# Biological Utilization of Fatty Acid Isomers

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FAT is more than a concentrated source of calories in the diet. It makes other important nutritional contributions, either directly as a vehicle for essential nutrients or as a sparer of other essential dietary components. It has also been amply demonstrated that animal and vegetable fats are equally effective in meeting nutritional requirements and that these two types of fat have the same ranges of digestibility. These facts have been discussed with supporting literature citations in a recent review paper (1). The results of experimental studies leading to the conclusions drawn have been summarized in a more extensive review article (2).

In all the studies covered by the review articles cited (1, 2), vegetable fats were compared with animal fats in nutritional studies using such indices as growth, work capacity, reproduction, lactation, and survival in rating performance. Some of the vegetable fats were hydrogenated under selective conditions, as for margarine manufacture, and under non-selective conditions, as for shortening manufacture. The primary difference between the two methods of hydrogenation is the preferential conversion under selective conditions of linoleic acid to oleic and iso-oleic acids rather than oleic to stearic acid (3). The conditions of hydrogenation employed in making fats for use in margarine or shortening manufacture find no counterpart in the animal organism. The related animal fat products, butter or lard, are naturally of a lower degree of unsaturation than the basic vegetable oils prior to hydrogenation. That fatty acid isomers are produced in the hydrogenation of vegetable oils is a well-known fact. It is conceivable that the effects of these isomers, as minor components of the hydrogenated fats, might have escaped detection when the fats as a whole were evaluated in the prior nutritional studies.

The present report presents the results of studies designed to determine more critically than hitherto attempted whether or not the fatty acid isomers, resulting from the hydrogenation of vegetable oils, differ from the naturally-occurring fatty acids in nutritional value.

### Fatty Acid Isomers Produced by Hydrogenation

The vegetable oils produced in the United States, prior to hydrogenation for margarine or shortening manufacture, are essentially mixed triglycerides of stearic, palmitic, oleic, linoleic, and linolenic acids in varying proportions. For the purpose of the present study, concerned with the biological utilization of the fatty acids, the $C_{16}$ saturated fatty acid (palmitic) and the $C_{18}$ saturated fatty acid (stearic) may be regarded as equivalent. No isomers of the saturated fatty acid obtain in the commercial hydrogenation of vegetable oils. Thus, to all intent and purpose, we are concerned with the fate of only three unsaturated fatty acids in commercial hydrogenations. These are oleic acid (9:10-octadecenoic acid), linoleic acid (9:10, 12:13-octadecadienoic acid), and linolenic acid (9:10, 12:13, 15:16-octadecatrienoic acid).

The naturally-occurring unsaturated fatty acids exist primarily in the *cis*-form. The juxtaposition of two double bonds with a single methylene ($CH_2$) group intervening, as in linoleic and linolenic acids, confers much greater reactivity that the presence of two more widely separated bonds. The reactivity ratios for oleic, linoleic, and linolenic acids for hydrogenation have been shown to be very similar to the corresponding ratios for oxidation (4). In each case reactivity is greatly increased, 8 to 30 times, when the second double bond is separated from the first by an active methylene group, and the latter is doubled when a third double bond is associated with two such groups (4). Linoleic and linolenic acids are essential fatty acids for the animal organism, *i.e.*, they cannot be synthesized *in vivo* but must be furnished preformed as a component of the ration (1, 2).

In the hydrogenation of vegetable oils there occurs not only a step-wise conversion of the more highly unsaturated fatty acids to the saturated fatty acid, but in the course of this addition of hydrogen at the double bonds there are formed in appreciable concentrations fatty acid isomers with physical and chemical properties different from the naturally-occurring unsaturated fatty acid. Conditions of hydrogenation will determine in large measure the relative quantities of the fatty acid isomers in a fat. Under selective conditions of hydrogenation (involving higher temperatures, reduced hydrogen pressure, minimal agitation, and increased catalyst concentration) there occurs preferential hydrogenation of the fatty acids containing active methylene groups (linoleic) in preference to acids devoid of such groups (oleic). Selectivity is never absolute but is relative. It is only possible to slow down the rate of hydrogenation of oleic acid in relation to the hydrogenation of linoleic acid. In commercial practices the ratio of reaction rates (oleic:linoleic acid) may be decreased from 1:4 in very non-selective hydrogenations to about 1:50 in very selective hydrogenations (3). It should therefore follow that in selective hydrogenations, with greater opportunities provided for the preferential hydrogenation of the polyunsaturated fatty acids, there occurs to a greater degree the development of fatty acid isomers.

In Table I are presented illustrative findings by a number of investigators on the development of isomeric unsaturated fatty acids during the hydrogenation of vegetable oils. These isomers originate from a) the addition of hydrogen at a double bond which is not normally saturated by natural processes, b) migration of double bonds with or without the addition of hydrogen to certain double bonds, and c) conversion of natural *cis*- to *trans*-forms.

In the hydrogenation of linolenic acid the hydrogen added to the middle double bond may amount to as much as 65% of the total (9). The resulting 9:10, 15:16-octadecadienoic acid is more desirable than natural linoleic acid in a fat designed to exhibit oxidative

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stability. There is no active methylene group in the linoleic acid isomer, with the result that this fatty acid resembles more closely oleic acid in resistance to oxidation. In soybean oil selectively hydrogenated to an iodine value of 70 to 100, the iso-linoleic acid content varies from 2.5 to 3.5% (3).

Migration of double bonds is not regarded as a major factor in the production of fatty acid isomers as a result of hydrogenation although it is a factor which operates to some degree (4). The end-products of such a change are not static since the double bond can migrate further in step-wise fashion. At least six different *trans* iso-oleic acids have been shown to occur in partially hydrogenated cottonseed or soybean oils (14). Of course, these acids may have originated from the hydrogenation of iso-linoleic acids as well as from oleic acid itself.

