mixture of essential amino acids in the proportions described by Steffee *et al.* (9). In this calculation it was assumed that the D enantiomorphs were not available, and thus nitrogen from this source was not included as part of the 160-mg. allowance. Actually the total daily nitrogen consumption increased to 190 mg. From the high weight gain of the animals in this group and in view of the work of Phillips and Berg (10), it is believed that nitrogen from the D isomers probably was converted or in some way utilized for growth by these depleted animals.

In the last three groups in Table IV the D amino acid nitrogen was all included as part of the 160-mg. daily allowance. Weight gains in these groups were relatively high as compared with group 2 fed re-extracted No. 1 meal. However a comparison of groups 46 and 47 indicates that the free gossypol causes a depression of weight gain even in the presence of a combination of amino acids which will support this high rate of gain. If a balance of amino acids is involved in counteracting effects of free gossypol, apparently the proper balance is not attained by this combination of amino acids and cottonseed meal. It appears that further study is needed on possible detoxifying effects resulting from mixed proteins or amino acids and proteins.

It is recognized that the differences in mean weight gain of rat groups may not differentiate between nutritional deficiency and toxicity effects. However comparisons which are permitted by the data offered here seem to allow a distinction between depression of weight gain due to intake of gossypol and that due to amino acid differences.

More work is necessary for better understanding of these apparent effects of total nitrogen intake and of difference in kind of protein on free gossypol toxicity. But the results described in the experiments reported here are significant in any attempt to establish dependable toxic levels of free gossypol for farm animals.

Summary and Conclusions

The rat-repletion method for protein assay has been used to estimate effects on tolerance for free gossypol of a) difference in source or kind of protein; b) level of total nitrogen or protein intake; and c) changes in amino acid content or balance in the nitrogen allowance. The data demonstrate that the first two of these factors at least influence the depression in weight repletion of these protein depleted rats which may be brought about by an intake of free gossypol. It has been shown that a daily intake of 9.5 mg. of free gossypol, when total daily protein intake is limited to 160 mg. of nitrogen, is a borderline level which depresses weight gain when the protein is supplied by some meal mixtures but is without effect with some others.

It has further been demonstrated that the toxic or weight-depressing effect of 9.5 mg. of free gossypol which has been obtained with a nitrogen intake of 160 mg. may be nullified by increasing the protein intake 50% or more. Attempts to render ineffective an intake of 9.5 mg. gossypol at the lower nitrogen level by replacing a part of the nitrogen allowance with different amounts of some amino acid or amino acid combinations have not been markedly successful. The data do not identify the factor or factors in these protein supplements which influence gossypol toxicity. They do however point to the importance of considering both the amount and kind of dietary protein in attempts to establish a minimum level of free gossypol which is toxic for farm species, or a maximum level which will be tolerated.

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Isomerization During Hydrogenation. IV. Methyl Eleostearate[1]

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THE PROCESS of hydrogenation of unsaturated fatty acids causes isomerization of the double bonds in the molecule even in monoenes (3, 13). As the number of double bonds in the molecule increases, the number of possible isomers increases because there are more positions at which the hydrogen can attack and also more possibilities for migration of the remaining bonds. The type of polyene system also affects the isomerizations that take place. The methylene-interrupted diene is believed to undergo isomerization, by a process of half hydrogenation-dehydrogenation, which can lead to isomeric monoenes (4), while the conjugated dienes show no evidence of dehydrogenation during the process but add hydrogen with equal ease to the 1,2, 1,4, and 3,4 positions of the diene (2).

To extend the study of isomerization during hydrogenation, the conjugated triene system is a logical subject. Fortunately this system occurs in nature in eleostearic acid (9,11,13-octadecatrienoic acid), which can be isolated from tung oil.

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Although there have been numerous studies of the hydrogenation of eleostearic acid or its esters, the literature reveals considerable confusion as to the products produced by the partial hydrogenation of this fatty acid. Boeseken (6) has shown that the addition of two moles of hydrogen to the conjugated triene occurs according to Thiele's principle. The extremities of the conjugated system become saturated first. Thus 1,6 addition followed by 1,4 addition to the new diene would form the 11-octadecenoic acid. Also Woltemate and Daubert (19) isolated what was believed to be 11-octadecenoic acid, after addition of two moles of hydrogen to β-eleostearic acid. However other investigators (5), using chromatographic analysis to identify the products, reported that addition of two moles of hydrogen to eleostearic acid produced not more than 30% of 11-octadecenoic acid.

