STUDIES ON THE MECHANISM OF FATTY ACID SYNTHESIS*

II. COFACTOR REQUIREMENTS OF THE SOLUBLE PIGEON LIVER SYSTEM

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In 1952 BRADY AND GURIN[5] reported the synthesis of long-chain fatty acids from acetate in a soluble extract prepared from pigeon liver. Addition of Mg^{++} and citrate markedly stimulated fatty acid synthesis. After the extract had been treated with charcoal[6] synthesis was minimal unless CoA§§, ATP and DPN were added in addition to Mg^{++} and citrate. However, after dialysis of the extract the activity declined almost completely and could not be restored by the addition of the above cofactors.

POPJÁK AND TIETZ[7,8] in 1955 reported that a soluble extract of mammary gland would synthesize fatty acids in the presence of added ATP and α-ketoglutarate. Citrate could not replace α-ketoglutarate. After treatment of the extract with Dowex-1 ion-exchange resin, a requirement for CoA and DPN emerged.

In the first paper of this series[9] the preparation and purification of a soluble enzyme system from pigeon liver which actively synthesizes long-chain fatty acids from acetate was described. Four enzyme fractions were prepared which only on recombination catalyzed fatty acid synthesis. The present paper describes in detail the cofactor requirements for synthesis. With the most purified enzyme fractions the following cofactors are necessary: ATP, CoA, DPNH, TPN, Mn^{++}, *iso*citrate and a suitable sulfhydryl compound such as glutathione or cysteine. In the unfractionated

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§§ The following abbreviations are used: ATP, adenosine triphosphate; CoA, CoASH, Coenzyme A; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN and TPNH oxidized and reduced triphosphopyridine nucleotide; GSH, glutathione; BAL, 2,3-dimercaptopropanol; PLS, pigeon liver supernatant.

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pigeon liver supernatant solution (PLS) several of these cofactor requirements could not be demonstrated. For example, with dialyzed PLS glucose-1-phosphate and DPN (in place of DPNH) were necessary for synthesis. Also citrate could be employed with dialyzed PLS but not in purified systems unless aconitase were added. Such variations are not unusual considering the multiplicity of enzymes involved in this complex system.

METHODS AND MATERIALS

Details of the preparation and fractionation of the pigeon liver supernatant (PLS) have been given in the previous communication of this series[9]. When the cofactor requirements of PLS were under study, the preparation was dialyzed against 20 volumes of $3 \cdot 10^{-3}$ *M* phosphate or phosphate–bicarbonate buffer of pH 7.0 for a total period of 4 hours at 0° and with a change of buffer at the end of 2 hours. The treatment of dialyzed PLS with Dowex-1-Cl was as follows: The dialyzed enzyme solution (30 ml containing 50 mg of protein per ml) was mixed with packed, wet Dowex-1-Cl (3 ml at pH 6.7). The mixture was stirred for 30 min at 0° and the resin was then removed by centrifugation.

The experimental procedures and details for the assay of fatty acid synthesis have been described fully in the first communication of this series[9].

The various chemicals used in this investigation were commercial products. ATP, TPN, DPN, and DPNH were obtained from the Sigma Chemical Co., TPNH and CoA from the Pabst Laboratories, glucose-1-phosphate and glutathione from the Schwartz Laboratories and lipoic acid, cysteine and *iso*citrate from the California Foundation for Biochemical Research. *Iso*citrate was obtained in the form of the *dl*-lactone, which was converted to the free acid by treatment with excess alkali. The concentration of *d-iso*citrate was determined in the *iso*citric dehydrogenase assay system[10]. The amount of TPN reduced to TPNH was taken to be stoichiometric with the amount of *d*-isocitrate in the sample which was being assayed. The light absorption at 260 mμ (adenine absorption) was taken as a measure of the concentration of ATP, DPN and TPN. The concentrations of DPNH and TPNH were determined from the absorbance at 340 mμ. CoASH was estimated by the nitroprusside reaction of GRUNERT AND PHILLIPS[11]. Aldolase and glyceraldehyde-3-phosphate dehydrogenase were obtained from the Worthington Biochemical Corporation and glucose-6-phosphate dehydrogenase was obtained from the Sigma Chemical Co.

