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Biosynthesis of Fatty Acids in Cell-free Preparations

2. SYNTHESIS OF FATTY ACIDS FROM ACETATE BY A SOLUBLE ENZYME SYSTEM PREPARED FROM RAT MAMMARY GLAND

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(*Received* 15 *November* 1954)

We have reported previously the synthesis of short- and long-chain fatty acids from acetate by cell-free suspensions (homogenates) of mammary gland of lactating rats and sheep (Popják & Tietz, 1954*a*). The following conditions were required for synthesis in the preparations of rat mammary gland: (*a*) aerobic incubation, (*b*) the co-oxidation of any of the three keto acids: pyruvate, oxaloacetate or α-oxoglutarate, and (*c*) the addition of adenosine triphosphate (ATP), although the last was not strictly required with pyruvate and α-oxoglutarate. The maximum synthesis of fatty acids from acetate occurred in the presence of acetate (0·02 M), oxaloacetate (0·02 M) and of ATP (0·01 M) and when the gas phase was air instead of pure O_2.

In this article the preparation and some of the properties of a soluble enzyme system from the mammary gland of lactating rats are described. The results have already been presented in a preliminary form (Popják & Tietz, 1953, 1954*b*).

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MATERIAL AND METHODS

Preparation of the soluble enzyme system. Homogenates of the mammary gland of lactating rats, 10–14 days after parturition, were prepared first as described previously (Popják & Tietz, 1954*a*) and were then fractionated by high-speed centrifuging. The homogenate, from which whole cells, cell debris, nuclei, etc., have been removed by preliminary centrifuging at 400 *g* for 10 min., was centrifuged first at 25 000 *g* for 30 min. and at 0°. A clear, transparent pink supernatant (Sp. I) was taken off and filtered through a small pad of cotton wool to remove a thin film of fat from the top. The sediment, designated as 'mitochondria', was washed twice by dispersion in fresh, ice-cold buffer and centrifuging at 25 000 *g* for 15 min. A sample of Sp. I was centrifuged further at 2–4° and at 104 000 *g* for 30 min. in a Spinco preparative centrifuge. The supernatant (Sp. II), which in appearance was similar to Sp. I, was taken off and filtered as described for Sp. I. The sediment, a pinkish brown translucent pellet, designated as 'microsomes', was washed once with fresh buffer and sedimented at 104 000 *g* for 10 min.

The 'mitochondria' obtained from 5 ml. of homogenate were suspended with the aid of a small glass homogenizer in 2·5 ml. of buffer or in the same volume of Sp. I, to provide 2 ml. for incubation and 0·5 ml. for determination of dry weight. The 'microsomes' obtained from 10 ml. of Sp. I were treated in the same way with buffer or Sp. II.

After it was found that all the enzymic activity required for fatty acid synthesis was present in Sp. II, the procedure was shortened. Homogenates were prepared in either phosphate buffer (0·154 M-KCl, 100 parts; 0·154 M-$MgCl_2$,

10 parts; 0·1 M potassium phosphate buffer, pH 7·4, 35 parts) or in THAM buffer (0·154 M-KCl, 9 parts; and 0·5 M aminotrishydroxymethylmethane buffer, pH 7·5, 1 part), and centrifuged immediately at 104 000 *g* for 1 hr. Most of the fat which separated on the surface was scooped off and the rest removed by filtering the supernatant through a small pad of cotton wool. The filtered supernatant provided the soluble mammary-gland enzyme system (MGE). The precipitate was discarded.

Since there was no observable difference between the enzymic activities of MGE's prepared in phosphate or in THAM buffer, no distinction will be made between the two types of preparation. However, when the MGE was prepared in THAM buffer, 50 μmoles of potassium phosphate buffer, pH 7·4, and 30 μmoles of $MgCl_2$ were added later to the incubation mixture (total volume, 3 ml.).

The synthetic activity of the various batches of MGE varied a good deal, although not as much as the activity of the homogenates (cf. Popják & Tietz, 1954*a*). These variations are not surprising in view of the crude nature of the enzyme preparations and since the synthesis of fatty acids depends probably on a number of enzymes and coenzymes. The synthetic activity of different batches of enzymes under various conditions may easily be compared since the results are expressed throughout this paper as μmoles (or 1×10^{-3} μmoles) of acetate incorporated into fatty acids per 100 mg. salt- and fat-free dry weight of the enzyme preparations.