Light-absorption measurements have been used to follow the hydrogenation of eleostearic acid since the conjugated triene has a well-defined absorption band at 2680–2700 Å while the conjugated diene absorbs at 2320–40 Å. Thus Moore (14) reported a decrease in the band at 2700 Å with an increase in absorption at 2320 Å during the hydrogenation of eleostearic acid. Later workers (8) also observed similar changes. However Lemon (12) found that hydrogenation caused a decrease in the band at 2700 Å but no increase at 2320 Å, which he believed was due to simultaneous hydrogenation of two of the double bonds in the conjugated triene.

The purpose of the present study was to gain a better understanding of the reactions occurring during the hydrogenation of methyl eleostearate by the use of newer chromatographic techniques.

Experimental

Preparation of Methyl Eleostearate. Methyl eleostearate was prepared from fresh tung oil by methanolysis, using KOH as a catalyst (11). The crude ester was recrystallized twice from Skellysolve F and was distilled in a short-path distillation apparatus at a pressure of 6 μ. Hydrogen-iodine value was 241 (theory = 260.4), sp $a_{2700} = 162$. Analysis for α and β isomers by the method of O'Connor *et al.* (15) indicated about 42% α- and 49% β-eleostearate and a total methyl eleostearate content of 93.8%.

The hydrogenations were carried out by two methods. The first was performed in ethyl acetate solution (4.18 g./100 ml.), using Raney nickel catalyst at room temperature (26°) and atmospheric pressure as described by Woltemate and Daubert (19). The second was carried out at a temperature of 120° by bubbling hydrogen through the pure ester in an open glass tube suspended in a constant-temperature oil bath. A reduced nickel formate catalyst was used. Samples were removed periodically, were filtered rapidly under nitrogen, and were kept frozen until analyses could be performed.

Analytical Procedures

Iodine values were determined by quantitative hydrogenation and by the modified Benham-Klee method (17). The results were found to agree within experimental error.

The positions of the double bonds in the partially hydrogenated samples were determined by oxidative scission of the unsaturated fatty acids, separation of the monobasic from the dibasic acids, and subsequent separation and determination of each monobasic and dibasic acid in the mixtures. A 1-g. sample of the fatty acids was dissolved in 15 ml. of acetone, 100% excess ozone was added, and the solution was refluxed for ½ hr. after the addition of 2 ml. of water. The solution was cooled and ozonized again for a short time. The acetone was removed by distillation on a steam bath, which left a mixture of mono- and dibasic acids in water. The mono- and dibasic acids were separated by liquid-liquid extraction. The mixture of acids was dissolved in 10 ml. of 1:4 (v/v) water:diethylene glycol and was continuously extracted for 4 hrs. with Skellysolve F. The dibasic acids remained in the aqueous layer, and the monobasic acids were extracted by the petroleum ether. Analyses of the two solutions were made by partition chromatography (7, 9); the results were expressed as mole percentage of total monobasic or dibasic acids. From these data the mole percentages of the positional isomeric monoenes were calculated by equating the mole percentages of mono- or dibasic acids to the monoenes (and dienes) which would produce them (1). Although the positions of three double bonds in a chain could not be determined by this method, the positions of the two outer double bonds could be located and the mole percentages estimated.

The percentage of isolated *trans* isomers was determined by the infrared spectrophotometric method described by Swern *et al.* (18), using the base-line technique (10).

