FAT METABOLISM IN HIGHER PLANTS

IX. ENZYMIC SYNTHESIS OF LONG CHAIN FATTY ACIDS BY AVOCADO PARTICLES*

By P. K. STUMPF and GEORGE A. BARBER

(From the Department of Agricultural Biochemistry, University of California, Berkeley, California)

(Received for publication, December 4, 1956)

The discovery and isolation of the multienzyme system responsible for β oxidation of fatty acids to acetyl CoA[1] have stimulated investigation of the synthesis of long chain fatty acids by multiple β condensation of the acetyl CoA. In 1953 Van Baalen and Gurin (1) were the first to demonstrate that acetate was incorporated into fatty acids by aqueous extracts of pigeon liver. In 1953 Stansly and Beinert (2), employing the purified enzymes of the β-oxidative system, showed the synthesis of butyryl CoA from acetyl CoA. In 1955 Hele and Popják (3) described soluble enzyme systems from rat and rabbit mammary glands which catalyze the synthesis of long chain fatty acids from acetate in the presence of ATP, CoA, and DPN. Langdon (4) has made the important observation that TPNH is required for the incorporation of acetate-C^{14} into the higher fatty acids by soluble liver systems. Recently Gibson and Jacob (5) and Wakil, Porter, and Tietz (6), using three different protein fractions obtained from pigeon liver and a mixture of cofactors and substrates, showed the synthesis of long chain fatty acids from acetate.

In higher plants, Newcomb and Stumpf (7) observed that slices of cotyledons of both germinating and developing peanuts had the capacity to incorporate acetate-C^{14} into long chain fatty acids. Gibble and Kurtz (8) demonstrated that acetate-1-C^{14} was incorporated by developing flax fruits into long chain fatty acids which were labeled predominantly in the odd-numbered carbon atoms. Sisakyan and Smirnov (9) supplied acetate-C^{14} to sunflower chloroplasts and isolated long chain fatty acids with relatively low radioactivity. No cofactor requirements were demonstrated.

* This work was supported in part by a grant from the National Science Foundation.

[1] The following contractions are employed: ATP, adenosine triphosphate; CoA, coenzyme A; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane; UTP, uridine triphosphate; GTP, guanosine triphosphate; CTP, cytosine triphosphate; ITP, inosine triphosphate; TCA, trichloroacetic acid.

This paper presents evidence that a cell-free particulate system, isolated from avocado mesocarp, catalyzes the synthesis of long chain fatty acids from acetate in the presence of ATP, CoA, and Mn^{++} under anaerobic conditions.

Methods and Materials

Preparation of Enzyme System—Avocado fruits (*Persea americana*) were purchased as needed from local food stores. The Fuerte variety was used in the majority of experiments. Generally one fruit, the mesocarp of which weighed about 200 gm., supplied sufficient particles for one set of experiments.

The fruit was peeled, the seed removed, and the remaining fleshy mesocarp was treated in the cold room (3–4°) as follows. The mesocarp was passed through a food grater and ground with acid-washed sand in a chilled mortar (10) with 1 volume of cold 0.4 M sucrose-0.2 M Tris buffer at pH 7.3. The homogenate was squeezed through four layers of cheesecloth, and the filtrate was centrifuged in a Servall superspeed angle centrifuge type SS-1 for 7 minutes at 500 × *g* to remove fine debris. The supernatant fluid was then centrifuged at 10,000 × *g* for 30 minutes, and the sediment was washed by resuspension in 50 ml. of sucrose buffer and by centrifugation again at 10,000 × *g* for 30 minutes. The final sediment was suspended in an appropriately small volume of sucrose-Tris buffer (containing about 30 mg. of protein per ml.) and used within a few minutes.

Procedure—Enzyme reactions were carried out in small Pyrex tubes containing the particulate suspension, cofactors, and substrate labeled with C^{14}. The tubes were flushed with nitrogen, stoppered, and shaken gently on a rotary shaker for 2 hours at 30°. After incubation, the contents of each tube were added to an equal volume of saturated $KHSO_4$ solution in a Maizel-Gerson tube. The lipides were extracted once from each tube with 1 ml. of chloroform. The chloroform extracts were evaporated at low heat on to aluminum planchets, and the fatty residues were counted directly with a thin window Geiger-Müller tube, assuming infinite thinness. Activity was expressed as the per cent of incorporation, counts per minute in chloroform extract × 100 divided by counts per minute in substrate. Repeated control experiments showed that unincorporated acetate-2-C^{14} was completely lost when the chloroform extract was evaporated on the aluminum planchets.

