5. Anaerobic incubation caused an inhibition of synthesis only in the presence of oxaloacetate; no inhibition was observed anaerobically in the presence of α -oxoglutarate plus malonate.

6. Hg^{2+} and arsenate almost completely abolished fatty acid synthesis; cyanide and 2:4-dinitrophenol caused a moderate (30–70%) inhibition, while azide and fluoride were without effect.

7. The implication of the results is discussed.

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Biosynthesis of Fatty Acids in Cell-free Preparations

3. COENZYME A DEPENDENT REACTIONS IN A SOLUBLE ENZYME SYSTEM OF MAMMARY GLAND

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In the preceding paper the biosynthesis of short- and long-chain fatty acids from acetate in a soluble enzyme system (MGE) prepared from the mammary gland of lactating rats was described, and favourable conditions for the synthesis were defined (Popják & Tietz, 1955). The discovery of coenzyme A (CoA) and of its participation in acetylation reactions by Lipmann and his colleagues (see Lipmann, 1948–9) resulted finally in the identification and isolation of acetyl-CoA as the active form of acetate by Lynen, Reichert & Rueff (1951). These discoveries and the subsequent extensive studies of β -oxidation of fatty acids led to the realization that

fatty acids are metabolized as CoA-derivatives (Lynen & Ochoa, 1953; Green, 1954). It became of interest therefore to establish whether or not fatty acid synthesis from acetate in the MGE likewise involved the intermediate formation of acetyl-CoA and of higher acyl-CoA homologues. The main object of the work reported here was the study of this question. The results have already been presented in a preliminary form (Popják & Tietz, 1954b).

MATERIAL AND METHODS

The preparation of the soluble enzyme system (MGE) from the mammary gland of lactating rats has been described in the preceding paper (Popják & Tietz, 1955).

Preparation and assay of coenzyme A. These were carried out by the method of Kaplan & Lipmann (1948). The crude CoA preparations were made from rat liver; they usually

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contained 3–7 Lipmann units/mg. CoA was removed from the sulphanilamide-acetylating enzyme system used for the assay of CoA by two treatments with Dowex-1 (mesh 200–400) ion-exchange resin, according to Chantrenne & Lipmann (1950), rather than by 'ageing' as originally described by Kaplan & Lipmann (1948).

Arylamine-acetylating enzyme (A_{80}). This was prepared from pigeon-liver acetone-powder as described by Chou & Lipmann (1952).

Diphosphopyridine nucleotide (DPN). This was made from dry yeast according to LePage (1951); it contained 35% DPN according to spectrophotometric estimation after reduction with hydrosulphite.

Preparation of hydroxamic acid standards. Acethydroxamic acid was made according to the method of Lipmann & Tuttle (1945).

Butyrylhydroxamic acid was prepared from ethylbutyrate. A 0.1M ethanolic solution of the ester was mixed with five equivalents of salt-free hydroxylamine (salt-free hydroxylamine prepared according to Beinert *et al.* 1953) and 1 equivalent of ethanolic NaOH. The mixture was incubated at 30° for 30 min., then acidified with HCl and the NaCl formed filtered off. The clear solution was assayed for its butyrylhydroxamic acid content (Kornberg & Pricer, 1953a) and kept at 4° until required. Hydroxamic acids from the ethyl esters of myristic (tetradecanoic), palmitic and stearic acids were made in a similar way.

Measurement of formation of hydroxamic acids in the MGE. MGE (0.5 ml.; 12.5 ± 1.5 mg. dry weight) was incubated at 37.5° for 1 hr. with the following reaction mixture: aminotrihydroxymethylmethane (THAM) buffer, pH 7.5–8.1, 200 μ moles; cysteine hydrochloride, 10 μ moles; $MgCl_2$, 10 μ moles; KF, 100 μ moles; salt-free hydroxylamine, 1 m-mole; adenosine triphosphate (ATP), 5–10 μ moles; CoA, 14–20 units; substrates (acetate, butyrate, pyruvate, α -oxoglutarate, malonate and hexadecanoate) in amounts indicated in the tables. Cysteine was added to the reaction mixture in order to protect coenzyme A against oxidation to the disulphide form and KF to inhibit pyrophosphatase (if present in the MGE). The quantitative determination of the water-soluble hydroxamic acids was made according to the method of Kornberg & Pricer (1953a) and of palmitohydroxamic acid according to the method of Lipmann & Tuttle (1950).

Identification of hydroxamic acids by paper chromatography. For the separation of acethydroxamic and butyrylhydroxamic acids on Whatman no. 1 filter paper *n*-butanol-acetic acid-water (63:10:27, by vol.) was used as the solvent system (Thompson, 1951). The chromatograms were developed overnight at 21° and then dried at room temperature. The dried papers were sprayed with a $FeCl_3$ reagent (Stadtman & Barker, 1950). In this system acethydroxamic acid and butyrylhydroxamic acid have R_f values of 0.47 and 0.76 respectively. Longer chain acylhydroxamates move with the solvent front.

For the separation and identification of higher acylhydroxamates (myristo-, palmito- and stearo-hydroxamic acids) a reversed-phase paper chromatographic method, suggested by Dr A. T. James, was used. Whatman no. 4 filter paper was impregnated with kerosene by immersion into a 10% kerosene solution in acetone; the acetone was then evaporated off. The mobile phase was a mixture of ethanol-m-acetic acid (57.5:42.5, by vol.) saturated with kerosene. The ratio of the two solvents was critical in that with higher concentrations of ethanol palmitohydroxamic acid moved with the solvent front and with lower concentrations it did not move at all. Ascending chromatography was preferred and in order to prevent 'tailing' not more than 0.3–0.5 μ mole of the hydroxamic acids was put on the paper. The chromatograms were developed as for the lower homologues (see above). Myristohydroxamic acid had an R_f value of 0.86, palmitohydroxamic acid of 0.64 and stearylhydroxamic acid of 0.1.