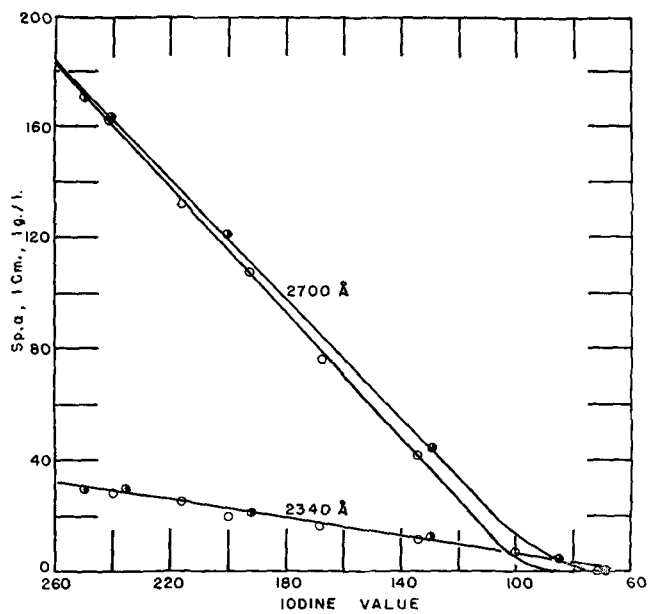

FIG. 1. Change in ultraviolet absorption spectra during hydrogenation of methyl eleostearate.

Open circles—Hydrogenated in ethyl acetate solution at 26°, 4% Raney Ni, atm. press.

Half-filled circles—Hydrogenated at 120°, 0.25% Ni, atm. press.

Results and Discussion

Figure 1 shows the change in ultraviolet-light absorption at 2700 Å and 2340 Å as hydrogenation proceeded. The decrease in the band at 2700 Å with a proportional decrease at 2340 Å indicates that the conjugated triene system was being destroyed but

was not forming a conjugated diene. The point at which all conjugated triene has disappeared corresponds to the addition of two moles of hydrogen. This can be interpreted in two ways, either all the triene has been reduced to monoene or a mixture of nonconjugated dienes, monoenes, and saturated acids has been produced. Table I shows the chromatographic analysis and calculation of the percentage of fatty acids which had double bonds at both the 9 and 13 positions compared to the amount of conjugated triene as determined by ultraviolet analysis. It is apparent from this table that during the hydrogenation at 26° in solution practically no 9,13 was found by chromatography that was not conjugated triene. However the hydrogenations at 120° formed some 9,13-diene. Thus, although no conjugated diene was found as an intermediate during the hydrogenation of a conjugated triene to a monoene, there was some non-conjugated diene formed.

TABLE I

Comparison of Values for Triene Content of Partially Hydrogenated Eleostearate Obtained by Different Methods

Iodine value	Ester with double bonds at both 9 and 13 positions found by chromatography	Triene found by ultraviolet analysis
26°, 4% Raney Ni, ethyl acetate soln., atm. press.		
216	71.0	74.5
192	55.2	60.8
168	37.7	39.5
99.2	9.7	7.7
68.2	0	0
120°, 0.25% Ni, atm. press.		
200	68.1	65.5
129	28.9	24.8
84.9	10.2	4.0
66.5	7.0	0

The percentages of the isomeric monoenes that were produced during the hydrogenation of methyl eleostearate are shown in Figures 2 and 3. As shown in Figure 2, an approximately equimolar mixture of all six possible monoenes is produced during hydrogenation at 26°. However hydrogenation at 120° produced somewhat less 9-octadecenoate than other isomers. These findings support the work of Begemann *et al.* (5) but contrast with the report of Woltemate and Daubert (19) that the hydrogenation of methyl eleostearate in solvent produced large amounts of the 11-octadecenoate. The double-bond migration that takes place during the hydrogenation is confined to the six carbons of the triene system. A similar observation was made during the hydrogenation of a conjugated diene (2).

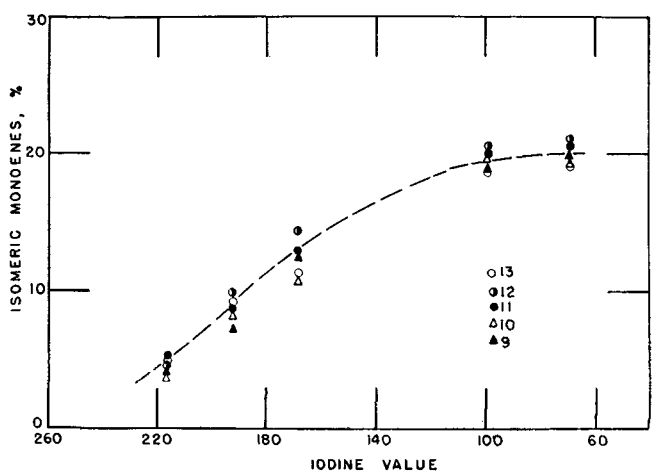

FIG. 2. Positional isomeric octadecenoates produced during the hydrogenation of methyl eleostearate in ethyl acetate soln. at 26°, 4% Raney Ni, atm. press.

