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REFERENCES

- Anderson, R. A., Enomoto, M., Miller, J. A. & Miller, E. C. (1963). *Proc. Amer. Ass. Cancer Res.* **4**, 2.
- Bayer, E. & Reuther, K.-H. (1956). *Chem. Ber.* **89**, 2541.
- Bradshaw, L. (1957). *Rep. Brit. Emp. Cancer Campgn*, **35**, 313.
- Bradshaw, L. & Clayson, D. B. (1955). *Nature, Lond.*, **176**, 974.
- Boyland, E., Dukes, C. E. & Grover, P. L. (1963). *Brit. J. Cancer*, **17**, 79.
- Boyland, E., Manson, D. & Nery, R. (1960). *Rep. Brit. Emp. Cancer Campgn*, **38**, 52.
- Boyland, E., Manson, D. & Nery, R. (1962). *J. chem. Soc.* p. 606.
- Boyland, E., Manson, D. & Nery, R. (1963). *Biochem. J.* **86**, 263.
- Boyland, E. & Nery, R. (1964). *Analyst*, **89**, 95.
- Bülow, C. & Baur, K. (1925). *Ber. dtsch. chem. Ges.* **58**, 1926.
- Clayson, D. B. (1959). *Acta Un. int. Cancer*, **15**, 581.
- Cramer, J. M., Miller, J. A. & Miller, E. C. (1960). *J. biol. Chem.* **235**, 885.
- Engelbertz, P. & Babel, E. (1953). *Zbl. Arbeitsmed.* **3**, 161.
- Fincham, J. R. S. (1954). *J. gen. Microbiol.* **11**, 236.
- Gibbs, H. D. (1927). *J. biol. Chem.* **72**, 649.
- Harley-Mason, J. & Archer, A. A. P. G. (1958). *Biochem. J.* **69**, 60 p.
- Irving, C. C. (1962*a*). *Biochim. biophys. Acta*, **65**, 564.
- Irving, C. C. (1962*b*). *Cancer Res.* **22**, 867.
- Kampffmeyer, H. & Kiese, M. (1963). *Arch. exp. Path. Pharmacol.* **244**, 375.
- Lossen, W. (1892). *Liebigs Ann.* **161**, 356.
- Lotlikar, P. D., Miller, E. C. & Miller, J. A. (1963). *Proc. Amer. Ass. Cancer Res.* **4**, 39.
- Miller, E. C. & Miller, J. A. (1960). *Biochim. biophys. Acta*, **40**, 380.
- Miller, E. C. & Miller, J. A. (1962). *Abstr. Pap. Amer. chem. Soc. 142nd Meet.*, p. 36 c.
- Miller, E. C., Miller, J. A. & Hartmann, H. A. (1961*a*). *Cancer Res.* **21**, 815.
- Miller, J. A., Wyatt, C. S., Miller, E. C. & Hartmann, H. A. (1961*b*). *Cancer Res.* **21**, 1465.
- Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
- Sciarini, L. J. & Meigs, J. W. (1961). *Arch. environmental Hlth*, **2**, 423.
- Stadtman, E. R. & Barker, H. A. (1950). *J. biol. Chem.* **184**, 769.
- Troll, W., Belman, S. & Rinde, E. (1963). *Proc. Amer. Ass. Cancer Res.* **4**, 68.
- Troll, W. & Nelson, N. (1961). *Fed. Proc.* **20**, 41.
- Uehleke, H. (1961). *Experientia*, **17**, 557.
- Uehleke, H. (1963). *Biochem. Pharmacol.* **12**, 219.
- Weisburger, J. H., Grantham, P. H. & Weisburger, E. K. (1962). *Proc. Amer. Ass. Cancer Res.* **3**, 371.

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Fatty Acid Synthesis in Cell-Free Preparations of Rat Liver containing Phosphorylating Mitochondria

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If rats are treated with thyroxine, fatty acid synthesis is depressed in cell-free fractions of their livers from which all the particles sedimenting at 10000*g* for 20 min., including the mitochondria, have been removed by centrifugation ('S₁₀ fractions') (Scaife & Migicovsky, 1957; Fletcher & Myant, 1960). Fletcher & Myant (1961*b*) have shown that the S₁₀ fraction from thyroxine-treated rats ('treated S₁₀ fractions') contains very little glycogen, and that synthesis of fatty acids can be partially restored by adding either glycogen or ATP *in vitro*. Since glycogen is the main source of

energy for the regeneration of ATP in S₁₀ fractions from untreated rats, Fletcher & Myant (1961*b*) suggested that the depression of fatty acid synthesis in the treated S₁₀ fractions was due indirectly to the fall in glycogen content of the liver brought about by thyroxine. They pointed out, however, that the energy for the regeneration of ATP in normally respiring liver cells is generally thought to be derived from oxidation in the mitochondria, and not from the breakdown of glycogen. It was therefore desirable to study the effect of thyroxine treatment on fatty acid synthesis in liver prepa-

rations that more closely resembled the intact cell, especially with regard to their source of energy for the regeneration of ATP.