RESULTS

It has been shown in a previous communication[9] that a system for synthesis of fatty acid from acetate can be reconstituted from four different fractions derived from pigeon liver supernatant. For maximal conversion of acetate into long-chain fatty acids the following cofactors were required (*cf.* Table I): ATP, CoA, GSH, DPNH, *iso*citrate, TPN, lipoic acid and Mn^{++}.

The requirement for ATP, CoA and GSH

Table I shows that very little or no synthesis takes place in the absence of any one of these components. The absolute requirement for ATP suggests that ATP is the main driving force for the synthesis. This can manifest itself in the activation of acetate prior to condensation. Since CoA is required for the synthesis (*cf.* Table I), it is reasonable to assume that acetyl CoA is the active form of acetate in the synthesis. ATP is required for this activation, as has been shown by many workers[12,13]. However, ATP might also be required at other stages of the synthetic process. Fig. 1 shows the effect of varying concentrations of ATP (curve D) and CoA (curve E) on the synthesis of fatty acids. It is of interest to note the inhibitory effect at higher levels of these components on the synthesis. At levels higher than maximal CoA is a much more potent inhibitor than ATP. The narrow limits within which CoA has to be used for

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maximal activity are shown by the appropriate curve of Fig. 1. The crude dialyzed PLS does not show a requirement for CoA unless it is first treated with Dowex-1-Cl. Then a stimulation by CoA can be demonstrated. This confirms the earlier findings