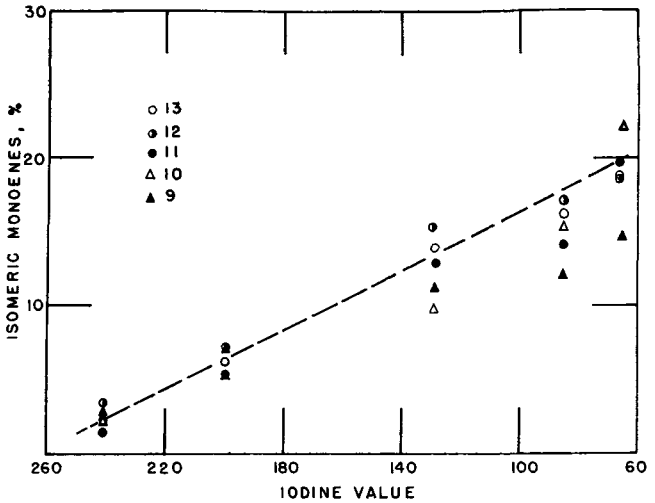

FIG. 3. Positional isomeric octadecenoates produced during the hydrogenation of methyl eleostearate at 120°, 0.25% Ni, atm. press.

From these data it appears that the first mole of hydrogen adds to the conjugated triene system in all possible positions. Thus from the 9,11,13-triene six different dienes would be produced: 9,13- would result from hydrogenation of the central double bond, 10,12- would result from 1,6 addition of hydrogen to the triene with a shift of the double bonds, similarly 10,13- and 9,12- would be formed by 1,4 addition, while 9,11- and 11,13- would result from saturation of the 13 and the 9 double bonds. Addition of a second mole of hydrogen to these dienes would form the isomeric monoenes that were found. Thus the 9,13- would produce the 9- and 13-monoenes, and, as shown in a previous report (2), the 9,11- produces the 9-, 10-, and 11-, the 10,12- gives 10-, 11-, and 12-, and the 11,13- forms the 11-, 12-, and 13-. Since the methylene-interrupted dienes produce isomeric monoenes (4), the 9,12- and 10,13-dienes would produce the 9-, 10-, 11-, 12-, and 10-, 11-, 12-, and 13-monoenes.

The failure to identify any but the 9,13-diene was probably due to the conditions of hydrogenation. The hydrogenation at 26° in solution would probably be very "non-selective," and therefore all the dienes that were formed, even those with several methylene groups between the double bonds, would be hydrogenated to monoenes at about the same rate. In other words, there would be no preferential hydrogenation of the more reactive types of dienes with a subsequent increase in relative amount of the less reactive diene. Although the mixtures probably contained other dienes in low concentration, the analytical methods employed made their detection uncertain. The higher temperature and lower catalyst concentration employed during the second hydrogenation would tend to make this reaction somewhat selective. Thus it would be expected that the conjugated dienes and dienes with a single methylene group between the double bonds would hydrogenate faster than the 9,13-diene with two methylene groups between the double bonds. The two double bonds in

this diene would each react like isolated monoenes. Therefore the 9,13-diene concentration would increase during the hydrogenation. In support of this view some 9,13-diene was detected by the chromatographic analysis of the products formed during the hydrogenation at 120°.

Added evidence for this mechanism was obtained by a determination of the geometrical configuration of the positional isomers produced during the hydrogenation. A sample of methyl eleostearate which contained approximately equal amounts of the *α* and *β* isomers was hydrogenated to an iodine value of 60. The positional isomers and the *trans*-isomer content were determined. The *trans* monoenes were isolated from the mixture by acetone crystallization, and the positional isomers in the pure *trans* were determined. The results of the analyses are shown in Table II.

