# STUDIES ON THE MECHANISM OF FATTY ACID SYNTHESIS*

## I. PREPARATION AND PURIFICATION OF AN ENZYME SYSTEM FOR RECONSTRUCTION OF FATTY ACID SYNTHESIS

SALIH J. WAKIL, JOHN W. PORTER** AND DAVID M. GIBSON
*Institute for Enzyme Research, University of Wisconsin, Madison, Wis. (U.S.A.)*

During the past few years the mechanism of fatty acid oxidation has been elucidated in several laboratories[1,2,3,4]. This was shown to follow the general scheme of β-oxidation as proposed by KNOOP in 1904[5]. The various enzymes involved in the fatty acid oxidation sequence have been prepared in a highly purified state and each step has been shown to be reversible. This fact led many workers to conclude that the synthesis of fatty acids occurs by the reversal of the β-oxidation in the presence of the appropriate enzymes and coenzymes[3].

Although it has been shown experimentally[6] that fatty acids are oxidized all the way to acetyl CoA in the presence of a combination of the purified enzymes, there has been no similar demonstration of the synthesis of long-chain fatty acids starting from acetate. STANSLY AND BEINERT[7] attempted to convert acetyl CoA to higher fatty acyl derivatives of CoA*** in the presence of DPNH, a reduced dye and the purified enzymes of the fatty acid oxidation cycle. They were not able to demonstrate any significant formation of a fatty acyl CoA of longer carbon chain than that of butyryl CoA. This result, therefore, suggested that the problem of fatty acid synthesis was not merely one of simple reversal of fatty acid oxidation.

STADMAN AND BARKER[8,9,10] were the first to demonstrate the conversion of of labeled acetate into short-chain fatty acids by a water-soluble enzyme preparation which they obtained from *Clostridium kluyveri*. Later on, BRADY AND GURIN[11,12,13] reported the synthesis of long-chain fatty acids from acetate, first in homogenates and later in a particle-free extract prepared from pigeon liver. This system incorporated labeled acetate predominantly into fatty acids rather than into glycerides. On treatment of the extract with charcoal it was possible to demonstrate a requirement for ATP, DPN and CoA. They also observed that citrate markedly stimulated the

* This work has been supported by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council; by a research grant, H-2236(C), from the National Heart Institute of the National Institutes of Health, Public Health Service; and aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Wisconsin, NR 120-264.

** Post-doctoral Trainee of the National Heart Institute, National Institutes of Health. Present address: Veterans' Administration Hospital and Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin.

*** The following abbreviations are used: coenzyme A (CoA), adenosine triphosphate (ATP), triphosphopyridinenucleotide (TPN), reduced TPN (TPNH), diphosphopyridinenucleotide (DPN), reduced DPN (DPNH), reduced glutathione (GSH), pigeon liver supernatant (PLS), $^{14}$C-carboxyl labeled acetate (Ac*).

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synthesis. Similar results were obtained by POPJÁK AND TIETZ[14,15,16] who used soluble preparations from the mammary gland of lactating rats and rabbits.

Since attempts to reverse the β-oxidation cycle have failed, the problem of synthesis has been re-examined. Our point of departure was a soluble enzyme system prepared from pigeon liver, which is similar to that described by BRADY AND GURIN[11]. In this and succeeding communications various aspects of the fatty acid synthesizing system will be considered—the preparation of the enzymes, the requirement for cofactors and the nature of the product. The first crude extract has been fractionated into three different fractions and each of these were further purified so that about a 50-fold purification was achieved with respect to the original extract[17]. The present communication will deal with the purification of the enzymic components and with the properties of the synthesizing system.