Incubations, extraction and purification of fatty acids and assay of ^{14}C . These were done as previously described (Popják & Tietz, 1954a, 1955).

RESULTS

Activation of acetate and of higher fatty acids in the MGE

Coenzyme A content of MGE. We have shown in our previous communication that fatty acid synthesis from acetate in the soluble enzyme preparation (MGE) could be activated by the addition of ATP alone, and that the requirement for ATP was absolute. In the activation of acetate by the mammalian acetate-activating enzyme, not only ATP but also coenzyme A (CoA) is an essential cofactor (Lipmann, 1948–9). It was of interest to know therefore if our MGE preparations contained CoA.

Samples of MGE were heated in boiling water for 1 min. and the coagulated protein centrifuged off; the supernatant was then assayed for its CoA content. Assays carried out on four preparations, nos. 150, 152, 154 and 159, gave values of 25, 17, 22 and 20 Lipmann units/ml. of the enzyme preparation corresponding to about 50 μ g. of CoA/ml.

Formation of acethydroxamic acid in the MGE. It has been shown by Chou & Lipmann (1952) that hydroxylamine, if present in high concentrations (0.5–1.0M), reacts non-enzymically with acetyl-CoA and with various other 'carboxyl-activated' substrates to form the corresponding hydroxamic acids. The formation of hydroxamic acids in the MGE preparations was therefore used as a measure of activation of acetate and of other fatty acids with CoA.

Table 1. *Acethydroxamic acid formation by the MGE*

The incubation mixture contained: THAM buffer, pH 8.1, 200 μ moles; cysteine hydrochloride, 10 μ moles; $MgCl_2$, 10 μ moles; KF, 100 μ moles; hydroxylamine, 1 m-mole; ATP, 5 μ moles; MGE, 0.5 ml. Additions: coenzyme A, 14 units; K acetate, 100 μ moles. Final volume, 2 ml. Incubation at 37.5° for 1 hr.

Preparation no.	Additions	Hydroxamic acid* found (μ moles)		Δ Hydroxamic acid† (μ moles) ($b - a$)
		Without acetate (a)	With acetate (b)	
150	None	0.80	1.54	+0.74
	CoA	0.90	1.89	+0.90
152	None	0.59	1.13	+0.54
	CoA	0.86	1.68	+0.82
153	None	0.50	1.22	+0.72
	CoA	0.68	1.60	+0.92

* Measured against acethydroxamic acid standards.

† Extra hydroxamic acid formed due to the addition of acetate to the incubation mixture.

Table 2. *Hydroxamic acid formation by the MGE (preparation no. 159)*

Experimental conditions as detailed in Table 1. Values given under t_0 represent amounts of hydroxamic acid detected at zero time and those under t_{60} found after 60 min. incubation.

Additions	Hydroxamic acid found (μ moles)		Hydroxamic acid formed ($t_{60} - t_0$) μ moles	Δ Hydroxamic acid* (μ moles)
	t_0	t_{60}		
None	0.35	0.88	0.53	—
Acetate	0.35	1.22	0.87	+0.34
CoA	0.63	1.22	0.59	—
CoA + acetate	0.63	2.36	1.73	+1.14

* Amount of hydroxamic acid formed due to addition of acetate to incubation mixture.

As can be seen from Table 1, hydroxamic acid formation was observed in the MGE preparations even when no substrate or extra CoA was added to the enzyme system. In the presence of 100 μ moles of acetate additional amounts (0.5–0.7 μ mole) of hydroxamic acid were formed, and by fortifying the MGE with 14 units of CoA the amounts of hydroxamic acids increased further. No hydroxamic acid formation was observed in the control tubes containing the reaction mixture only, i.e. in the absence of MGE.

In order to account for the formation of hydroxamic acids in the absence of added substrates ('endogenous' formation), the amounts of hydroxamic acids detected at zero time (t_0), i.e. on immediate deproteinization of the enzyme-assay mixture, were compared with those found after 1 hr. of incubation (t_{60}). The results showed (Table 2) the genuine increase of hydroxamic acids in the enzyme preparation and also that the addition of 20 units of CoA stimulated the activation of added acetate, but had little or no effect in this particular experiment on the 'endogenous' formation of hydroxamic acids (see also Table 3). The relatively

Table 3. *Effects of ATP and of CoA on acetate activation by the MGE as measured by formation of hydroxamic acids*

The incubation mixture contained: THAM buffer, pH 8.1, 200 μ moles; cysteine hydrochloride, 10 μ moles; $MgCl_2$, 10 μ moles; KF, 100 μ moles; hydroxylamine 1 m-mole; MGE, 0.5 ml. Additions: K acetate, 100 μ moles; CoA, 20 units; ATP, 5–10 μ moles as shown. Final volume, 2 ml.

Additions	Hydroxamic acid formed* ($t_{60} - t_0$) μ moles		Δ Hydroxamic acid† (μ moles)
	Without acetate	With acetate	
None	0.00	0.00	0.0
CoA	0.00	0.00	0.0
ATP, 5 μ moles	0.26	1.53	+1.27
CoA + ATP, 5 μ moles	0.33	2.00	+1.67
CoA + ATP, ‡ 5 μ moles	0.57	2.17	+1.60
CoA + ATP, ‡ 10 μ moles	0.57	3.64	+3.07

* Zero time values have been subtracted from those found after 60 min. of incubation at 37.5°.

† Amount of hydroxamic acid formed due to addition of acetate to the incubation mixture.

‡ KF was omitted.

high t_0 values for hydroxamic acids may be due to a non-specific development of colour with the reagents, or to the presence of acyl-CoA compounds in the MGE and in the crude CoA preparations used. It is to be noted that when the system was fortified with 20 units of CoA (crude liver preparation), the t_0 values for hydroxamic acids were nearly twice as large as those found without the extra CoA.

