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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[CONTRIBUTION FROM THE RUSSELL-MILLER MILLING COMPANY AND THE UNIVERSITY OF MINNESOTA]

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, $C_{29}H_{50}O$.

fat containing 4% unsaponifiable material, the amount of combined sterols was calculated as follows: (4.00 × 0.7077) − 1.59 = 1.24%. In summary the results were

	%
Unsaponifiable matter	4.00
Sterols in unsaponifiable matter	70.77
Free sterols in wheat germ lipids	1.59
Combined sterols in wheat germ lipids	1.24

About 56.2% of the sterols of germ occur in the free state and about 43.8% in some combination. Phytosterolin was isolated from wheat germ by Nakamura and Ichiba.[11] On hydrolysis the ester gave a sterol and glucose. The exact nature of the sterol was not investigated since it was thought to be the definite homogeneous phytosterol. No other esters of sterols have been reported as found in the wheat embryo.

Measurement of the Unsaturation of the Sterol Fraction and of the Total Unsaponifiable Fraction.—Many investigators continue to report iodine values on sterol fractions and use these as indices of unsaturation. While it is common knowledge that certain methods for determining iodine values of sterols (for example, the Wijs method) give high results, one frequently sees statements in the literature that methods such as the Hanus or the Rosenmund–Kuhnhenn give theoretical values when properly conducted.[12] *A priori*, the addition of halogens to compounds having an aromatic ring would be thought to give rise to substitution as well as additional reactions and hence should be unreliable. Such was found to be the case in measuring the iodine as well as the thiocyanogen values of the unsaponifiable fraction of wheat germ. Irregular results were obtained regardless of modifications of the Hanus and Rosenmund methods that were employed. Iodine values on the sterol fraction ranged from 100 to 250 depending on the temperature and reaction time. Another method was tried using a solution of bromine in carbon tetrachloride at 25° for twelve hours. After titration with thiosulfate, the mixture was shaken with water and the water extracted titrated with 0.05 *N* sodium hydroxide using methyl red as the indicator. An appreciable titration was found after correction for the blanks indicating that hydrogen bromide had been liberated by some substitution reaction. The unsaturation of this fraction was measured by per acids which have been shown to be reliable for the sterol group. Benzoperacid, peracetic acid and camphoric acid peracid have been recommended for the measurement of unsaturation. Since camphoric acid peracid has been shown to be more stable at 25° than benzoperacid, the method of Milas and Cliff[13] was chosen. The camphoric acid peracid was prepared according to the directions given by Milas and McAlevy.[14] The sterols used were obtained by recrystallization of the unsaponifiable material three times from ethanol. The mixture had an m. p. of 138° and a rotation of $[\alpha]^{26}D$ −24.5°. A chloroform solution of the peracid (10 cc.) was added to a known weight of the sample (0.1–0.2 g.) and allowed to react for one hour at 26°. Blanks were run using the same amount of camphoric acid per acid. At the end of the reaction period, 10 cc. of 10% potassium iodide and 2 cc. of 0.5% starch solution were added and the solution titrated with 0.09537 *N* sodium thiosulfate. An average of five closely agreeing determinations on the mixed sterols gave 3.91% available oxygen.

G.	$Na_2S_2O_3$ req., cc.	% Available oxygen
0.1089	5.65	3.960
.1753	9.02	3.986
.1003	5.02	3.818
.1271	6.37	3.823
.1255	6.50	3.951
	Average	3.907

The theoretical available oxygen for sitosterol ($C_{29}H_{50}O$) is 3.72%. Since the saturated dihydrositosterol represents an appreciable part of the sterol fraction and would absorb no oxygen, it follows that the value of 3.91% shows the presence of a sterol having a greater degree of unsaturation than those already reported as being present in wheat germ.

The same method was used to measure the unsaturation of the total unsaponifiable fraction. Two determinations gave the following results

	% Available oxygen
0.1709 g. required 13.0 cc. of $Na_2S_2O_3$	= 5.804
0.1522 g. required 11.05 cc. of $Na_2S_2O_3$	= 5.539

An average of 5.671% available oxygen was found for the total unsaponifiable material, showing that the yellow oil likewise contains some unsaturated compounds. Since 70.77% of the total unsaponifiable fraction was found to be sterols, the following calculation was made for the amount of oxygen required by the yellow oil. 5.671 − (3.907 × 0.7077) = 2.91% oxygen required by the non-sterol fraction of the unsaponifiable material.