## EXPERIMENTAL

### *Assay*

The synthesis of fatty acids was measured by the extent to which radioactive acetate (carboxyl-labeled) was incorporated into fatty acids. For routine assays the following components were pipetted into a glass-stoppered test tube (16 × 150 mm): CoA 0.02 μmoles, ATP 2.5 μmoles, $Mn^{++}$ 0.02 μmoles, *iso*citrate 6.0 μmoles, TPN 0.2 μmoles, glucose-1-phosphate 15 μmoles, DPN 0.2 μmoles, lipoic acid 0.15 μmoles, reduced glutathione 8.0 μmoles, phosphate buffer (pH 6.5) 30 μmoles, and acetate-1-$^{14}$C 4 μmoles (150,000 to 300,000 c.p.m.). The final volume was 0.5 ml. The reaction was started by the addition of the enzyme (0.6 to 3.0 mg). The tube was flushed with a gentle stream of $N_2$ gas for 15 to 30 seconds, stoppered and incubated in a water bath at 38° for 2 hours. At the end of the incubation time the reaction was stopped by the addition of 0.5 ml of 10% alcoholic KOH and the tube was put into a boiling water bath for half an hour. At the end of this time saponification was complete, and 0.5 ml of 2 *N* HCl solution was added to bring the pH to 2 or 3. Four ml of *n*-pentane were added, the tube was stoppered tightly and the mixture was shaken for about 1.5 minutes*. The two phases were allowed to separate and the pentane layer was transferred by means of a medicine dropper into a 15 ml graduated centrifuge tube. The extraction procedure was repeated twice in exactly the same manner. The pooled pentane extracts were concentrated in a stream of hot air from a hair drier which was passed over the test tubes until a volume of less than 1.0 ml was reached. The air stream was stopped, the final volume was adjusted to 1.0 ml, and 50 μl of the pentane solution were plated on an aluminum plate. The radioactivity of the aliquot was then measured. The radioactivity measured in this way represents the amount of acetate incorporated into the long-chain fatty acids, since under the above conditions only the long-chain fatty acids are extracted into the pentane. As shown in Table I, the recovery of $^{14}$C-palmitic acid from the reaction mixture was satisfactory, when the mixture was subjected to the described procedures. Only the $C_{10}$ to $C_{18}$ acids were extracted in this way as indicated by paper chromatography in the butanol–ammonia[18] and kerosene–acetic acid[19] systems. Full details of the nature of the reaction products will be discussed in a later communication[20].

### *Unit of enzyme activity*

The unit of enzyme activity has been defined as the amount of enzyme necessary to incorporate 1 μmole of radioactive acetate into long-chain fatty acids per two hours under the above conditions. The specific enzyme activity, therefore, has the dimensions of units of enzyme per 1 mg of protein.

### *Plating and counting*

The fatty acids were dissolved in pentane and known aliquots (50 to 100 μl) were plated on aluminum discs. The spots of infinite thinness had a diameter of 5 to 10 mm. The radioactivity was measured with a thin (1.5 mg per cm$^2$) end window G.M. tube. Apart from the usual statistical error of the counting equipment which is less than 3% the variation in counting between duplicate samples which had been submitted to the extraction procedure was less than 5%. This, therefore, gives an overall error of less than 15%. When the radioactivity on the paper chromatogram was measured, the paper strip was cut into small strips 1 cm in length and the radioactivity on each strip was measured directly, in the end window counter.

* The tubes were purchased from the Ace Glass Company and were fitted with hollow glass stoppers. There was no leakage of the pentane on shaking.

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*Chemicals*

The chemicals used in this investigation were commercial products. ATP, DPN and TPN were obtained from the Sigma Chemical Company and isocitrate from the California Foundation for Biochemical Research in the form of *dl*-lactone. The lactone was converted to the free acid by treatment with excess alkali. Lipoic acid was obtained from the California Foundation for Biochemical Research and CoA from the Pabst Laboratories.

