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# Fat Metabolism in Higher Plants

## XXIII. PROPERTIES OF A SOLUBLE FATTY ACID SYNTHETASE FROM AVOCADO MESOCARP*

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The conversion of malonyl coenzyme A to long chain fatty acids has been studied in enzyme systems from various tissues (1–7). Attempts to purify the enzymes involved in this conversion revealed an important difference between these systems. Thus the highly purified synthetase from yeast (8, 9) and from liver (10, 11) behaves as a single complex; its fractionation has not yet been accomplished. However, extracts of *Clostridium kluyveri* and *Escherichia coli* can be resolved into several components which on combination catalyze the synthesis of fatty acids (7, 12–16). One of these components has the rather unusual property of being relatively stable to heat and dilute hydrochloric acid (7, 12, 13). Acetyl and malonyl moieties can be transferred from their coenzyme A derivatives to this protein. The acyl complexes are then condensed by suitable enzymes to form acetoacetyl-enzyme, which is subsequently converted to butyryl-enzyme (15–19). These findings support the scheme proposed earlier for the synthetase from yeast (8).[1]

Extracts from avocado mesocarp contain, in addition to the previously studied particulate system (5, 20), a soluble enzymatic system, which converts malonyl coenzyme A to saturated long chain fatty acids (21). This soluble enzyme has as one of its components a heat-stable protein and therefore resembles the bacterial systems. Some of the data discussed here have already been reported elsewhere (22).

### EXPERIMENTAL PROCEDURE

#### *Materials and Methods*

CoA, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, papain, D-pantethine, and DPNH were purchased from Sigma Chemical Company; TPN was purchased from C. F. Boehringer und Soehne. $Ba^{14}CO_3$ was a product of the Oak Ridge National Laboratories. Malonyl-2-$^{14}$C-CoA was prepared according to the method of Eggerer and Lynen (23), starting with 100 to 200 mg of malonic acid-2-$^{14}$C (New England Nuclear Corporation). Malonyl-CoA and malonylpantetheine were prepared by the same method. Caproic anhydride was prepared by the method of Antenrieth (24). Pantetheine was prepared either by saponification of *S*-benzoylpantetheine (mp. 113–116°)[2] or by reduction of D-pantethine with 2% NaHg in methanol at pH 8 to 9 (23). Acetyl-CoA, caproyl-CoA, and caproylpantetheine were prepared according to Simon and Shemin (25). The thioesters were assayed by conversion into the corresponding hydroxamic acids (26). Protein was determined either by the biuret reaction (27) or spectrophotometrically (28).

#### *Enzyme Assays*

*Fatty Acid Synthesis*—Typical conditions for the reaction are described in the legend to Fig. 2. The incubation was stopped by the addition of 1.0 ml of 15% methanolic KOH. After 3 hours at 70°, the samples were acidified with 6 N HCl and extracted twice with 10 ml of chloroform. The chloroform layer was washed with 10 ml of a solution containing 1% malonic and 1% acetic acid. An aliquot was then dried in a scintillation vial and counted in a Packard liquid scintillation spectrometer.

*Malonyl-CoA-$CO_2$ Exchange*—The assay for the exchange reaction was performed as described by Alberts, Goldman, and Vagelos (12). The reaction was stopped by the addition of 0.2 ml of acetic acid. An aliquot was then dried on a strip (2.5 × 7.5 cm) of Whatman No. 1 paper, which was counted directly in the scintillation counter.

#### *Enzyme Preparations*[3]

*E. coli Enzymes*—Fraction A and enzyme II were prepared according to Goldman, Alberts, and Vagelos (13). We are indebted to Dr. Peter Goldman for providing us with a sample and a culture of *E. coli* K12 Hfr Thia⁻ and unpublished details for the improved separation and purification of these enzymes. The units of activity used are the same as given by Goldman *et al.* (13).

*Enzyme Preparations from Avocado*—Avocados of the Fuerte variety were customarily used, but other varieties were occasionally tested. For the preparation of crude extract, 150 g of mesocarp were homogenized with 300 ml of 0.02 M potassium phosphate-0.001 M cysteine, pH 7.3, for 1 minute in a Waring Blendor. The homogenate was centrifuged for 10 minutes at 13,000 × *g* in a Servall RC-2 centrifuge. The middle layer of extract was separated from the lipid floating on top and from the pellet, and centrifuged for 10 minutes at 37,000 × *g*. Residual lipid and large particles were removed by filtration through

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[1] F. Lynen, lecture given at the *Gemeinsame Tagung der Gesellschaft für physiologische Chemie und der Oesterreichischen biochemischen Gesellschaft*, Vienna, September 26, 1962.

[2] We are indebted to Dr. Alexander Hagen, Munich, Germany, for sending us a sample of *S*-benzoylpantetheine.

[3] Unless stated otherwise, all enzyme preparations were obtained at 0–4°.

a Büchner funnel. The extract had 2 to 4 mg of protein per ml. For the preparation of boiled juice, the extract was heated for 3 minutes in a boiling water bath and separated from the precipitate by filtration.

In the experiment described in Table I, 20 ml of extract were passed over a Sephadex G-25 column (medium grade, 2.5 × 25 cm, equilibrated against 0.005 M potassium phosphate-0.001 M 2-mercaptoethanol, pH 7.2); 25 ml of eluent with a protein content of 2 to 3 mg per ml were collected. In the experiments described in Fig. 2 and Table II, 5.82 g of ammonium sulfate were added with stirring to 20 ml of this solution (0 to 50% saturation) in 30 minutes. Throughout this procedure the pH was maintained at 7 (adjustment against Hydrion pH paper, 6.0 to 8.0, Micro Essential Laboratory) by addition of ammonium hydroxide. After 15 minutes the solution was centrifuged for 10 minutes at 37,000 × *g*. The precipitate was discarded. To the clear supernatant (22.4 ml), 4.4 g of ammonium sulfate were added under the conditions described above (50 to 80% saturation). The precipitate formed was centrifuged for 10 minutes at 105,000 × *g* in a Spinco ultracentrifuge. The protein was dissolved in 4 ml of 0.02 M potassium phosphate-0.001 M cysteine, pH 7.3. This preparation was used in Fig. 2 and Table II.