Formation of Hydroxamic Acid Derivatives—Hydroxylamine with low salt content was prepared by adjusting a concentrated solution of hydroxylamine sulfate with saturated $Ba(OH)_2$ to pH 6.5 and precipitating the $BaSO_4$. Water was then added to yield a molar solution of hydroxylamine. Hydroxylamine was added to reaction mixtures at various stages in their

incubation, and the hydroxamic acid derivatives were isolated by the method of Stadtman and Barker (11). Colorimetric analyses for hydroxamic acid derivatives formed from non-radioactive substrates were carried out by the method of Lipmann and Tuttle (12).

Identification of C^{14}-Labeled Lipides—Portions of the chloroform extract were fractionated on MgO-Celite and $CaCO_3$-Celite columns, as described by Strain (13). The radioactivity of each fraction was determined by counting the eluates on aluminum planchets.

Other portions of the chloroform-soluble lipide material were saponified by being refluxed in 10 per cent ethanolic KOH for 5 hours. Fatty acids were separated from the acidified saponification mixtures by steam distillation. Long chain fatty acids tended to collect in the condenser and were washed out with a few ml. of ethyl ether. The aqueous solutions were then made slightly alkaline and reduced to small volumes in a flash evaporator, model FE-2 (Laboratory Glass Supply Company), at 40°. The concentrates were acidified with 6 N H_2SO_4 and extracted with ethyl ether. The ether was evaporated to dryness, and the residues were redissolved in minimal volumes of petroleum ether. The fatty acids were chromatographed by the reversed phase method of Kaufmann and Nitsch (14). In this method, Whatman No. 3 paper is immersed in kerosene for 15 minutes and then dried in a well ventilated hood for 4 hours at room temperature. Fatty acids are applied as free acids dissolved in hexane, and the paper was developed in a descending direction with a 9:1 acetic acid-water solvent system at room temperature. The fatty acids and their R_F values are as follows: capric 0.60, lauric 0.50, myristic 0.39, palmitic 0.23, oleic 0.24, and stearic 0.15.

With short chain fatty acids, a portion of the above ethyl ether extract was treated with concentrated NH_4OH solution to form the ammonium salts of the fatty acids which were chromatographed by the method of Kennedy and Barker (15).

Radioactive spots were located by exposure of the papers to x-ray film for from 5 to 10 days, depending upon the activity of the material chromatographed. In addition, long chain fatty acids were located by treating the paper with a 1 per cent lead acetate solution, washing it with water, and exposing the lead salts of the fatty acids to H_2S. Fatty acids appeared as dark spots of lead sulfide. This method is sensitive to 0.1 μmole quantities of fatty acid (16).

Unsaturated fatty acids were demonstrated by the change in their chromatographic movement after reduction by hydrogen with a platinum catalyst in a pressure reaction apparatus (Parr Instrument Company).

Aliquots of fatty acids, synthesized by the avocado particulate system from carboxyl- and methyl-labeled acetate, were decarboxylated by the

Schmidt azide reaction by the method of Phares (17). The CO_2 released was collected in 20 per cent KOH, precipitated with barium acetate, and counted as $BaCO_3$.

Reagents—Carboxyl- and methyl-labeled acetate-C^{14}, both with a specific activity of 1.0 mc. per mmole, were purchased from Tracerlab, Inc., Boston. CoA and ATP were purchased from the Pabst Brewing Company and the Sigma Chemical Company, and reduced glutathione from the Nutritional Biochemicals Corporation.