TABLE I

PER CENT RECOVERY OF $^{14}$C-PALMITIC ACID* FROM THE ASSAY MIXTURE

| *Expt. No.* | *Total counts added* | *Total counts recovered* | *Per cent recovered* |
|---|---|---|---|
| 1 | 2,500 | 2,000 | 81 |
| 2 | 5,000 | 4,600 | 92 |
| 3 | 10,000 | 10,600 | 106 |
| 4 | 15,000 | 17,100 | 114 |
| 5 | 20,000 | 20,300 | 101 |
| 6 | 30,000 | 31,400 | 104 |
| 7 | 40,000 | 39,100 | 98 |
| 8 | 50,000 | 51,900 | 104 |

Each tube contained all the cofactors routinely used in the assay of fatty acids synthesis (see section on assay) plus 3 mg of enzyme system and variable amounts of $^{14}$C-palmitic acid in a total volume of 0.5 ml. The tubes were saponified immediately with alcoholic KOH and the fatty acids were isolated as discussed in assay.

* The palmitic acid added had a specific activity of 50,000 c.p.m. per μmole.

*Preparation of the fatty acid synthesizing enzyme system*

*Pigeon liver extract.* After the pigeons were decapitated, the livers were removed and chilled in crushed ice. All subsequent operations were carried out at 0 to 4° except when otherwise stated. 200 g of chilled liver were blended with 300 ml of phosphate–bicarbonate buffer[11] (final concentration of 0.1 *M* potassium phosphate and 0.07 *M* $KHCO_3$ pH 9.0) in a Waring blendor at full speed for 30 seconds. The homogenate was centrifuged for 15 minutes at 1,000 × *g*. The supernatant was decanted through a cheese cloth and was further centrifuged at 100,000 × *g* for half an hour in the Spinco preparative centrifuge. The clear supernatant was collected. This fraction is referred to as "pigeon liver supernatant" (PLS). It contains all the necessary enzymes for the synthesis of fatty acids and has a specific activity of 0.02 to 0.05.

*Fractionation of the pigeon liver supernatant (PLS).* The PLS was fractionated with solid ammonium sulfate into four different fractions as follows. For each 100 ml of PLS, 13.4 g of solid ammonium sulfate were added slowly with constant stirring. This gave a solution which was 25% saturated with respect to ammonium sulfate. The mixture was stirred for an additional 15 minutes in order to complete the precipitation of the protein. The precipitate was separated by centrifugation at 20,000 × *g* for 15 min, taken up in 10 to 15 ml of 0.04 *M* $KHCO_3$, and the solution was dialyzed against 4 l of the same buffer for 3 to 4 hours. This fraction (designated as $R_1$) was orange-yellow in color and milky in appearance.

The supernatant was further fractionated with solid ammonium sulfate and the fraction precipitating between the limits of 25 to 40% saturation with respect to ammonium sulfate was isolated in the same manner as described above. The residue was taken up in 10 to 15 ml of 0.04 *M* $KHCO_3$ and the solution was dialyzed against the same buffer for 3 to 4 hours. This fraction (called $R_2$) was orange in color and opaque in appearance.

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To the remaining supernatant solution solid ammonium sulfate was added in sufficient amount to obtain 50% saturation and the mixture was allowed to stand with constant stirring for at least 15 minutes. The mixture was centrifuged as above, the supernatant was saved, and the residue ($R_3$) was taken up in 0.04 *M* $KHCO_3$ solution. This fraction was clear and red in color.

The supernatant was treated further with solid ammonium sulfate. The saturation level was brought to 65%. The precipitate obtained by centrifugation was taken up in 10 to 15 ml of 0.04 *M* $KHCO_3$ and the solution was dialyzed against the same buffer for 3 to 4 hours. This fraction (called $R_4$) was transparent and deep red. The remaining supernatant fluid was discarded.

*Reconstitution of the fatty acid synthesizing system*

The four fractions obtained were used to reconstruct the fatty acid synthesizing system. It was found that none of these fractions alone nor any combination of any two of these fractions would catalyze the synthesis of fatty acids from acetate (see Table II). The only combination that catalyzed an active synthesis of long-chain fatty acids from acetate was $R_1 + R_2 + R_4$, as shown in Table II. Fraction $R_3$ has no effect at all on the synthesis. If anything it is slightly inhibitory. The specific activity calculated on the basis of total protein of the combined ractions shows about a 10-fold purification as compared to PLS (*cf.* Table II). The reconstituted system shows a more clearly defined requirement for the various components than the original extract. The cofactor requirements will be discussed in more detail in the second paper of this series[21].