For the preparation of avocado Fraction I and avocado Fraction II,[4] 15 kg of avocado mesocarp were worked up batchwise in the following manner. The crude extract was prepared from 1.5 kg of mesocarp as described above. The resulting 2.2 liters of crude extract were saturated with ammonium sulfate with thorough stirring for 1 hour. The pH was adjusted to 7 as described above. After stirring for another hour, the precipitate which formed was collected by centrifugation for 30 minutes at 37,000 × *g*. The turbid supernatant was discarded. The pellet was dissolved in 80 ml of 0.1 M Tris-HCl buffer at pH 7.9, containing 0.01 M 2-mercaptoethanol. The buffer was changed after the first 4 hours. Ten such preparations were pooled, yielding 1820 ml of concentrate which lost no activity when stored frozen for several months.

The above concentrate (198 ml) was freed from insoluble material by centrifugation to give 178 ml of solution containing 18.7 mg of protein per ml. This solution was separated into three fractions by addition of solid ammonium sulfate at pH 7. The protein was sedimented by centrifugation for 10 minutes at 78,000 × *g* and taken up in a small amount of 0.05 M potassium phosphate-0.01 M 2-mercaptoethanol at pH 7.2. The fraction from 0 to 20% saturation was discarded, the fraction from 20 to 60% saturation was designated Fraction $I_{AV}$, and the fraction from 60 to 90% saturation was named Fraction $II_{AV}$. Both fractions were dialyzed for 4 hours against 0.01 M potassium phosphate-0.01 M 2-mercaptoethanol, pH 7.9, and then were stored frozen. Fraction $II_{AV}$ was routinely heated for 1 minute in a boiling water bath to destroy traces of Fraction $I_{AV}$ activity.

### *Identification of Products of Fatty Acid Synthesis and Malonyl-CoA-$CO_2$ Reaction*

*Fatty Acids*—The fatty acids were identified by a combination of the following procedures. The free fatty acids were separated by reverse phase chromatography on siliconized Whatman No. 1 paper according to the method of Mangold, Lamp, and Schenk (29) and Buchanan (31) with the modification of Yang and Stumpf (21). Polar and nonpolar acids were separated as methyl esters by thin layer chromatography on silicic acid with *n*-hexane-diethyl ether (10:6) as a solvent system (31). Saturated and unsaturated acids were separated as methyl esters on silicic acid thin layer plates impregnated with silver nitrate (32). Gas-liquid chromatography was performed with a Wilkens Aerograph A-90P instrument. The conditions are described in the legend for Fig. 5. The effluent vapors were passed directly into a Nuclear-Chicago proportional tube radioactive counter for continuous $^{14}C$ monitoring.

*Malonyl-CoA-$CO_2$ Exchange*—The product of the exchange reaction was identified as malonyl thioester by the following criteria. The samples of the experiments described in Table V were saponified by heating for 10 minutes at 100° in 0.4 N KOH. They were then acidified with HCl, and 100 mg of malonic acid were added as carrier. After an aliquot was counted on paper in the scintillation counter, the samples were passed through a column (0.7 × 10 cm) of Dowex 50-$H^+$ and lyophilized. The malonic acid was sublimed at 90° and a pressure of 0.05 mm of Hg. The specific activity was determined, and the samples were recrystallized from ethyl acetate-petroleum ether. The specific activity was the same as before the recrystallization. All the radioactivity fixed from $^{14}CO_2$ into nonvolatile material was associated with malonic acid.

After treatment of the samples with hydroxylamine, the thioesters were converted to their hydroxamates (26). After removal of the salt by use of Dowex 50 in the way outlined above, the samples were subjected to paper electrophoresis in pyridine-acetic acid-water (100:10:890) for 2 hours at about 40 volts per cm on Whatman No. 1 paper and to paper chromatography in pyridine-isopropyl alcohol-water (1:1:1) (33). In both systems over 90% of the radioactivity moved to the same spot as authentic malonic monohydroxamate.

## RESULTS

*Requirements for Fatty Acid Synthesis*—When malonyl-CoA, acetyl-CoA, and a TPNH-generating system are incubated with varying amounts of a crude extract of avocado mesocarp, fatty acid synthesis proceeds at an increasing rate. It was found that DPNH meets the requirement of a dissociable cofactor, as shown in Fig. 1. In the absence of DPNH an upward curvature is obtained, whereas with the addition of DPNH the incorporation increases linearly with increasing protein concentration.

After passage through a column of Sephadex G-25, the extract showed several cofactor requirements (Table I). The system is completely dependent on TPNH. Although the requirement for DPNH was not absolute in the crude extracts, it became more pronounced after fractionation of the system (see below). Addition of boiled extract to the complete system resulted in a 65% stimulation. A requirement for acetyl-CoA was not demonstrable, presumably owing to the presence of malonyl-CoA decarboxylase, which has been shown to occur in a variety of plants, including avocado (34). Since it has already been established (21) that fatty acids are synthesized *de novo*, an elongation mechanism with malonyl-CoA is ruled out.

An enzyme dependence curve of the Sephadex-treated extract again showed an upward curvature, which could be overcome by the addition of boiled crude extract. Since all the other

[4] The terms Fraction $I_{AV}$, Fraction $II_{AV}$, Fraction $A_{EC}$, and enzyme $II_{EC}$ will be used to indicate the source of each fraction. AV represents avocado enzymes, and EC, *E. coli*.

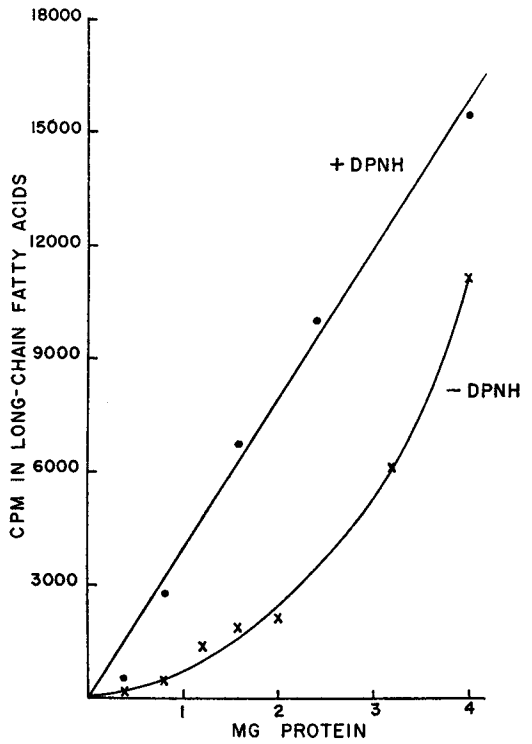


FIG. 1. Requirement of DPNH for fatty acid synthesis. The complete system contained potassium phosphate buffer, pH 7.2, 100 μmoles; malonyl-2-$^{14}$C-CoA (83,000 c.p.m.), 0.104 μmole; acetyl-CoA, 0.1 μmole; $TPN^+$, 0.2 μmole; glucose 6-phosphate, 1 μmole; glucose 6-phosphate dehydrogenase, 0.1 unit; and varying amounts of crude extract from avocado. The two curves were obtained with different extracts. DPNH (1 μmole) was added as indicated; total volume, 1.5 ml; time of incubation, 30 minutes at 37°.