Incubations. Aerobic and anaerobic incubations were made in Warburg vessels as described previously for homogenates (Popják & Tietz, 1954*a*). In the anaerobic incubations ATP and [^{14}C]acetate were tipped in from the side arm after the enzyme preparations had been incubated under N_2 for 10 min. Commercial N_2 and N_2 purified by passing through an ammoniacal cuprous chloride solution were used.

Extraction of fatty acids. This was done as described previously for homogenates (Popják & Tietz, 1954*a*) except that an ethanol–ether (3:1, v/v) extract of the mammary-gland fat, obtained in the course of the centrifuging, was added at the end of the incubation and before saponification to the preparations of Sp. I, Sp. II and MGE on account of the very low fat content of these. The amount of fat added was equivalent to 10 mg. of mixed fatty acids.

[*carboxy*-^{14}C]Acetate (obtained from the Radiochemical Centre, Amersham, Bucks) was used as precursor of fatty acids. The purification of fatty acids from contaminating [^{14}C]acetate and the methods of ^{14}C counting and calculations were described in our previous communication (Popják & Tietz, 1954*a*).

RESULTS

A comparison of the various fractions obtained from the centrifugal fractionation of mammary-gland homogenates for their ability to synthesize fatty acids from acetate is shown in Table 1. The conditions of these experiments were those found optimal for fatty acid synthesis by the homogenates. It can be seen from Table 1 that both supernatants, Sp. I and Sp. II, were more active (on a dry weight basis) than the full homogenates from which they were obtained. The mitochondrial fraction showed only a very slight synthetic activity. Moreover, when it was combined with Sp. I, fatty acid synthesis by the mixture was depressed to the originally lower level of the full homogenates. The microsomes completely lacked the ability to synthesize fatty acids and, in the few experiments tested (nos. 116 and 117), seemed to depress slightly the activity of Sp. II.

The soluble enzyme preparations (MGE), with the exception of one experiment (no. 142), were also

Table 1. *Fatty acid synthesis from* [carboxy-^{14}C] *acetate by fractions of rat mammary-gland homogenates*

Each flask contained: K acetate, 60 μmoles (5 μc ^{14}C); K oxaloacetate, 0·02 M; ATP, 0·01 M, and 2 ml. of the indicated preparation; final volume, 3 ml. Fatty acid synthesis expressed as μmoles $\times 10^{-3}$ acetate incorporated/100 mg. dry weight (corrected for salt and fat content).

Fraction incubated	Preparation no. 112	114	116	117	119	120
Full homogenate	6·95	21·2	8·9	12·1	2·5	3·6
Sp. I	35·20	32·4, 27·8	90·0	75·0	9·1	17·3
Mitochondria	6·20	8·48, 9·30	—	10·3	—	—
Mitochondria + Sp. I	3·85	12·90, 14·10	—	4·54	—	—
Sp. II	—	—	48·5	18·5	41·5	62·1
Microsomes	—	—	0·0	0·0	—	—
Microsomes + Sp. II	—	—	25·6	12·4	—	—

Table 2. *Comparison of fatty acid synthesis from* [carboxy-^{14}C]*acetate by full homogenate and soluble mammary gland enzyme system* (*MGE*)

Experimental details as in Table 1. Results expressed as μmoles $\times 10^{-3}$ acetate incorporated into fatty acids/100 mg. dry weight.

Preparation no.	Fatty acid synthesis: Full homogenate	MGE
131	7·5	72·5
134	22·5	47·3
135	31·1	(*a*) 56·0
		(*b*) 62·0
141	18·2	165·0
142	107·0	94·4
143	8·2	59·0

Table 3. *The effect of short and long homogenization on fatty acid synthesis by full homogenate and MGE*

Experimental details as in Table 1. Results expressed as μmoles $\times 10^{-3}$ acetate incorporated into fatty acids/100 mg. dry weight.

Preparation no.	Mode of homogenization: 1 min. in a loose homogenizer* — Full homogenate	1 min. in a loose homogenizer* — MGE	1 min. in loose homogenizer* + 1 min. in tight homogenizer† — Full homogenate	1 min. in loose homogenizer* + 1 min. in tight homogenizer† — MGE
138	187·0	—	75·0	325·0
132	76·0	130·5	11·4	135·0
140	18·8	73·0	4·9	92·0

* Difference between diameter of tube of homogenizer and that of the pestle, 0·4 mm.
† Difference between the diameter of tube and that of the pestle, 0·2 mm.

Table 4. *The effect of freezing and thawing on fatty acid synthesis by the MGE*

Each flask contained: K acetate, 60 μmoles (5 μc ^{14}C); ATP, 0·01 M; 2 ml. of MGE; further additions were as follows: K oxaloacetate, 0·02 M; K α-oxoglutarate, 0·02 M and K malonate 0·05 M; final volume, 3 ml. Results expressed as μmoles acetate incorporated into fatty acids/100 mg. dry weight.