In the present paper we describe a cell-free preparation of rat liver that is capable of synthesizing long-chain fatty acids from acetate and in which all the ATP required for synthesis is generated by phosphorylating mitochondria, the preparation containing no added cofactors.

METHODS

Cell fractionation. The rats were albino males of an inbred strain, weighing 150–250 g. They were given a diet of dried pellets (M.R.C. diet no. 18; Bruce & Parkes, 1946) and were allowed to feed *ad libitum* until they were used for the experiment. They were killed by breaking their necks; the livers were then removed immediately and minced as rapidly as possible in an ice-cold stainless-steel mincer. The minced tissue was stirred into 20 ml. of sucrose solution [sucrose (250 mM), EDTA (2 mM) and nicotinamide (5 mM), adjusted to pH 7.4 with KOH] in a weighed beaker. The beaker plus minced tissue was then weighed rapidly, and enough sucrose solution added to give a final volume of 4 ml. of sucrose solution/g. of liver. The suspension of minced tissue was homogenized in a glass tube with a loose-fitting Teflon plunger driven up and down by hand 20 times. For the preparation of the standard mitochondrial system the homogenate was spun at 700g for 10 min. and the supernatant ('S_{0.7} fraction') decanted from the sediment of nuclei and cell debris. To remove the mitochondria, the S_{0.7} fraction was spun at 9000g for 20 min. The supernatant from this spinning was then decanted from the mitochondrial pellet. When isolated mitochondria were required, they were washed twice by resuspension in the sucrose solution, followed by centrifugation at 9000g for 10 min. After the second wash they were suspended in a volume of the sucrose solution equal to the original volume of S_{0.7} fraction from which they were prepared. They were used within 1 hr. of their preparation, and were kept on ice until the beginning of the incubation. All the procedures for cell fractionation were carried out at 1°. In the preliminary stage of the work the distilled water was obtained from a metal still; later deionized glass-distilled water was used for making up all solutions, since this gave more consistent results.

Conditions of incubation. The standard incubation mixture contained 2.0 ml. of S_{0.7} supernatant in a final volume of 4.5 ml. with tris-HCl buffer, pH 7.4 (45 mM), KCl (40 mM), MgCl₂ (4 mM), potassium acetate (2.5 or 5.0 mM), sodium [1-¹⁴C]acetate (0.01 mM, 2 μ C/flask), K₂HPO₄ (1.0 mM), GSH (5.0 mM), neutralized with KOH, and potassium succinate (15 mM). Each flask contained the equivalent of 400 mg. wet wt. of liver. The concentration of total protein in the incubation mixture, estimated by the method of Cleland & Slater (1953), averaged 39.1 mg./ml. The incubations were carried out in paired flasks at 37° for 3 hr.

Estimation of adenosine triphosphate. Samples (0.5 ml.) of the incubation mixtures were pipetted into 0.5 ml. of ice-cold 20% (w/v) trichloroacetic acid and left at 0° for 10 min., neutralized with NaOH and made up to 10 ml. with distilled water. If necessary, they were diluted further to give a final concentration of about 2 μ C. of ATP/ml.

The ATP in the extract was estimated from the light emitted by the fire-fly enzyme system (Strehler & Totter, 1954; Fletcher & Myant, 1961 b).

Isolation and radio-assay of fatty acids. At the end of the incubation, 10 mg. of soap prepared from ox fat was added to each flask as carrier for the isolation of fatty acids. Then 1 ml. of saturated NaOH and 5 ml. of ethanol were added and the flasks heated on a steam bath for 3 hr. Unsaponifiable lipids were removed by two extractions with 50 ml. of light petroleum (b.p. 40–60°), and the incubation mixture was acidified with conc. HCl. The fatty acids were extracted twice with 50 ml. of redistilled light petroleum and the petroleum was evaporated under reduced pressure. Two methods were used for radio-assay. In some cases, the dried residue of fatty acids was converted into soap and assayed for radioactivity with an end-window Geiger-Müller counter (Fletcher & Myant, 1960). All counting rates were converted into the rate at 'infinite thickness' by means of a standard self-absorption curve. The values so obtained were converted into μ C of ¹⁴C by comparison with the counting rate given by an 'infinitely thick' standard sample of polymethyl [methyl-¹⁴C]methacrylate containing 1 μ C/g. The amount of acetate incorporated into fatty acids was calculated from the specific activity of the acetate in the incubation medium and the amount of ¹⁴C incorporated. In other cases, the fatty acids were washed into glass vials with 10 ml. of a solution of 2,5-diphenyloxazole in toluene (3 g./l.) and 2 ml. of ethanol. Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer. The percentage of the dose of [1-¹⁴C]acetate incorporated into fatty acids was estimated by comparing the counting rate given by the sample with that given by a known proportion of the dose, in 0.05 ml. of water, dissolved in 10 ml. of 2,5-diphenyloxazole in toluene (3 g./l.) and 2 ml. of ethanol. Endogenous acetate, acetyl-CoA and malonyl-CoA were neglected in all calculations of the amounts of these substances incorporated into fatty acids.