RESULTS AND DISCUSSION

Properties of Enzyme System—A study of various centrifugal fractions of the avocado mesocarp homogenate showed that the particles most active in incorporating acetate into the lipide fraction were sedimented in 30 minutes at 10,000 × *g*. When examined under the light microscope, this fraction appeared to consist mainly of particles at the limit of optical resolution in addition to a small number of chloroplasts. When the mesocarp was divided into the green outer and the yellow inner sections, the green tissue contained far more chloroplasts than the yellow, but, according to weight, particles prepared from each section gave about equal incorporation of acetate. Since the procedure used to prepare the particles was identical with that of Millerd *et al.* for isolating avocado mitochondria (17), the particles comprising the enzyme system in question appear to be "mitochondria" in the generally accepted sense of that term. Addition of supernatant solution to mitochondrial preparations caused no stimulation, but instead inhibition of acetate incorporation. This is in contrast to the work of Gibson and Jacob (5) and of Hele and Popják (3). The former found a requirement for both soluble and particulate fractions of pigeon liver for fatty acid biosynthesis, whereas the latter demonstrated all activity to be in the soluble portion of rat and rabbit mammary gland homogenates.

The enzyme system in avocado mitochondria is quite labile. Heating at 50° for 5 minutes at pH 7.0 destroyed its activity completely; freezing overnight at −20° reduced the activity 80 per cent; merely allowing the preparation to stand at 0° for 2 hours lowered its ability to incorporate acetate into lipides by 50 per cent.

Anaerobic incubation of the reaction mixtures resulted in greater incorporation of acetate than did aerobic incubation. However, the difference between the two was not as large as was expected, *i.e.* incubation in Warburg vessels exposed to the air showed 9 per cent incorporation against 15 per cent in tubes flushed with nitrogen. In other experiments, reactions carried out either in Warburg vessels flushed with nitrogen and containing Oxsorbent (Burrell Corporation) in the center well or in Thunberg tubes

flushed carefully with nitrogen gave no greater acetate incorporation than did reactions in small tubes flushed with nitrogen and closed with rubber stoppers.

A time study of the reaction system showed that after 90 minutes there was no further acetate incorporation. The mixtures were routinely incubated for 2 hours.

Cofactor Requirements—Cofactor requirements for the mitochondrial system are shown in Table I. The requirements for ATP, CoA, and Mn^{++} are clearly evident. The effect of ATP is particularly striking, although a rather high concentration was required for optimal activation. This

TABLE I

Cofactor Requirements for Incorporation of Acetate-2-C^{14} into Lipides by Particulate System of Avocado Fruit

The complete reaction mixture contained 1.0 ml. of mitochondrial suspension (about 15 mg. of protein) in 0.4 M sucrose-0.2 M Tris buffer, ATP 5.5×10^{-3} M, CoA 3×10^{-4} M, glutathione 2.7×10^{-3} M, $MnSO_4$ 5×10^{-4} M, NaF 2.7×10^{-3} M, acetate-2-C^{14} 5×10^{-5} M, 0.05 μc., 35,000 c.p.m., phosphate buffer 5.5×10^{-3} M. Total volume in each tube, 1.8 ml. The tubes were flushed with nitrogen and incubated for 2 hours at 30°.

Components	Per cent substrate acetate-2-C^{14} incorporated into lipides
Complete system	27.5
Without ATP	0.1
" CoA	4.1
" $MnSO_4$	1.4
" glutathione	25.2
" NaF	22.5

suggested a contaminating nucleotide other than ATP as the active nucleotide. As a source of such a nucleotide, a complex of mononucleotides, isolated by charcoal adsorption from acid-extracted yeast cells,[2] was added to ATP. No stimulation was observed. Other nucleotides such as UTP, GTP, CTP, and ITP were ineffective. The specific requirement for Mn^{++} is also rather remarkable. Although different metal ions were tested for activity, either alone or in combination with Mn^{++}, none of them stimulated acetate incorporation significantly. Substrate concentration was also critical. Increases in concentration of acetate over about 2.0×10^{-4} M caused inhibition of the incorporating system. Inhibition of a mitochondrial system by short chain fatty acids was also observed in the β oxidation of butyrate by mitochondria of germinating peanut cotyledons (18).

[2] The authors are indebted to Dr. Rao Sanadi for this preparation.

It should be pointed out that throughout the course of these experiments there was considerable variation from day to day in the activity of the particulate preparations. This was undoubtedly related to the condition of the avocado fruit and its history of handling and storage. Nonetheless, although activity varied quantitatively (1 to 30 per cent incorporation of acetate), no instance was ever found of qualitative difference in cofactor requirements.