Preparation no.	No. of days kept frozen	Additions	Fatty acid synthesis: Fresh MGE	Fatty acid synthesis: Frozen MGE
135	6	α-Oxoglutarate	0·168	0·053
136	2	α-Oxoglutarate	0·069	0·064
139	4	Oxaloacetate	0·135	0·182
140	2	Oxaloacetate	0·092	(*a*) 0·214 (*b*) 0·233
141	2	Oxaloacetate	0·165	0·187
152	2	α-Oxoglutarate	0·077	0·145
	5	α-Oxoglutarate + malonate		0·146
162	27	α-Oxoglutarate + malonate	0·760	0·281

more active than the homogenates in synthesizing fatty acids from acetate (Table 2). It is of interest to note that, while prolonged homogenizing impaired very considerably the synthetic activity of homogenates, there was no impairment in the activity of the MGE (Table 3). A poorly active homogenate could still yield a highly active MGE. The change produced in the homogenates by prolonged grinding leading to a decreased synthetic activity is unknown at present.

The MGE retained its enzymic activities after being kept frozen in solid CO_2 for several days (Table 4). Surprisingly, the synthetic activity of the preparations was frequently greater after a few (2–5) days' storage in solid CO_2 than when freshly tested. This change is attributed to the precipitation of some inactive protein, which almost invariably occurred on thawing. Further experiments, which will be described below, were carried out with either fresh or stored preparations as convenient. It should be pointed out that experiments made with any one batch of MGE are entered in the tables under the same number, but these were not necessarily carried out on the same day; this is the explanation for the apparent variation in the synthetic activity of the same batch of MGE under otherwise identical conditions.

The rate of fatty acid synthesis by the MGE

The effect of varying times of incubation on the amounts of acetate incorporated into fatty acids is illustrated in Fig. 1. These experiments were set up

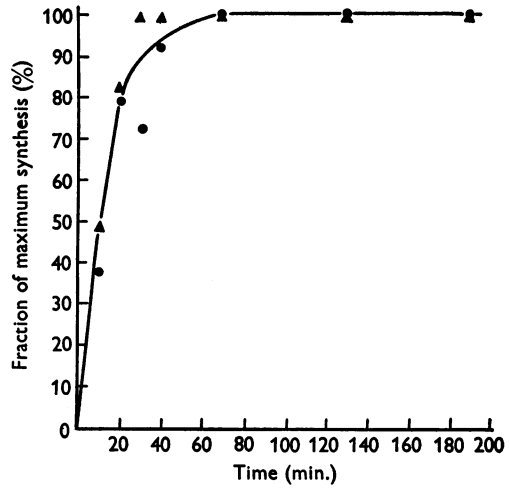

Fig. 1. Rate of fatty acid synthesis by soluble mammary gland enzyme system (MGE).

in such a way that 2 ml. of MGE were mixed with 1 ml. of additions in Warburg vessels and incubation was started immediately. The first vessel was removed after 10 min. (the time interval usually allowed for equilibration before O_2 uptake measurements were started). Further vessels were removed 10, 20, 30 min., 1, 2 and 3 hr. later. It can be seen from Fig. 1 that 50% of maximum incorporation was reached about 15 min. after the incubation had been started and maximum incorporation of [^{14}C]-acetate into fatty acids was attained in 1 hr.; once the maximum was reached no further changes in the specific activity of the fatty acids occurred. Incubation for 70 min. was therefore used.

Type of fatty acids synthesized

The fatty acids recovered from preparation no. 140 after incubation with [*carboxy*-^{14}C]acetate in the presence of oxaloacetate and ATP were resolved into two groups by reversed-phase chromatography (Howard & Martin, 1950). The first group contained all the acids which were removed from the column with 45% (v/v) aqueous acetone; i.e. the first band which contains butyric, caproic (hexanoic), and caprylic (octanoic) acids, plus a second band of capric (decanoic) acid. On changing the solvent to 75% (v/v) aqueous acetone all long-chain fatty acids with a chain length between C_{12} and C_{18} emerged in one band.

Both acid groups were radioactive: $5{\cdot}34 \times 10^{-3}$ μc (75·8%) were recovered in the first fraction and $1{\cdot}70 \times 10^{-3}$ μc (24·2%) in the second fraction. Since an unknown mixture of fatty acids was added before saponification, the true specific activity of each fraction in the preparation could not be calculated. The results, however, indicate that the MGE synthesizes both short- and long-chain fatty acids from acetate, with a preference for short-chain acids.

