TABLE I

Reacting ethylene-diamine	Resulting nicotinamide	Recrystallizing solvent mixture: Alcohol (vol.)	Benzene (vol.)	Yield, %	M. p., °C. (cor.)	Nitrogen, % Calcd.	Found
Acetyl-	N-(2-Acetylaminoethyl)-	1	3	59	170–171	20.3	20.3
Propionyl-	N-(2-Propionylaminoethyl)-	1	3	68	126–127	19.0	19.2
Butyryl-	N-(2-Butyrylaminoethyl)-	1	9	63	157–159	17.9	17.8
Valeryl-	N-(2-Valerylaminoethyl)-	1	20	58	141–142	16.9	16.8
Caproyl-	N-(2-Caproylaminoethyl)-	0	1	47	124–125	16.0	16.0

Pfeiffer of the research laboratories of Parke, Davis and Co., Detroit, Michigan. The authors acknowledge this valuable assistance of Parke, Davis and Co.

All of these nicotinamides are convulsant stimulants. Compared with coramine (N,N-diethylnicotinamide), to which they bear some structural resemblance, they are less toxic but lack potency. The mouse LD-50's are given in Table II. The toxicity increases with the lengthening of the carbon chain of the acyl group but the potency of coramine is not attained.

TABLE II

Empirical formula of nicotinamide	LD-50's of nicotinamide (g./kg.)[a]	Picrate M. p., °C. (cor.)	Hydrochloride M. p., °C. (cor.)	% Cl Calcd.	Found
$C_{10}H_{14}N_2O_2$	4.0	178–180	222–226	14.5	14.5
$C_{11}H_{16}N_2O_2$	4.0	177–179	177–180	13.8	13.7
$C_{12}H_{17}N_2O_2$	3.5	163–166	186–189	13.1	13.1
$C_{13}H_{18}N_2O_2$	1.5	170–172	182–186	12.4	12.1
$C_{14}H_{21}N_2O_2$	1.0	168–170	174–177	11.8	11.7

[a] Coramine (N,N-diethylnicotinamide), 250 mg./kg.

The acetyl derivative and the propionyl derivative are not analgesic in subcutaneous doses as high as 600 mg./kg., which is the effective oral dosage of aspirin. The acetyl derivative and the caproyl derivative are inactive as analeptics against barbiturate depression at a dosage level of 150 mg./kg., whereas coramine shortens the sleeping time by thirty minutes. The lack of analeptic power is also evident by the short convulsant time before death occurs. On the contrary, the prelethal period of convulsions is prolonged when coramine is administered.

Summary

A synthesis of N-(2-acylaminoethyl)-nicotinamides has been developed and properties of members of the lower aliphatic series have been investigated.

The compounds are convulsant stimulants. They are less toxic than coramine but lack potency.

TALLAHASSEE, FLA. RECEIVED OCTOBER 13, 1942

[CONTRIBUTION FROM THE DEPARTMENTS OF CHEMISTRY AND OF PHYSIOLOGICAL CHEMISTRY, THE OHIO STATE UNIVERSITY]

Application of the Mercaptalation Assay to Synthetic Starch[1]

BY M. L. WOLFROM, C. S. SMITH AND A. E. BROWN

The role of the Cori ester in carbohydrate metabolism has been made clear by the reversible, enzymic transformation: glycogen + free phosphate ⇄ Cori ester. Using a phosphorylase preparation isolated from heart and liver tissue, Cori[2] and others[3] have succeeded in preparing a polysaccharide similar to glycogen. In the vegetable world, Hanes[4] has demonstrated that a phosphorylase, capable of converting starch to the Cori ester and of resynthesizing the latter to a polysaccharide resembling starch by reversal of the enzymic action, is present in pea and potato extracts. It is therefore definitely established that phosphorylases acting *in vitro* on the Cori ester, which has been shown to be α-*d*-glucopyranose 1-phosphate,[5] are able to synthesize polysaccharides resembling, or identical with, the natural products.

Since the discovery of synthetic starch there have been attempts to correlate this material with natural starch. Hanes[4] showed that syn-

(1) Presented before the Division of Sugar Chemistry and Technology at the 103rd meeting of the American Chemical Society, Memphis, Tennessee, April 23, 1942.

(2) Gerty T. Cori, C. F. Cori and G. Schmidt, *J. Biol. Chem.*, **129**, 629 (1939).