The MGE cannot activate either the endogenous substrates present or the added acetate in the absence of ATP (Table 3). The activation of acetate

fied; the spot corresponding to the acethydroxamic acid became, however, very prominent.

Activation of acyl homologues of acetate in the MGE. Since it has been suggested that higher fatty acids must also be activated with CoA before they can condense with acetyl-CoA to form the next acyl-CoA homologue (Lynen & Ochoa, 1953), it was of interest to determine if—in addition to acetate—other fatty acids with an even number of carbon atoms are activated by the MGE. As can be seen from Table 5, small amounts of butyrate and of

Table 4. *Comparison of the effect of ATP and of ADP on acetate activation by the MGE as measured by formation of hydroxamic acid*

The incubation mixture contained: THAM buffer, pH 7.5, 200 μ moles; cysteine hydrochloride, 10 μ moles; $MgCl_2$, 10 μ moles; KF, 100 μ moles; hydroxylamine, 1 m-mole; CoA, 20 units; MGE, 0.5 ml. Additions: K acetate, 100 μ moles; ATP and ADP as indicated. Final volume, 2 ml.

Preparation no.	Additions	Hydroxamic acid formed ($t_{90} - t_0$) μ moles		Δ Hydroxamic acid* (μ moles)
		Without acetate	With acetate	
160	ATP, 5 μ moles	0.81	2.06	+1.25
	ATP, 10 μ moles	0.85	4.18	+3.33
	ADP, 5.5 μ moles	0.77	2.95	+2.18
162	ATP, 10 μ moles	0.68	3.59	+2.91
	ADP, 8 μ moles	0.65	2.86	+2.21

* Amount of hydroxamic acid formed due to the addition of acetate to the incubation mixture.

and of endogenous substrates by the MGE when only ATP is added can, of course, be accounted for by the presence of CoA in the enzyme preparation. The dependence of the acetate-activating reaction on ATP is shown further by the observation that, in the presence of added acetate, the amount of hydroxamic acids formed was nearly doubled when the amount of ATP in the incubation mixture was increased from 5 to 10 μ moles.

The dependence on ATP of acetate activation by the MGE is in complete harmony with the dependence on ATP of fatty acid synthesis from acetate. In further agreement with previous results on fatty acid synthesis (cf. Popják & Tietz, 1955), adenosine diphosphate (ADP) can replace ATP in the MGE as activator of acetate; ADP (purified by the method of Cohn & Carter, 1950) was at least as effective as ATP in the hydroxamic acid test (Table 4).

The hydroxamic acids formed in these and similar experiments were analysed by paper chromatography (Thompson, 1951) with *n*-butanol-acetic acid-water solvent system. The hydroxamates formed when substrates were not added to the MGE separated into two spots: one had an R_f value of 0.47 and corresponded to acethydroxamic acid, the other spot migrated with the solvent front (R_f 0.91) and represents probably a mixture of the homologous acyl derivatives with a chain-length of 6 carbons or more. When acetate (100 μ moles) was added to the MGE the same two spots were identi-

Table 5. *Activation of substrates by the MGE*

The experimental reaction mixture contained: THAM buffer, pH 7.5, 200 μ moles; cysteine hydrochloride, 10 μ moles; $MgCl_2$, 10 μ moles; hydroxylamine, 1 m-mole; ATP, 10 μ moles; CoA, 20 units; MGE, 0.5 ml. Additions: acetate, pyruvate, butyrate, as the potassium salts, 100 μ moles each; K palmitate, 10 μ moles. Final volume, 2 ml.

Preparation no.	Additions	Hydroxamic acids formed ($t_{90} - t_0$) μ moles		Δ Hydroxamic acid* (μ moles)
157	None	0.59	—	—
	Acetate	3.00	—	+2.41
	Pyruvate	2.27	—	+1.68
	Butyrate	0.85	—	+0.26
	Palmitate	0.70	—	+0.11
160	None	0.50	—	—
	Acetate	3.41	—	+2.91
	Pyruvate	2.68	—	+2.18
	Butyrate	0.71	—	+0.21
	Palmitate	0.56	—	+0.06
162	None	0.80	—	—
	Acetate	3.50	—	+2.70
	Pyruvate	2.86	—	+2.06
	Butyrate	1.08	—	+0.28
	Palmitate	0.95	—	+0.15
163	None	0.69	—	—
	Acetate	3.59	—	+2.90
	Butyrate	0.95	—	+0.26
	Palmitate	0.79	—	+0.10

* Amount of hydroxamic acid formed due to the addition of substrates to the incubation medium.

palmitate were activated by the MGE, as measured by the formation of hydroxamic acids; at the same time the enzyme preparations formed relatively large amounts of acethydroxamic acids.

Both the butyro- and palmito-hydroxamic acids were identified by paper chromatography. The hydroxamates formed by the MGE in the presence of butyrate were separated on Whatman no. 1 paper by the *n*-butanol-acetic acid-water solvent system, as used for the identification of acet-hydroxamic acid. In addition to the two spots obtained from the endogenous substrates of the MGE (see above), a third spot with an R_f value of 0.76, corresponding to butyrohdroxamic acid, also appeared, indicating the formation of butyryl-CoA.

When the reversed-phase paper chromatographic technique (see under Methods) was applied to the hydroxamates formed in the MGE from endogenous substrates or in the presence of added palmitate, two spots were found: one had an R_f value of 0.64, corresponding to palmitohydroxamic acid, and the other an R_f value of 0.91. The latter probably represents a mixture of acylhydroxamates containing 2-14 carbon atoms. The formation of palmityl-CoA in the MGE may thus be inferred.