The effect of ATP, pyruvate and some intermediates of the citric acid cycle on fatty acid synthesis by the MGE

In the experiments described so far the experimental conditions were those found optimal for the full homogenates. It was found, however, that whereas in the full homogenate the addition of pyruvate or oxaloacetate or α-oxoglutarate and of ATP was required to activate fatty acid synthesis from acetate, in the soluble preparation ATP alone was sufficient for activation. An absolute requirement for ATP was demonstrated; only traces of [^{14}C]acetate were incorporated into fatty acids unless ATP was added to the incubation mixture. In contrast to observations with the full homogenate, even α-oxoglutarate could stimulate fatty acid synthesis only in the presence of ATP. When increasing amounts of ATP were added (in the presence of acetate plus oxaloacetate) the amounts of acetate incorporated into fatty acids also increased, a concentration of 0·01 M being optimum (Table 5).

The effects of pyruvate and of some intermediates of the citric acid cycle (added in the presence of ATP) were further studied. Table 6

Table 5. *The effect of varying concentrations of ATP on fatty acid synthesis by the MGE*

Each flask contained: K acetate, 0·02 M (5 μc ^{14}C); K oxaloacetate, 0·02 M; MGE, 2 ml. (prepared in phosphate buffer); ATP was added as indicated; final volume, 3 ml. Fatty acid synthesis expressed as μmoles $\times 10^{-3}$ acetate incorporated/100 mg. dry weight.

Preparation no.	Final concentration of ATP added					
	None	0·001 M	0·002 M	0·005 M	0·01 M	0·02 M
120	4·7	5·9	—	13·25	62·1	—
142	4·45	10·7	16·9	26·2	59·5	60·0

Table 6. *The effect of pyruvate and of some intermediates of the citric acid cycle on fatty acid synthesis by the MGE*

Each flask contained: K acetate, 60 μmoles (5 μc ^{14}C); ATP, 0·01 M; THAM buffer, pH 7·6, 0·025 M; MGE (in phosphate buffer), 2 ml.; total volume, 3 ml. Additions were as indicated. Fatty acid synthesis expressed as μmoles acetate incorporated into fatty acids/100 mg. dry weight.

Preparation no.	Additions, final concn., 0·02 M				
	None	K pyruvate	K oxaloacetate	K α-oxoglutarate	K succinate
134	0·026	0·032	0·047	0·118	0·041
135	0·058	0·038	0·059	0·168	0·070
136	0·035	0·027	0·026	0·065	0·044
139	0·022	—	0·182	0·086	—
140	0·031	—	0·224	0·505	—
143	0·014	0·014	0·047	0·031	0·045
145	0·018	—	0·073	0·052	0·052
146	0·045	—	0·014	0·163	0·070

shows that oxaloacetate stimulated fatty acid synthesis in most of the preparations although it seemed to inhibit others. α-Oxoglutarate had a marked stimulating effect in all preparations, augmenting the incorporation of [^{14}C]acetate into fatty acids 2 to 5 times. In one preparation (no. 140) a 15-fold increase was observed. Succinate seemed to stimulate fatty acid synthesis also, while citrate, fumarate and malate (not shown in the table) were without effect. Pyruvate, in contrast to its behaviour in the full homogenate, did not stimulate fatty acid synthesis by the MGE; in two experiments (nos. 135 and 136) a slight inhibitory effect was observed.

Oxygen consumption by the MGE

As already described for the full homogenates (Popják & Tietz, 1954*a*) only a very low endogenous O_2 uptake was observed. ATP, however, very markedly stimulated the O_2-consumption. The

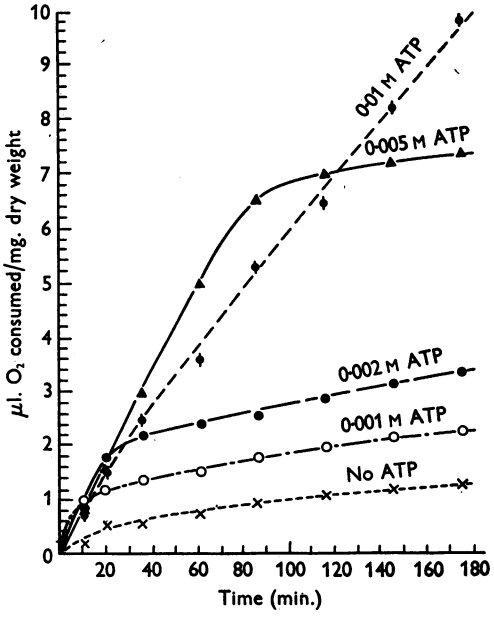

Fig. 2. The effect of varying concentrations of ATP in the presence of acetate (0·02 M) and oxaloacetate (0·02 M) on oxygen uptake by MGE.