(3) A. Schäffner and H. Specht, *Naturwissenschaften*, **26**, 494 (1938); W. Kiessling, *Biochem. Z.*, **302**, 50 (1939); P. Ostern, D. Herbert and E. Holmes, *Biochem. J.*, **33**, 1858 (1939).

(4) C. S. Hanes, (a) *Proc. Roy. Soc.* (London), **B128**, 421 (1940); (b) **B129**, 174 (1940).

(5) (a) M. L. Wolfrom and D. E. Pletcher, THIS JOURNAL, **63**, 1050 (1941); (b) M. L. Wolfrom, C. S. Smith, D. E. Pletcher and A. E. Brown, *ibid.*, **64**, 23 (1942).

thetic starch is very similar to natural starch in certain properties, and Astbury, Bell and Hanes[6] reported that native potato starch and the synthetic polysaccharide give the same general type of X-ray pattern. However, Hanes does point out that synthetic starch is less soluble in water, is granular, and rapidly retrogrades from solution and that the color with iodine is a more brilliant and intense blue than that given by natural starch. Synthetic starch is also quantitatively hydrolyzed to maltose by β-amylase whereas natural starch is only 60% hydrolyzed. Hanes therefore claims that his material is very similar in behavior to the amylo-amylose fraction of natural starch. Bear and Cori[7] state that the X-ray pattern of their polysaccharide synthesized from the Cori ester by muscle phosphorylase is similar to that of natural starch.

When Haworth and co-workers[8] applied the tetramethylglucopyranose end-group assay method[9] to the starch synthesized *in vitro* by the agency of potato phosphorylase, they obtained a small amount (1.5%) of tetramethylglucose, corresponding to a minimum chain length of 80–90 anhydroglucose units. A like amount (1.5%) of dimethylglucose was isolated. On the other hand, Hassid and McCready[10] found no tetramethylglucose and thus suggested the presence of continuous loops or of chains too long to allow the isolation of determinable quantities of tetramethylglucose.

For natural starch, it is claimed[11] that the main portion consists of repeating units of 24–30 glucose residues joined together by 1,4 α-linkages in each repeating unit. These in turn are assumed to be joined together to high molecular weight aggregates by true glucosidic linkage between the reducing end of the repeating unit and the sixth carbon atom of a glucose residue in the chain. Evidence exists that there may be present in natural starches a less-branched portion, sometimes termed the amylo-amylose fraction.

The immediate objective of the work herein reported was to apply the mercaptalation molecular size assay[12] to the starch synthesized *in vitro* by the action of potato phosphorylase upon the Cori ester. This assay is independent of branching, or closed rings; it merely requires that the bonds hydrolyze into two entities with no significant differences in rate. If such differences are present and are large, the kinetic assay then follows the slowest reaction and an extrapolation of the data to zero time will indicate the average molecular size of the fragments present at the beginning of the slowest reaction. The mercaptalation assay is thus independent of branching, whereas the tetramethylglucopyranose end-group assay measures only the length of straight chain molecules or of the straight chain portions of branched molecules. The mercaptalation procedure is limited by extrapolation difficulties to degrees of polymerization under *ca.* 500 anhydroglucose units. The validity of the kinetic data obtained is tested by the straightness of the line function plotted (Fig. 2).

Synthetic starch was prepared from α-*d*-glucopyranose 1-phosphate by the action of potato phosphorylase. At no time during its preparation was it subjected to acid or alkaline conditions and there was thus no possibility of degradation. The purified material showed no detectable Fehling reduction, gave a blue-black color with iodine, and gave a clear solution in both acid and alkali. In connection with this preparation work, it was noted that the unnatural or β-form of *d*-glucopyranose 1-phosphate[5b] did not undergo polysaccharide formation with the enzyme preparation used, but that hydrolysis of the phosphate group occurred with the liberation of reducing sugar (Table II).

For the mercaptalation work the synthetic starch was hydrolyzed at 0° and the degree of hydrolysis at various time intervals was determined by mercaptalation, as previously described[13] for natural potato starch. The course of the hydrolysis of the synthetic starch was followed polarimetrically in the same concentration of hydrochloric acid, and the data are diagrammed in Fig. 1. A smooth continuous curve was obtained, and on extrapolation of the curve to zero time (time of the addition of the acid to the synthetic starch), an initial specific rotation of approximately +205° was obtained for the

(6) W. T. Astbury, F. O. Bell and C. S. Hanes, *Nature*, **146**, 558 (1940).