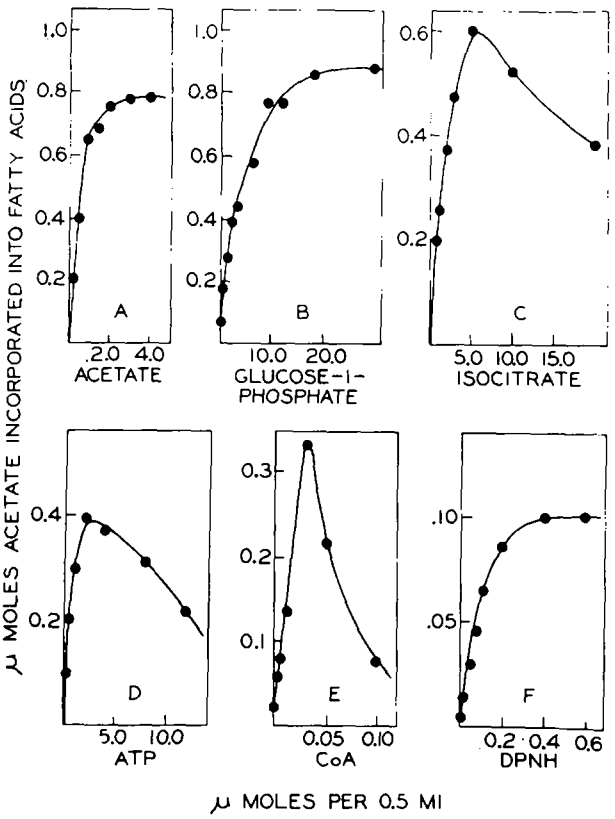

Fig. 1. Incorporation of acetate into fatty acids as a function of cofactor concentration. Curve A shows the effect of acetate concentration on the synthesis of fatty acids. Each tube contained the following components: 30 μmoles of K phosphate buffer pH 6.5; 18.0 μmoles of glucose-1-phosphate; 4.0 μmoles of ATP; 8.0 μmoles of GSH; 0.01 μmoles of CoA; 0.15 μmoles of lipoic acid; 0.2 μmoles of DPN; 0.2 μmoles of TPN; 0.1 μmoles of $MgCl_2$; 0.2 μmoles of $MnCl_2$; and 5.0 μmoles of *iso*citrate. Various amounts of acetate-1-^{14}C were added as indicated with a total radioactivity of 100,000 c.p.m. in each case. The reaction was started by the addition of 1.0 mg of R_1 (gel treated), 1.0 mg of R_2 and 0.8 mg of R_4. Total volume was 0.5 ml and the tubes were incubated for 2 hours at 38°. Curve B: The effect of glucose-1-phosphate concentration on the incorporation of acetate into fatty acids. Each tube contained the same components as described for curve A except that 2.5 μmoles of acetate-1-^{14}C (150,000 c.p.m.) and varied amounts of glucose-1-phosphate were added as indicated. The reaction was started by the addition of the same amounts of R_1, R_2 and R_4 as in curve A. The total volume was 0.5 ml and the tubes were incubated for 2 hours at 38°. Curve C: The effect of *iso*citrate concentration on the incorporation of acetate into fatty acids. Each tube contained the same components as described in curve A except that 2.5 μmoles of acetate-1-^{14}C (150,000 c.p.m.) and varied amounts of *iso*citrate were added as indicated. The same amounts of enzyme fractions were added and the final volume was 0.5 ml. The tubes were incubated for 2 hours at 38°. Curve D: The effect of ATP concentration on the incorporation of acetate into fatty acids. Each tube contained the same components as in curve A except that 2.5 μmoles of acetate-1-^{14}C (150,000 c.p.m.) and varied amounts of ATP were added as indicated. The reaction was started by the addition of the same amounts of the enzyme fractions as in curve A. The final volume was 0.5 ml and the tubes were incubated for 2 hours at 38°. Curve E: Each tube contained the same components as in curve A except that 2.5 μmoles of acetate-1-^{14}C (150,000 c.p.m.) and varied amounts of CoA were added as indicated. The reaction was started by the addition of the same amounts of enzyme fractions as in curve A. The final volume was 0.5 ml and the tubes were incubated for 2 hours at 38°. Curve F: The effect of DPNH concentration on the incorporation of acetate into fatty acids. Each tube contained the following: 30 μmoles of K phosphate pH 6.5; 4.0 μmoles of ATP; 8.0 μmoles of GSH; 0.02 μmoles of CoA, 0.15 μmoles of lipoic acid; 0.2 μmoles of $MnCl_2$; 0.2 μmoles of TPN; 5.0 μmoles of *iso*citrate; and 2.5 μmoles of acetate-1-^{14}C (150,000 c.p.m.). Varied amounts of DPNH were added as indicated. The reaction was started by the addition of 0.8 mg of R_1 (gel-treated), 0.9 mg of R_{23} and 0.4 mg each of R_{43} and R_{46} (these are alcohol fractions of R_2 and R_4 as described in the first paper of this series[9]). The final volume was 0.5 ml and the tubes were incubated for 1.5 hours at 38°. When glucose-1-phosphate (15.0 μmoles) and DPN (0.2 μmole) were employed in place of DPNH under the conditions of this experiment 0.10 μmole of acetate was incorporated into fatty acids.

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of GURIN and co-workers[6]. High levels of CoA are also inhibitory to fatty acid synthesis in the crude unfractionated system. Recently BRADY and co-workers[14] have reported similar inhibitory effect of CoA and they have contrasted this inhibition with the stimulation of fatty acid oxidation by CoA. They suggested that the level of CoA might play an important role in the biological control of fat metabolism.

TABLE I

COMPONENTS REQUIRED FOR FATTY ACID SYNTHESIS

Cofactor omitted	*Acetate incorporated into fatty acids µmoles*
None	0.32
ATP	0.00
CoA	0.02
GSH	0.01
DPNH	0.03
*Iso*citrate	0.01
TPN	0.15
Lipoic acid	0.18
Mn^{++}	0.07
Mg^{++}	0.34

Each tube (except when otherwise omitted) contained: 25 µmoles of K phosphate buffer pH 6.5; 5.0 µmoles of Na *iso*citrate; 5 µmoles of ATP; 0.02 µmoles of CoA; 1.0 µmoles of DPNH, 0.02 µmoles of $MnCl_2$; 8.0 µmoles of GSH; 0.20 µmoles of TPN; 0.15 µmoles of lipoic acid, 0.05 µmoles of $MgCl_2$; and 2.5 µmoles of K acetate (150,000 c.p.m.). The reaction was started by the addition of 1.0 mg gel-treated R_1; 0.7 mg R_2; and 1.0 mg of R_4. Total volume 0.5 ml. The tubes were incubated at 38° for 2 hours.

GSH could have two distinct roles: first, that of maintaining CoA in the reduced SH form—the only form in which it is available for the activation of acetate—and second, that of maintaining the sulfhydryl groups of the enzymes in their reduced and active states[9]. GSH can be replaced by any other sulfhydryl compounds such as D- and L-cysteine, 2,3 dimercaptopropanol and 1,3 dimercaptopropanol[9].