Conjugation of double bonds in the fatty acids is favored by the higher temperatures of hydrogenation. Indeed it occurs in the presence of a hydrogenation catalyst induced by heat alone (7, 16). The conjugated double bonds are very highly reactive toward oxygen; thus elaeostearic acid (9:10, 11:12, 13:14-octadecatrienoic acid) is more susceptible to oxidation than linolenic acid. The same is true for ease of hydrogenation. The conjugated linolenic acid reacts almost spontaneously with hydrogen at a rate about 20 times that of normal linolenic acid (17). Thus, in oils hydrogenated for margarine or shortening manufacture, fatty acids with conjugated double bonds are formed initially but are then hydrogenated further so that the end-products of the hydrogenation are free of such isomers. In lightly hydrogenating soybean oil, *viz.*, to an iodine value of 115, to obtain a superior mayonnaise oil following winterization, appreciable quantities of fatty acids with conjugated double bonds (up to 5%) may be obtained under selective conditions of hydrogenation (18); under non-selective conditions of hydrogenation oils free of conjugated fatty acids are obtainable (19). Fatty acids with conjugated double bonds (conjugated dienes) have been shown to occur in all food fats in amounts of from 0.1 to 1.0%. The latter value was obtained in the analysis of butter fat (20, 21).

In *cis-trans* isomerization induced to a greater degree by employing selective conditions of hydrogenation, substantial concentrations of the *trans*-isomers remain in the end-product. In oils such as cottonseed, peanut, or soybean, hydrogenated with normal catalysts, the *trans* iso-oleic acid content may be as low as 4-5% or as high as 40%, depending upon conditions of hydrogenation (4, 14, 22). The *trans* iso-acids are more resistant to oxidation than the natural *cis*-acids. An oil high in *trans* iso-oleic acid content contains less of the readily oxidizable natural linoleic acid. The *trans* iso-oleic acids are of higher melting point than the *cis*-oleic acids (see Tables II and III) but about 20 to 25°C. less than that of stearic acid (M.P. of 69.6°C.). These three factors have led to the universal adoption of selective conditions in hydrogenating oils for margarine manufacture. The resulting fat is resistant to oxidative deterioration, "stands up" well on the table at room temperature, and melts readily in the mouth. Under non-selective conditions of hydrogenation, such as used in shortening manufacture, fats of an equivalent iodine value are more susceptible to oxidation, are more plastic at lower temperatures, but tend to be salvy in the mouth. The increased concentration of stearic rather than iso-oleic acid interferes with mouthing properties.

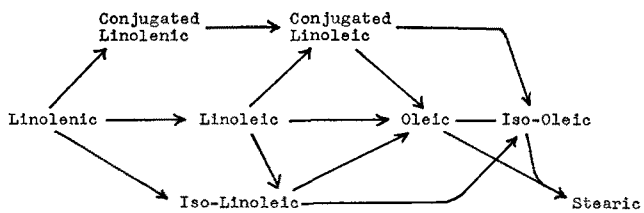


FIG. 1. Fatty acids formed during hydrogenation of vegetable oils.

In Figure 1 is shown diagramatically the reactions in which the unsaturated fatty acids in an oil participate during hydrogenation. Many of the fatty acid isomers are intermediates in the development of other isomers and of the more-saturated natural fatty acids. It is apparent that at a given time a highly complex mixture of fatty acids are present in a hydrogenated oil. Only the starting oils and the completely hydrogenated fat comprise relatively simple mixtures of the fatty acids.

## Biological Activity of Iso-oleic Acids

Oleic acid is not an essential fatty acid for the animal organism. However it is required as a growth factor by a number of microorganisms. Other microorganisms, such as *Lactobacillus arabinosus,* are able to utilize oleic acid in place of biotin (a member of the vitamin B complex) in the medium. Recent publications in this field (23, 24) adequately review this subject.

In the past 15 years there has developed an extensive literature on the use of microorganisms for

TABLE I

Illustrative Findings on Isomeric Unsaturated Fatty Acids Formed During Hydrogenation of Fats

| Cause | Changes | References |
|---|---|---|
| Addition of hydrogen at a double bond which is not normally saturated by natural processes | 9:10, 12:13, 15:16 linolenic → 9:10, 15:16 iso-linoleic | 5-10 |
| | 9:10, 12:13 linoleic → 12:13 iso-oleic | 5 |
| Migration of double bonds with or without addition of hydrogen | 9:10, 12:13, 15:16 linolenic → 10:11, 12:13, 14:15 conjugated linolenic | 11 |
| | 9:10, 12:13, 15:16 linolenic → 8:9, 14:15 iso-linoleic | 10 |
| | 9:10, 12:13, 15:16 linolenic → 10:11, 14:15 iso-linoleic | 10 |
| | 9:10, 12:13, 15:16 linolenic → 10:11, 12:13 conjugated linoleic | 11 |
| | 9:10, 12:13, 15:16 linolenic → 12:13, 14:15 conjugated linoleic | 11 |
| | 9:10, 12:13 linoleic → 8:9 and 10:11 and 11:12 iso-oleic acids | 12, 13 |
| | 9:10 oleic → 8:9 and 10:11 and 11:12 iso-oleic acids | 12, 13 |
| Conversion of *cis*- to *trans*-forms | 9:10 *cis*-oleic → 9:10 *trans*-oleic (elaidic) | 14 |
| | 9:10 *cis*-oleic → 10:11 *trans*-oleic | 14, 15 |

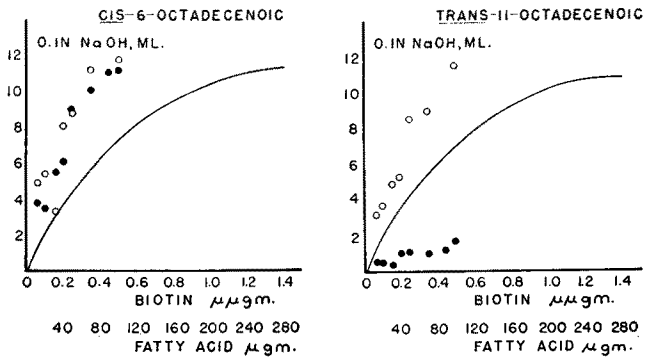


FIG. 2. Biotin-like activity for *cis*-6:7-(left) and *trans*-11:12-octadecenoic acids (right). The solid curve represents the response to graded doses of biotin when added to the medium and incubated with *L. arabinosus* for 48 hours. ●, response to graded doses of fatty acids without biotin. ○, response to graded doses of fatty acids incubated simultaneously with graded doses of biotin at a constant ratio; however the values plotted are for the fatty acids after subtraction of the activity contributed by the biotin moiety as determined from the biotin curve. [From Journal of Biological Chemistry (23).]

revealing the presence of new growth factors in foods, for the assay of these factors, and for determining the metabolic reactions in which these factors participate. The results of these studies have been followed by animal studies with astonishing agreement in results. It is true that certain microorganisms exhibit requirements for factors not essential to the animal organism. Since in such cases the factors are normal components of animal tissues, it has been concluded that the animal organism is able to synthesize these factors whereas they must be furnished preformed to the microorganism.