TABLE II

RECONSTITUTION OF THE FATTY ACID SYNTHESIZING SYSTEM

| *Fraction* | *Acetate converted into fatty acids in μmoles* | *Specific activity μmoles Ac*/mg prot./2 h* |
|---|---|---|
| PLS | 0.21 | 0.032 |
| $R_1$ | 0.006 | |
| $R_2$ | 0.002 | |
| $R_3$ | 0.000 | |
| $R_4$ | 0.000 | |
| $R_1 + R_2$ | 0.010 | |
| $R_1 + R_2 + R_3$ | 0.080 | |
| $R_1 + R_2 + R_4$ | 0.920 | 0.310 |
| $R_1 + R_2 + R_3 + R_4$ | 0.600 | |
| $R_2 + R_4$ | 0.002 | |

Each tube contained all the cofactors required (see assay) and the reaction was started by the addition of the various fractions shown above. 6.5 mg of PLS were used and 1 mg of protein of each of the subfractions ($R_1$, $R_2$, $R_3$ and $R_4$) was added whenever mentioned. Total volume was 0.5 mg and the tubes were incubated at 38° under $N_2$ for 2 hours.

The optimum amount that had to be added of each component used was determined in experiments in which two of the enzyme components were kept constant at a given level while the concentration of the third was varied (*cf.* Fig. 1). The results show that the conversion of acetate into long-chain fatty acids is proportional to the amount of each fraction added over a limited range beyond which saturation is reached. The specific activity of each fraction can, therefore, be calculated from the

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straight portion of the curve. This specific activity has been defined as the number of μmoles of radioactive acetate converted to fatty acids per mg of the limiting fraction per two hours of incubation. This type of assay was used in further purification and separate study of each fraction as will be shown later.

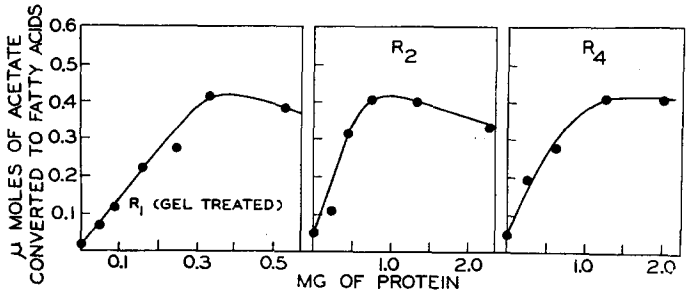


Fig. 1. Each tube contained all the cofactors routinely used in the assay of fatty acid synthesis (see section on assay). To $R_1$ curve was added 1.3 mg protein of $R_2$ plus 1.10 mg of $R_4$. The reaction was started by the addition of varied amounts of purified $R_1$ as indicated. Total volume was 0.5 ml and the tubes were incubated at 38° under $N_2$ for 2 hours. To $R_2$ curve was added 1.7 mg of purified $R_1$, 1.10 mg of $R_4$ and varied amounts of $R_2$ as indicated. The total volume was 0.5 ml and the tubes were incubated at 38° under $N_2$ for 2 hours. To $R_4$ curve was added 1.7 mg of purified $R_1$, 1.3 mg $R_2$ and varied amounts of $R_4$ as indicated. The total volume was 0.5 ml and the tubes were incubated at 38° under $N_2$ for 2 hours.