dependence of the O_2 uptake on ATP added (in the presence of acetate plus oxaloacetate) can be seen from Fig. 2. With the lower concentrations of ATP (0·001 M–0·005 M) the curves flattened out after an interval and became parallel with the curve obtained in the absence of ATP. This sudden inflexion of the curves suggests that ATP was used up during the preceding periods. Although with 0·01 M ATP the initial rate of O_2 uptake was lower than with 0·005 M ATP, the O_2 uptake continued unchecked for 3 hr. The addition of substrates alone (acetate, pyruvate, oxaloacetate or α-oxoglutarate) had no effect on the O_2 uptake unless ATP was also added. Under this condition, oxaloacetate invariably very markedly stimulated the O_2 uptake. α-Oxoglutarate and pyruvate had a more moderate effect. A typical experiment is illustrated by Fig. 3.

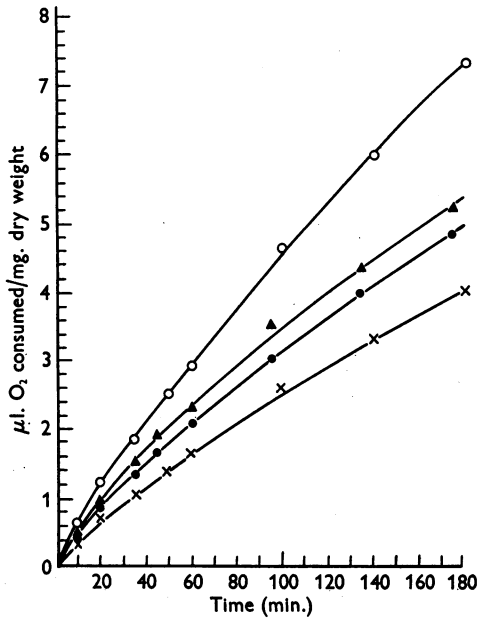

Fig. 3. The effects of pyruvate (0·02 M), oxaloacetate (0·02 M) and of α-oxoglutarate (0·02 M) on the oxygen uptake by MGE in the presence of acetate (0·02 M) plus ATP (0·01 M). ○, Acetate + ATP + oxaloacetate; ▲, acetate + ATP + α-oxoglutarate; ●, acetate + ATP + pyruvate; ×, acetate + ATP.

Acetate and other intermediates of the citric acid cycle: citrate, succinate, fumarate and malate were without effect.

The effect of inhibitors on fatty acid synthesis

In order to get some insight into the enzymic systems involved in the biosynthesis of fatty acids by the MGE, an attempt was made to inhibit fatty acid synthesis with various compounds known to inhibit some vital process. The following substances were chosen: $HgCl_2$ (1×10^{-4} M), arsenate (0·05 M), 2:4-dinitrophenol (2×10^{-4} M), cyanide (0·01 M), azide (0·01 M), fluoride (0·05 M) and malonate (0·05 M). As can be seen from the results shown in Table 7, mercury and arsenate almost completely inhibited the incorporation of acetate into fatty

Table 7. *The effect of inhibitors on fatty acid synthesis and on oxygen uptake by the MGE*

Each flask contained: K acetate, 60 μmoles (5 μc ^{14}C); K oxaloacetate, 0·02 M; ATP, 0·01 M; MGE, 2 ml. (prepared in phosphate buffer); final volume, 3 ml. Fatty acid synthesis expressed as μmoles $\times 10^{-3}$ acetate incorporated/100 mg. dry weight.

Substance added and concentration	Preparation no. 141 $-Q_{O_2}$	141 Fatty acid synthesis	142 $-Q_{O_2}$	142 Fatty acid synthesis
None	2·61	186·5	2·41	35·2
2:4-Dinitrophenol, 2×10^{-4} M	4·46	118·0	1·43	24·6
KF, 0·05 M	2·11	169·0	2·01	53·4
Na_3AsO_4, 0·05 M	1·95	14·4	1·69	11·7
NaN_3, 0·01 M	3·15	184·0	—	—
KCN, 0·01 M	—	57·6	—	25·8
$HgCl_2$, 1×10^{-4} M	2·48	9·8	1·93	9·7
K malonate, 0·05 M	2·43	328·0	2·30	214·0

acids. Of the two substances, only arsenate reduced the O_2 uptake. Cyanide caused a 30–70 % inhibition, while azide was without effect. 2:4-Dinitrophenol caused a 30 % inhibition of fatty acid synthesis and a 40 % inhibition in O_2 uptake. Fluoride was without effect. Malonate, however, markedly stimulated fatty acid synthesis, without affecting the O_2 uptake. These effects of malonate were most surprising and further investigations on this finding were undertaken.