The microorganisms have proved to be especially useful in determining metabolite-antimetabolite relationships. Structural analogues of vitamins and the metabolites have been shown repeatedly to inhibit the growth of microorganisms requiring these factors. These inhibitory effects can be negated by adding the normal metabolites in adequate concentration. An excess of an antimetabolite can be added to negate the effects of the metabolite. In fact, the growth of the microorganism can be employed as a means for titrating metabolite *vs.* antimetabolite. Confirmation of these findings in animal studies has been repeatedly obtained.

With the above as background, investigations were conducted to determine the relative biotin-like activity of the positional and stereoisomers of oleic acid as demonstrated by the effect on acid production from glucose by *Lactobacillus arabinosus;* acid production is a sequence to microbiological growth. The details of the microbiological assay procedure have been presented elsewhere (23).

Most of the *cis*- and *trans*-octadecenoic acids used in this study were prepared by W. Frederick Huber of the Procter and Gamble Company. All the *cis*-acids were synthetic, except for *cis*-6:7 and *cis*-9:10-octadecenoic acids, while all the *trans*-acids were prepared from the corresponding *cis*-compounds by selenium elaidinization. The method of synthesis and the physical properties of these acids have been reported by Huber (25). The sample of *cis*-9:10-octadecenoic acid (oleic) was obtained from the Hormel Foundation.

Typical variations in response by *Lactobacillus arabinosus* to *cis*- and *trans*-octadecenoic acids in the medium, with and without added biotin, are graphically illustrated in Figure 2. In Table II are listed

TABLE II

Biotin-Like Effect of *Cis*-Octadecenoic Acids Alone and With Biotin; *L. Arabinosus* Assay

| Fatty Acid | Average M. P. | Biotin Equivalent of Fatty Acid | |
|---|---|---|---|
| | | Fatty Acid Alone | In Presence of Biotin[a] |
| | °C. | *mμgm. per mg.* | *mμgm. per mg.* |
| *cis*-6:7 octadecenoic | 29.8 | 11.6 | 12.4 |
| *cis*-8:9 octadecenoic | 23.3 | 14.7 | 13.4 |
| *cis*-9:10 octadecenoic (oleic) | 13.0 | 10.3 | 10.2 |
| *cis*-11:12 octadecenoic | 13.5 | 12.0 | 11.4 |
| *cis*-12:13 octadecenoic | 27.2 | 10.8 | 13.4 |

[a] 5.0 mμgm. of biotin per 1.00 mg. of fatty acid.

the biotin-like activities of various *cis*-acids tested alone and with supplementary biotin; corresponding values for the *trans*-acids are given in Table III. Repetitive assays were conducted on each acid, with only the average figure reported.

TABLE III

Biotin-Like Effect of *Trans*-Octadecenoic Acids Alone and With Biotin; *L. Arabinosus* Assay

| Fatty Acid | Average M.P. | Biotin Equivalent of Fatty Acid | |
|---|---|---|---|
| | | Fatty Acid Alone | In Presence of Biotin[a] |
| | °C. | *mμgm. per mg.* | *mμgm. per mg.* |
| *trans*-6:7 octadecenoic | 53.0 | 0.4 | 3.7 |
| *trans*-7:8 octadecenoic | 44.0 | 2.7[b] | 15.2 |
| *trans*-8:9 octadecenoic | 51.9 | 2.8[b] | 9.1 |
| *trans*-9:10 octadecenoic (elaidic) | 43.5 | 10.6 | 10.1 |
| *trans* 10:11-octadecenoic | 52.3 | 6.1[b] | 11.0 |
| *trans*-11:12 octadecenoic (vaccenic) | 41.5 | 2.1 | 7.3 |
| *trans*-12:13 octadecenoic | 52.5 | 2.7[b] | 15.2 |
| 17-octadecenoic | 55.8 | 0.0 | 1.8 |

[a] 5.0 mμgm. of biotin per 1.00 mg. of fatty acid.
[b] "Toxic" effect on microorganism noted; the response, calculated to a common dilution, decreased uniformly from the lowest to highest concentrations.

No great differences in biotin-like activity were noted for any of the *cis*-octadecenoic acids tested although the *cis*-8:9 acid possessed a statistically higher activity than any of the other acids. The responses following the administration of biotin with the fatty acids were additive; that is, the effect of the biotin plus the fatty acid, at the various levels fed, was equivalent to the sum of the activities of the two supplements when administered separately. Some synergistic stimulation was observed with the *cis*-12:13-octadecenoic acid and biotin supplement.

The *trans*-octadecenoic acids reacted quite differently. The *trans*-9:10-octadecenoic acid (elaidic) possessed the greatest biotin-like effect, equal to that of normal oleic acid. From position 9, at which the unsaturation is moving on either side toward the end carbon atoms, the biotin-like potency of the *trans*-acids decreased in step-like fashion until no or very little measurable activity remained. *Trans*-7:8-, -8:9-, -10:11-, and -12:13-octadecenoic acids showed progressively increasing "toxic" effects for the microorganism as the dosages increased. Responses calculated to a common dilution decreased uniformly from lowest to highest concentrations of these *trans*-acids.

TABLE IV

Biotin-Like Activity of Fatty Acids Obtained from Natural and Hydrogenated Oils; *L. Arabinosus* Assay

| Fatty Acids from | Iodine Value | Spectrophotometric Analysis | | | Biotin Equivalent | |
|---|---|---|---|---|---|---|
| | | Linoleic | Oleic | Saturated | Anticipated[a] | Found |
| | | *per cent* | *per cent* | *per cent* | *mμgm. per mg.* | *mμgm. per mg.* |
| Coconut oil, hydrogenated | 0.3 | 0 | 0 | 100 | 0.0 | 0.0 |
| Coconut oil | 9.3 | 2 | 6 | 92 | 0.7 | 2.0 |
| Cottonseed oil, winterized | 115.4 | 52 | 22 | 26 | 5.0 | 9.2 |
| Olive oil | 85.6 | 7 | 81 | 12 | 8.7 | 12.0 |
| Soybean oil | 138.5 | 62[b] | 25 | 13 | 5.5 | 7.7 |
| Butter oil | 40.6 | 3 | 37 | 59 | 4.0 | 9.1 |
| Margarine oil | 76.4 | 5 | 74 | 21 | 7.8 | 11.8 |
| Shortening I[c] | 62.8 | 7 | 55 | 38 | 6.1 | 8.2 |
| Shortening II[d] | 61.2 | 2 | 66 | 32 | 6.9 | 10.6 |

[a] See text.
[b] Including 7% of linolenic acid.
[c] Cottonseed oil shortening hydrogenated under non-selective conditions; m.p. = 42.8°C.
[d] Same cottonseed oil as in Shortening 1 but hydrogenated under selective conditions; m.p. =39.6°C.