*Purification of $R_1$*

The protein concentration of $R_1$ was adjusted to 30 to 40 mg per ml by the addition of 0.04 *M* $KHCO_3$ solution. To this enzyme solution was then added slowly 3 parts (v/v) of precooled calcium phosphate gel[22] (27 mg per ml) with gentle stirring. The mixture was allowed to stand for three minutes with occasional stirring and was then centrifuged for 2 minutes at 20,000 × *g*. The supernatant was saved and the precipitate was discarded. The supernatant solution was treated again with a fresh charge of calcium phosphate gel as before and the mixture was centrifuged for 3 minutes. The supernatant was saved and the precipitate was discarded. The volume was then measured and enough solid ammonium sulfate was added to bring the degree of saturation to 30%. Sufficient time (about 20 min) was allowed for the precipitate to form maximally and this was separated by centrifugation at 20,000 × *g* for 15 minutes. The supernatant solution was discarded and the residue was dissolved in 0.04 *M* phosphate buffer of pH 7.0. The final solution was clear yellow and had a protein concentration of 30 to 40 mg per ml. When this preparation was assayed in the usual system with $R_2$ and $R_4$ as supplementary enzymes, it showed a specific activity of 4.0 μmoles of radioactive acetate converted to fatty acids per mg protein per 2 hours or a purification of about 5-fold over $R_1$ with a 90% recovery of the active proteins. At this stage the activity of this fraction declined rapidly when it was frozen and thawed repeatedly. But the material is stable for 2 to 3 days if kept frozen at —20°. In the presence of GSH at a concentration of 0.005 *M* the fraction was more stable and could be stored at —20° for 4 to 6 days without appreciable loss in activity. With some calcium phosphate gel preparations the active enzyme was adsorbed on the gel when $R_1$ was treated as described above. Under these conditions, however, the active protein is eluted from the gel by repeated washing of the gel (5 to 7 times) with 10 to 20 ml of 0.1 *M* phosphate buffer pH 7.0. The eluates were pooled and the enzyme was

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precipitated with solid ammonium sulfate in exactly the same manner as described previously. This modification resulted in approximately the same degree of purity of the enzyme.

The clear yellow solution of the enzyme can be further purified by centrifugation in the Spinco preparative centrifuge at 141,000 × *g* for 2 to 3 hours. The supernatant had no activity and was discarded. The yellowish-brown residue was dissolved in 0.04*M* $KHCO_3$ buffer and enough GSH was added to a final concentration of $5 \cdot 10^{-3} M$. The resulting solution had a concentration of 25 to 35 mg of protein per ml, and the full activity of $R_1$. This treatment yielded 1.5 to 2 fold purification with no loss in total units.

*Alcohol fractionation of $R_2$*

$R_2$ was adjusted to a protein concentration of 30 to 40 mg per ml and was fractionated with ethanol. During the addition of ethanol, the temperature of the solution was kept as low as possible without freezing the solution. At the end of each addition of alcohol, the solution was allowed to stand for 5 minutes and was then centrifuged at low temperature for 10 minutes at 20,000 × *g*. The precipitate was taken up in 0.04 *M* $KHCO_3$ and the solution was immediately dialyzed against the same buffer. Care should be exercised to avoid long exposure of the enzyme to ethanol. The supernatant solution was then used in the next fractionation step. Fractions were obtained at 0 to 17, 17 to 22, 22 to 30, 30 to 40 and 40 to 50% ethanol concentration. Only the fraction precipitating between 22 and 30% alcohol was found to be active. This fraction ($R_{23}$) was clear and brown-yellowish in color.

*Alcohol fractionation of $R_4$*

Fraction $R_4$ was fractionated with alcohol in the same manner as $R_2$ and fractions were prepared at 0 to 20, 20 to 30, 30 to 35, 35 to 40, 40 to 50 and 50 to 60% ethanol concentration. Only the fractions precipitating between alcohol concentrations of 20 to 30 and 50 to 60% were found to be required for the synthesis of long-chain fatty acids. These fractions were designated $R_{43}$ and $R_{46}$ respectively.

*Dependence on the concentration of the enzymic components*

As stated above, only $R_1$, $R_2$ and $R_4$ or the mentioned subfractions were required for the complete synthesis of fatty acids from acetate. The optimum concentration for each component fraction was determined as described above and the results are shown in Fig. 1. Any one fraction in large excess inhibited the activity of the system. However, if the amounts of all three fractions were increased proportionately, the amount of acetate converted into fatty acids showed a proportional increase. The reason for the observed inhibition is not clear. However, in a complex system of many components such an observation is not surprising.