A marked requirement for a reducing system could not be shown although the addition to the reaction mixtures of DPN and TPN with α-keto glutaric acid gave a slight but consistent stimulation of acetate incorporation. DPN and the reducing system, ethanol dehydrogenase and ethanol,

TABLE II

Effect of CoA and ATP on Formation of Acetyl Hydroxamates by Avocado Particulate System

The reaction systems were as in Table I except for the addition of 0.5 mmole of hydroxylamine, pH 6.5, and 0.2 μmole of acetate-2-C^{14}, 54,000 c.p.m. The hydroxamic acids were chromatographed, detected, and eluted as described in the text. In each case only one spot, corresponding to the acetyl hydroxamate, appeared on the chromatogram.

Reaction mixture	Radioactivity of eluted material
	c.p.m.
Complete	842
Without CoA	138
" " ATP	0
" ATP	0

alone or in combination with TPN and its reducing system, glucose-6-phosphate dehydrogenase and glucose 6-phosphate, produced no stimulation. Moreover, DPNH alone had no effect on the incorporation of acetate. This lack of effect by DPN and TPN reduced outside the mitochondrion may be related to a permeability barrier into the particle.

Fatty Acid Activation—To demonstrate the enzymes of fatty acid activation, the mitochondrial system was incubated with hydroxylamine and either labeled or non-labeled fatty acid substrates. Acyl CoA derivatives will form hydroxamates which can be detected by their production of a colored complex with $FeCl_3$ and by their chromatographic movement. In experiments with labeled acetate, the reaction mixtures were chromatographed on paper with water-saturated butanol, and the spots were located on x-ray film, eluted with ethanol, and counted (11). As indicated in Table II, only the acetyl hydroxamate was evident on the chromatograms. It is of

interest to point out that the condensation products of acetyl CoA, C4, C6, C8, etc. (CoA derivatives), did not accumulate in hydroxylamine-trapping experiments in which hydroxylamine was added at different time intervals. In other experiments with 5 μmoles of non-radioactive acetate, butyrate, and octanoate, colorimetric analysis gave evidence for the formation of acetyl, butyryl, and octanyl hydroxamates. Palmitic and oleic acids gave no reaction (Table III). Tris buffer extracts of acetone powder of the fruit mesocarp also formed hydroxamic acid derivatives when incubated with cofactors and non-radioactive acetate, but the activity of the powder decreased rapidly with storage. From the above evidence it was concluded that avocado mitochondria possess a fatty acid-

TABLE III

Specificity of Fatty Acid-CoA-Activating System of Avocado Particles

The reaction systems were as in Table I except that 0.5 μmole of non-labeled fatty acid substrate was substituted for acetate-2-C^{14} and 0.5 mmole of hydroxylamine, pH 6.5, was added at the beginning of the reaction. Quantitative estimations of hydroxamic acid derivatives are described in the text.

Substrate	Hydroxamate*
	μmole
Acetate	0.84
Butyrate	0.49
Octanoate	0.38
Palmitate	0.00
Oleate	0.00

* Succinyl hydroxamate as standard.

activating system which in our preparations is able to activate acetate, butyrate, and octanoate in a decreasing order of activity. These results are similar to those in plant extracts (19) and in bacteria and animals (20).

Identification of C^{14}-Labeled Reaction Products—Since chloroform extracts obtained from mitochondrial mixtures were green in color, it was decided to determine first whether any of the activity was present in pigmented compounds. Portions of the labeled chloroform-soluble materials were fractionated on $CaCO_3$-Celite and MgO-Celite columns by us and by Dr. Chichester, Department of Food Technology, University of California, Davis. Activity was found to be associated with the esterified xanthophyll fraction in all cases. However, upon elution and saponification, the colored fraction lost its activity. This radioactivity was then recovered from the colorless aqueous extracts of the saponified eluate. It was concluded from this evidence that the fatty acids synthesized by the reaction mixtures were esterified in part with the naturally occurring xanthophylls. This

conclusion is tentative since it is possible that triglycerides are associated with esterified xanthophylls on the chromatographic columns. Experiments in which the particulate enzymes were incubated with cofactors, non-radioactive acetate, and glycerol-1-C^{14} indicated that glycerol could serve as an acceptor of fatty acids. The question of whether the final occurrence of the newly synthesized fatty acid is as a xanthophyll ester, a triglyceride, or both, requires further investigation.