(7) R. S. Bear and C. F. Cori, *J. Biol. Chem.*, **140**, 111 (1941).

(8) W. N. Haworth, R. L. Heath and S. Peat, *J. Chem. Soc.*, 55 (1942).

(9) W. N. Haworth and H. Machemer, *ibid.*, 2270 (1932).

(10) W. Z. Hassid and R. M. McCready, THIS JOURNAL, **63**, 2171 (1941).

(11) C. C. Barker, E. L. Hirst and G. T. Young, *Nature*, **147**, 296 (1941); K. Freudenberg and H. Boppel, *Ber.*, **73B**, 609 (1940); K. Freudenberg and G. Hüll, *ibid.*, **74B**, 237 (1941).

(12) M. L. Wolfrom, J. C. Sowden and E. N. Lassettre, THIS JOURNAL, **61**, 1072 (1939).

(13) M. L. Wolfrom, D. R. Myers and E. N. Lassettre, *ibid.*, **61**, 2172 (1939).

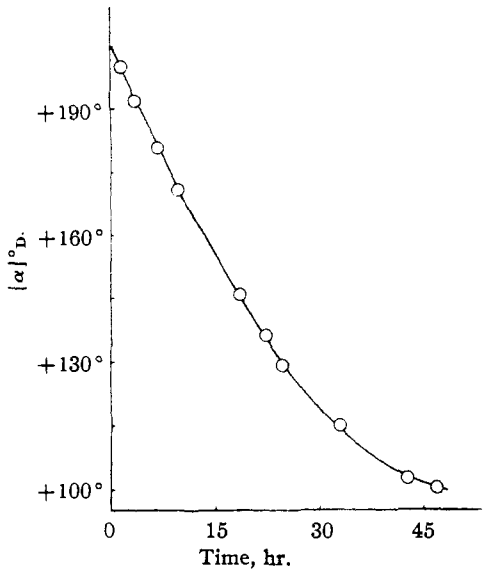

Fig. 1.—Mutarotation of synthetic starch (*c*, 4.04 g. per 100 cc. soln.) in hydrochloric acid (37.3 g. HCl per 100 cc. soln.) at 0°.

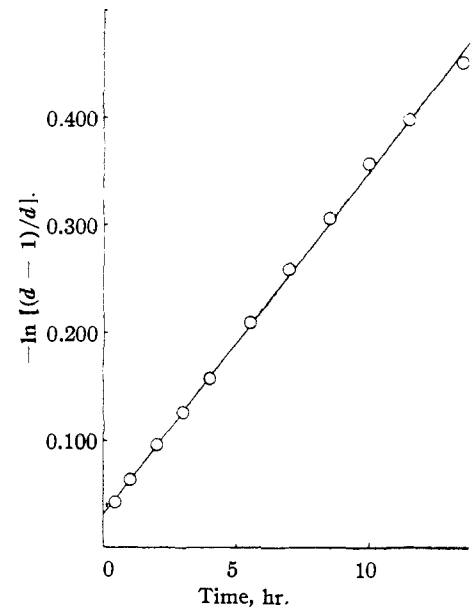

Fig. 2.—Extrapolation of data for initial D.P. of synthetic starch.

original material in the given concentration of hydrochloric acid. The shape of this curve, and the extrapolated value, were almost identical with those previously found for natural potato starch in this Laboratory.

Pertinent data of the mercaptalation assay are tabulated in Table I. These data were then subjected to a graphic analysis as previously described.[12] Figure 2 represents a straight line plot of the function $-\ln [(d-1)/d]$ against time, wherein d (total number of glucose units ÷ number of polymers) is the average degree of polymerization at time t. The intercept gives the initial degree of polymerization and the slope is $-k$, where k is the specific rate constant for the rate of change in the degree of polymerization with time. The value of the specific rate constant, k (hours^{-1}), at 0° was found to be 3.2×10^{-2}, in good agreement with the value 2.7×10^{-2} for natural potato starch. The average degree of polymerization, d_0 for the original synthetic starch is 32 ± 1, the uncertainty being estimated graphically. This value is slightly higher than the value 20 ± 4 found for a commerical potato starch in this Laboratory[13] by the same method and is in agreement with the value of 24–30 glucose units found on employment of the end-group assay by Hirst and Young[14] for the repeating unit of a whole series of natural starches from different sources. It is not in agreement with the value of *ca.* 150 found[15] by the mercaptalation method for a high viscosity methylated starch prepared in low yield from potato starch by the method of Hess and Lung.[16] Here a particular long-chain fraction of potato starch may have been isolated or methylation may have stabilized an otherwise more easily hydrolyzable bond occurring once in periods of 25–30 glucose units apart.