Formation of acetyl-CoA from pyruvate in the MGE. On the basis of manometric measurements we have previously concluded that pyruvate is oxidized in the MGE (Popják & Tietz, 1955). Investigations on the mechanism of pyruvate oxidation (Korkes, del Campillo & Ochoa, 1952; Littlefield & Sanadi, 1952) have shown that acetyl-CoA

was an intermediate in the process. The results shown in Table 5 demonstrate also that large amounts of hydroxamic acid were trapped on addition of pyruvate to the MGE. The hydroxamic acid formed from pyruvate was identified by paper chromatography as acethydroxamic acid. The results indicate the presence of a pyruvic oxidase system in the MGE, which forms acetyl-CoA from pyruvate. It should, however, be mentioned that, in contrast to the results of Korkes *et al.* (1952) and of Littlefield & Sanadi (1952) (who demonstrated that pyruvic oxidase, as opposed to the acetate-activating enzyme, did not require ATP for the formation of acetyl-CoA), the MGE system required ATP for the oxidation of pyruvate to acetyl-CoA. Acethydroxamic acid was not formed from pyruvate in our system unless ATP was added.

The effect of α -oxoglutarate and of malonate on activation of acetate by the MGE

We have shown previously that α -oxoglutarate and malonate markedly stimulated fatty acid synthesis from acetate in the MGE (Popják & Tietz, 1955). This stimulation might have been brought about by an effect on the activation of acetate or on the utilization of acetyl-CoA in the later stages of fatty acid synthesis. In order to distinguish between these alternatives, we studied the effects of α -oxoglutarate and of malonate on the activation of acetate.

It is obvious from the results shown in Table 6 that α -oxoglutarate and malonate, when added

Table 6. *Effects of α -oxoglutarate and of malonate on activation of acetate as compared with their effect on fatty acid synthesis from acetate*

The incubation mixture used for measuring hydroxamic acid formation contained: MGE, 0.5 ml.; THAM buffer, pH 8.1, 200 μ moles; cysteine hydrochloride, 10 μ moles; $MgCl_2$, 10 μ moles; KF, 100 μ moles; hydroxylamine, 1 m-mole; ATP, 5 μ moles; CoA, 14 units. Additions: K acetate, K α -oxoglutarate and K malonate, 100 μ moles each. For measurement of fatty acid synthesis 2 ml. of MGE were incubated at 37.5° for 60 min. with K [*carboxy*- ^{14}C]acetate (0.02 M, 5 μC ^{14}C) and ATP (0.01 M). Final concentration of additions, when made, were: K α -oxoglutarate, 0.02 M; and K malonate, 0.05 M.

Preparation no.	Additions	Hydroxamic acid formed ($t_{60} - t_0$) μ moles	Acetate incorporated into fatty acids (μ moles/100 mg. dry wt. of MGE)
150	None	0.74	—
	Acetate	1.50	—
	α -Oxoglutarate	0.68	—
	Malonate	0.74	—
152	None	0.73	—
	Acetate	1.61	0.062
	α -Oxoglutarate	0.73	—
	Acetate + α -oxoglutarate	1.61	0.338
154	None	0.68	—
	Acetate	1.40	0.008
	α -Oxoglutarate	0.65	—
	Malonate	0.71	—
	Acetate + α -oxoglutarate	1.31	0.020
	Acetate + malonate	1.43	0.106
	Acetate + α -oxoglutarate + malonate	1.27	0.134

either alone or in combination to the MGE, had no effect on acetate activation as measured by the formation of hydroxamic acids either from endogenous substrates in the MGE or from added acetate. The characteristic effect on fatty acid synthesis was observed at the same time. It would seem, therefore, that both α -oxoglutarate and malonate increase fatty acid synthesis from acetate by enhancing in some way the utilization of acetyl-CoA.

A comparison of the amount of acetate activated (measured as μ moles of hydroxamic acid formed) with the amounts incorporated into fatty acids (cf. Table 6) revealed that only a fraction of the acetate activated was incorporated into fatty acids. This is particularly clear from the fact that hydroxamic acid formation was measured with an amount of MGE containing only 12.5 ± 1.5 mg. dry weight as compared with 100 mg. dry weight used to measure fatty acid synthesis. Yet it should be pointed out that the experimental conditions in the two kinds of measurement were not identical in that the MGE was not fortified with extra CoA when fatty acid synthesis was measured, and it will be shown later

that the addition of CoA to the preparations enhanced the incorporation of acetate into fatty acids considerably.

Effects of inhibitors on acetate activation by the MGE

It was shown in the previous paper (Popják & Tietz, 1955) that Hg^{2+} and arsenate almost completely abolished fatty acid synthesis from acetate. It was of interest to determine if these inhibitors had a similar effect on activation of acetate by the MGE.

The experiments were set up in such a way that two equal samples of the same enzyme preparation were incubated: in one sample the inhibitor was added to the MGE, together with the reaction mixture for the assay of acethydroxamic acid formation, and in the other the MGE was preincubated for 5 min. with the inhibitor, and the reaction mixture was added afterwards.

The results showed (Table 7) that Hg^{2+} stopped almost completely the formation of acethydroxamic acid when the enzyme was preincubated with this inhibitor. When Hg^{2+} was added, together with the

Table 7. *The effect of mercury and of arsenate on acetate activation by the MGE as measured by the formation of acethydroxamic acid*

The reaction mixture contained: THAM buffer, pH 7.5, 200 μ moles; cysteine hydrochloride, 10 μ moles; MgCl_2 , 10 μ moles; ATP, 10 μ moles; CoA, 20 units; K acetate, 100 μ moles; hydroxylamine, 1 m-mole. 0.5 ml. of MGE was used. Inhibitors were added in concentrations shown. Final volume 2.0 ml.