The effect of malonate on fatty acid synthesis by the MGE

It can be seen from Table 8 that malonate markedly stimulated fatty acid synthesis when only acetate and ATP were added. However, no effect was observed in the absence of ATP (cf. Table 9). Comparing the stimulating effect of malonate or of α-oxoglutarate, malonate seemed to have an even stronger effect than α-oxoglutarate, especially when added at a concentration of 0·05 M instead of 0·02 M.

Table 8. *The effect of malonate and of α-oxoglutarate on fatty acid synthesis by the MGE*

Each flask contained: K acetate, 60 μmoles (5 μc ^{14}C); ATP, 0·01 M; THAM buffer, pH 7·6, 0·025 M; MGE, 2 ml. (prepared in phosphate buffer); additions were as indicated. Fatty acid synthesis expressed as μmoles $\times 10^{-3}$ acetate incorporated/100 mg. dry weight.

Preparation no.	Additions, final concn. 0·02 M: None	K α-oxoglutarate	K malonate
140	14·4	31·0	93·0
145	18·2	51·7	33·9
			118·0*
146	45·0	162·5	67·5
153	8·0	20·2	106·0*

* 0·05 M.

We further compared the effect of malonate on fatty acid synthesis in the presence of oxaloacetate or of α-oxoglutarate. When malonate was added together with either of the two substrates, the stimulating effect was higher, particularly with

Table 9. *Comparison of the effect of oxaloacetate, α-oxoglutarate and of malonate on fatty acid synthesis by the MGE*

Each flask contained: K acetate, 60 μmoles (5 μc ^{14}C); THAM buffer, pH 7·6, 0·025 M; MGE, 2 ml. (prepared in phosphate buffer); final concentration of additions: K oxaloacetate and K α-oxoglutarate, 0·02 M; K malonate, 0·05 M; ATP, 0·01 M. Results expressed as μmoles $\times 10^{-3}$ acetate incorporated into fatty acids/100 mg. dry weight.

Additions	Fatty acid synthesis: Without ATP	With ATP
None	—	18·2
Malonate	12·6	118·0
Oxaloacetate	—	72·7
Oxaloacetate + malonate	10·9	241·0
α-Oxoglutarate	—	51·7
α-Oxoglutarate + malonate	11·3	517·0

Table 10. *The effect of malonate in the presence of α-oxoglutarate and of ATP on fatty acid synthesis by the MGE*

Each flask contained: K acetate, 60 μmoles (5 μc ^{14}C); ATP, 0·01 M; K phosphate buffer, pH 7·4, 0·015 M; $MgCl_2$, 0·01 M; MGE, 2 ml. (prepared in THAM buffer); final concentration of additions: K α-oxoglutarate, 0·02 M; K malonate, 0·05 M. Fatty acid synthesis expressed as μmoles acetate incorporated/100 mg. dry weight.

Preparation no.	Additions: None	α-Oxoglutarate	α-Oxoglutarate + malonate
154	0·053	0·427	1·560
156	0·020	0·115	1·200
157	0·010	0·018	0·144

α-oxoglutarate, than would be expected if the effects of the two substances in combination were additive (Tables 9 and 10). While α-oxoglutarate caused a threefold increase, malonate a sixfold increase, α-oxoglutarate and malonate when added together augmented fatty acid synthesis 30 times.

The effect of anaerobic incubation on fatty acid synthesis

As can be seen from the results shown in Table 11, aerobic incubation is not essential for fatty acid synthesis in the presence of ATP, α-oxoglutarate and malonate. Except in one experiment (no. 162) incubation under N_2 did not inhibit fatty acid synthesis; in two experiments (nos. 158 and 164) even a slight stimulation was observed. With oxaloacetate plus ATP as sparkers, however, fatty acid synthesis was inhibited by anaerobic incubation (prep. no. 140), just as was found for the full homogenates (Popják & Tietz, 1954*a*).