The requirement for DPNH

In the purified system DPNH was essential for the fatty acid synthesis (*cf.* Table I). Such a requirement could not be demonstrated in crude dialyzed PLS nor in the first ammonium sulfate fractionated system[9]. Instead, these less purified systems showed a requirement for glucose-1-phosphate plus DPN (*cf.* Table II), which was only partly replaceable by glucose-6-phosphate and less by fructose-1,6-diphosphate. However, glucose-1-phosphate could be replaced fully by a combination of 3-phosphoglyceraldehyde dehydrogenase, fructose-1,6-diphosphate, and aldolase, as shown in Table II. This replacement suggested that the function of glucose-1-phosphate might be that of generating DPNH though it is not at all clear which specific dehydrogenase system ultimately is involved. The dependence of fatty acid synthesis on the concentration of DPNH (curve F) and of glucose-1-phosphate (curve B) is shown graphically in Fig. 1.

The requirement for isocitrate and TPN

BRADY AND GURIN[5] were the first to show the requirement of citrate for the synthesis of fatty acids in a soluble enzyme system. This was confirmed with the PLS. Furthermore, *iso*citrate could replace citrate and was indeed more active[2]. The requirement for *iso*citrate persisted throughout the purification of PLS, whereas citrate was active only in the crude system. Table I shows the requirement for *iso*citrate in the reconstituted purified system and Table III the components which may replace *iso*citrate.

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TABLE II

REPLACEMENT OF GLUCOSE-1-PHOSPHATE

Cofactor system	*Acetate incorporated into fatty acids μmoles*
Control	0.02
Control + glucose-1-phosphate	0.60
Control + glucose-6-phosphate	0.40
Control + fructose-1,6-diphosphate	0.18
Control + fructose-1,6-diphosphate + aldolase	0.40
Control + fructose-1,6-diphosphate + aldolase + glyceraldehyde phosphate dehydrogenase	0.70

All tubes contain the same components reported in Table I except no DPNH was added. Instead 0.2 μmoles of DPN were added. Whenever stated the following additions were made: 6.0 μmoles of glucose-1-phosphate; 6.0 μmoles of glucose-6-phosphate; 6.0 μmoles of fructose-1,6-diphosphate; 0.2 mg crystalline aldolase; and 0.2 mg glyceraldehyde phosphate dehydrogenase. The reaction was started by the addition of 3.4 mg of $R_1 + R_2 + R_4$. Total volume 0.5 ml. All samples were incubated at 38° for 2 hours.

TABLE III

ATTEMPTED REPLACEMENT OF *iso*CITRATE

Cofactor system	*Acetate incorporated into fatty acids μmoles*
Control	0.02
Control + *iso*citrate	0.60
Control + citrate	0.02
Control + citrate + aconitase	0.02
Control + glucose-6-phosphate	0.03
Control + glucose-6-phosphate + glucose-6-phosphate dehydrogenase	0.03
Control + α-ketoglutarate	0.03
Control + $KHCO_3$	0.01
Control + glucose-6-phosphate + glucose-6-phosphate dehydrogenase + α-ketoglutarate	0.05
Control + glucose-6-phosphate + glucose-6-phosphate dehydrogenase + α-ketoglutarate + $KHCO_3$	0.50

To the control tubes the following components were added: 30 μmoles of K phosphate buffer, pH 6.5; 8.0 μmoles of GSH; 0.02 μmoles of CoA; 2.5 μmoles of ATP; 0.2 μmoles of $MnCl_2$; 0.15 μmoles of lipoic acid; 0.4 μmoles of DPN; 15.0 μmoles of glucose-1-phosphate; 0.2 μmoles of TPN; and 2.5 μmoles of K acetate (150,000 c.p.m.). Whenever indicated, the following additional components were added: 6.0 μmoles of Na *iso*citrate; 10.0 μmoles of citrate; 0.3 mg of aconitase; 10.0 μmoles of α-ketoglutarate; 5.0 μmoles of glucose-6-phosphate; 0.2 mg of glucose-6-phosphate dehydrogenase; and 20 μmoles of $KHCO_3$. The reaction was started by the addition of 1.0 mg of gel-treated R_1, 0.8 mg of R_2 and 0.8 mg R_4. Total volume 0.5 ml. The tubes were incubated at 38° for 2 hours.