Preparation no.	Inhibitor added	Inhibitor added together with reaction mixture		Enzyme preincubated with inhibitor for 5 min.	
		Acethydroxamic acid formed ($t_{80} - t_0$) μ moles	% inhibition	Acethydroxamic acid formed ($t_{80} - t_0$) μ moles	% inhibition
160	None	3.40	—	2.68	—
	HgCl_2 (10^{-3} M)	2.42	29.5	0.05	98
	Na_3AsO_4 (0.05 M)	2.42	29.5	1.95	27.5
163	None	3.31	—	2.93	—
	HgCl_2 (10^{-3} M)	1.17	64.5	0.04	99
	HgCl_2 (10^{-4} M)	3.40	0.0	0.72	75.5
	Na_3AsO_4 (0.05 M)	1.84	44.0	1.70	42.0

Table 8. *Comparison of the effect of arsenate on fatty acid synthesis and on hydroxamic acid formation by the MGE*

The experimental details for measuring formation of acethydroxamic acid were as shown in Table 7, and for fatty acid synthesis as shown in Table 6.

Preparation no.	Inhibitor added	Hydroxamic acid formation		Fatty acid synthesis	
		μ moles formed	% inhibition	μ moles acetate incorporated into fatty acids/100 mg. dry wt.	% inhibition
162	None	2.93	—	0.281	—
	Na_3AsO_4 (0.05 M)	2.16	21	0.067	76
163	None	3.31	—	0.088	—
	Na_3AsO_4 (0.05 M)	1.84	44	0.026	70
164	None	3.02	—	0.200	—
	Na_3AsO_4 (0.05 M)	2.38	21	0.084	58

reaction mixture, only a moderate inhibitory effect was observed. This observation can probably be explained by the presence of cysteine in the reaction mixture which, by combining with Hg^{2+} , may protect the enzyme(s). The inference is that the acetate-activating enzyme is an SH-enzyme.

Arsenate exerted only a moderate inhibitory effect on the formation of acetylhydroxamic acid, and the degree of inhibition was the same whether the MGE was preincubated with arsenate or not. The data of Table 8 show further that the process of fatty acid synthesis was more susceptible to the inhibitory action of arsenate than the activation of acetate.

Acetylation of sulphanilamide by combination of MGE with arylamine-acetylating enzyme from pigeon liver

A further proof of activation of acetate with CoA in our MGE preparations was provided by the acetylation of sulphanilamide when the MGE was combined with the arylamine-acetylating enzyme (A_{80}) prepared from pigeon liver according to Chou & Lipmann (1952). The acetylation reaction proceeds in two stages: (a) the activation of acetate by the acetate-activating enzyme with CoA, and (b) the transfer of acetyl from CoA to sulphanilamide catalysed by the arylamine-acetylating enzyme (Chou & Lipmann, 1952). Since this latter enzyme alone cannot acetylate sulphanilamide unless acetyl-CoA is being provided from another source, it may conveniently be used to test other enzyme systems for their ability to form acetyl-CoA.

As can be seen from the results shown in Table 9, neither the arylamine-acetylating enzyme (A_{80}) nor

the MGE alone could acetylate sulphanilamide. However, a combination of the two preparations (MGE + A_{80}) afforded acetylation. It may be noted that when the amounts of the MGE relative to that of A_{80} were increased, progressively less and less sulphanilamide was acetylated. These observations indicate that the MGE can synthesize acetyl-CoA very efficiently, but that it contains enzymes which compete with the arylamine-acetylating enzyme for the acetyl-CoA formed. For acetylation a combination of 0.1 ml. of MGE with 1.0 ml. of A_{80} was found optimal and was used in further experiments.

Table 10. *The effect of CoA and of ATP on the acetylation of sulphanilamide by a combination of the MGE and the arylamine-acetylating enzyme (A_{80})*

The reaction mixture contained: THAM buffer, pH 8.1, 200 μ moles; cysteine hydrochloride, 10 μ moles; MgCl_2 , 10 μ moles; KF, 100 μ moles; and sulphanilamide, 1 μ mole. Additions: MGE, 0.1 ml.; A_{80} , 1 ml.; ATP, 5 μ moles; CoA, 14 units (crude liver preparation); K acetate, 100 μ moles. Final volume, 2.4 ml. Incubation at 37.5° for 1 hr.

Additions	Free sulphanilamide present (μ g.)	Sulphanilamide acetylated (μ g.)
Incubation mixture alone	163	0
MGE + ATP + CoA + acetate	163	0
A_{80} + ATP + CoA + acetate	159	4
MGE + A_{80}	163	0
MGE + A_{80} + acetate	165	0
MGE + A_{80} + acetate + ATP	82.5	80.5
MGE + A_{80} + CoA + ATP	52.5	110.5
MGE + A_{80} + CoA + ATP + acetate	35.5	127.5

Table 9. *Acetylation of sulphanilamide by a combination of MGE and the arylamine-acetylating enzyme (A_{80})*

The incubation mixture contained: THAM buffer, pH 8.1, 200 μ moles; cysteine hydrochloride, 10 μ moles; MgCl_2 , 10 μ moles; KF, 100 μ moles; K acetate, 100 μ moles; Na-citrate, 50 μ moles; ATP, 5 μ moles; CoA, 14 units (crude liver preparation); and sulphanilamide, 1 μ mole. Enzymes in volumes indicated. Total volume 2.4 ml. Incubation at 37.5° for 1 hr.

Volume of arylamine-acetylating (A_{80}) enzyme (ml.)	Volume of MGE (ml.)	Sulphanilamide acetylated (μ g.)
(a) $\left\{ \begin{array}{l} 0.5 \\ 0.0 \\ 0.5 \\ 0.5 \\ 0.5 \end{array} \right.$	0.0	4
	0.5	1
	0.5	56.0
	1.0	28.5
	1.5	8.5
(b) $\left\{ \begin{array}{l} 0.5 \\ 0.5 \\ 0.5 \\ 1.0 \\ 1.0 \\ 1.0 \end{array} \right.$	0.1	59.5
	0.2	58.0
	0.5	55.0
	0.1	104.5
	0.2	101.5
	0.5	80.0

It may be seen from Table 10 that both CoA and ATP are required for acetylation of sulphanilamide by the combined enzyme preparations. Since the MGE, and probably the A_{80} preparation also, contained CoA, it is not surprising that the addition of ATP alone to the MGE + A_{80} system caused some acetylation; by increasing the concentration of CoA in the system, however, a substantial rise in acetylation was observed. It appears that the activation of acetate (and of other fatty acids) by the MGE is in no way different from that of other activating enzymes of animal origin (Chou & Lipmann, 1952; Beinert *et al.* 1953; Kornberg & Pricer, 1953a; Mahler, Wakil & Bock, 1953) except that ADP can replace ATP.