From these data it would appear that synthetic starch has the same type of bond as the repeating units in natural starch. We may eliminate the long chain structure for synthetic starch, except for the possibility that the 32 glucose unit may still be joined to other such units by linkages more easily hydrolyzed than the 1,4-linkage in the glucose chain. The possibility of a closed loop of *ca.* 32 α-*d*-glucopyranose units in 1,4-linkage is not eliminated. Such a loop or helical structure is not unreasonable when viewed in the light of the helical structure for starch elaborated by Freudenberg[17] on the basis of the original postulate of Hanes.[18]

Experimental

Preparation and Characterization of Synthetic Starch.—Synthetic starch was prepared from pure Cori ester (spec. rot. +78.5°, D-line, water) and purified potato phosphoryl-

(14) E. L. Hirst and G. T. Young, *J. Chem. Soc.*, 951 (1939).

(15) M. L. Wolfrom and D. R. Myers, THIS JOURNAL, **63**, 1336 (1941).

(16) K. Hess and K. H. Lung, *Ber.*, **71B**, 815 (1938).

(17) K. Freudenberg, H. Boppel and Margot Meyer-Delius, *Naturwissenschaften*, **26**, 123 (1938).

(18) C. S. Hanes, *New Phytologist*, **36**, 101, 189 (1937).

ase according to the method of Hanes[4b] with slight modifications in the purification. The crude polysaccharide obtained after removing iodine completely from the starch–iodine complex was purified by dissolving the starch in hot neutral calcium chloride solution (sp. gr. 1.32), the insoluble fraction that remained behind after five extractions being discarded. The starch was then precipitated from the combined extracts with 70% ethanol, suspended in warm water, and reprecipitated. After washing with ethanol (70, 90%, and absolute) the product was dried. The calcium chloride purification was then repeated to eliminate further protein impurities. This differs from the procedure of Hanes[4b] in that a strong alkaline solution was used in his purification. We thus had a product which throughout its preparation was never exposed to acidity or alkalinity, but was always kept near the *p*H 7 range when in solution. The polysaccharide gave no detectable Fehling reduction and gave a blue-black color with iodine. It was very slightly soluble in water (retrograded) and gave a clear solution in dilute alkali. The specific rotation in *N* sodium hydroxide was +165° (D-line, *c*, 0.2, 25°).

Anal. Calcd. for $C_6H_{10}O_5$: C, 44.5; H, 6.23. Found[19]: C, 44.53; H, 6.66; N, 0.02; P, 0.02; ash, 1.0.

Action of Potato Phosphorylase on β-*d*-Glucopyranose 1-Phosphate.—A solution of the dipotassium salt of β-*d*-glucopyranose 1-phosphate (regenerated from the crystalline dibrucine salt[5b]) was treated with potato phosphorylase. The data of Table II were obtained from such a digest. Simultaneous experiments using the dipotassium salt of the Cori ester (regenerated from the crystalline dibrucine salt) and a trace of natural starch in place of the β-form gave good synthetic starch formation. Hydrolysis of the labile β-form was found to be negligible at *p*H 6.0 in the absence of the enzyme. At 100 minutes, 1 cc. of the digest was removed and treated for 30 minutes at 60° with *N* hydrochloric acid (total volume, 3 cc.). This treatment hydrolyzed the labile β-form completely to glucose and inorganic phosphate. Another 1 cc. of the digest removed at the same time was hydrolyzed in *N* hydrochloric acid (total volume, 3 cc.) for two and one-half hours at 100°. The reduction values were the same for both samples, showing that no maltosidic linkages were present. No iodine colorations were observed at any time during the course of the reaction. The addition of starch to the digest had no effect.