When biotin was given with these acids, the inhibitory effects of higher concentrations of the acids were not apparent.

The administration of biotin concurrently with the *trans*-octadecenoic acids (except elaidic acid) and with 17-octadecenoic acid revealed a considerable degree of synergism. Maximum synergistic effects were noted with the *trans*-7:8 and *trans*-12:13 acids, both of which had exhibited, in the absence of biotin, inhibition of microbiological growth with increasing dosage. In fact, the most pronounced synergism between the *trans*-acids and biotin occurred with those acids which are most "toxic" when employed in the absence of biotin. In Figure 3 are plotted the results obtained with the *trans*-acids.

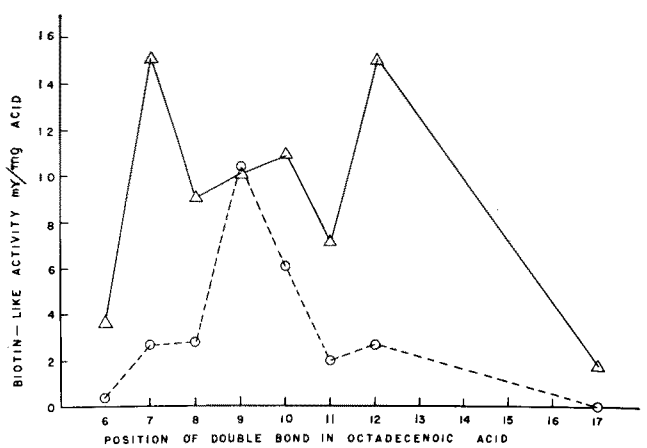


FIG. 3. Biotin-like activity of *trans*-octadecenoic acids as affected by the position of the double bond ○---○, and the activity exhibited by the fatty acids alone following biotin supplementation, 5.0 mμgm. per 1.00 mg. of fatty acid △———△.

A study was next conducted of the biotin-like activities of the fatty acids obtained from natural and hydrogenated oils. These were subjected to spectrophotometric assay (26) for the determination of fatty acid composition. The assay involves alkali isomerization to convert the non-conjugated fatty acids to their light-absorbing conjugated forms. Calculations of the oleic acid (including the iso-acids) and saturated acid contents were made (27). Based upon a 10.3:5.3:2.6 ratio for the biotin-like activity of oleic *vs.* linoleic *vs.* linolenic acids (23) and the assumption that the iso-oleic acids formed during hydrogengenation are as active as natural oleic, calculations were made of the anticipated biotin-like activities of the mixed fatty acids derived from the natural and hydrogenated oils. The results of this study are summarized in Table IV.

In every case the values found for biotin-like activity were definitely greater than those anticipated from the fatty acid composition. The percentage deviation of the found from the anticipated values in general increased as the mixed fatty acids contained more of the saturated fatty acids. Calculations indicated that on the average, for each per cent of saturated fatty acid, the biotin-like activity of the mixed fatty acids increased by 2.3% over that anticipated from the type and amounts of unsaturated fatty acids present. This finding of an augmentation of the microbiological response by saturated fatty acids, which in themselves are inactive (*viz.*, in assays of saturated coconut oil), is confirmatory of the findings by others (28). The results of the present study have permitted calculation of the biotin-like activity of the fatty acids of limpid and hydrogenated oils from their fatty acid composition according to the following formula.

Biotin equivalent, as millimicrograms of biotin per mg. of fatty acids equals

$$\frac{(\%\ \text{oleic} \times 10.3) + (\%\ \text{linoleic} \times 5.3)}{100} \times \left(1.00 + 2.3\,\frac{(\%\ \text{saturated fatty acid})}{100}\right)$$

The agreement between values thus calculated and those found was surprisingly good (23), considering the precision of the methods employed in determining the fatty acid composition of the samples and the accuracy of the microbiological assay for estimating biotin-like activity.

The results of the present study indicate that, in in the absence of biotin, the *trans* iso-oleic acids, with the exception of elaidic acid, are not efficiently utilized by the microorganism. The addition of biotin to the medium is very effective in allowing proper utilization of these iso-acids. The *cis* iso-acids are equally effective as oleic acid in promoting growth and acid production by the microorganism in the presence or absence of biotin. Biotin might well be required for the isomerization of the *trans*-acids or in the enhancement of the absorption of the acid by the organism.

The need for biotin in studies with animals was first demonstrated by feeding large amounts of raw egg. This effect was shown to be due to a heat-sensitive protein, avidin, in raw egg white which combines

with biotin so firmly as to make it unavailable for absorption. Biotin is normally synthesized in the digestive tract of both animal and man in more than ample quantities for satisfying nutritional needs. The inclusion in the diet of various sulfa drugs in quantity can inhibit microbiological synthesis of biotin in the intestine. Thus under only most unusual conditions can a need for biotin be demonstrated (29). For this reason biotin is not regarded as one of the B-vitamins essential for man.

Even if biotin were an essential vitamin, the studies conducted on the natural and hydrogenated fats have shown that the iso-oleic acids obtained during hydrogenation are utilized by the microorganism as effectively as natural oleic acid. Moreover the iso-acids formed during hydrogenation exhibited no antimetabolite activity toward the natural oleic acid remaining, nor did the mixed fatty acids derived from the hydrogenated fats show any tendency to inhibit growth of the microorganisms at any of the dosage levels employed.

## Biological Activity of Fatty Acids with Conjugated Double Bonds

The rapid conversion of the conjugated triene in tung oil, elaeostearic acid, to conjugated diene in the organs of the rat, followed by a gradual decline in the amount of both acids, has been reported (30). Conjugated linoleic acid taken orally by the hen is deposited in the egg yolk lipids as such or in a partially oxidized form (31). The conjugated isomer of linolenic acid, eleostearic acid, also appears in the egg yolk lipids along with large quantities of newly synthesized conjugated dienoic acid (31). An increase in the concentration of tetraenoic, pentaenoic, and hexaenoic acids in the egg yolk lipids has also been reported (32). In these studies on the metabolism of eleostearic acid, tung oil was fed the animals, the oil containing from 75 to 95% of the conjugated trienoic acid. Two per cent tung oil in the ration of the hen produced no obvious adverse effects over the test period of 14 weeks (32). Elaeostearic acid as the methyl ester was shown to be non-toxic for the mouse in acute and long-term feeding experiments (33). These findings have prompted the use of elaeostearic acid in tracing normal fat absorption in both the rat and the human (33).

