anal. Calcd. for $C_{18}H_{36}O_6$: C, 62.07; H, 10.43. Found: C, 61.90; H, 10.56.

α-Sativic acid is reported in the literature as melting from 153–157°. Earlier workers report melting points as high as 163° for α-sativic acid, doubtless due to contamination with the β-isomer.

The fraction (b) insoluble in water acidified with hydrochloric acid also weighed 1.4 g., indicating that the α- and β-forms of linolic acids are present in the unsaturated fraction in about equivalent quantities. This fraction melted at 171–172°. It was recrystallized twice from glacial acetic acid. The compound melted sharply at 172.5°.

Anal. Calcd. for $C_{18}H_{36}O_6$: C, 62.07; H, 10.43. Found: C, 61.27; H, 10.20.

β-Sativic acid is reported as melting at 173° by Meyer and Beer[32] and at 170° by Nicolet and Cox.[31]

Linusic and Isolinusic Acid (Hexahydroxystearic Acid).—The filtrate from the original precipitate of the oxidized acids was neutralized and concentrated *in vacuo* from 5 liters to about 500 cc. After standing at room tempera-

(31) Nicolet and Cox, THIS JOURNAL, **44**, 144 (1922).
(32) Meyer and Beer, *Monatsh.*, **33**, 311 (1912).

ture, a precipitate settled which was collected, dried in a vacuum desiccator and extracted with ether in order to remove any possible split products of the fatty acids resulting from the oxidation with permanganate. The precipitate was recrystallized once from hot alcohol (m. p. 198–200°) and twice from water, giving rhombic plates of linusic acid melting at 201–202°.

Anal. Calcd. for $C_{18}H_{36}O_8$: C, 56.84; H, 9.55. Found: C, 56.36; H, 9.38.

The filtrates from the linusic acid precipitates were evaporated to a small volume and 0.03 g. of the characteristic needles of isolinusic acid were obtained, which, after one recrystallization from water, had a sharp melting point of 173° which is correct for isolinusic acid. There was not enough material left for combustion.

The hydroxy acids obtained, *i. e.*, dihydroxystearic, α-sativic, β-sativic, linusic and isolinusic acids, show the presence of oleic, α-linolic, β-linolic, α-linolenic and β-linolenic acids in the unsaturated acids from the wheat embryo. No oxidation of the unsaturated acids from wheat germ has been reported previously.

Proof of the Presence of Oleic Acid by Separation as the Lithium Salt.—A partial separation of certain of the fatty acids can be made by fractional crystallization of their salts or on the basis of the varying solubility of certain of the salts with different solvents. Moore[33] recommended the use of the lithium salt for the preparation of pure oleic acid. Neither the lithium nor the barium salts can effect a quantitative removal of oleic acid from a mixture of unsaturated acids. Both procedures, however, are very useful in the preparation of pure oleic acid and for the qualitative separation of oleic acid from a mixture. A 2.79-g. portion of the mixed unsaturated acids was dissolved in 12.5 cc. of hot absolute alcohol and 0.55 g. of lithium hydroxide in 12.5 cc. of boiling water was added. The solution was refluxed, cooled slowly and allowed to stand overnight in a refrigerator. The precipitate was filtered by suction, washed with cold 50% alcohol and then recrystallized from the same solvent, 0.31 g. of oleic acid being recovered on acidification with hydrochloric acid. The acid had an iodine number of 88. The iodine number indicates pure oleic acid. As can be judged by the amount recovered, a quantitative yield of oleic acid was not attained. The experiment simply serves as another proof of the presence of oleic acid in the fatty acids recovered from the wheat embryo.

The Measurement of the Fully Saturated Glycerides of the Wheat Germ Lipids.—Hilditch and his collaborators[34,35,36,37] have advocated the use of a method designed for estimating the fully saturated glycerides present in natural fats. They calculated the so-called "association ratio" of a large number of plant and animal fats and found it to be distinctly characteristic of the biological origin of the fat. It is well known that, in general, the mixed fatty acids tend toward uneven distribution in the glyceride molecule. According to Hilditch, the simplest form of triglyceride (*i. e.*, containing only one fatty acid) is formed in any quantity only if no other method of combining the fatty acids is at the disposal of the plant or animal. Since the saturated acids from the wheat embryo amount to, at most, 17–18%, it would not be expected that any fully saturated glycerides would be present. It was, however, a matter of some interest to ascertain if such were the case since the Hilditch procedure is seldom tried on seed fats containing a relatively small proportion of saturated acids.