The reconstituted system of $R_1$ (gel-treated), $R_{23}$, $R_{43}$ and $R_{46}$ under optimal conditions incorporates 1 to 2 μmoles of acetate into fatty acids per mg of total protein in 2 hours at 38°. This corresponds to an average of 50-fold purification of the system above the initial liver extract (PLS).

*Cofactor requirements*

A detailed account of the cofactor requirements and the dependence of the system on the concentration of these factors will be presented in the second paper of this series[21].

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Sufficient to say here that the following cofactors are needed for optimal synthesis of fatty acids from acetate: ATP, CoA, DPNH, TPN, *iso*citrate, $Mn^{++}$, lipoic acid and GSH.

*Effect of pH on the synthesis*

The pH of the incubation mixture has a profound effect on the synthesis of fatty acids from acetate. Fig. 2 demonstrates the results of such studies. The system has a very sharp optimum at pH 6.5 in presence of phosphate or histidine ions. Tris(hydroxymethyl)aminomethane inhibits the system very strongly and therefore cannot be used as a buffer. It is of interest to note here that the system is very inefficient at pH 8 in contrast to the behavior of the HELE AND POPJÁK system of mammary gland[23].

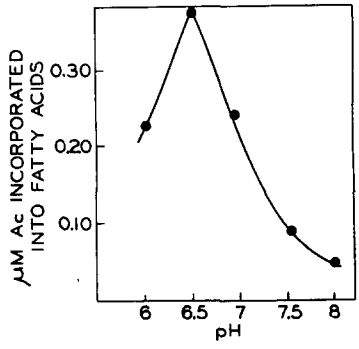


Fig. 2. The concentration of the various cofactors added was exactly as described in section on assay except for the phosphate buffer. To each tube was added 30 μmoles of potassium phosphate buffer at the pH indicated. The reaction was started by the addition of 1.5 mg of $R_2$, 0.5 mg $R_2$ and 0.7 mg of $R_4$. The total volume was 0.5 ml and the tubes were incubated for 2 hours at 38°.

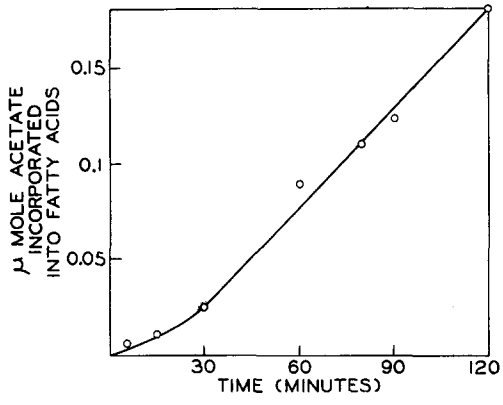


Fig. 3. The effect of time on the incorporation of acetate into fatty acids. The various components added were exactly as described in the section on assay. The reaction was started by the addition of 0.5 mg of $R_1$, 0.6 mg of $R_2$ and 0.5 mg of $R_4$. The total volume was 0.5 ml and the tubes were incubated at 38° for the time indicated on the graph.

*Dependence of synthesis on time*

The rate of incorporation of acetate into fatty acids as a function of time is shown graphically in Fig. 3. There is a lag period during the first 30 minutes. This lag period cannot be attributed to the accumulation of intermediates which are needed for synthesis, since no such intermediates could be isolated or demonstrated. The evidence for non-accumulation of intermediates will be considered in a later communication of this series[20].

*Effect of SH-compounds*

There is an absolute requirement for some suitable SH-compounds in fatty acid synthesis (*cf.* Table III). Various SH-compounds could be used in this capacity. These SH-compounds may either maintain CoA in its reduced form or reduce SH groups of the enzymes or both. Since CoA is required in the system it is reasonable to assume that the reduced form of the coenzyme is used for the synthesis of the acyl CoA derivatives[24,25,26]. However, it was found that the SH-compounds have an effect on the state of the enzyme. This conclusion emerged from the studies on the lag period.