In the present investigation studies were conducted to determine through other approaches whether or not fatty acids with conjugated double bonds were nutritionally undesirable. Elaeostearic acid is an isomer of linolenic acid, one of the essential fatty acids. If elaeostearic acid were an antimetabolite for the essential fatty acids, the 11-12 weeks required for depletion of the rats on a fat-free diet should be substantially reduced. The growth of the animals, subsisting on a ration containing both the isomer and the essential fatty acid, should improve as the ratio of the isomer to normal metabolite decreased. Sorbic acid, a $C_6$ fatty acid containing conjugated double bonds (2:3, 4:5-hexadienoic acid), was also included in the present study for evaluation of possible antimetabolite function.

The basal diet employed in the present study is complete in all nutrients with the exception of fat (and the essential fatty acids). Its composition is shown in Table V. The dietary supplements are described in Table VI.

TABLE V

Fat-Free Basal Diet Employed in Producing an Essential Fatty Acid Deficiency in Rats

| Component | Per Cent | Vitamins[a] | Per Kg. |
|---|---|---|---|
| Casein (vitamin-free) | 20 | Thiamine | 36 mg. |
| Cellulose | 4 | Riboflavin | 72 mg. |
| Salt mixture (O-M) | 4 | Pyridoxine | 27 mg. |
| Sucrose | 72 | Ca Pantothenate | 67 mg. |
| | | Menadione | 5 mg. |
| [a]Each rat received in addition per day about: | | Biotin | 1 mg. |
| | | Folic acid | 10 mg. |
| 200 USP units of vitamin A | | Niacin | 60 mg. |
| 20 USP units of vitamin D | | P-amino benzoic acid | 1 gm. |
| 2 mg. of α-tocopherol | | Inositol | 0.5 gm. |
| in propylene glycol solution; dose administered twice each week. | | Choline | 2.0 gm. |
| | | Vitamin $B_{12}$ | 0.01 mg. |

The test animals were male weanling rats. They subsisted on the basal and test rations *ad libitum*; 20 animals on the basal and 10 animals on each of the test rations. The latter included the dietary supplements (listed in Table VI) at three different levels. The addition of the supplements was at the expense of the sucrose component of the ration. The studies with the sorbic acid rations permitted an evaluation of growth responses of the animals ingesting a fat-free diet and containing a single pure fatty acid with conjugated double bonds. The studies with the natural and hydrogenated tung oils permitted an evaluation of the growth responses of the animals ingesting the same basal ration but supplemented with both conjugated and nonconjugated fatty acids in decreasing ratio but with the apparent linoleic acid content more or less constant.

In Table VII are summarized the results of the present investigation. After 11 weeks on the experiment it became obvious that there would be no significant decrease in depletion time with any of the diets. In fact, at 11 weeks, when depletion had occurred in the control group, there were no symptoms indicating that essential fatty acid depletion had occurred in the groups receiving sorbic acid or any of the tung oils. All animals were then transferred to the control diet

TABLE VI

Composition of Dietary Supplements Containing Fatty Acids with Conjugated Double Bonds

| Dietary Supplement | M.P. | Iodine Value | | Triene Conjugated Acids[d] | Diene Conjugated Acids[d] | Linoleic Acid[d] | Ratio of Conjugated to Linoleic |
|---|---|---|---|---|---|---|---|
| | | Theoretical | Wijs | | | | |
| | °C. | | | *per cent* | *per cent* | *per cent* | |
| Tung oil | 4.0 | 250[a] | 161 | 87.8 | 0.0 | 4.9 | 18:1 |
| Tung oil, hydrogenated | 27.8 | 145[b] | 94 | 10.4 | 29.3 | 7.9 | 5:1 |
| Tung oil, hydrogenated further | 31.6 | 87[b] | 83 | 0.8 | 4.7 | 5.2 | 1:1 |
| Sorbic acid[c] | 133.5 | 453 | 209 | 0.0 | 100.0 | 0.0 | ∞ |

[a] Method of Klee and Benham (34).

[b] Calculated from the value for the natural tung oil and the hydrogen pressure drop following hydrogenation (temperature of 185-195°C., pressure of 45 pounds per square inch, nickel catalyst in 0.1% concentration).

[c] $CH_3$—CH=CH—CH=CH—COOH.

[d] Spectrophotometric analysis (35).

TABLE VII

Growth Response of Rats on a Fat-Free Diet to Supplements of Fatty Acids with Conjugated Double Bonds

| Diet | | | Supplement in Diet | Average Body Weight | | | |
|---|---|---|---|---|---|---|---|
| No. | Supplement | Subgroup | | At Start | After 6 wks. | After[a] 11 wks. | After 19 wks. |
| | | | % | gm. | gm. | gm. | gm. |
| 35 | Tung oil | A | 0.6 | 35 | 158 | 212 | 241 |
| | | B | 1.2 | 34 | 165 | 201 | 254 |
| | | C | 6.0 | 36 | 157 | 172[b] | 263 |
| 36 | Tung oil, hydrogenated | A | 0.6 | 35 | 172 | 214 | 260 |
| | | B | 1.2 | 36 | 172 | 226 | 268 |
| | | C | 6.0 | 33 | 171 | 224 | 307 |
| 37 | Tung oil, hydrogenated further | A | 0.6 | 33 | 149 | 197 | 232 |
| | | B | 1.2 | 36 | 171 | 220 | 270 |
| | | C | 6.0 | 35 | 180 | 254 | 292 |
| 38 | Sorbic acid | A | 0.5 | 34 | 146 | 171 | 206 |
| | | B | 1.0 | 34 | 155 | 186 | 221 |
| | | C | 5.0 | 36 | 154 | 183 | 233 |
| 39 | Fat-free | Control | 0.0 | 36 | 154 | 189 | 217 |

[a] After 11 weeks all animals subsisted on the fat-free basal diet alone.