A 95.1-g. charge of fresh wheat embryo fat (obtained by ether extraction at room temperature) was dissolved in 1 liter of acetone in a 5-liter round-bottomed flask. The solution was gently refluxed on a steam-bath using an air condenser while 500 g. of very finely pulverized potassium permanganate was added gradually with shaking over a period of one and one-half hours. The mixture was then refluxed for six hours with occasional shaking and allowed to stand overnight. The acetone was evaporated as far as possible and the residue mixed carefully with 450 g. of sodium bisulfite. One liter of distilled water was added, the solution carefully decolorized by the addition of dilute sulfuric acid, cooled, transferred to a separatory funnel and thoroughly extracted with ether. The ether extract was washed with water until free from acid, dried with sodium sulfate, filtered and 50.15 g. was recovered. This product was transferred to the original flask and the oxidation repeated in the same manner using 1 liter of acetone, 200 g. of potassium permanganate and 250 g. of sodium bisulfite. After extraction with ether, 3.85 g. of semi-oxidized product was obtained which had an iodine value of 60. The oxidation was repeated again using 500 cc. of acetone and 50 g. of potassium permanganate. The ether extract, after washing with water, was shaken with dilute ammonia to remove acidic products resulting from the oxidation and then re-extracted with ether. The ether solution was washed with water until free from ammonia and dried. It weighed 0.7 g. and the material had a negligible iodine number (under 3). Three oxidations were found necessary. On the basis of the original fat 0.74% of supposedly completely saturated glycerides was obtained. No correction was made for the unsaponifiable fraction or its oxidized products, part of which is undoubtedly included in this result. From the amount obtained, it is evident that wheat germ fat contains only a trace, if any at all, of fully saturated glycerides. This would be expected in view of the preponderance of unsaturated acids in the lipids from the wheat embryo. It is, therefore, concluded that wheat germ fat is made up of mixed saturated-unsaturated glycerides with the very likely presence of tri-unsaturated glycerides.

Glycerol.—Glycerol was estimated on the fat by the titration of the oxalic acid formed on oxidation with permanganate. Values on various samples ranged from 7–10%.

Summary

The presence of a new carbohydrate in the wheat embryo which has not yet been identified completely is reported. The saturated acid fraction from the wheat germ lipids was found to be composed largely of palmitic acid together with

(33) Moore, *J. Soc. Chem. Ind.*, **38**, 320 (1919).
(34) Hilditch and Lea, *J. Chem. Soc.*, 3106 (1927).
(35) Collin and Hilditch, *J. Soc. Chem. Ind.*, **47**, 261 (1928).
(36) Collin and Hilditch, *Biochem. J.*, **23**, 1273 (1929).
(37) Hilditch and Salatore, *J. Soc. Chem. Ind.*, **50**, 468 (1932).

smaller amounts of stearic and lignoceric acids. The unsaturated acids found were oleic, α- and β-linolic and α- and β-linolenic. No fully saturated glycerides were present in wheat germ fat, the latter probably being composed of mixed unsaturated-saturated as well as some tri-unsaturated glycerides.

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The Lipids of the Wheat Embryo. II. The Unsaponifiable Fraction[1]

BY B. SULLIVAN AND C. H. BAILEY

About 4% of the fat from the wheat embryo consists of unsaponifiable material, of which about 70% is a mixture of sterols. The remaining 30% of the unsaponifiable fraction consists of a yellow, viscous oil, the composition of which has never been determined.[2]

The excellent work of Anderson, Shriner and Burr[3] showed conclusively that the sterol fraction of wheat germ could not be considered as one distinct sitosterol, as was believed by some earlier workers, but rather was a mixture of dihydrositosterol, and three isomeric sitosterols containing one double bond, called α-, β- and γ-sitosterols. The saturated sterol was found to melt at 143–144° and had a rotation of +22.2°. The α- and β-sitosterols could not be obtained in pure form. The γ-sitosterol was isolated in a fairly pure state, m. p. 147–148°, $[\alpha]_D$ −42°. Sandquist and Bengstsson[4] and Windaus, Werder and Gschaider[5] have since found that sitosterol is a homolog rather than an isomer of cholesterol and is best represented as $C_{29}H_{50}O$ instead of the previously used formula $C_{27}H_{46}O$.