TABLE I

*Cofactor requirement*

Incubation conditions and components were the same as in Fig. 1; enzyme (2.9 mg) was used after treatment with Sephadex (see "Materials and Methods").

| System | Incorporation into fatty acids |
|---|---|
| | *c.p.m.* |
| Complete | 8,424 |
| −Acetyl-CoA | 9,630 |
| −$TPN^+$ − glucose-6-P dehydrogenase | 376 |
| −DPNH | 4,148 |
| −Enzyme | 46 |
| +Boiled extract (0.5 ml) | 13,907 |

cofactors were present at saturating amounts, another dissociable cofactor, which was present in the boiled extract, appeared to be required. In order to obtain an enzyme preparation which would be completely dependent on boiled juice, the Sephadex-treated extract was fractionated with $(NH_4)_2SO_4$. A fraction was obtained between 50 and 80% saturation which had essentially no activity without addition of boiled extract. In Fig. 2 the dependence of fatty acid synthesis on added boiled extract is shown.

With this $(NH_4)_2SO_4$ fraction it was possible to study the properties of the cofactor in the boiled extract in more detail. The factor could not be replaced by flavin mononucleotide or flavin adenine dinucleotide. The activity was lost on ashing and was not dialyzable. Table II summarizes some further properties of the factor. Whereas the cofactor is stable when exposed to 100° at pH 6.9 with little loss in activity, it is destroyed when heated at pH 5.3. The activity was lost com-

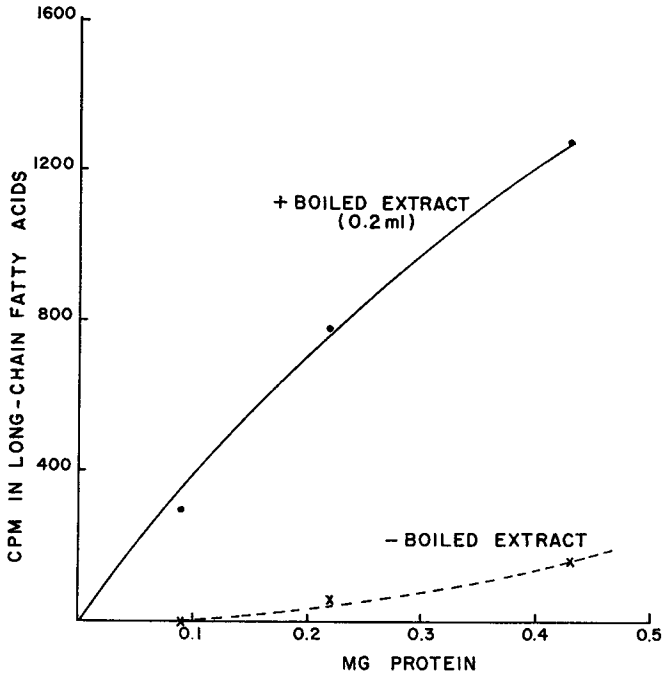


FIG. 2. Stimulation of the $(NH_4)_2SO_4$ fraction by boiled extract. The complete system contained potassium phosphate buffer, pH 7.2, 40 μmoles; malonyl-2-$^{14}$C-CoA (29,500 c.p.m.), 0.038 μmole; acetyl-CoA, 0.036 μmole; $TPN^+$, 0.07 μmole; glucose 6-phosphate, 0.3 μmole; glucose 6-phosphate dehydrogenase, 0.07 unit; and DPNH, 0.3 μmole. The enzyme was obtained by $(NH_4)_2SO_4$ fractionation of the Sephadex-treated crude extract (see "Materials and Methods"). Total volume, 0.5 ml; time of incubation, 30 minutes at 37°.

TABLE II

*Properties of heat-stable cofactor*

The complete system contained the same components as in Fig. 1. The $(NH_4)_2SO_4$ fraction (0.89 mg) (see "Materials and Methods") and boiled extract (0.5 ml) were used. Preincubation: 0.5 ml of boiled extract with 0.15 mg of papain in a total volume of 0.55 ml for 30 minutes at 37°.

| System | Incorporation into fatty acids |
|---|---|
| | *c.p.m.* |
| Complete | 134 |
| +Boiled extract (3 min, 100°, at pH 6.9) | 1282 |
| +Boiled extract (3 min, 100°, at pH 5.3) | 67 |
| +Dialyzed boiled extract (3 min, 100°, at pH 6.9) | 1596 |
| +Boiled extract (3 min, 100°, at pH 6.9) preincubated with papain and again heated for 5 min at 100° | 4 |
| +Boiled extract (3 min, 100°, at pH 6.9) preincubated and again heated for 5 min at 100° | 877 |
| +Preincubated with papain, heated for 5 min at 100° + boiled extract (3 min, 100°, at pH 6.9) | 1143 |

TABLE III

*Separation of two enzymatic activities by fractionation with $(NH_4)_2SO_4$*

Incubation conditions were the same as in Fig. 2, except that the tubes contained 0.47 mg of Fraction $I_{AV}$ and 0.11 mg of Fraction $II_{AV}$ where indicated.

| Enzyme | Incorporation into fatty acids |
|---|---|
| | *c.p.m.* |
| Fraction $I_{AV}$ (20 to 60% saturation) | 161 |
| Fraction $II_{AV}$ (60 to 90% saturation) | 18 |
| Fraction $I_{AV}$ + Fraction $II_{AV}$ | 2025 |
| Fraction $I_{AV}$ + boiled Fraction $II_{AV}$ (3 min, 100°) | 1243 |
| Boiled Fraction $I_{AV}$ + Fraction $II_{AV}$ (3 min, 100°) | 19 |

pletely when the boiled extract was incubated with papain. The two last experiments in Table II show that this loss of activity is due neither to the inactivation of the cofactor during the second heat treatment nor to an inhibition by the inactivated papain. It seemed likely, therefore, that a heat-stable protein similar to the protein found in bacterial systems (7, 12) is involved in the avocado synthetase.

*Separation of Two Enzymatic Activities*—The procedure for the preparation of the $(NH_4)_2SO_4$ fraction used in Fig. 2 and Table II proved to be rather difficult to reproduce. The stimulation achieved by the addition of the boiled juice varied considerably. This was mainly caused by differences in the crude extracts, which were prepared from several varieties of avocado, the exact ripening stage of which was difficult to determine. Furthermore, accurate fractionations were made impossible by the low protein content of the crude extract (2 to 4 mg per ml).