TABLE II

Monoenoic Fatty Acid Composition of Partially Hydrogenated Methyl Eleostearate [a]

Double bond position	% total unsaturated (72.5% *trans*)	% *trans* fraction	% total unsaturated in *trans* form	% positional isomer in *trans* form
9	21.3	14.1	10.2	48.0
10	20.6	20.8	15.1	73.2
11	20.0	21.2	15.4	77.0
12	18.1	19.8	14.3	79.0
13	20.0	24.1	17.5	87.4

[a] 48% methyl *β*-eleostearate, 43% methyl *α*-eleostearate.

The last column in the table, which shows the percentage of each positional isomer that is *trans*, indicates that practically all the 13-octadecenoate is *trans* while the *trans* content of the 11 is somewhat less and the *trans*-9 much less. These observations are believed to be related to the geometrical configuration of the original conjugated triene. If the configurations of the *α*- and *β*-eleostearic acids as proposed by Paschke (16) are considered, it is apparent that hydrogenation of the 9 and 11 bonds of eleostearate would produce only *trans*-13. However, since the 11-monoene may be produced not only by saturation of the original 9 and 13 bonds but also from the intermediate dienes, some *cis* is formed even though the original 11 bond in the triene was *trans*. The percentage of the 9-monoene that is *trans* supports the proposed *cis*-9, *trans*-11, *trans*-13 geometrical configuration of *α*-eleostearic acid (16) since the 9 double bond would not undergo geometrical isomerization during the hydrogenation. The observed 48% *trans*-9 monoene is due mainly to the fact that methyl *β*-eleostearate was present in the original sample.

Further investigation of this proof of structure involved addition of two moles of hydrogen to a sample of *α*-eleostearic acid prepared by the method of O'Connor (15). The *trans*-octadecenoic acids were separated by acetone crystallization, and the positional isomers were measured. Only a very small amount of *trans*-9-octadecenoic acid was found. This procedure offers a method by which the configuration of the double bonds of a conjugated system in an aliphatic compound may be determined.

Summary

The hydrogenation of methyl eleostearate with and without solvent has been studied. The data indicate the stepwise addition of two moles of hydrogen to the conjugated triene to produce equimolar amounts of 9-, 10-, 11-, 12-, and 13-octadecenoates. Additional evidence for the *cis*-9, *trans*-11, *trans*-13 structure of *α*-eleostearic acid was obtained.

Acknowledgment

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Report of the Uniform Methods Committee

Business Session, Philadelphia, October 12, 1955

BOTH OF THE RECOMMENDATIONS for changes in A.O.C.S. Methods were by the Color Committee, of which R. C. Stillman is chairman.

1. The Color Committee recommends the adoption, as tentative, of a new method for the "Determination of Chlorophyll in Parts per Million in Refined and Refined and Bleached Oils." This method will be tentative and, if adopted, will be coded Cc 13d-55. The detailed procedure was published in the Journal of the American Oil Chemists' Society for September, 1955 (vol. 32, pp. 503-505). This recommendation is supported by a considerable amount of excellent cooperative work by the Color Committee.

   Approved by U.M.C. Adopted.

2. The Color Committee recommends several changes in Official Method Cc 13b-45 for "Color of Fats and Oils—Wesson Method Using Lovibond Glasses." These changes, relating to preparation of oils for color determination, are as follows:

   B. Procedure: Change paragraph 1 to read:

   "Crude, raw, and refined oil samples must be treated with 0.5 g. of official diatomaceous earth per 300 g. of oil. Add the diatomaceous earth to the oil and agitate for 2.5 min. at 250 r.p.m. at room temperature, or at no more than 10° to 15°C. above the melting point of the fat, if necessary, and filter through an approved paper.

   "Oils which have just been bleached in the laboratory, in accordance with A.O.C.S. methods Cc 8a-52, Cc 8b-52, or Cc 8d-48, normally are sufficiently clear for the color determination. Suspended material, even if of colloidal size, will cause light scattering. If the sample is not absolutely clear, treat