[b] This low value was due to the very low food consumption by the animals during the two weeks just preceding the last week on the 6% tung oil diet; the high level of polyunsaturated fatty acids in this ration was responsible for a rancidity having developed. The freshly prepared ration fed during the eleventh week was readily eaten and growth resumed.

to determine the rapidity with which further depletion would be obtained. In all cases the animals on the tung oil diets continued to grow after this change in regimen. The response of the animals confirmed the spectrophotometric assays (see Table VI), indicating essential fatty acid activity in the tung oil supplements. The highest average weights in all groups were obtained by those animals receiving the 6% fat. The next highest and lowest weight averages were found on the 1.2 and 0.6% levels, respectively, in all cases. The sorbic acid group was not significantly different from the control animals at any of the levels.

With the exception of those animals on Diet 35C, quantity of food consumption was not significantly different between any of the test groups and the control group. During the two weeks just preceding the last week on Diet 35C the animals consumed very little of the ration due to the development of a rancidity. The freshly-prepared ration fed this group during the 11th week was readily eaten, and growth was resumed.

One might argue that the growth performance of the animals on a diet supplemented with the higher levels of tung oil (a fat containing from 5 to 8% of apparent linoleic acid) should have been superior to the performance of the control animals to a greater degree than that noted. If this were true, the growth rates of the animals on the progressively hydrogenated tung oil should also have been improved since ratio of conjugated to linoleic acid content was strikingly reduced with hydrogenation. Such differences in growth performance, particularly during the subsequent feedings on the control diet, were not noted. It must therefore be concluded that the conjugated fatty acids are not antimetabolites for the essential fatty acids. No hydrogenated fat prepared for human consumption would contain the very high ratios of conjugated to linoleic acid noted in the test fats employed in this study. The conjugated fatty acids are preferentially hydrogenated as shown in Table VI. The linoleic acids in the tung oils might very well be in large part non-conjugated iso-linoleic, a form of linoleic acid which has no (or very little) biological activity but which produces a conjugated acid on alkali isomerization.

If it could be demonstrated that a conjugated fatty acid is metabolized in the animal body in the same manner as the normally-occurring saturated fatty acid, then one would have proof of its complete utilization for the production of energy. The best way to approach the solution of this problem is to determine to what extent the conjugated fatty acid in question contributes to the development and excretion of ketone bodies in comparison with the normally-occurring saturated fatty acid. A ketonuria does not occur normally with the rat or the human when adequate carbohydrate is present in the diet. However in the absence of the latter foodstuff all fatty acids of even number carbon atoms are converted to ketone bodies which accumulate and are then excreted as such in the urine. These ketone bodies, acetoacetic acid, $\beta$-hydroxybutyric acid, and acetone, represent intermediates in the oxidation of fatty acids *in vivo* and are completely metabolized to carbon dioxide and water when carbohydrate is simultaneously metabolized.

*In vitro* studies with liver tissue have suggested that the metabolism of sorbic acid and the naturally-occurring hexanoic acid, caproic acid, is qualitatively and quantitatively similar (36). In the present investigation *in vivo* experiments were conducted on fasting rats to evaluate the ketonuria resulting when equimolecular doses of sorbic and caproic acid were administered as their sodium salts without or with added glucose. For a more extended discussion of the rationale of this experiment, experimental details, and results, the reader is referred to the original publication (37); the objectives of that study were different from those presented earlier in this paper. For the purpose of the present report only the pertinent high-lights of the original investigation are presented.

In Tables VIII and IX are summarized the results of the ketonuria studies. The results obtained indicate that the path of metabolism of sorbic acid is identical with that of the corresponding 6-carbon saturated acid, caproic acid. This conclusion is based on the fact that, when corresponding doses of the sodium salts of these acids were fed at two different levels to the fasting rats, the proportion excreted in the urine as ketone bodies was approximately the same, *i.e.*, within experimental error. Limited doses of glucose were effective in suppressing the ketonurias. Practically complete disappearance of ketone bodies from the urine was obtained when 1,000 mg. of glucose was administered to each rat.

These results indicate that the ketonuria observed after sorbate administration is identical with that produced after the administration of the normally-occurring fatty acid. Under conditions of normal alimentation it is evident that the conjugated fatty acid is completely oxidized to carbon dioxide and water, as are the fatty acids normally present in unhydrogenated fats. Under such circumstances the conjugated fatty acid serves as a source of calories.

The findings reported here are pertinent to the human since there are several facts which point to the similarity of ketonuria in man with that in the rat. In the first place, an exogenous source of fatty acids will increase the degree of ketonuria in both species. Secondly, the ketonuria in both the human and the rat is abolished by concomitant ingestion of metabolizable carbohydrate. Thirdly, the same sex

TABLE VIII

Ketone Body Excretion in the Urine of Fasting Female Rats Fed $C_6$ Fatty Acids Without and With Conjugated Double Bonds

| Category | Fasting Rats | | Glucose-Fed Rats | |
|---|---|---|---|---|
| | Caproate | Sorbate | Caproate | Sorbate |
| Ketogenic acid fed: | | | | |
| Calculated as acetone, *mg./100 sq. cm.* | 75 | 75 | 75 | 75 |
| Calculated as acid, *mg./100 sq. cm.* | 150 | 145 | 150 | 145 |
| Average total daily dose fed, *mg.* | 482 | 458 | 474 | 463 |
| Glucose fed, *mg./100 sq. cm.* | 0 | 0 | 75 | 75 |
| Urinary ketone bodies, *mg./100 sq. cm.* | | | | |
| 2nd day | 17.8 | 12.1 | 8.5 | 3.3 |
| 3rd day | 21.9 | 18.8 | 11.6 | 5.0 |
| 4th day | 26.3 | 25.0 | 14.3 | 9.8 |
| 5th day | 29.9 | 23.8 | 16.2 | 6.8 |
| Average, 4-5 days | 28.1 | 24.4 | 15.2 | 8.3 |
| Reduction from control, *mg.* | ..... | ..... | 12.9 | 16.1 |