These difficulties were overcome in the following way. The crude extract was saturated with $(NH_4)_2SO_4$, and the protein was collected as quantitatively as possible (49% yield) by centrifugation. The protein was dissolved in a small volume of Tris buffer and dialyzed (see "Materials and Methods"). In this concentrated extract (19 mg of protein per ml), two fractions could be separated by precipitation with $(NH_4)_2SO_4$. Table III shows the result of a typical fractionation. The heat-labile fraction (Fraction $I_{AV}$) previously precipitated from dilute extracts between 50 and 80% saturation appeared now between 20 and 60% saturation. The heat-stable protein previously lost by the $(NH_4)_2SO_4$ fractionation was now precipitated between 60 and 90% saturation (Fraction $II_{AV}$).

After the separation of the two fractions, a determination of their activities in the crude extract was undertaken. When an excess of either Fraction $I_{AV}$ or $II_{AV}$ was used under conditions of fatty acid synthesis (see legend for Fig. 2 for details), it was observed that in the crude extract, Fraction $I_{AV}$ (6.3 units[5] per ml; specific activity, 1.9), was about 8 times more active than Fraction $II_{AV}$ (0.78 unit per ml; specific activity, 0.23). However, the same assay is restrictive when used for the mutual determination of the two fractions themselves. This is evident from the *curves* with the *solid dots* in Figs. 3 and 4. In Fig. 3, Fraction $I_{AV}$ was varied in the presence of an excess of Fraction

[5] One unit is defined as 1 mμmole of malonyl-CoA incorporated into fatty acids per minute at 37° under the conditions of Fig. 2.

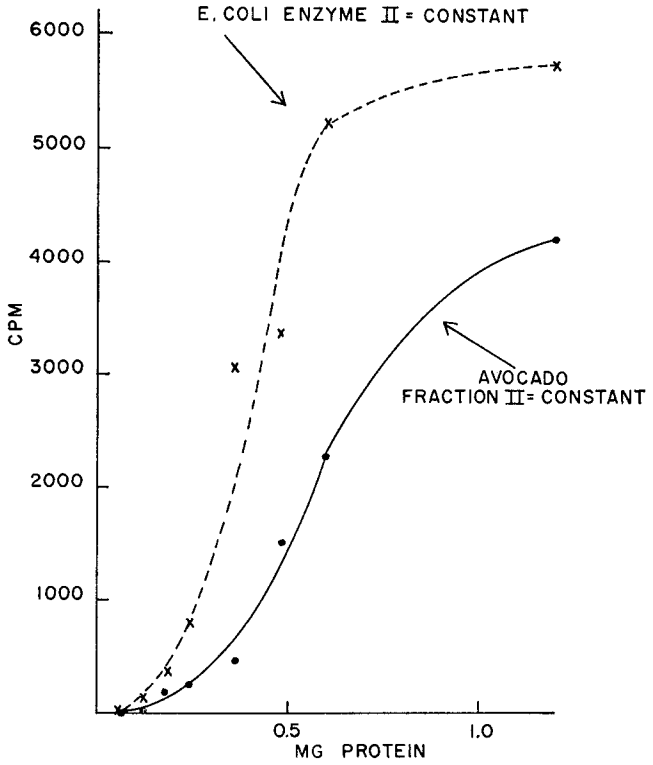


FIG. 3. The dependence of fatty acid synthesis on Fraction $I_{AV}$ in the presence of an excess of Fraction $II_{AV}$ or enzyme $II_{EC}$. The complete system was the same as in Fig. 2 with the exception that 0.044 μmole of malonyl-2-$^{14}$C-CoA (34,400 c.p.m.) was used. Enzyme $II_{EC}$ ($2.5 \times 10^{-2}$ unit, 0.57 unit per mg of protein) or Fraction $II_{AV}$ (1.1 mg of protein) was added to the corresponding tubes. Fraction $I_{AV}$ was added as shown on the *abscissa*.

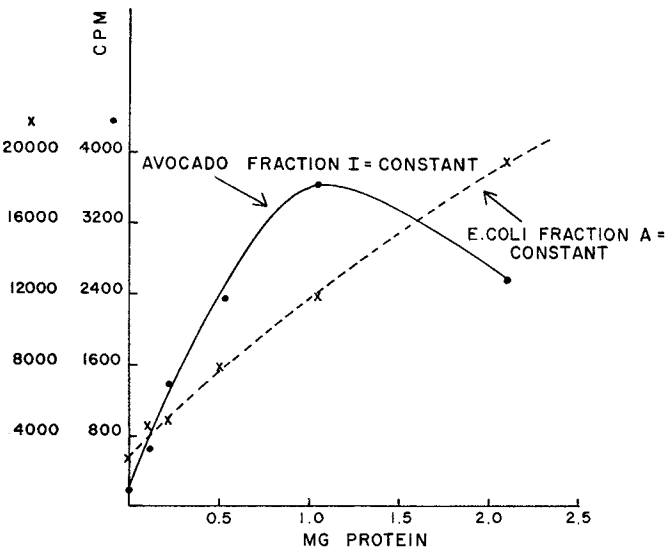


FIG. 4. The dependence of fatty acid synthesis on Fraction $II_{AV}$ in the presence of an excess of Fraction $I_{AV}$ or Fraction $A_{EC}$. The complete system was the same as in Fig. 3. Fraction A from *E. coli* ($1.7 \times 10^{-2}$ unit, $10.4 \times 10^{-3}$ unit per mg of protein) or Fraction $I_{AV}$ (1.2 mg) was added to the corresponding tubes. Fraction $II_{AV}$ was added as indicated on the *abscissa*.

$II_{AV}$. The first part of this curve shows an upward curvature, which makes an accurate determination of Fraction $I_{AV}$ impossible. The shape of the curve would indicate another dissociable cofactor. Additions of flavin mononucleotide, boiled extract, 2-mercaptoethanol, or bovine serum albumin were tested, but proved to be without effect. In Fig. 4, Fraction $II_{AV}$ was varied in the presence of an excess of Fraction $I_{AV}$. Whereas the first part of the curve seems to be fairly linear, it falls off rather abruptly with higher concentrations of Fraction $II_{AV}$. This would suggest an inhibitor in Fraction $II_{AV}$, which probably also decreases the rate in the first part of the curve (see below). Thus Fraction $I_{AV}$ can be assayed with Fraction $II_{EC}$ but not with Fraction $II_{AV}$, and Fraction $II_{AV}$ can be assayed with Fraction $I_{EC}$ but not with Fraction $I_{AV}$. Interactions appear to occur when Fractions $I_{AV}$ and $II_{AV}$ are combined at high concentrations. The nature of these interactions awaits the further purifications of the two fractions.