Table 11. *The effect of anaerobic incubation on fatty acid synthesis from* [^{14}C]*acetate by the MGE*

Each flask contained: K acetate, 60 μmoles (5 μc ^{14}C); ATP, 0·01 M; MGE, 2 ml.; final concentration of additions: K oxaloacetate, 0·02 M; K α-oxoglutarate, 0·02 M; K malonate, 0·05 M; final volume, 3 ml. Fatty acid synthesis expressed as μmoles acetate incorporated/100 mg. dry weight.

Preparation no.	Additions	Fatty acid synthesis: In air	Fatty acid synthesis: In N_2
140	Oxaloacetate	0·246	0·029
158	α-Oxoglutarate + malonate	0·146	0·371*
			0·256
162	α-Oxoglutarate + malonate	1·910	0·469
163	α-Oxoglutarate + malonate	0·281	0·202
164	α-Oxoglutarate + malonate	0·142	0·200

* Purified N_2.

The effect of adenylic acid, adenosine diphosphate and of ATP on fatty acid synthesis by the MGE

Since an absolute requirement of ATP for fatty acid synthesis was demonstrated (cf. Table 5) it was of interest to determine whether muscle adenylic acid (AMP) or adenosine diphosphate (ADP) could replace ATP. In all experiments in which the effect of AMP, ADP and of ATP was compared, fluoride (0·05 M) was added to inhibit adenosinetriphosphatase, myokinase and pyrophosphatase (if present in the MGE). Table 12 shows that AMP could not effect fatty acid synthesis while ADP was at least as effective as ATP. All three compounds, however, stimulated O_2-consumption. In this experiment and in all preceding experiments commercial samples of AMP, ADP and of ATP of unknown purity were used. For further comparisons the commercial preparations were purified chromatographically on a Dowex-1 ion-exchange column as described by Cohn & Carter (1950). The results obtained (Table 13) were identical with those found when the commercial preparations were used.

Table 12. *The effects of AMP, ADP and of ATP on fatty acid synthesis by the MGE (preparation no. 147)*

Each flask contained: K acetate, 60 μmoles (5 μc ^{14}C); K α-oxoglutarate, 0·02 M; $MgCl_2$, 0·01 M; KF, 0·05 M; MGE, 2 ml. (prepared in THAM buffer); final volume, 3 ml. Fatty acid synthesis expressed as μmoles acetate incorporated/100 mg. dry weight.

Additions	$-Q_{O_2}$	Fatty acid synthesis
None	0·62	0·004
AMP (0·01 M)	1·78	0·008
ADP (0·01 M)	2·63	0·061
ATP (0·01 M)	1·69	0·040

Table 13. *Comparison of the effect of ADP and of ATP on fatty acid synthesis by the MGE*

Conditions as in Table 12, plus 0·05 M K malonate. Fatty acid synthesis expressed as μmoles acetate incorporated/100 mg. dry weight of the preparation.

Additions	Preparation no. 158	Preparation no. 162
None	0·007	—
Commercial ATP (0·01 M)	0·150	1·320
Purified ATP (0·0012 M)	0·022	—
Purified ATP (0·006 M)	0·103	—
Purified ADP (0·0012 M)	0·017	0·125
Purified ADP (0·006 M)	0·125	1·225

DISCUSSION

The distribution of fatty acid-synthesizing enzymes in the mammary gland cells seems to differ from that in liver. Brady & Gurin (1952) and Dituri & Gurin (1953) demonstrated that, in pigeon and rat liver, enzymes of both the mitochondria and the cytoplasmic phase were required for fatty acid synthesis from acetate. In the mammary gland, however, the cytoplasmic fraction alone could effect fatty acid synthesis from acetate, although the possibility that some enzymes were extracted

from the mitochondria in the course of the preparation and were thus contained in the MGE cannot be excluded. It is worth emphasizing, however, that the addition of the 'mitochondrial' fraction to the soluble preparations inhibited fatty acid synthesis. Recently Kornberg & Pricer (1953*a*, *b*) demonstrated that in guinea-pig liver preparations enzymes activating fatty acids with coenzyme A were present both in the mitochondrial and soluble fractions. We have detected similar enzymes in the MGE (Tietz & Popják, 1955). There is now ample evidence available to show that biochemical reactions involving fatty acids require the previous activation of the acids with coenzyme A (for review see Green, 1954). One of the reactions leading to such activation is represented by equation (1) (Mahler, Wakil & Bock, 1953; Kornberg & Pricer, 1953*a*):

$$\text{Fatty acid} + \text{ATP} + \text{HS-CoA} \rightleftharpoons \text{fatty acyl-S-CoA} + \text{AMP} + \text{pyrophosphate} \quad (1)$$