TABLE IX

Ketone Body Excretion in the Urine of Fasting Female Rats Fed at a Higher Dose Level $C_6$ Fatty Acids Without and With Conjugated Double Bonds

| Category | Fasting Rats | | Glucose-Fed Rats | |
|---|---|---|---|---|
| | Caproate | Sorbate | Caproate | Sorbate |
| Ketogenic acid fed: | | | | |
| Calculated as acetone, *mg./100 sq. cm.* | 150 | 150 | 150 | 150 |
| Calculated as acid, *mg./100 sq. cm.* | 300 | 290 | 300 | 290 |
| Average total daily dose fed, *mg.* | 990 | 983 | 1,030 | 990 |
| Glucose fed: | | | | |
| 2nd-4th days, *mg./100 sq. cm.* | 0 | 0 | 100 | 100 |
| 5th day, *mg.* | 0 | 0 | 1,000 | 1,000 |
| Urinary ketone bodies, *mg./100 sq. cm.* | | | | |
| 2nd day | 34.5 | 34.9 | 14.8 | 9.4 |
| 3rd day | 55.1 | 39.9 | 16.4 | 20.0 |
| 4th day | 67.9 | 58.6 | 22.2 | 27.8 |
| 5th day | 74.5 | 63.0 | 4.5 | 4.9 |
| Average, 2-4 days | 52.5 | 44.5 | 17.8 | 18.7 |
| 4-5 days | 71.2 | 60.8 | ..... | ..... |
| Reduction from control, *mg.* | | | | |
| 2-4 days | ..... | ..... | 34.7 | 25.8 |
| 5th day | ..... | ..... | 70.0 | 58.1 |

difference—a markedly higher level of ketonuria occurring in women during fasting as contrasted with that in men under the same conditions—is exhibited also by the rat. And lastly, the greater ketolytic action of galactose in comparison to that of glucose is noted with both species. Thus it is evident that the identity of the metabolism of the conjugated fatty acid (sorbic acid) and the normally-occurring fatty acid (caproic acid), as demonstrated by the experiments on rats, must also be true for the human.

## Hydrogenated Fats as a Source of Essential Fatty Acids

A biological assay procedure was recently described (38) for the determination of the essential fatty acids in oils and fats. This assay method is based upon the gain in weight of male rats, previously depleted of essential fatty acid stores and now subsisting on the same fat-free diet (see Table V) but supplemented with graded doses of linoleic acid. Body weight increase is a linear function of the logarithm of the dosage with linoleic acid as shown in Figure 4. In order for the test to be valid it is necessary that the substance to be assayed be given at more than one level in the range of critical growth response following dosage with the reference material (linoleic acid).

A number of natural and hydrogenated fats were subjected to biological assay for determination of essential fatty acid content, expressed as linoleic acid. These included a limpid cottonseed oil (non-hydrogenated), two butter oils of different iodine values, four margarine oils (a hydrogenated cottonseed and a hydrogenated soybean oil blend), and two cottonseed oil shortenings of similar iodine value. The latter products were the same used in the microbiological studies of iso-oleic acids (see Table IV); one shortening was hydrogenated under selective, and the other under non-selective conditions. All fats were first subjected to spectrophotometric analyses (26, 27) for fatty acid composition in order to establish proper feeding levels. The butter oils, margarine oils, and shortenings were fed at two levels, namely, 250 and

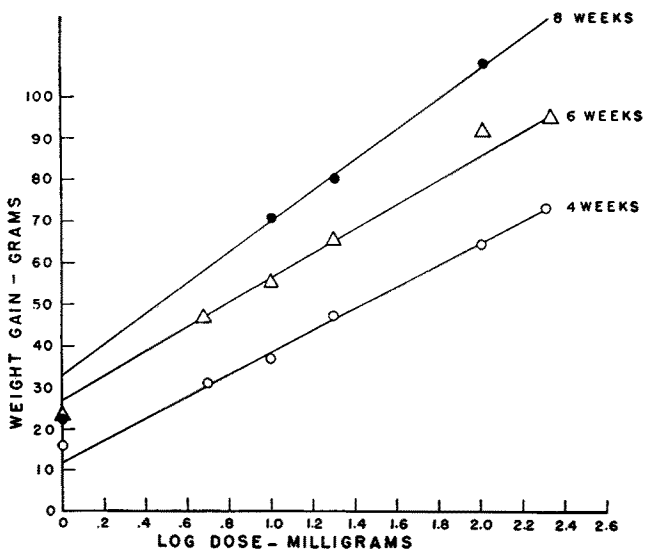


FIG. 4. The average gain in weight of male rats plotted against log dose of the daily linoleate supplement per rat after 4, 6, and 8 weeks. [From Journal of Nutrition (38).]

TABLE X

The Essential Fatty Acid Content of Natural and Hydrogenated Fats

| Sample | Melting Point | Iodine Value (Wijs) | Fatty Acid Composition[a] | | | Essential F. A. Content[b] | |
|---|---|---|---|---|---|---|---|
| | | | Saturated | Oleic | Linoleic | Linoleic Reference | Linoleic Coconut Oil Reference |
| | °C. | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| Cottonseed oil | ..... | 109.0 | 24.7 | 21.3 | 49.5 | 48.0 | ..... |
| Margarine oil | | | | | | | |
| I | 34.2 | 72.8 | 19.5 | 71.2 | 4.8 | 2.4 | 3.0 |
| II | 34.1 | 74.0 | 18.9 | 71.1 | 5.5 | 2.4 | 4.3 |
| III | 33.9 | 71.8 | 20.0 | 71.1 | 4.4 | 3.5 | 5.0 |
| IV | 33.9 | 72.0 | 20.3 | 70.3 | 4.9 | 2.4 | 4.3 |
| Butter Oil | | | | | | | |
| I | 34.2 | 37.5 | 59.0 | 32.2 | 3.1[c] | 1.1 | 2.3 |
| II | 34.4 | 44.6 | 53.0 | 37.5 | 3.8[c] | 1.5 | 3.6 |
| Shortening | | | | | | | |
| Selective | 39.6 | 60.6 | 30.4 | 63.0 | 2.1 | 1.0 | 2.5 |
| Non-selective | 42.8 | 59.4 | 36.3 | 52.4 | 6.8 | 10.2 | 13.0 |

[a] Based upon spectrophotometric analysis (26, 27); calculated to a triglyceride basis.
[b] Based upon the biological assay; expressed as the linoleic acid equivalent.
[c] In addition, 1.0% linolenic and 0.2% arachidonic acids present. These more highly unsaturated fatty acids were absent from the other oils.

500 mg. per rat per day while the cottonseed oil was administered at dosages of 25 and 50 mg. per day. The bioassays were carried out for an 8-week period, following the 12-week period required to deplete the animals. In order to compensate for a potential variable in the assay, two reference curves were obtained: one for the rats receiving linoleic acid in graded doses as the sole supplement to the fat-free diet, and the other for the rats receiving not only linoleic acid in the same graded doses but also completely hydrogenated coconut oil (fat without essential fatty acid activity) at the same two levels corresponding to the dosage levels in the assays of the solid fats.