*Mutual Replacement of Fractions from Avocado and E. coli in Fatty Acid Synthesis*—As mentioned previously, a heat-stable (enzyme $II_{EC}$) and a heat-labile (Fraction $A_{EC}$) protein fraction were shown to be required for fatty acid synthesis in *E. coli* (13). From the comparative point of view, it seemed of considerable interest to study the cross-reactions of the bacterial enzymes with the avocado fractions. Moreover, the *E. coli* fractions would be useful for the purification of the avocado enzymes. Table IV summarizes an experiment in which the two fractions from avocado were combined with Fraction $A_{EC}$ and enzyme $II_{EC}$. It is seen that all fractions alone and the combinations Fraction $A_{EC}$ + Fraction $I_{AV}$ and enzyme $II_{EC}$ + Fraction $II_{AV}$ give little or no activity which is extractable with chloroform. The combinations of Fraction $A_{EC}$ + Fraction $II_{AV}$ and Fraction $I_{AV}$ + enzyme $II_{EC}$ give good activity. This result suggests, therefore, that the two heat-stable proteins have the same function.

The curves marked by *crosses* in Figs. 3 and 4 show the enzyme dependences of Fraction $I_{AV}$ and Fraction $II_{AV}$ in the presence of an excess of enzyme $II_{EC}$ and Fraction $A_{EC}$, respectively. The shape of the curve in Fig. 3 seems to be the same as the corresponding curve with Fraction $II_{AV}$. Fraction $I_{AV}$, however, is somewhat more active in the presence of enzyme $II_{EC}$. The leveling off of the curve with enzyme $II_{EC}$ is not related to a saturation effect. This becomes evident when the same amount of enzyme $II_{EC}$ is tested with Fraction $A_{EC}$. As shown already by Goldman *et al.* (13), the fatty acid synthesis of *E. coli* is linearly dependent on Fraction $A_{EC}$ in the presence of an excess of enzyme $II_{EC}$. Under the conditions of Fig. 3, such a dependence levels off after an incorporation 3 times greater than that catalyzed by Fraction $I_{AV}$. One would thus conclude that Fraction $I_{AV}$ becomes inhibitory at higher concentrations.

In the presence of an excess of Fraction $A_{EC}$ (Fig. 4, curve marked with *crosses*), a linear relationship exists between the concentration of Fraction $II_{AV}$ and incorporation of malonyl-CoA over a considerable range. The activity of Fraction $II_{AV}$ is greater in the presence of Fraction $A_{EC}$ than in the presence of Fraction $I_{AV}$.

*Analysis of Products (see Table IV)*—The products of the various combinations shown in Table IV were analyzed for the type and the amount of fatty acids formed. A combination of thin layer, gas-liquid, and paper chromatography was used.

*Fraction $I_{EC}$ + Enzyme $II_{EC}$*—In agreement with Goldman *et al.* (13), it was found that the main product of the *E. coli* synthetase is a monounsaturated fatty acid with 18 carbon atoms, presumably vaccenic acid. In addition, stearic and palmitic acids were formed in about equal amounts. All three chromatographic methods mentioned above lead to the same conclusion.

TABLE IV

*Mutual replacement of fractions from avocado and E. coli for fatty acid synthesis*

The conditions were the same as in Fig. 2 except that the amount of malonyl-2-$^{14}$C-CoA was 0.061 $\mu$mole (38,000 c.p.m.) and 5 $\mu$moles of 2-mercaptoethanol were added. Enzymes: Fraction $A_{EC}$, $1.7 \times 10^{-3}$ unit (10.4 units per mg of protein); Enzyme $II_{EC}$, $1.3 \times 10^{-2}$ unit (0.57 unit per mg of protein); Fraction $I_{AV}$, 1 mg; and Fraction $II_{AV}$, 0.6 mg. The samples were incubated for 60 minutes at 37°.

| Enzyme | Fatty acid synthesis | Product |
|---|---|---|
| | *c.p.m.* | |
| Fraction $A_{EC}$ | 164 | |
| Enzyme $II_{EC}$ | 2 | |
| Fraction $I_{AV}$ | 86 | |
| Fraction $II_{AV}$ | 0 | |
| Fraction $A_{EC}$ + enzyme $II_{EC}$ | 26,691 | $C_{18:1}$* (49%), $C_{16:0}$ (20%), $C_{18:0}$ (20%) |
| Fraction $I_{AV}$ + Fraction $II_{AV}$ | 1,303 | $C_{18:1}$ (82%), $C_{18:0}$ (18%) |
| Fraction $A_{EC}$ + Fraction $I_{AV}$ | 30 | |
| Fraction $A_{EC}$ + Fraction $II_{AV}$ | 2,20 | · compounds (90%) |
| Fraction $I_{AV}$ + enzyme $II_{EC}$ | 4,62 | $C_{18:1}$ (73%), $C_{18:0}$ (27%) |
| Enzyme $II_{EC}$ + Fraction $II_{AV}$ | 0 | |

* The subscript preceding the colon denotes the number of carbon atoms; the subscript following the colon, the number of double bonds.

*Fraction $I_{AV}$ + Fraction $II_{A_4}$*—In agreement with Yang and Stumpf (21), it was found that the products of the avocado synthetase are stearic and palmitic acids. It might be mentioned, however, that the ratio $C_{18}$:$C_{16}$ is about 4 in the crude extract, whereas it is about 0.5 under the conditions used in Table IV.

*Fraction $A_{EC}$ + Fraction $II_{A_4}$*—It has been shown (15, 16, 18, 19) that enzyme $II_{EC}$ functions as the carrier for the intermediates whereas Fraction $A_{EC}$ supplies all the heat-labile enzymes necessary for fatty acid synthesis in *E. coli*. If one assumes the same to be true for the avocado fractions, then a combination of Fraction $A_{EC}$ and Fraction $II_{AV}$ should yield vaccenic, stearic, and palmitic acids. Surprisingly, the expected results were not obtained.