Hydrolysis and Mercaptalation.—The synthetic starch (25.8 g., moisture-free basis) was placed in a 2-liter, 3-necked flask at 0°, equipped for rapid mechanical stirring and for rapid removal of sample through a delivery tube, and was treated with water (51 cc.) previously undercooled to 0°. The starch was suspended by rapid stirring and then fuming hydrochloric acid (584 cc., d^0_4 1.214), previously cooled to 0°, was added. As soon as solution (4.0 g. of starch, moisture-free, per 100 cc. of solution) at 0° was complete, well-purified ethyl mercaptan (100 g.), previously cooled to 0°, was added, and the stirring was continued at 0°. Samples of 50 cc. (first four samples were 60 cc. to get more product due to low sulfur content) were withdrawn after the time intervals recorded in Table I, and immediately poured, with stirring, into mixtures of sodium bicarbonate (54 g.) and water (185 cc.). The neutralization mixtures were then concentrated to dryness under reduced pressure at 37–40°, and the resulting mixture of salt and hydrolyzed starch mercaptals was dried in a vacuum oven at 40°.

Acetylation of Mercaptalated Products.—The mixtures of salt and mercaptalated products were soaked in dry pyridine (40 cc.) for twelve hours. Acetic anhydride (20 cc.) was then added to each sample and the acetylation was allowed to continue at room temperature with occasional shaking for five days. The resulting pyridine solutions were poured into ice and water (1000 cc.) and the insoluble acetates extracted with chloroform. The chloroform extracts were washed successively with hydrochloric acid (5%), saturated sodium bicarbonate, and water. Concentration of the dried extracts under reduced pressure at 37–40° produced the acetates, in all cases save the last two samples (sirups), as amorphous solids. Several of these products were analyzed for acetyl content by the Malm and Clarke[20] procedure. The results are tabulated in Table I.

Optical Rotation of Synthetic Starch in Hydrochloric Acid at 0°.—The polarimeter sample was maintained at 0° and its optical rotation observed at various time intervals. The polarimetric data are plotted in Fig. 1 and values corresponding to the times of sample removal as obtained from the graph are recorded in Table I.

Isolation of *d*-Glucose Diethyl Mercaptal.—The polarimeter sample after reaching constant rotation (spec. rot. +100.9°) was mercaptalated by shaking for fifteen minutes with an excess of ethyl mercaptan. The acid was neutralized by pouring into a suitable mixture of sodium bicarbonate and water, the final solution being alkaline to congo red. Evaporation to dryness at 37–40° under diminished pressure was followed by drying for twenty-four hours under reduced pressure at 40°. The glucose diethyl mercaptal was extracted with acetone in a Soxhlet apparatus, the acetone was removed, and the residue dis-

TABLE I

Degree of polymerization of synthetic starch (*c*, 4.04) after hydrolysis with hydrochloric acid (37.3 g. HCl per 100 cc. soln.) at 0° for various time intervals.

Time of hydrolysis, hr.[a]	$[\alpha]^{0}D$	Acetylated, mercaptalated product from 2.0 g. starch, wt., g.	S, %	D. P.[d] by S content	Acetyl content (cc. 0.1 *N* NaOH per 100 mg.) Calcd.	Found
0.0	+205°[b]	...	..	..	...	...
.42	204	1.3[c]	0.90	24.0	...	...
1.00	202	2.4	1.30	16.4	...	...
2.00	197	3.5	1.89	11.1	...	...
3.00	193	3.9	2.40	8.6	10.35	10.90
4.00	189	3.2	2.91	6.9	...	...
5.50	185	3.1	3.68	5.3	10.31	10.24
7.00	178	3.2	4.33	4.4	...	...
8.50	174	3.3	4.93	3.8	10.28	10.68
10.00	170	3.7	5.47	3.4	...	...
11.50	165	3.3	5.91	3.1	...	...
13.50	161	3.5	6.35	2.8	10.24	10.29

[a] Initial time taken as time of addition of acid to the starch. [b] By extrapolation. [c] For the first four samples, *i. e.*, through the three-hr. sample, the weight is the product from 2.4 g. of starch. [d] Average degree of polymerization in glucose units, assuming complete acetylation.

(19) Recorded on an ash-free and moisture-free basis. Moisture on air-dried sample was 9.2%.

(20) C. J. Malm and H. T. Clarke, THIS JOURNAL, **51**, 274 (1929).

solved in water, decolorized and recrystallized; m. p. 127°, mixed m. p. with authentic sample, no depression.