No sterols of a greater degree of unsaturation than one double bond have been reported as occurring in wheat germ oil.

The yellow oil remaining after the removal of the sterols is particularly important because of its richness in Vitamin E. Evans and Burr[6] and Olcott and Mattill[7] have reported on certain physical and chemical characteristics of the oil in connection with their biological studies but no compounds were isolated from this fraction. Martin, Moore, Schmidt and Bowden[8] have noted an association between the Vitamin E activity of this oil and an absorption band at 294 mμ.

Experimental

The fraction of the wheat germ lipids which cannot be saponified with alcoholic potassium hydroxide has been found by various investigators to range from 3.5–4.7%. Values of approximately 4% were found consistently in this investigation. It was observed that the preliminary treatment of the germ with alcohol previous to ether extraction did not increase the amount of unsaponifiable material appreciably over that found by ether extraction alone.

Since it is known that part of the sterols occur in combination with a sugar and possibly also in ester formation with the fatty acids, the percentage of free and combined sterols was measured by the method of Abderhalden.[9]

Free Sterols of the Wheat Germ Lipids.—A sample of 4.9124 g. of the fat was dissolved in 50 cc. of 95% alcohol at 70° and 0.493 g. of digitonin (Merck) in 50 cc. of 90% alcohol was then added in a thin stream to the fat solution, the precipitation being made at 70°. After standing overnight, the precipitate was filtered on a sintered glass crucible (Schott G 3) and washed with 90% alcohol, petroleum ether and ethyl ether until free from fat. After drying at 105°, the digitonin steride weighed 0.3086 g. or the equivalent of 1.61% free sterol.[10] A duplicate determination conducted on 1.8885 g. of fat yielded 0.1157 g. of digitonin steride or 1.57% free sterol. The average of the two determinations was 1.59%.

The determination of the total sterol in the unsaponifiable fraction was conducted with 0.1500 g. of the unsaponifiable matter (recovered from the same fat on which the determination of free sterol was made). This was dissolved in 15 cc. of 95% aldehyde free alcohol. At 70°, 0.5 g. of digitonin in 50 cc. of 90% alcohol was added slowly. The solution stood for forty-eight hours. It was filtered through a sintered glass crucible, washed with 90% ethanol and then ether and dried at 105°. The precipitate weighed 0.4142 g. or 70.77%. On the basis of the

(1) Condensed from one section of a thesis presented by B. Sullivan to the Faculty of the Graduate School of the University of Minnesota in partial fulfilment for the degree of Doctor of Philosophy, June, 1935. Paper No. 1396, Journal Series, Minnesota Agricultural Experiment Station.

(2) This work was completed before the appearance of the paper by Drummond, Singer and MacWalter, *Biochem. J.*, **29**, 456 (1935).

(3) Anderson, Shriner and Burr, THIS JOURNAL, **48**, 2987 (1926).

(4) Sandquist and Bengstsson, *Ber.*, **64**, 2167 (1931).

(5) Windaus, Werder and Gschaider, *ibid.*, **65**, 1006 (1932).

(6) Evans and Burr, "Memoirs," University of California, University of California Press, Berkeley, California, 1927.

(7) Olcott and Mattill, *J. Biol. Chem.*, **104**, 423 (1934).

(8) Martin, Moore, Schmidt and Bowden, *Nature*, **134**, 214 (1934).

(9) E. Abderhalden, "Handbuch der biologischen Arbeitsmethoden, Fette," Urban and Schwarzenberg, Berlin, 1925, pp. 490–491.

(10) The factor 0.2563 used in all these calculations is based on the new formula for sitosterol, $C_{29}H_{50}O$.