In reverse phase paper chromatography, the product (free acids) moved with an $R_F$ of 0.89 in a solvent system of 85% acetic acid, indicating that the compound (or compounds) was polar in nature. This was confirmed by thin layer chromatography of the methyl esters on silica gel G with hexane-diethyl ether (10:6) as a solvent; most of the radioactivity had an $R_F$ of 0.26. This $R_F$ was identical with authentic $\beta$-hydroxy acid methyl esters with 10 to 14 carbon atoms. When this spot was eluted and subjected to thin layer chromatography on $AgNO_3$, part of the polar methyl esters were retained near the origin. This would indicate that a proportion of the polar acids were unsaturated. Fig. 5 shows gas-liquid chromatograms of the methyl

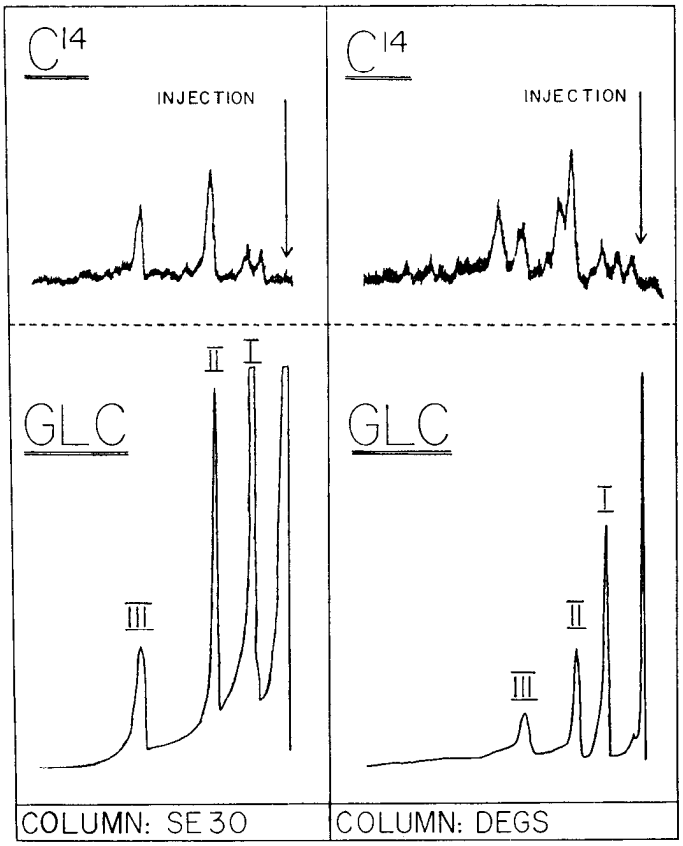


FIG. 5. Gas-liquid chromatography of the methylated compounds formed by the combination of Fraction $A_{EC}$ and Fraction $II_{AV}$. The carrier Peaks I, II, and III are the methyl esters of β-hydroxydecanoic, β-hydroxylauric, and β-hydroxymyristic acids, respectively. The SE-30 column (5 feet, ¼ inch, stainless steel) was operated at 222°. The diethylene glycol succinate (*DEGS*) column (6 feet, ¼ inch, stainless steel) had a temperature of 182°. Effluents from an Aerograph A-90P were passed into a Nuclear-Chicago continuous radioactive monitoring unit, and both the gas-liquid chromatographic (*GLC*) mass peaks and the radioactive peaks were recorded by a Texas Servo-Rite dual chart recorder.

ester of the product on two columns with concomitant $^{14}C$ tracings by a radioactive monitor system. On the SE-30 column, most of the radioactivity had the same retention times as the carrier peaks β-hydroxylauric acid methyl ester (Peak II) and β-hydroxymyristic acid methyl ester (Peak III). On the diethylene glycol succinate column, a further resolution of these peaks was obtained. Smaller peaks were now observed near Peaks II and III. The slower moving peaks behind II and III again would indicate unsaturation. β-Hydroxymyristic acid and β-hydroxylauric acid[6] have been definitely identified by recrystallization with authentic carrier acids to constant specific activity (solvents, ethanol-$H_2O$ and hexane). Further work will lead to the identification of the other peaks.

*Fraction $I_{AV}$ + Enzyme $II_{EC}$*—In this combination, the enzymes in Fraction $I_{AV}$ would be expected to determine the type of fatty acids synthesized. It was observed that the products of the combination were almost identical with the product of the combination of both avocado fractions, namely, palmitic acid.

[6] We are greatly indebted to Mr. Robert Simoni for his assistance in these experiments.

*Malonyl-CoA-$CO_2$ Exchange Reaction*—The acyl-CoA-dependent exchange of malonyl-CoA and $CO_2$ proved to be a very useful tool for the elucidation of the mechanism of the condensation reaction (12, 13). In analogy to the behavior of the enzymes from *E. coli* and *C. kluyveri*, it could be expected that the malonyl-CoA-$CO_2$ exchange reaction would also be operative with the avocado synthetase and that moreover both fractions would be required. This reaction would also provide a simple and rapid assay for the purification of the heat-stable Fraction $II_{AV}$. Table V summarizes the results of attempts to demonstrate the exchange reaction in the avocado system and cross-reactions with the corresponding *E. coli* fractions. In the presence of phosphate buffer at pH 6.3, the various fractions alone show little or no activity. Combination of the two fractions from avocado gives no activity. With the exception of the combined *E. coli* fractions, the only system which gives exchange is the combination of Fraction $A_{EC}$ with Fraction $II_{AV}$. The reaction is dependent on malonyl-CoA and caproyl-CoA. Experiments in imidazole buffer give essentially the same general picture as with phosphate buffer. However, now Fraction $I_{AV}$ catalyzes an exchange activity, which is not dependent on the presence of either Fraction $II_{AV}$ or enzyme $II_{EC}$. It is also worth mentioning that since Fraction $I_{AV}$ has little if any inhibitory effect when added to the exchange system of the combined *E. coli* fractions, the lack of exchange activity of the type described by Alberts

TABLE V

*Mutual replacement of fractions from avocado and E. coli for malonyl-CoA-$CO_2$ exchange reaction*

The complete system contained 50 μmoles of potassium phosphate buffer at pH 6.3 or 50 μmoles of imidazole-HCl buffer at pH 6.2. The other components were the same as described with by Alberts *et al.* (12) the exception that only 5 μmoles of 2-mercaptoethanol were used in the experiment with phosphate buffer. Enzymes: Fraction $A_{EC}$, $0.9 \times 10^{-3}$ unit ($4.3 \times 10^{-3}$ unit per mg of protein); enzyme $II_{EC}$, $2.6 \times 10^{-3}$ unit ($74 \times 10^{-3}$ unit per mg of protein); Fraction $I_{AV}$, 0.5 mg; and Fraction $II_{AV}$, 0.6 mg. The samples were incubated for 60 minutes at 30° and counted as described in "Experimental Procedure."