Acetylation of sulphanilamide was observed in the combined enzyme system even when no substrate (acetate) was added (cf. Table 10), and this confirmed the conclusion presented earlier that acetyl-CoA was formed in the MGE from 'endogenous' substrates. On addition of 100 μ moles of acetate (in the presence of ATP and CoA) the amount of

sulphanilamide acetylated increased by only 15% in contrast to the large amount of extra acetylhydroxamic acid formed when the active acetate was trapped with hydroxylamine under similar conditions. It seems that the activity of the A_{60} preparation was the limiting factor in these experiments, although it should also be pointed out that in the acetylation experiments only 0.1 ml. of MGE was used, whereas in the hydroxamic acid tests 0.5 ml. was employed.

Table 11. *The effect of α -oxoglutarate and of malonate on the acetylation of sulphanilamide by a combination of MGE and arylamine-acetylating enzyme (A_{60})*

The incubation mixture contained: THAM buffer, pH 8.1, 200 μ moles; cysteine hydrochloride, 10 μ moles; $MgCl_2$, 10 μ moles; KF, 100 μ moles; Na-citrate, 50 μ moles; ATP, 5 μ moles; CoA, 14 units; sulphanilamide, 1 μ mole; MGE, 0.1 ml. and A_{60} , 1 ml. Additions: K acetate, K α -oxoglutarate, K malonate, 100 μ moles each. Final volume, 2.4 ml. Incubation at 37.5° for 1 hr.

Additions	Sulphanilamide acetylated (μ g.)	
	Expt. 150	Expt. 152
None	85.0	89.5
Acetate	88.0	94.5
α -Oxoglutarate	73.0	93.0
Acetate + α -oxoglutarate	—	81.0
Malonate	78.0	—

In further experiments the influence of α -oxoglutarate and of malonate was tested on the acetylation reaction, but they had no effect on this reaction in the MGE + A_{60} system (Table 11). This finding corroborates the conclusion drawn earlier, that these substrates did not influence acetate activation.

The effect of coenzyme A and of diphosphopyridine nucleotide (DPN) on fatty acid synthesis from [carboxy- ^{14}C]acetate by the MGE

We have shown already that the MGE preparations contained significant amounts of CoA. To show therefore the dependence of fatty acid synthesis on CoA it was necessary to remove this cofactor from the MGE. Dowex-1 ion-exchange resin (mesh size 200–400) was used for this purpose, as described by Chantrenne & Lipmann (1950); the treatment of the MGE with this resin removes not only CoA but also DPN (Kornberg & Pricer, 1953b).

MGE (10 ml.) was mixed with about 1 g. of Dowex-1 resin (prepared by washing the resin first thoroughly with 1N-HCl and then with water until the washings became neutral), and the mixture was left standing, packed in ice, with occasional stirring

for 30 min., after which the resin was centrifuged off. The removal of the CoA was checked by assaying the original and resin-treated enzyme preparation for their CoA content. In the experiment shown in Table 12 the original MGE contained 20 units of CoA/ml.; after treatment with resin this was reduced to 3.8 units/ml.

Using the same preparation (no. 154) we compared the fatty acid synthesis in the original MGE with the synthesis in the Dowex-1-treated MGE (Table 12). The treatment with resin resulted in an

Table 12. *The effects of CoA and of DPN on fatty acid synthesis by the MGE (preparation no. 154)*

Each flask contained: K [carboxy- ^{14}C]acetate, 60 μ moles, 5 μ C ^{14}C ; K α -oxoglutarate, 0.02M; K malonate, 0.05M; K phosphate buffer, pH 7.4, 0.015M; $MgCl_2$, 0.01M; ATP, 0.01M; MGE (prepared in KCl in THAM buffer), 1.1 ml. Additions and their final concentration: cysteine hydrochloride, 0.005M; CoA (crude liver preparation) 40 units; DPN, 1.5 mg. (5 mg. of a 35% pure preparation). Final volume, 3 ml. Incubation at 37.5° for 70 min.

Additions	Untreated MGE (20 units CoA/ml.)	Dowex-1- treated MGE (3.8 units CoA/ml.)
	0.231	0.024
None	0.231	0.024
Cysteine-HCl	0.175	0.024
CoA	1.600	1.430
DPN	0.827	0.053
Cysteine-HCl + CoA	1.625	2.200
Cysteine-HCl + CoA + DPN	1.500	2.440
Boiled MGE (1.1 ml.)	—	0.293
Boiled MGE (1.1 ml.) + cysteine-HCl + CoA + DPN	—	3.210

almost complete loss of fatty acid synthesis. Full reactivation was, however, obtained by the addition of an equal volume of boiled MGE (thus making the content in heat-stable cofactors the same as that in the vessel with untreated MGE). A crude liver CoA preparation could replace the boiled MGE, and in addition strongly stimulated fatty acid synthesis in the original and in the Dowex-treated MGE. Diphosphopyridine nucleotide (DPN) alone could not reactivate the system, although it markedly stimulated fatty acid synthesis by the non-treated MGE. The addition of CoA plus DPN in the presence of boiled MGE enhanced very markedly fatty acid synthesis by the Dowex-1-treated enzyme.