Sulfur Analyses of Acetylated, Mercaptalated Products.—The sulfur analyses were performed by the Parr bomb method, employing a total sample of approximately 1 g., on a moisture-free basis, in the manner previously described.[13]

In Table I are recorded the sulfur analytical data and the corresponding average degrees of polymerization calculated from them. The degree of polymerization (D. P.) may be calculated from the sulfur content by the equation

$$\text{D. P.} = 2 + \left(\frac{6412}{\%\text{S} \times \text{C}_{12}\text{H}_{16}\text{O}_8}\right) - 2\left(\frac{\text{C}_{14}\text{H}_{19}\text{O}_9 + \text{C}_2\text{H}_5\text{S}}{\text{C}_{12}\text{H}_{16}\text{O}_8}\right)$$

$$\text{or D. P.} = \frac{22.25}{\%\text{S}} - 0.72$$

where $C_{14}H_{19}O_9$ is the molecular weight of the end structural units and $C_{12}H_{16}O_8$ is the molecular weight of the intervening units.

TABLE II

HYDROLYSIS OF β-*d*-GLUCOPYRANOSE 1-PHOSPHATE WITH POTATO PHOSPHORYLASE

Enzymic digest contained 28.5 mg. of the dipotassium salt of β-*d*-glucopyranose 1-phosphate prepared from the crystalline dibrucine salt,[5b] 3 cc. of enzyme, and 2 cc. of 0.5 *M* citrate buffer of *p*H 6.0; total volume, 10 cc.

Time, min.	0	30	60	100
Free P, mg. per 100 cc. of digest	0.3	1.2	1.4	1.8
Free + ester P[a]	2.6	2.6	2.6	2.6

[a] Ester P is the phosphorus liberated as phosphate after three minutes of hydrolysis with *N* perchloric acid at 100°. All phosphorus determinations were made by the method of Allen.[21]

The assistance in the laboratory of Mr. Irving Auerbach is acknowledged.

(21) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).

Summary

1. Synthetic starch prepared by the action of potato phosphorylase on the Cori ester *in vitro* has been hydrolyzed with a solution of concentrated hydrochloric acid at 0° in the presence of an excess of ethyl mercaptan. The resulting mercaptalated mixtures of hydrolyzed products were isolated as their acetates at various time intervals.

2. Sulfur analytical data indicated that the average degree of polymerization of the mercaptalated products varied from 24 glucose units after 0.4 hour to 2.8 glucose units after thirteen hours.

3. The course of the hydrolytic reaction (without mercaptalation) at 0° was followed by optical rotation measurements, and was found to be similar to that of natural potato starch.

4. A graphic analysis of the data yielded a value of 3.2×10^{-2} for the specific rate constant (hours^{-1}) of the rate of change of the degree of polymerization in concentrated hydrochloric acid at 0°, a value similar to the *k* for natural potato starch.

5. By graphic analysis the value 32 ± 1 glucose units was obtained for the initial average degree of polymerization of the synthetic potato starch.

6. β-*d*-Glucopyranose 1-phosphate did not undergo polysaccharide formation with potato phosphorylase.

COLUMBUS, OHIO RECEIVED AUGUST 5, 1942

[CONTRIBUTION FROM THE LABORATORY OF PHYSIOLOGICAL CHEMISTRY, THE OHIO STATE UNIVERSITY]

Studies on the Chemistry of the Fatty Acids. XI. The Isolation of Linoleic Acid from Vegetable Oils by Low Temperature Crystallization

BY JEROME S. FRANKEL, WESLEY STONEBURNER AND J. B. BROWN

In 1941 we reported the isolation of pure linoleic acid from corn oil by a low temperature crystallization procedure.[1] This was the first time this acid had been prepared pure, as evaluated from the iodine number, by a method other than the classical bromination–debromination technique of Rollett.[2] In the present work we have applied the crystallization method to four additional oils of high linoleic acid content: sesame, cottonseed, grapeseed and poppyseed, and in addition to olive oil which contains comparatively small amounts of linoleic acid. Preparations of linoleic acid, 97–100% pure, were obtained from the first four oils mentioned. Purities, calculated from the iodine numbers as binary mixtures of oleic and linoleic acids, were compared to those calculated from the tetrabromide numbers, based on the factor 102.9 for pure linoleic acid.[3] The latter values were 2–6% lower. These differences may be due to the presence of small amounts of octadecadienoic acids which do not yield petroleum ether insoluble tetrabromides. We feel, however, that with the present evidence, this state-

(1) Frankel and Brown, THIS JOURNAL, **63**, 1483 (1941).

(2) Rollett, *Z. physiol. Chem.*, **62**, 410 (1909).

(3) Matthews, Brode and Brown, THIS JOURNAL, **63**, 1064 (1941).