| Enzyme | Malonyl-CoA-$CO_2$ exchange reaction in | |
|---|---|---|
| | Phosphate buffer, pH 6.3 | Imidazole buffer, pH 6.2 |
| | *c.p.m.* | *c.p.m.* |
| Fraction $A_{EC}$ | 246 | 410 |
| Enzyme $II_{EC}$ | 0 | 0 |
| Fraction $I_{AV}$ | 276 | 2130 |
| Fraction $II_{AV}$ | 0 | 0 |
| Fraction $A_{EC}$ + enzyme $II_{EC}$ | 3900 | 8250 |
| Fraction $I_{AV}$ + Fraction $II_{AV}$ | 291 | 2110 |
| Fraction $A_{EC}$ + Fraction $I_{AV}$ | 629 | 2640 |
| Fraction $A_{EC}$ + Fraction $II_{AV}$ | 4610 | 4850 |
| Fraction $I_{AV}$ + enzyme $II_{EC}$ | 368 | 2340 |
| Enzyme $II_{EC}$ + Fraction $II_{AV}$ | 0 | 28 |
| Fraction $A_{EC}$ + enzyme $II_{EC}$ − malonyl-CoA | 0 | |
| Fraction $A_{EC}$ + enzyme $II_{EC}$ − caproyl-CoA | 1140 | |
| Fraction $A_{EC}$ + Fraction $II_{AV}$ − malonyl-CoA | 0 | |
| Fraction $A_{EC}$ + Fraction $II_{AV}$ − caproyl-CoA | 964 | |

*et al.* (12) cannot be ascribed to possible inhibition by avocado proteins.

The product of the reaction of Fraction $I_{AV}$ alone and the combination of Fraction $A_{EC}$ and Fraction $II_{AV}$ (incubation in imidazole buffer under conditions identical with those in Table V) is a malonyl thioester, presumably malonyl-CoA. Saponification of the product with alkali and subsequent isolation of the labeled malonic acid in the presence of carrier showed that essentially all the activity remained in this acid. After conversion of the product to the hydroxamate, over 90% of the radioactivity behaved like malonic monohydroxamate, as shown by paper chromatography and paper electrophoresis.

The demonstration of an exchange reaction with Fraction $A_{EC}$ and Fraction $II_{AV}$ provides a simple assay for the determination

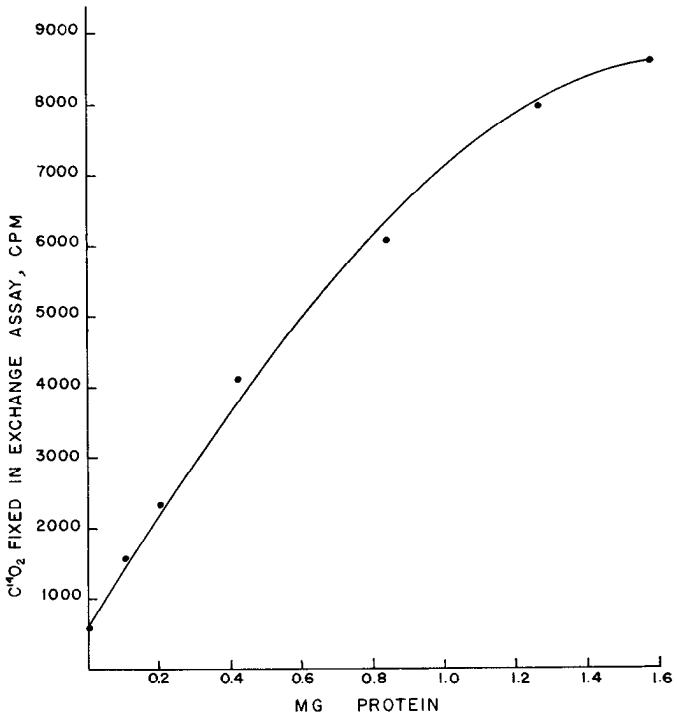


FIG. 6. Dependence of the malonyl-CoA-$CO_2$ exchange reaction on Fraction $II_{AV}$ in the presence of an excess of Fraction $A_{EC}$. The incubation conditions were the same as described by Alberts *et al.* (12); $3.4 \times 10^{-3}$ unit of Fraction $A_{EC}$ ($10.4 \times 10^{-3}$ unit per mg of protein) and the indicated amounts of Fraction $II_{AV}$ were added.

TABLE VI

*Replacement of CoA by pantetheine derivatives in exchange reaction*

The complete system contained the same components as given by Alberts *et al.* (12); 0.7 μmole of malonyl thioester and 0.1 μmole of caproyl thioesters were added. Enzymes: Fraction $A_{EC}$, $2.0 \times 10^{-3}$ unit ($10.4 \times 10^{-3}$ unit per mg of protein); Fraction $II_{AV}$, 0.5 mg of protein.

| Substrate | Radioactivity in malonyl thioester |
|---|---|
| | *c.p.m.* |
| Malonyl-CoA + caproyl-CoA | 3140 |
| Malonylpantetheine + caproylpantetheine | 5400 |
| Malonylpantetheine | 330 |
| Caproylpantetheine | 220 |

of Fraction $II_{AV}$. It can be seen from Fig. 6 that the exchange is linearly dependent on Fraction $II_{AV}$ in the presence of an excess of Fraction $A_{EC}$. As in the bacterial systems (34), the CoA derivatives can be replaced by the corresponding pantetheine derivatives. Table VI summarizes these data.

## DISCUSSION

Unlike the animal and yeast fatty acid synthetases, but like the bacterial systems, the soluble synthetase from avocado mesocarp is not a single complex but can be resolved into at least two components. The heat-stable Fraction II of the plant system has stability properties strikingly similar to enzyme $II_{EC}$ (13). In both the malonyl-CoA-$CO_2$ exchange reaction and the fatty acid synthetase system, Fraction $II_{AV}$ replaces enzyme $II_{EC}$ in the system in which enzyme $A_{EC}$ serves as the source of heat-labile enzymes. Conversely, enzyme $II_{EC}$ fully substitutes for Fraction $II_{AV}$ in a fatty acid-synthesizing system with Fraction $I_{AV}$ as the source of the heat-labile enzymes.

However, in the fatty acid-synthesizing system consisting of enzyme $A_{EC}$ and Fraction $II_{AV}$, the final products are a mixture of β-hydroxy long chain fatty acids rather than the expected products, namely, vaccenic, stearic, and palmitic acids. This unexpected result suggests that Fraction $II_{AV}$ inhibits an enzyme in enzyme $A_{EC}$ which normally converts the β-hydroxy acids to long chain fatty acids. Likely candidates for such an inhibition are enzymes $III_{EC}$ or $IV_{EC}$ derived from Fraction $A_{EC}$, the absence of which gives rise to polar acids (16).

