(Fig. 2) were constructed. Herein each unit of the coordinates is equal to 1 g.; for the curves A, B, and C it is however, as mentioned above, 0.1 g.

Summary

The presence of oleic acid in mixtures containing palmitic and stearic acids has the effect, within certain limits, of lowering the melting point without the irregularities observed in those cases where the former is absent. Experiments have been made which show that this behavior of oleic acid has a counterpart, insofar as solubilities are concerned, in its influence on the solubility of the same ternary system in methanol. Thus the quantities of the solid fatty acids dissolved are, within commercially important ranges, proportional to the amount of oleic acid present in the mixture.

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A Procedure for the Determination of Total Unsaturation in the Products of *in Vitro* Oxidation of Fatty Acids in Biological Systems

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NUMEROUS halogen absorption methods are available for measuring the unsaturation of fats and fatty acids and the majority of customary methods are satisfactory when the fat is available in an anhydrous state. The estimation of the absolute amount of unsaturation produced in the course of the biological oxidation of fatty acids *in vitro* in the presence of mold suspensions presents a problem of considerable difficulty. The prevalent methods for estimating unsaturation of fatty substances, viz., those of Wijs, Hanus, and Hubl, that have been used by previous investigators in the field of fat metabolism entail the laborious process of extraction of the fatty acids from the reaction mixture by the ether-alcohol mixture, evaporation of the solvent, and the determination of the iodine absorption of the residual fat, which must be present in an absolute anhydrous state. The difficulty experienced in complete extraction of the fatty matter and the consequent inaccuracy of the results obtained has been mentioned again and again, and so far absolutely no attempt has been made to devise a method for the determination of unsaturation produced in biological systems without involving the extraction methods. The interest in evolving a suitable method for the estimation of the unsaturation of the reaction fluid obtained in the biochemical oxidation of the fatty acids in *in vitro* experiments lies in the fact that the absolute quantities of the fatty acids employed in such experiments are exceedingly small to effect an efficient extraction by the prevalent technique with ether-alcohol mixture and that for easy manipulation such estimation of unsaturation should only be made in aqueous solutions of salts of the fatty acids. Goswami and Basu (1) employed hypochlorous acid reagent for determining the unsaturation of oils and fats, and their method consisted in first preparing the soap and then conducting the absorption of the hypochlorous acid for effecting the saturation of the double bond.

The method originally employed by them has however been modified greatly by the present author, and an extensive investigation regarding the concentration of the acid, the relative amounts of reagents, and the time of reaction has resulted in a more simplified method.

Experimental

The acid is weighed directly into a 250-ml. reagent bottle, provided with a ground glass stopper, and treated with 5 ml. of glacial acetic acid (A. R.); 10 ml. of sodium hypochlorite solution (approximately 0.14 N) is added by a pipette and the bottle stoppered immediately. The stopper is sealed by moistening it with potassium iodide solution. A definite reaction time is allowed for the absorption of HOCl, after which 10 ml. of a 15-p.c. solution of potassium iodide is added and the flask is kept in the dark for 5 minutes. Distilled water (100 ml.) is added and the liberated iodine titrated with 0.1 N thiosulfate. The amount of HOCl absorbed is determined by carrying out a blank experiment. The results can be expressed in terms of iodine equivalents of the HOCl absorbed by the fatty acid, and the absolute amount of acid can be calculated directly from the volume of thiosulfate consumed.

A study of the relative effect of time, reaction temperature, sample size, and excess of reagents, factors which are of primary importance in the addition of halogen to double bonds, has been made, using pure oleic acid (I.V—89.86) as a substrate. Table I indicates that halogen absorption is almost complete within 5-10 minutes at 37°C. when 0.1-0.12 gm. of the sample are used.

The amount of reagent used in excess varied from 210 to 236%. As a safe limit a 15-minute reaction

TABLE I

Effect of Time and Temperature on the Determination of Unsaturation of Oleic Acid by Use of Hypochlorous Acid Reagent

Temp. (°C.)	5 min.	10 min.	15 min.	30 min.	60 min.	120 min.	24 hr.
5±1	89.40	89.60	89.60	89.64	89.80	89.81	90.0
16±1	89.43	89.65	89.80	89.86	89.90	89.90	89.96
37±1	89.72	89.87	89.87	89.89	89.90	89.90	89.94

TABLE II

Effect of Excess Reagent and Sample Size on Determination of Iodine Number by Hypochlorous Acid Method