Bromination of the Acetylated Sterols.—A 15-g. sample of the sterols (m. p. 138°, $[\alpha]^{26}D$ −24.5°) was treated with 150 cc. of acetic anhydride and heated under reflux on the steam-bath for one hour. The acetylated sterols were then recrystallized twice from ethanol. They exhibited a melting point of 124° and a rotation of $[\alpha]^{26}D$ −29.1°. The acetylated sterols were dissolved in 150 cc. of dry ethyl ether and 1.5 cc. of bromine in 100 cc. of glacial acetic acid was added slowly to the solution at 0° according to the procedure of Windaus and Hauth.[15] The solution was allowed to stand at 0° for one hour and then placed in an ice box. Even after several days' standing, no tetrabromide separated, showing the absence of stigmasterol and zymosterol. Neither Windaus and Hauth nor Anderson, Shriner and Burr could find stigmasterol in wheat germ. On the addition of water to the bromination mixture, crystals separated after several days. After several recrystallizations from ethanol and methanol, the crystals were fractionated into two compounds, one of which had the correct positive rotation and a m. p. 139–140°. This proved to be dihydrositosterol acetate.

Anal. Calcd. for $C_{31}H_{52}O_2$: C, 81.50; H, 11.48. Found: C, 81.20; H, 11.51.

(11) Nakamura and Ichiba, *Sci. Papers Inst. Phys. Chem. Research* (Tokyo), **15**, 137 (1931).

(12) Dam, *Biochem. Z.*, **152**, 101 (1924).

(13) Milas and Cliff, THIS JOURNAL, **55**, 352 (1933).

(14) Milas and McAlevy, *ibid.*, **55**, 349 (1933).

(15) Windaus and Hauth, *Ber.*, **39**, 4378 (1906).

The other sterol fraction melted at 100–101° and showed a positive Liebermann–Burchard reaction. It did not contain bromine and has not yet been identified. *Anal.* Found: C, 81.13; H, 11.33. No insoluble bromides separated from the mixture even on addition of water.

Ergosterol.—Brückner[16] has published recently a qualitative test for ergosterol which gave negative results on the mixed sterols from the wheat embryo. No addition product of ergosterol with maleic anhydride was found by heating the mixed sterols (15 g.) with maleic anhydride and xylene at 135° according to the directions of Windaus and Lüttringhaus.[17]

Yellow Oil Fraction

This fraction which comprises about 30% of the unsaponifiable material of the wheat embryo presents great interest since it is a rich source of Vitamin E and its composition is largely unknown. It was found to be practically ash free (0.16%) and to contain only a trace of nitrogen (0.056%). Carbon, hydrogen and a small amount of oxygen (by difference) are found on combustion. The type of compounds most likely to be present would be hydrocarbons (either saturated or unsaturated), higher alcohols or ketones.

The promising method of Chibnall, *et al.*,[18] for the separation of unsaponifiable constituents of waxes was tried using 38 g. of total unsaponifiable material and two successive treatments with phthalic anhydride at 120°. No paraffins or ketones were found on taking up the sodium salts of the secondary phthalates in alcohol. A brown oil settled out from the solution on long standing. On hydrolysis of the solution only sterols were recovered.

Distillation of the Oil.—Since even qualitative tests for functional groups of the yellow oil were always complicated by the presence of the sterols, a more complete separation was effected by distillation *in vacuo*. The sterols were removed as far as possible from a fresh lot of 40 g. of the unsaponifiable fraction by repeated recrystallizations from ethanol until only traces remained. The oil was then distilled *in vacuo* in a Hickman molecular still. A pressure of only a fraction of a millimeter was attained by the use of a Hyvac pump and a liquid air trap. Cooling was achieved by placing dry ice in the indentation of the still. Five fractions were obtained as follows.

Fraction	Temp. range, °C.	Weight, g.	Description
1	55– 70	5.2	Light reddish-brown
2	77– 85	1.7	Bright yellow
3	85–100	1.5	Light brown
4	100–106	2.1	Light brown
5	Material remaining in the still was slightly carbonized at the completion of the distillation and probably consisted of traces of decomposed sterols		

The temperature ranges at which the oil distilled were not sharp and there was some bumping during the distillation. The only fraction which was significantly different from the others in appearance was fraction 2 which was a bright yellow. All the fractions partly solidified on intense cooling (CO_2) but precipitates could not be obtained by this means. From the results obtained on fractional distillation, the yellow oil would appear to be a mixture of several compounds.

Tests for Functional Groups.—The primary purpose of the distillation was the removal of the sterols and the purification of the yellow oil for qualitative tests for functional groups. Certain arbitrarily selected fractions, however, had the same behavior and are probably made up of the same class of compounds. All fractions slowly decolorized a 5% bromine solution in carbon tetrachloride and also decolorized a 2% potassium permanganate solution, showing that all the fractions contain unsaturated compounds. This was further verified by results obtained on titration with per acids.

2,4-Dinitrophenylhydrazine gave no derivative when added to a sample of fraction 1, indicating no aldehydes or ketones.

Fraction 2 was heated in the usual way with semicarbazide hydrochloride and sodium acetate. The original oil was recovered, showing the absence of ketones.

Hydroxylamine hydrochloride and sodium acetate were added to an alcohol solution of a portion of fraction 4 and after heating the solution was diluted to turbidity. No oxime was formed and the original oil was recovered.