In the purified system *iso*citrate cannot be replaced by citrate, *cis*-aconitate or by any other member of the citric acid cycle. However, in the presence of aconitase either citrate or *cis*-aconitate can replace isocitrate. One possible role of *iso*citrate is that of generating TPNH in the presence of the *iso*citric dehydrogenase and TPN. This enzyme could be detected in the crude PLS and it persists throughout the purification. However, *iso*citrate could not be replaced by TPNH or by a TPNH-generating dehydrogenase system (*e.g.* glucose-6-phosphate dehydrogenase plus substrate and TPN). But if a TPNH generating system is supplemented with α-ketoglutarate and

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HCO_3^-, then such a combination can replace *iso*citrate completely (*cf.* Table III). This is in essence a resynthesis of *iso*citrate by the carboxylation of α-ketoglutarate by CO_2 in the presence of TPNH (generated in this case from the glucose-6-phosphate dehydrogenase system) as was shown by ADLER *et al.*[15] and OCHOA[16]. The observation of BRADY *et al.*[14] that a combination of the glucose-6-phosphate dehydrogenase system and α-ketoglutarate can substitute for citrate in the crude enzyme preparation, might be explained in terms of enzymic synthesis of *iso*citrate. Since *iso*citrate cannot be replaced by any other component (other than these which give rise to *iso*citrate) it would follow that *iso*citrate must have a role additional to that of generating TPNH. The relationship between the *iso*citrate concentration and fatty acid synthesis is shown in Fig. 1 (curve C).

The requirement for TPN in the synthesis of fatty acids by the purified system is only partial, as shown in Table I. In the absence of TPN the system is about 30 to 50% as efficient as with TPN. No such requirement for TPN can be shown in the dialyzed crude PLS. However, on treatment of the dialyzed crude PLS with Dowex-1-Cl it was possible to obtain considerable stimulation of fatty acid synthesis by the addition of TPN. A similar requirement for TPN has been reported recently by LANGDON[17] in experiments with rat liver preparations. Furthermore, he was able to demonstrate that TPNH is oxidized by crotonyl CoA in the presence of such preparations. In contrast, HELE AND POPJÁK[18] reported that enzyme preparations from rabbit mammary gland after purification with ammonium sulfate do not require either TPN or *iso*citrate but do require DPNH for the synthesis of short-chain fatty acid from acetate.

The requirement for lipoic acid

Table I shows that lipoic acid stimulates the synthesis of fatty acids from acetate. When the reconstituted purified system was preincubated with a sulfhydryl compound (GSH, D- or L-cysteine) the lag phase in fatty acid synthesis was abolished[9]. Such a treatment also abolished the requirement for lipoic acid. The chicken liver system according to TIETZ[19] does not show any requirement for lipoic acid. Therefore, the significance of the requirement for lipoic acid in the pigeon system is not yet clear.

Metal requirement

Mn^{++} is an absolute requirement for fatty acid synthesis from acetate by the purified pigeon liver system, as shown in Table I. The crude dialyzed PLS showed a requirement for both Mg^{++} and Mn^{++}. In the purified system only Mn^{++} is required. Mn^{++} inhibits fatty acid synthesis at levels higher than 10^{-3} *M*.

Acetate concentration

Fig. 1 (curve A) shows the effect of acetate concentration on the incorporation of acetate into fatty acids. The results show that the optimal concentration of acetate is about 2 to 5 μmoles in 0.5 ml of reaction mixture.

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SUMMARY

The synthesis of long-chain fatty acids from acetate by the reconstituted fractions of pigeon liver supernatant requires the following cofactors: ATP, CoA, GSH, DPNH, Mn^{++}, *iso*citrate and TPN. Glucose-1-phosphate is needed in crude systems but this component can be replaced by DPNH in the more purified system.

*Iso*citrate cannot be replaced by TPNH or a TPNH-generating system such as glucose-6-phosphate dehydrogenase system plus substrate. However, it can be replaced by a combination of α-ketoglutarate, CO_2, and a TPNH generating system.

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III. PRODUCTS OF ENZYMIC SYNTHESIS OF FATTY ACIDS

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Considerable experimental evidence has been obtained which supports the concept that fatty acids are synthesized by successive head-to-tail condensations of two carbon (acetate) units[3,4,5]. Much of this information has originated from studies with

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