Amount of acid taken	15-minute reaction period at 37°C.	
	Percent excess reagent	Iodine No.
0.0832	176	89.18
0.0893	291	89.87
0.0882	368	89.90
0.1415	182	89.12
0.1515	296	89.85
0.1476	377	89.86
0.1973	152	88.82
0.1920	273	89.78
0.2315	351	89.83

period can be recommended for the absorption of hypochlorous acid. From the data of Table II it appears that when about 300% excess reagent and a 15-minute time period is used, this procedure gives reproducible results for the iodine number. The sample size is relatively unimportant provided a large excess reagent is used. The following table (Table III) records the results of the estimation of oleic, linoleic, crotonic, and ricinoleic acids by use of this procedure. The acids were employed in the form of their sodium salts, an approximately N/10 solution being prepared in each case and different volumes pipetted in order to vary the weight of the acid taken. The solution was acidified with glacial acetic acid and treated with 0.14 N sodium hypochlorite solution as before. Duplicate experiments were performed with the same volume of soap solution and the mean values tabulated.

TABLE III

Hypochlorous Acid Method for Estimation of Fatty Acid Weights by Iodine Equivalents

Acid added		Acid calculated from titrations (mg.)
Name	Amount (mg.)	
Oleic	90.0	90.5
	180.0	180.0
	225.0	226.2
	450.0	450.0
Linoleic	102.5	102.8
	205.0	203.0
	401.0	401.0
	256.3	256.2
Crotonic	141.6	141.7
	140.2	139.5
	2.80	2.79
Ricinoleic	151.0	150.2
	302.0	301.7
	226.5	226.5

The results of Table III clearly show the accuracy of the method in estimating the concentration of a known fatty acid in terms of iodine equivalents. The following table records the results of estimation of crotonic acid on a semi-micro scale by use of the hypochlorous acid method.

The ease with which HOCl adds at the double bond quantitatively, as evidenced by experimental results in Tables III and IV, places in the hands of future investigators in the field of fat metabolism a very useful tool for exploring such reaction products.

Consideration should be given to the interference of other intermediaries of fatty acid oxidation with the HOCl reaction, viz., ketone bodies and beta-hydroxy acids. Experiments conducted with crotonic acid in the presence of different amounts of acetone and beta-hydroxybutyric acid lead one to the conclusion that acetone must be removed from the reaction mixture before HOCl estimations may be taken to give an accurate measure of the quantity of unsaturation. The presence of beta-hydroxybutyric acid has no interfering influence on the reaction. This is further substantiated by experiments with 12-hydroxy oleic acid (ricinoleic acid) when the amount of HOCl absorbed gives a true measure of the double bonds. The hydroxyl group is left unattacked (Table III).

The results of the experiments with ricinoleic acid conclusively prove that with HOCl reagent no substitution reactions take place.

For the estimation of unsaturation in the product of biological oxidation of fatty acids, it is only necessary to precipitate the proteinacious substances with trichloracetic acid, remove the ketone bodies, if any, and then directly apply the hypochlorous acid procedure for estimation of the unsaturation.

TABLE IV

Semi-Micro Estimation of Crotonic Acid by HOCl Method

Acid added		Acid calculated from titrations (mg.)
Name	Amount (mg.)	
Crotonic	0.1290	0.1284
	0.0258	0.0256
	0.0129	0.0130
	0.00645	0.0063
	0.3096	0.3096
	0.0172	0.0172
	0.02150	0.02152

Recommended Procedure. The reaction fluid (5 ml.), of which the unsaturation is to be measured, is treated with trichloracetic acid (1 ml. of 100% solution) to precipitate the protein matter and centrifuged for 5 minutes, and the clear solution made up to volume in a 10-ml. graduated cylinder with one or two washings. An aliquot part is used for the estimation of unsaturation. A 2-ml. aliquot of the solution is pipetted into a 150-ml. Erlenmeyer flask provided with ground glass stopper and acidified with 5 ml. of glacial acetic acid (A. R.) and the solution gently heated, neutralized with Na_2CO_3, and evaporated nearly to dryness on a water bath to ensure complete removal of acetone. The contents are dissolved in 5 ml. of distilled water, made strongly acid with 5 ml. of glacial acetic acid (A. R.), and 2.0 ml. of standard 0.1 N HOCl solution added and the reaction carried out as before. The liberated iodine is titrated against N/500 thiosulfate. The amount of HOCl absorbed can be obtained by running a blank experiment with 2.0 ml. of the HOCl reagent. The result can be expressed as percentage total unsaturation in terms of iodine equivalents of the HOCl absorbed.