This experiment was repeated with another enzyme preparation (no. 159, Table 13). In this instance the resin removed only 50% of the CoA from the MGE, and fatty acid synthesis was reduced to 30% of that observed with the non-treated MGE. In this case DPN alone, besides CoA, could reactivate the system completely. The addition of both CoA and DPN caused further stimulation of fatty acid synthesis in both the treated and non-

treated MGE. In this experiment a CoA preparation, free from DPN and reputed to be 50% pure, was used (see note to Table 13). A crude CoA preparation made from rat liver was, however, more effective in stimulating fatty acid synthesis by the resin-treated and non-treated MGE than the purified CoA preparation, the comparison being based on the number of CoA units added to the enzyme system.

Parallel with the studies on fatty acid synthesis from [*carboxy*-¹⁴C]acetate in the Dowex-treated and non-treated MGE, the formation of acethydroxamic

acid was also followed. Table 14 shows that only small amounts of acetate were activated in the Dowex-treated MGE and that full reactivation and stimulation were observed when CoA was added.

DISCUSSION

The results presented have demonstrated that the soluble enzyme preparations made from homogenates of rat mammary gland contained CoA and enzymes capable of activating acetate and also other fatty acids with this coenzyme. The activation of acetate, however, exceeded several times the activation of other fatty acids (e.g. butyrate and palmitate), a fact which may at least partly account for the predominance of short-chain fatty acids synthesized in the preparations (cf. Popják & Tietz, 1955).

The specificity of the acetate and fatty acid activating reaction for ATP in the presence of purified or partially purified enzymes is well established (Jones, Lipmann, Hiltz & Lynen, 1953; Mahler *et al.* 1953; Kornberg & Pricer, 1953*a*; Beinert *et al.* 1953). Our observation that ADP (but not AMP) can effectively replace ATP in the MGE for the activation reaction is not incompatible with the findings of these workers and, as has already been discussed, may be due to myokinase activity in our preparations (Popják & Tietz, 1955).

The two tests (hydroxamic acid test, acetylation of sulphanilamide) used for the demonstration of formation of acetyl-CoA in our soluble enzyme system indicated the presence of endogenous substrates giving rise to the formation of acetyl-CoA and higher acyl-CoA derivatives. Whether the source of this acetyl is preformed acetate or some other substrate yielding acetate through oxidative reactions cannot be decided at present. However, the consequence of this 'endogenous' formation of acetyl-CoA in our enzyme preparations is that in the measurements of fatty acid synthesis from [¹⁴C]-acetate we must, inevitably, have underestimated the amounts of acetate converted into fatty acids, since the calculations were based on the specific radioactivity of the acetate added. We think that the values for fatty acid synthesis given in this and the preceding paper are probably too low by about 30%. Even without this correction, the amounts of acetate converted into fatty acids by the MGE preparations in the presence of α -oxoglutarate plus malonate, and especially when the MGE was fortified with CoA and DPN, exceeded by a factor of about ten the amounts of acetate converted into fatty acids by mammary-gland slices under similar conditions (cf. Popják & Tietz, 1954*a*), the comparison being made on a dry-weight basis.

The fact that α -oxoglutarate and malonate stimulated only fatty acid synthesis from acetate,

Table 13. *Effects of CoA and of DPN on fatty acid synthesis by the MGE (preparation no. 159)*

The experimental conditions were as shown in Table 12 except that 2 ml. of MGE were used. Additions were made as indicated. Final volume, 3 ml. Incubation at 37.5° for 70 min. Fatty acid synthesis expressed as μ moles acetate incorporated/100 mg. dry weight of the MGE preparation.

Additions	Untreated MGE (21 units CoA/ml.)	Dowex-1- treated MGE (10.5 units CoA/ml.)
None	0.270	0.084
CoA, 32 units ('50% pure')*	0.419	0.234
DPN, 1.5 mg.	0.377	0.320
CoA, 32 units ('50% pure')* + DPN, 1.5 mg.	0.421	0.412
CoA, 40 units (crude liver preparation) + DPN, 1.5 mg.	0.876	0.880

* 3.0 mg. of a '50% pure' CoA preparation (dated 4 April 1952) were kindly given to us by Dr J. Baddily of the Lister Institute, Chelsea Bridge Road, London. Taking the activity of pure CoA to be 400 units/mg., we expected to find a total of 600 units in the 3 mg. sample. According to our assay on 15 February 1954 by the method of Kaplan & Lipmann (1948) only 160 units were found. It is probable that the sample deteriorated on long storage. The numbers of units stated in the Table are based on our assay.

Table 14. *The effects of CoA on acetate activation by the MGE*

The incubation mixture contained: THAM buffer, pH 8.1, 200 μ moles; cysteine hydrochloride, 10 μ moles; MgCl₂, 10 μ moles; hydroxylamine, 1 m-mole; ATP, 10 μ moles; K acetate, 100 μ moles; MGE, 0.5 ml. Final volume, 3 ml. Incubation at 37.5° for 1 hr.

Preparation no.	Additions	Acethydroxamic acid formed ($t_{80} - t_0$), μ moles	
		Untreated MGE	Dowex-1-treated MGE
154*	None	0.80	0.17
	CoA, 20 units	1.17	1.07
159*	None	0.34	0.19
	CoA, 20 units	1.14	1.36

* For the CoA content of the untreated and Dowex-1-treated MGE cf. Tables 12 and 13.

but not the formation of acetyl-CoA, indicates that the action of these two substances on fatty acid synthesis must be sought in the utilization of acetyl-CoA. The stimulating action of added DPN on fatty acid synthesis underlines our previous suggestion that in the MGE system DPNH is generated through the action of α -oxoglutaric dehydrogenase and acts as hydrogen donor for the reduction of some of the intermediates in fatty acid synthesis. As to the action of malonate, only highly speculative views could be advanced at present.

Of the two main inhibitors of fatty acid synthesis in the MGE, Hg^{2+} seems to inhibit the primary step, i.e. the formation of acetyl-CoA, whereas arsenate acts both on this and on subsequent reactions concerned with the utilization of acetyl-CoA. It should be mentioned, however, that Chou & Lipmann (1952) reported that arsenate did not inhibit formation of acetyl-CoA by the pigeon liver acetate-activating enzyme.