A negative test for phenols using ferric chloride was obtained on all fractions.

When concentrated sulfuric acid was added to these fractions, they dissolved and turned reddish-brown.

Sodium hydroxide did not dissolve any of the fractions but produced a bright red color.

No color was developed on heating a sample of fraction 4 in benzene solution with maleic anhydride.

Antimony trichloride in chloroform gave an orange brown color with all fractions.

When portions of fractions 3 and 4 were treated with α-naphthyl isocyanate, a precipitate formed on heating (together with α-dinaphthyl urea) showing the presence of an alcohol. The precipitate was small in amount and there was not enough material for identification.

Judging from these qualitative tests as well as the results of the Chibnall procedure, the following conclusions were drawn in regard to the composition of the yellow oil. It consists of a mixture of several substances. There is no paraffin, no aldehyde nor ketone present in this fraction. The yellow oil probably contains unsaturated hydrocarbons of the polyene type with conjugated double bonds and no functional groups. In addition to xanthophyll, there are probably one or more compounds containing an alcoholic OH group. There are no nitrogen-containing compounds present in this fraction.

Work on the unsaponifiable constituents of the wheat embryo is being continued.

Summary

The unsaponifiable fraction of the wheat embryo amounts to about 4%. Approximately 70%

(16) Brückner, *Biochem. Z.*, **270**, 346 (1934).

(17) Windaus and Lüttringhaus, *Ber.*, **64**, 850 (1931).

(18) Chibnall, Piper, Pollard, Smith and Williams, *Biochem. J.*, **25**, 2094 (1931).

of this fraction consists of a mixture of sterols about 56% of which occur in the free state. Measurements of unsaturation of the sterol fraction by per acids show the presence of an unsaturated sterol with at least two double bonds in addition to the isomeric sitosterols and dihydrositosterol. Determination of unsaturation on sterols by iodine values was found to be unreliable.

The non-sterol fraction of the unsaponifiable material required 2.91% available oxygen. Preliminary work on this fraction indicates the presence of polyene hydrocarbons as well as an alcohol.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF CALIFORNIA]

The Heats of Activation of the Related Reactions Involved when *l*-Bromosuccinic Acid is Treated with Chloride Ion

BY A. R. OLSON AND F. A. LONG

In a previous article[1] we reported on an experimental determination of the mechanism of reactions that involve substitution on a carbon atom. We showed that, for the reactions which we studied, every substitution on the carbon atom was accompanied by a configurational inversion of the molecule. A quantitative proof of this inversion was obtained from the results for the reaction which occurs at 50° between *l*-bromosuccinic acid and chloride ion. However, in order to study the kinetics of this reaction quantitatively it was necessary to study the rates of all the other reactions which can take place in an acidified solution of *l*-bromosuccinic acid and chloride ion. Thus it was necessary to know the rate of decomposition of both *l*-bromo- and *l*-chlorosuccinic acids and the rates of reaction of these two optically active substances with both bromide ions and chloride ions. The previous article contained the results for the rate constants that were obtained for these six reactions at 50°.

The present paper deals with an extension of these studies to other temperatures in order to determine the heats of activation and the collision factors for these related reactions.

Experimental

The preparation of the materials and the methods of investigation were the same as in the previous article. The temperatures are all accurate to within ±0.05°. In obtaining the concentrations at the various temperatures the expansion of the solvent has been taken into account.

For all the rate constants, the unit of time is the minute, but for the collision factors that are given in the various Arrhenius equations, the unit of time is the second.

Experimental Results

1. The Decomposition of *l*-Bromosuccinic Acid, $COOHCH_2CHBrCOOH \xrightarrow{k_2} COOHCH{=}CHCOOH + H^+ + Br^-$.—This reaction was followed by titration of the produced bromide ion with silver nitrate, using chromate indicator. Before titrating the excess acid, the solutions were chilled with ice to minimize the error due to production of further bromide ion through lactone formation, etc. This titration, of course, corresponds to the total amount of fumaric acid and malic acid formed. However, at these high hydrogen-ion concentrations, the amount of malic acid that is produced is small.

Samples for analysis were withdrawn from each run at four different times, the last sample being taken when about four-fifths of the original *l*-bromosuccinic acid had disappeared. Assuming a unimolecular reaction, the result from the bromide ion titration for each sample allows a value for the specific rate constant to be calculated. That the observed rate was indeed unimolecular may be seen from the deviations tabulated in Table I, which lists the results for this reaction at the various temperatures. For all these, the initial concentration of *l*-bromosuccinic acid was 0.180 molar.

The calculated rate constants that are given in the last column of Table I have been obtained from the expression

$$k_1 = 0.755 \times 10^{10}\, e^{-24{,}010/RT} \qquad (1)$$

The agreement between the calculated and observed rate constants indicates that the heat of

(1) Olson and Long, THIS JOURNAL, **56**, 1294 (1934).