Although both fractions from avocado are required for fatty acid synthesis, the requirement of both fractions for the malonyl-CoA-$CO_2$-exchange could not be demonstrated. The reason for this remains obscure. The condensation of acetyl-CoA and malonyl-CoA must occur under the conditions for fatty acid synthesis. Since Fraction $II_{AV}$ catalyzes the exchange effectively when combined with Fraction $A_{EC}$, Fraction $I_{AV}$ may be the site of the difficulty. The kinetics of Fig. 3 suggests a strong inhibitory effect by Fraction $I_{AV}$. At least two possibilities may exist: (*a*) the β-ketoacyl intermediate is inactivated, and in the presence of TPNH the reduction might be faster than the inactivation, or (*b*) the condensing enzyme in Fraction $I_{AV}$ may not catalyze the back reaction at all or at the required rate.

Since the malonyl-CoA-$CO_2$ exchange reaction with Fraction $A_{EC}$ depends on the presence of Fraction $II_{AV}$, a rapid assay for the purification of the heat stable enzyme from avocado is now available. Furture investigations will be directed toward the comparative aspects of the plant and the bacterial synthetases. It is of interest that lettuce chloroplast preparations incorporate malonyl-CoA to a slight extent in the absence of the heat-stable protein, but when either Fraction $II_{AV}$ or enzyme $II_{EC}$ is added, marked incorporation is noted.[7] The presence of the heat-stable protein would seem to be a limiting factor in the capacity of chloroplasts to synthesize fatty acid.

## SUMMARY

1. The soluble fatty acid synthetase from avocado mesocarp has been fractionated into a heat-labile (Fraction $I_{AV}$) and a heat-stable (Fraction $II_{AV}$) component. Either component alone is inactive in condensing malonyl coenzyme to form long chain fatty acid, but when combined they catalyze the synthesis of palmitic and stearic acids.

[7] J. Brooks and P. K. Stumpf, unpublished observations.

2. Fraction $II_{AV}$ appears to be a heat-stable protein which can replace enzyme $II_{EC}$ in the *Escherichia coli* synthetase system and the malonyl coenzyme A-$CO_2$ exchange.

3. When Fraction $II_{AV}$ is added to Fraction $A_{EC}$, $\beta$-hydroxylauric and $\beta$-hydroxymyristic acids are the major products rather than the expected products for Fraction $A_{EC}$, namely, vaccenic, palmitic, and stearic acids.

## REFERENCES


1. Brady, R. O., *Proc. Natl. Acad. Sci. U. S.*, **44,** 993 (1958).
2. Wakil, S. J., and Ganguly, J., *J. Am. Chem. Soc.*, **81,** 2597 (1958).
3. Lynen, F., *J. Cellular Comp. Physiol.*, **54** (Suppl. 1), 33 (1959).
4. Martin, D. B., Horning, M. G., and Vagelos, P. R., *J. Biol. Chem.*, **236,** 663 (1961).
5. Barron, E. J., Squires, C., and Stumpf, P. K., *J. Biol. Chem.*, **236,** 2610 (1961).
6. Goldman, P., Alberts, A. W., and Vagelos, P. R., *Biochem. and Biophys. Research Communs.*, **5,** 280 (1961).
7. Lennarz, W. J., Light, R. J., and Bloch, K., *Proc. Natl. Acad. Sci. U. S.*, **48,** 840 (1962).
8. Lynen, F., *Federation Proc.*, **20,** 941 (1961).
9. Lynen, F., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. V*, Academic Press, Inc., New York, 1962, p. 443.
10. Brady, R. O., Bradley, R. M., and Trams, E. G., *J. Biol. Chem.*, **235,** 3093 (1960).
11. Bressler, R., and Wakil, S. J., *J. Biol. Chem.*, **236,** 1643 (1961).
12. Alberts, A. W., Goldman, P., and Vagelos, P. R., *J. Biol. Chem.*, **238,** 557 (1963).
13. Goldman, P., Alberts, A. W., and Vagelos, P. R., *J. Biol. Chem.*, **238,** 1255 (1963).
14. Norris, A. T., and Bloch, K., *J. Biol. Chem.*, **238,** PC3133 (1963).
15. Alberts, A. W., *Federation Proc.*, **23,** 166 (1964).
16. Pugh, E. L., Sauer, F., and Wakil, S. J., *Federation Proc.*, **23,** 166 (1964).
17. Goldman, P., and Vagelos, P. R., *Biochem. and Biophys. Research Communs.*, **7,** 414 (1962).
18. Goldman, P., Alberts, A. W., and Vagelos, P. R., *J. Biol. Chem.*, **238,** 3579 (1963).
19. Majerus, P. W., and Vagelos, P. R., *Federation Proc.*, **23,** 166 (1964).
20. Stumpf, P. K., and Barber, G. A., *J. Biol. Chem.*, **227,** 407 (1957).
21. Yang, S. F., and Stumpf, P. K., *Biochim. et Biophys. Acta*, **84,** No. 6 (1964).
22. Overath, P., and Stumpf, P. K., *Federation Proc.*, **23,** 166 (1964).
23. Eggerer, H., and Lynen, F., *Biochem. Z.*, **335,** 540 (1962).
24. Antenrieth, W., *Ber. deut chem. Ges.*, **34,** 182 (1901).
25. Simon, E. J., and Shemin, D., *J. Am. Chem. Soc.*, **75,** 2520 (1953).
26. Stadtman, E. R., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. III*, Academic Press, Inc., New York, 1957, p. 938.
27. Beisenherz, G., Boltze, H. J., Bucher, T., Czok, R., Garbade, K. H., Meyer-Arendt, E., and Pfleiderer, G., *Z. Naturforsch., Pt. b,* **8,** 555 (1953).
28. Kalckar, H. M., *J. Biol. Chem.*, **167,** 461 (1947).
29. Mangold, H. K., Lamp, B. G., and Schenk, H., *J. Am. Chem. Soc.*, **77,** 6070 (1955).
30. Buchanan, M. A., *Anal. Chem.*, **31,** 1616 (1959).
31. Mangold, H. K., *J. Am. Oil Chemists' Soc.*, **38,** 708 (1961).
32. Morris, L., *Chem. and Ind. (London)*, 1238 (1962).
33. Vagelos, P. R., *J. Biol. Chem.*, **235,** 346 (1960).
34. Hatch, M. D., and Stumpf, P. K., *Plant Physiol.*, **37,** 121 (1962).