the prototype reaction being the activation of acetate (Jones, Lipmann, Hilz & Lynen, 1953; Beinert *et al.* 1953; Hele, 1954). The absolute requirement for ATP in our MGE preparations is in harmony with this concept. The fact, however, that ATP could be replaced by equimolar amounts of ADP (but not by AMP) suggests the presence of either powerful myokinase activity in our preparations, or of some other reaction leading to the formation of ATP from ADP. It might be mentioned here that the fatty acid-synthesizing soluble preparations of Dituri & Gurin (1953) differ from the MGE in that the addition of ATP is not required and maximum stimulation of the synthesis is obtained in their preparation with citrate, which has no effect in the MGE.

Measurements of oxygen uptake by the MGE indicated that oxaloacetate, α-oxoglutarate and also pyruvate were oxidized by the preparations if ATP was added. The presence of pyruvic oxidase was also inferred from the formation of acetyl-coenzyme A from pyruvate in the MGE (Tietz & Popják, 1955). Since neither the succinic oxidase nor cytochrome *c* oxidase systems were detected in the soluble preparations, it is suggested that the relatively high oxygen uptakes were catalysed by flavine-enzymes.

The fact that in the full homogenate fatty acid synthesis can be linked with the oxidation of α-oxoglutarate or pyruvate, even in the absence of added ATP, suggests that the generation of energy-rich phosphate bonds is coupled to the oxidation of these substrates, whereas in the MGE oxidative phosphorylation does not occur, hence ATP is required. Moore & Nelson (1952) and Terner (1954*a*, *b*) have demonstrated oxidative phosphorylation in homogenates of mammary gland. A lack of apparent stimulation of fatty acid synthesis from [^{14}C]acetate by pyruvate in MGE preparations may be attributed to the dilution of the [^{14}C]acetyl-coenzyme A, formed directly by the acetate activating enzyme, with unlabelled acetyl-coenzyme A derived from the oxidative decarboxylation of pyruvate (see above).

The stimulating effect of α-oxoglutarate on fatty acid synthesis by the MGE can be most readily explained by the generation of reduced diphosphopyridine nucleotide (DPNH) through reaction (2) (Kaufman, Gilvarg, Cori & Ochoa, 1953; Sanadi & Littlefield, 1953; Hift, Quellet, Littlefield & Sanadi, 1953), thus furnishing a hydrogen donor for the reduction of the intermediary products of synthesis. The lack of inhibition of fatty acid synthesis in the MGE by anaerobic conditions in the presence of α-oxoglutarate plus ATP, and the stimulating effect of added DPN on the synthesis (Tietz & Popják, 1955) are in harmony with this conclusion.

$$\alpha\text{-Oxoglutarate} + \text{HS-CoA} + \text{DPN} \xrightleftharpoons[\text{dehydrogenase}]{\alpha\text{-oxoglutaric}} \text{succinyl-S-CoA} + \text{CO}_2 + \text{DPNH} + \text{H}^+. \quad (2)$$

No satisfactory explanation can as yet be offered for the stimulating effects of succinate, oxaloacetate and of malonate on fatty acid synthesis. Further work is planned to elucidate this question.

The mechanism of action of some of the inhibitors, e.g. Hg^{2+} and arsenate, will be analysed in the following communication (Tietz & Popják, 1955).

SUMMARY

1. A soluble enzyme system, synthesizing short- and long-chain fatty acids from acetate, has been prepared by high-speed centrifugal fractionation of homogenates of mammary gland of lactating rats.

2. Neither the 'mitochondrial' nor 'microsomal' fractions of the mammary-gland homogenates were required for fatty acid synthesis. The ability of the soluble supernatant to synthesize fatty acids was usually greater than that of the full homogenate.

3. In the soluble enzyme preparations ATP alone was sufficient to activate synthesis from acetate and the requirement for ATP was absolute. ADP could effectively replace ATP.

4. Fatty acid synthesis was markedly stimulated in the presence of ATP by oxaloacetate, α-oxoglutarate and to a lesser extent by succinate. A particularly strong stimulation was caused by malonate; a combination of α-oxoglutarate with malonate elicited on occasion as much as a 30-fold increase in the amount of [^{14}C]acetate incorporated into fatty acids.

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.

We wish to thank Miss Mary O'Donnell for her technical assistance and Mr J. Orr in the Biophysics Division for carrying out all the ultracentrifugal operations. Miss Maureen Clevely drew the diagrams.

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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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(*Received* 15 *November* 1954)

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, 1954*b*).

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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