For preliminary identification of the fatty acids synthesized by the particulate preparations, the reversed phase paper chromatography procedure developed by Kaufmann and Nitsch (14) was employed. When the saponified lipide was chromatographed, two radioactive spots were observed by exposure of the chromatogram to x-ray film. One spot (R_F 0.22) was larger and considerably more radioactive than the other (R_F 0.14). These R_F values correspond to the positions of palmitic or oleic and stearic acids, respectively. When an aliquot of the saponified material was hydrogenated and chromatographed, the 0.22 area decreased while the 0.14 area increased in size and radioactivity. This would indicate the accumulation of stearic acid which originated from the reduction of oleic acid present in the saponified lipide material. All the radioactivity of newly isolated lipide material, when chromatographed by the Kaufmann and Nitsch (14) system, remained at the origin. This indicated the absence of free fatty acid and the occurrence of the radioactive acids as neutral fats.

A more precise analysis was made by separating the saponified acids by gas-liquid partition chromatography. We are greatly indebted to Dr. K. P. Dimick of the Western Utilization Research Laboratory, Albany, California, for his cooperation in this phase of the work. Enzymically prepared radioactive fatty acids were esterified to the methyl esters by treatment with diazomethane, and 2.5 mg. each of methyl laurate, methyl myristate, methyl palmitate, methyl stearate, and methyl oleate were added as carriers. The ester mixture dissolved in *n*-heptane was injected into the column heated to 214° (General Electric silicone supported in brick dust in a 5 foot column). As the peaks appeared on the Brown recorder, the samples were collected and assayed for radioactivity (Table IV). Although only 20 per cent of the total applied radioactivity was recovered, it can be clearly seen that higher fatty acids became labeled. It was not possible to separate stearic and oleic acids by this method, but because stearic acid is never labeled to any noticeable extent on the basis of reversed phase chromatography and does not occur in avocado fruits in significant amounts (21), it is concluded that the major radioactivity of the stearic-oleic fraction is contributed by oleic acid.

No labeled short chain fatty acids accumulate in reaction mixtures as evidenced by the Kennedy and Barker method (15). Water-soluble

compounds that were left after chloroform extraction were recovered by Soxhlet ether extraction. Upon chromatography with butanol-formic acid-water, the presence of labeled citric, malic, succinic, and probably α-ketoglutaric acids was observed. Since labeling in these TCA cycle acids of the tricarboxylic acid cycle was reduced considerably by rigorously anaerobic incubation of the reaction mixtures, it is assumed that acetate-C^{14} enters the TCA system through the presence of residual oxygen. Neither butyrate-1-C^{14} nor propionate-1-C^{14} was incorporated into chloroform-soluble compounds by the particulate system.

In view of these experiments it is concluded that palmitic and oleic acids are the only fatty acids synthesized in detectable quantity by the avocado mitochondrial system. Similar preliminary studies have been made with

TABLE IV

Gas-Liquid Chromatography of Enzymically Synthesized Fatty Acids

Radioactive fatty acids were isolated from the avocado particulate system and treated as described in the text.

Fatty acid (methyl ester)*	Emergence time	Radioactivity
	min.	*c.p.m.*
Lauric	7.0	0
Myristic	14.2	18
Palmitic	28	280
Oleic†	55	469
Stearic†	60	

* 2.5 mg. of the methyl esters of these fatty acids added as carriers.

† Stearic and oleic acids were not separable. See the text.

mitochondria isolated from cotyledons of germinating peanuts. Acetate-2-C^{14} was readily incorporated into the chloroform-extractable lipides when the mitochondria were incubated in the presence of ATP, CoA, and Mn^{++}. Without the cofactors no incorporation was observed.

The results of the Schmidt decarboxylation of fatty acids synthesized from carboxyl-labeled acetate are shown in Table V. Fatty acids were converted by catalytic reduction to the saturated fatty acids before decarboxylation to avoid oxidation at points of unsaturation by reagents of the Schmidt reaction. The results of the decarboxylation suggest strongly that the fatty acids are labeled along the whole chain. If the fatty acids are synthesized by the condensation of 2-carbon units, every other carbon in the chain should be labeled. Thus, in a 16-carbon fatty acid synthesized from carboxyl-labeled acetate the carboxyl group should contain one-eighth the total radioactivity in the molecule. The experimental results in Table V agree with this hypothesis.