*References p. 460/461.*

TABLE III

| *Expt. No.* | *SH-Compounds* | *Amount added µmoles* | *Acetate incorporated in fatty acid µmoles* |
|---|---|---|---|
| 1 | none | | 0.000 |
| 2 | GSH | 12 | 0.41 |
| 3 | cysteine | 12 | 0.50 |
| 4 | 2,3 dimercaptopropanol (BAL) | 4 | 0.30 |
| | | 12 | 0.14 |
| 5 | 1,3 dimercaptopropanol | 4 | 0.14 |
| | | 12 | 0.35 |
| 6 | thioglycol | 12 | 0.10 |

Each tube contained all the cofactors required (see assay) except the GSH as in experiment number 1. To the rest of the tubes, a given amount of an SH-compound was added respectively. The reaction was started by the addition of the enzyme fractions (total protein 2 mg) and the tubes were incubated at 38° under $N_2$ for two hours.

When cysteine was used in the system instead of glutathione, the lag period was shortened or completely eliminated. This result was also noted when the gel-treated $R_1$ that was stored in the presence of GSH was used in the synthetic system. This pointed to the presence of essential SH-groups in at least one of the enzyme fractions, namely $R_1$, as indicated by these effects of cysteine and GSH on the lag period. When gel-treated $R_1$ was prepared under carefully controlled conditions designed to minimize exposure to air, it was possible to eliminate the lag period during the synthesis of fatty acids. However when such a preparation was treated with hydrogen peroxide the lag period was again observed. It could then again be eliminated by preincubation of the gel-treated $R_1$ with either GSH, cysteine or BAL. This study suggests the role of enzyme-bound SH-groups in the synthesis of the fatty acids from acetate.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. DAVID E. GREEN for his advice and encouragement in this investigation. We wish to express our appreciation to Mrs. MARY H. BENOWITZ for her expert technical assistance.

SUMMARY

The synthesis of long-chain fatty acids ($C_{16}$) from acetate has been shown to be catalyzed by at least four different subfractions derived from a soluble fraction (100,000 × *g*) of pigeon liver. Methods for the preparation of each fraction have been described. About a 50-fold purification of the composite synthetic system over the original supernatant was achieved.

The composite system shows an optimum pH of 6.5 and the activity is proportional to both protein concentration and time under appropriate conditions. SH-compounds are needed for activity. It was shown that these compounds, at least in part, are active by virtue of their capacity to maintain the SH-groups of the component enzymes in reduced form.

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Received February 5th, 1957

# STUDIES ON THE BIOCHEMISTRY OF CONTRACTION AND RELAXATION IN GLYCERINATED MUSCLE

## THE EFFECTS OF PHOSPHOENOLPYRUVATE

C. MOOS*,**

*Department of Chemistry, Northwestern University, Evanston, Ill.*
*and Subcommittee on Biophysics, Columbia University, New York City (U.S.A.)*

and

L. LORAND

*Department of Chemistry, Northwestern University, Evanston, Ill. (U.S.A.)*

### INTRODUCTION

In 1949 SZENT-GYÖRGYI[1] introduced a new approach to the biochemical study of muscle by extracting whole fibers in cold 50% aqueous glycerol. With this procedure, the basic structure of the contractile apparatus remains essentially unaltered, while some constituents are removed. The effects of the removal of these constituents, as well as of returning them individually to the muscle, may be observed. SZENT-GYÖRGYI showed that rabbit psoas fibers extracted in the above manner contract

* Submitted by C. Moos in partial fulfillment of the requirements for the degree of Doctor of Philosophy, under the Joint Committee on Graduate Instruction, Columbia University.
** Interdisciplinary Predoctoral Fellow, Columbia University, 1953–1954.

*References p. 473/474.*