Summary

1. The unsaturation produced during the biological oxidation of fatty acids *in vitro* can be determined by use of a hypochlorous acid reagent, which readily adds at the double bond of the system as follows:

$$\begin{array}{l} -CH \\ \quad \| \\ -CH \end{array} + HOCl \longrightarrow \begin{array}{l} -CH-OH \\ \quad | \\ -CH-Cl \end{array}$$

2. The addition is quantitative and complete within 10 minutes. The use of 15-minute reaction time at 37°C. is recommended.

3. The method can be employed for the semi-micro estimation of unsaturated acids.

4. There is no chance of substitution and hence prolonged reaction times are without any influence.

5. The results are affected by the presence of ketone bodies, but beta-hydroxy acids cause no interference with the estimation.

The method has been successfully extended for the estimation of total unsaturation of oils and fats, which will form the subject matter of a future communication.

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A Comparison of Several Methods for Determination of Non-Oil Constituents of Raw Linseed Oil

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DURING the year of 1950 a total of 1 billion, 87 million pounds of linseed oil was produced (1). A large portion of this oil was sold and consumed as raw linseed oil. In establishing quality, a grading factor such as refining loss is not applicable. Raw linseed oil must be evaluated by tests showing its quality as a raw oil.

Both the American Society for Testing Materials and the Federal Government have set up similar specifications for the evaluation of raw linseed oil. With one exception the tests such as specific gravity, acid value, iodine value, saponification value, etc., are common to the grading of all vegetable oils. The exception is the "Foots" test. This test, the Walker-Wertz Method of Foots Determination, has been in use for approximately 22 years, and during this period the only modification has been the inclusion of a heated oil and chilled oil section, a change made within five years of the initial adoption of the test.

TABLE I

A.S.T.M. and Federal Specifications for Raw Linseed Oil

Test	Specification
Specific Gravity at 15.5/15.5°C	0.931 to 0.936
Acid Value, Maximum	4.0
Saponification Value	189.0 to 195.0
Unsaponifiable, Maximum, %	1.50
Iodine Value, Wijs, Minimum	177.0
Loss on Heating at 105 to 110°C., Maximum, %	0.3
Appearance	Clear and transparent at 65°C.
Color	Not darker than a freshly prepared solution of 1.0 g. of $K_2Cr_2O_7$ in 100 ml. of pure H_2SO_4 (Sp. Gr. 1.84)
Foots, Maximum, %	
Heated Oil	1.0
Chilled Oil	4.0

In 1923 and 1924 the ASTM Subcommittee V of D-1 spent considerable time on collaborative work on the Foots Determination (4, 5). Nine samples of oil from various sources and of different ages were examined by seven laboratories during each year. Various modifications were considered, including preheating of the oil in a water bath at 65.5°F. for one hour or pre-cooling of the oil in an ice bath for 12 hours before carrying out the Foots Determination. A centrifuge modification was also considered.

After collecting all of the data and examining it very critically, the subcommittee reached this conclusion in 1923: "There is but one conclusion that can be drawn from the data . . . and that is that in all modifications and upon all types of oil the (Foots) test is very inaccurate. The average error varies from 55% to 130% when the results of the determinations by the several modifications of the methods are considered . . . therefore the subcommittee concludes that the ASTM and the Sutherland modifications (centrifuge procedure) of the ASTM Foots test are both incapable of yielding accurate results in the hands of skilled operators. Consequently it is recommended that this test not be advanced to standard at this time." And in 1924 the subcommittee came to this conclusion: "The variables in the method seem to be too great to allow for satisfactory results. The figures speak for themselves. The subcommittee, therefore, must reluctantly reaffirm the position taken in its last year's report, namely, that neither this test, nor any modification of it proposed to date, is sufficiently accurate for use in the standard specifications."

However, in spite of the above conclusion, the Foots test was made a part of the tentative specifications for linseed oil in 1926 and shortly thereafter became a standard specification, which has remained essentially unchanged to this date.

Original Foots Test. American Society Materials, D51-18T (6)

I. Percentage of Foots

1. The amount of Foots in properly clarified pure raw linseed oil from North American seed, as determined by the test specified below, shall not exceed 2% by volume.

II. Method of Determination

2. The following reagents are required:

a. Acetone that will pass United States Pharmacopoeia specifications.