In Table X are summarized both the results of physico-chemical and biological assays for essential fatty acid content. The results by the latter assays were obtained after interpolating the growth responses of the test animals on each of the two reference curves described above. For a more detailed description of the assay techniques and interpretation of the data obtained, the reader is referred to the original article (38).

Several interesting findings were apparent from the present study. The apparently higher essential fatty acid contents of the fats when read against the linoleic acid-coconut oil reference curve were due to the fact that these reference animals did not grow as well as those receiving only the linoleic acid supplement. The completely hydrogenated coconut oil (no fatty acid isomers present) is either toxic to the rats depleted of essential fatty acids, or the requirement for essential fatty acids for growth is increased with the concomitant ingestion of fat. These two possibilities are under current investigation. In the absence of an unequivocal answer both sets of values have been reported in this paper.

Regardless of which set of bioassay values is accepted, the conclusion remains that the selectively hydrogenated oils for margarine manufacture were somewhat better sources of the essential fatty acids than the natural butter oils.

The most striking effect noted was the marked variation in biological potency between shortenings hydrogenated under selective and under non-selective conditions. The concentration of essential fatty acids in the shortening prepared by non-selective hydrogenation was between 10 and 13% as determined by biological assay while in the shortening selectively hydrogenated to about the same iodine value it was 1 to 2.5%. Spectrophotometric analyses indicated a possible 3 to 1 ratio in essential fatty acid content in favor of the non-selectively hydrogenated shortening. It is apparent that, in preparing the latter shortening, isomers of linoleic acid were formed, which are biologically active but which do not respond to the spectrophotometric method, *i.e.*, double bonds so far removed from each other that conjugation cannot be effected by the alkali treatment. Inasmuch as the base oil used in making the shortening was cottonseed oil, the iso-linoleic acid with biological activity must have been derived from linoleic acid.

The over-all conclusion to be drawn from this phase of the present study is that hydrogenated fats compare favorably with a natural fat of comparable firmness in serving as a source of essential fatty acids. Hydrogenation, of course, lowers markedly the essential fatty acid content of vegetable oils, but, in the course of the hydrogenation, linoleic acid isomers can be formed which exhibit essential fatty acid activity.

## Summary

A review of the literature has shown that in the hydrogenation of vegetable oils, positional and stereoisomers of the unsaturated fatty acids are formed in appreciable quantities and that some isomers are intermediates in the development of others as hydrogenation proceeds. Using a microbiological assay technique, it was demonstrated that the iso-oleic acids formed during hydrogenation are not antimetabolites for natural oleic acid but are utilized as nutrients. Fatty acids with conjugated double bonds are not antimetabolites for the essential fatty acids but are readily metabolized to carbon dioxide and water. Hydrogenated fats compare favorably with a natural fat of comparable firmness in serving as a source of essential fatty acids. In hydrogenating vegetable oils, isomers of linoleic acid can be formed which resist spectrophotometric detection but exhibit essential fatty acid activity.

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# Tall Oil Studies. II. Decolorization of Polyethenoxy Tallates with Ozone and Hydrogen Peroxide

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DURING a study on the production of nonionic detergents in this laboratory by the condensation of ethylene oxide with various grades and samples of tall oil (1), a product of the paper pulp industry, the resultant products were contaminated with a dark brown coloration sometimes exhibiting a greenish fluorescence. Decolorization of these products however could not be effected by the usual means, such as the use of various chemical decolorizing agents or adsorption with activated charcoals or other adsorbents. It was also evident that tall oils of various compositions and origins gave products which varied in the intensity of this contaminant coloration: in general, those tall oils containing a larger proportion of rosin acid developed substantially darker colors and were more difficult to decolorize than those containing a larger proportion of fatty acids. This led us to suspect that the color formation might be caused by oxidation of the rosin acid perhydrophenanthrene ring to unsaturated quinoid phenanthrene derivatives which would be capable of resonance and hence color formation. Since the resonating structures might be decolorized by peroxidation with a reagent such as hydrogen peroxide or by ozonization, both reagents were tried and found successful in removing the color from the polyethenoxy tallates. The details of the decolorization of the tall oil-ethylene oxide nonionic detergents are described herein.

## Experimental

*Preparation of Polyethenoxy Tallates.* A three-necked flask with standard-tapered ground glass joints was fitted with a fritted-glass gas inlet tube, a thermometer, and an outlet tube. The flask was externally heated with a glas-col mantle, and provisions were made for convenient weighing of the flask and its contents during the actual condensation. Various compositions and grades of tall oil (100 g.) (see Table I) were placed in the flask with 0.5 g. of potassium carbonate catalyst and heated to 190°C. with a current of nitrogen gas flowing through the mixture. At 190° the passage of nitrogen was discontinued, and ethylene oxide gas was bubbled through the liquid. The excess was vented through the outlet tube. The temperature generally increased with the heat of the reaction and was maintained between 170-210°C. When a total weight of 200 g. of ethylene oxide had been taken up by the tall oil, the gasification was discontinued, the polyethenoxy tallate was allowed to cool to room temperature, and the dark-colored fluorescent product was decolorized with hydrogen peroxide and ozone as described below:

TABLE I

Preparation of Polyethenoxy Tallates

| Exp. No. | Composition Ratio | | Approx. Molecular Weight | | % Transmission (Red Filter) |
|---|---|---|---|---|---|
| | Rosin Acid | Fatty Acid | Tall Oil | Polyethenoxy Tallate | |
| A | 10 | 90 | 289 | 867 | 48 |
| B | 30 | 70 | 288 | 864 | 47 |
| C | 45 | 55 | 291 | 873 | 38 |
| D | 55 | 45 | 293 | 879 | 24 |
| E | 90 | 10 | 300 | 900 | 4 |

Twenty grams of each sample of polyethenoxy tallate (Table I) were weighed into a Coleman colorimeter test tube, and the intensity of the color was measured on a Coleman electric colorimeter, Model 8, with a red filter No. 8-215, using the null method with the reading for distilled water arbitrarily set at 100% transmission. A transmission value of 90% was considered desirable for adequate decolorization of the products.

*Ozone Generator.* Ozone was generated in the usual manner by passing oxygen through an air space charged with an alternating current potential of 15,000 volts, using as an inner electrode a mercury