The experiments reported have also shown the complete dependence of fatty acid synthesis on CoA and DPN, since the MGE depleted of these coenzymes by treatment with Dowex-1 ion-exchange resin lost its ability to synthesize fatty acids but could be fully reactivated by the addition of CoA and DPN. It should be mentioned that Van Baalen & Gurin (1953) have arrived at similar conclusions while working with enzyme preparations made from pigeon liver. These authors used charcoal for the removal of CoA and of DPN from their enzyme preparations but succeeded only in partial reactivation of their system afterwards. The charcoal treatment may have caused a loss of enzymes. Dowex-1 ion-exchange resin had no deleterious effect whatever on the MGE.

Admittedly, some of our experiments about the demonstration of the role of CoA and DPN in fatty acid synthesis have their shortcomings in that impure coenzyme preparations were mostly used. Nevertheless, the results were quite clear cut. Not only were our enzyme preparations fully reactivated by CoA and DPN, but they were markedly stimulated by these cofactors.

On the basis of *in vivo* studies of fatty acid synthesis from $[^{14}\text{C}]$ acetate in the mammary gland, it was concluded that fatty acid synthesis proceeds by a stepwise building up of fatty acid chains from acetate, that the short-chain acids (or at least the carbon skeletons of these) are intermediates in the formation of longer acids and that the process is analogous to the reversal of β -oxidation (Popják, French, Hunter & Martin, 1951; Popják, 1952). On the basis of these *in vivo* studies and of the work with the soluble preparations, the over-all reaction

has been suggested (Popják, 1953). The present studies fully support such a scheme and are in complete agreement with the very beautiful work of Green and Lynen and their colleagues with purified enzymes of fatty acid metabolism (see Lynen & Ochoa, 1953; Green, 1954). So far, only synthesis of butyryl-CoA has been achieved with individual purified enzymes (Stansly & Beinert, 1953). The use of the mammary-gland enzyme system, which seems to be the only preparation of animal origin synthesizing the whole range of *n*-saturated acids, may ultimately reveal the process of fatty acid synthesis in greater detail.

SUMMARY

1. A soluble enzyme system (MGE), prepared from the mammary gland of lactating rats, which synthesizes fatty acids from acetate on addition of ATP, was shown to contain about 50 μg . of CoA/ml.

2. The activation of acetate and of other *n*-fatty acids with CoA in the MGE was inferred from the formation of hydroxamic acids, some of which were identified by paper chromatography. The activation of acetate predominated. ADP could replace ATP in the activation reaction.

3. The MGE contained 'endogenous' substrates from which acetyl-CoA and higher acyl-CoA compounds were formed under appropriate conditions.

4. The presence of a pyruvic oxidase system in the MGE was inferred from the formation of acethydroxamic acid from pyruvate in the presence of ATP.

5. The MGE could act as acetyl-donor for acetylation of sulphanilamide when it was coupled with the arylamine-acetylating enzyme (A_{60}) of Chou & Lipmann (1952).

6. α -Oxoglutarate and malonate, which powerfully stimulated fatty acid synthesis from acetate in the MGE, had no effect on the formation of acetyl-CoA.

7. Hg^{2+} inhibited fatty acid synthesis from acetate by virtue of abolishing formation of acetyl-CoA. Arsenate caused only a moderate inhibition of formation of acetyl-CoA, but had a strong inhibitory effect on fatty acid synthesis.

8. Removal of CoA and DPN from the MGE by treatment with Dowex-1 ion-exchange resin abolished fatty acid synthesis from acetate. The system was fully reactivated by addition of boiled MGE or of CoA and DPN.

9. Addition of excess CoA and DPN to fresh MGE, or to MGE treated with Dowex-1 resin, enhanced considerably the incorporation of acetate into fatty acids.

10. The bearing of the results on the mechanism of biosynthesis of fatty acids is discussed.

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Studies on the Use of Sulphonated Cation-Exchange Resins for the Hydrolysis of Ovomucoid

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Acid hydrolysis of mucoproteins in order to liberate their sugar components tends at the same time to destroy the sugars produced. The carbohydrate residues present (as judged by colorimetric estimations) are not all recovered as monosaccharides after hydrolysis. During such hydrolysis there is usually a formation of black insoluble, or yellow soluble, humins, which probably arise in part from condensation of liberated neutral sugars with amino sugars or amino acids. Tryptophan is particularly liable to combine with neutral sugars in this manner (Lugg, 1938). Underwood & Deatherage (1952) heated the sulphonated cation-exchange resin Dowex-50 with coffee proteins and obtained a good yield of amino acids which were absorbed by the resin. Humins were not formed. Since neutral sugars would not be absorbed, the resin method seemed applicable to mucoproteins, in that the products of hydrolysis would be immediately separated, and their further chemical interaction less likely. Moreover, neutral sugars would be exposed to a high concentration of

hydrogen ions only in the immediate vicinity of the insoluble resin: this also might reduce sugar destruction. Preliminary experiments (A. St. J. Dixon & R. W. Jeanloz, unpublished) confirmed that Dowex-50 resin hydrolysis compared favourably with previously used acid-hydrolytic methods in liberating neutral sugars from mucoproteins isolated from human plasma. Accordingly, the following study of the method was undertaken, using ovomucoid as a model.

MATERIALS AND METHODS

Ovomucoid was obtained from Worthington Biochemical Corporation, Freehold, New Jersey, prepared according to Lineweaver & Murray (1947), and had a moisture content of 14.6% as determined by drying to constant weight under 2.0 mm. Hg at 60°. Its composition, corrected for moisture content, was as follows: acetyl, 4.3; N, 13.4, 13.6; hexosamine 9.0, 9.4 (2N-HCl hydrolysis); hexose (expressed as mannose), 10.0 ± 0.3 (average of five estimations, anthrone and orcinol methods, $\pm$ standard deviation).