The evidence of C^{14} distribution combined with the experiments demonstrating the presence of a conventional CoA-fatty acid-activating system supports the concept that fatty acid biosynthesis in the avocado system is through a mechanism essentially the reverse of β oxidation. This is the expected mechanism since β oxidation of fatty acids is known to occur in plants (18), and the condensation of acetyl CoA has been demonstrated to be the route of fatty acid biosynthesis in other organisms (20).

TABLE V

Schmidt Decarboxylation of Enzymically Synthesized Fatty Acids

Radioactive fatty acids were prepared and isolated from the avocado particulate system as described in the text. No attempt was made to separate the acids since only palmitic and oleic acids are the major ones. Control experiments with different concentrations of palmitic-1-C^{14} consistently gave 60 per cent yields. Therefore, a correction factor was applied to the radioactivity of CO_2 evolved. By assuming equal alternate labeling in the side chain of the C_{16} and C_{18} fatty acids and an average carbon chain length of 17, a factor of 1 to 8.5 was derived to relate the radioactivity in the terminal carbon (carboxyl) to that of the whole molecule. The result of decarboxylation of fatty acid enzymically synthesized from methyl-labeled acetate-C^{14} is presented as a control.

Experiment No.	Substrate enzymically incorporated into fatty acid	Total C^{14} in enzymically synthesized fatty acid	Corrected radioactivity of CO_2 evolved from carbon 1	Calculated radioactivity in carbon 1 of enzymically synthesized fatty acid
		c.p.m.	*c.p.m.*	*c.p.m.*
1	Acetate-1-C^{14}	1440	171	170
2	"	960	138	113
3	Acetate-2-C^{14}	1195	5	0

SUMMARY

Particles from avocado fruit mesocarp incorporate C^{14}-labeled acetic acid into esterified long chain fatty acids. Adenosine triphosphate, coenzyme A, and Mn^{++} are essential components of the system. Palmitic and oleic acids were the only labeled acids found to accumulate. Evidence is presented to support the theory that the mechanism of fatty acid formation in the avocado is by the condensation of coenzyme A-activated acetate units. The identity of the natural acceptor of fatty acids in the avocado mitochondrion is in doubt but may be a xanthophyll or a glycerol.

BIBLIOGRAPHY

1. Van Baalen, J., and Gurin, S., *J. Biol. Chem.*, **205**, 303 (1953).
2. Stansly, P. G., and Beinert, H., *Biochim. et biophys. acta*, **11**, 600 (1953).
3. Hele, P., and Popják, G., *Biochem. J.*, **60**, p. xxxii (1955).

4. Langdon, R. G., *J. Am. Chem. Soc.*, **77,** 5190 (1955).
5. Gibson, D. M., and Jacob, M. I., *Federation Proc.*, **15,** 261 (1956).
6. Wakil, S. J., Porter, J. W., and Tietz, A., *Federation Proc.*, **15,** 377 (1956).
7. Newcomb, E. H., and Stumpf, P. K., *J. Biol. Chem.*, **200,** 233 (1953).
8. Gibble, W. P., and Kurtz, E. B., Jr., *Arch. Biochem. and Biophys.*, **64,** 1 (1956).
9. Sisakyan, N. M., and Smirnov, B. P., *Biokhimiya*, **21,** 273 (1956).
10. Millerd, A., Bonner, J., and Biale, J. B., *Plant Physiol.*, **28,** 521 (1953).
11. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **184,** 769 (1950).
12. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159,** 21 (1945).
13. Strain, H. H., in Chromatographic adsorption analysis, New York (1945).
14. Kaufmann, H. P., and Nitsch, W. H., *Fette u. Seifen*, **57,** 473 (1955).
15. Kennedy, E. P., and Barker, H. A., *Anal. Chem.*, **23,** 1033 (1951).
16. Ashley, B. D., and Westphal, U., *Arch. Biochem. and Biophys.*, **56,** 1 (1955).
17. Phares, E. F., *Arch. Biochem. and Biophys.*, **33,** 173 (1951).
18. Stumpf, P. K., and Barber, G. A., *Plant Physiol.*, **31,** 304 (1956).
19. Millerd, A., and Bonner, J., *Arch. Biochem. and Biophys.*, **49,** 343 (1954).
20. Green, D. E., *Biol. Rev. Cambridge Phil. Soc.*, **29,** 330 (1954).
21. Hilditch, T. P., in The chemical constitution of natural fats, London (1940).