Gas-liquid chromatography. The proportion of the total radioactivity in each fatty acid fraction was determined by gas-liquid radiochromatography (Popják, Lowe, Moore, Brown & Smith, 1959) after converting the fatty acids into their methyl esters with diazomethane. The conditions used were those described by Dils & Popják (1962). When the incubation mixture was to be analysed by gas-liquid chromatography, no soap was added before saponification. Identification of the radioactive C₁₂–C₁₈ fatty acids was confirmed by adding enough of their methyl esters to the sample to give well-defined peaks in the record from the gas-density balance. These peaks could be correlated with the successive steps in the record of radioactivity.

Materials. Sodium [1-¹⁴C]acetate and sodium [2-¹⁴C]-malonate were obtained from The Radiochemical Centre, Amersham, Bucks. [1-¹⁴C]Acetyl-CoA was obtained from Tracerlab, Waltham, Mass., U.S.A.; [2-¹⁴C]malonyl-CoA was prepared essentially by the method of Trams & Brady (1960). Avidin was either prepared from fresh hen's eggs by the method of Woolley & Longworth (1942) or obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. The activity of the preparations was assayed by Dr R. H. Pritchard, using a biotin-requiring strain of *Aspergillus nidulans*. Dried fire-fly tails were obtained from Schwartz BioResearch Inc., Mount Vernon, N.Y., U.S.A. ATP, NADH and NADP⁺ were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. CoA, GSH and

glucose 6-phosphate were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Citric acid and trisodium DL-isocitrate were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex. Biotin was obtained from Roche Products Ltd., Welwyn Garden City, Herts.

RESULTS

Incorporation of acetate into fatty acids in the standard incubation mixture. The amount of acetate incorporated into fatty acids in the standard incubation mixture reached a maximum of 100–200 μ m-moles when the concentration of acetate was between 5 and 10 mM (Fig. 1). Incorporation followed a sigmoid course: a lag phase of 1 hr., during which little activity appeared in the fatty acids, was followed by a period of rapid incorporation reaching 90 % of completion within 3 hr. of zero time (Fig. 2). The lag phase was not due to the time required for the mitochondria to build up an adequate concentration of ATP, since the ATP concentration in the incubation mixture was maintained at a high level from zero time (Fig. 2); nor could the lag be abolished by adding exogenous ATP.

[1- 14 C]Acetyl-CoA and [1- 14 C]acetate were incorporated into fatty acids in the standard incubation mixture with the same efficiency when added in equimolar concentrations; in one experiment in which the concentration of both substrates was 0.1 mM, 0.92 % of the acetyl-CoA and 1.15 % of the acetate were incorporated during 3 hr. incubation periods.

Analysis of the fatty acids by gas-liquid chromatography showed that most of the total activity incorporated from [1- 14 C]acetate was in the

C₁₄–C₁₈ fatty acids. Up to 24 %, however, was present in the C₁₈ fatty acid fraction (Table 1).

Relation between the supply of adenosine triphosphate and the incorporation of acetate. In all experiments, the concentration of ATP was measured either serially throughout the incubation, or once at the beginning and once at the end. In the standard mixture the initial concentration of ATP was usually between 0.15 and 0.30 mM. The concentration was almost always maintained at or above its initial level throughout the incubation, sometimes rising during the first 1–2 hr. (as in Fig. 2).

Table 1. Recovery of 14 C from [1- 14 C]acetate in fatty acids of different chain length in a cell-free rat-liver preparation

The standard incubation mixture was used. Experimental details are given in the Methods section. Up to 50 % of the total activity in the C₁₈ fatty acid fraction was due to oleic acid. This has been included in the total activity of the C₁₈ fatty acid fraction. Radioactive palmitoleic acid was not detected.

Expt. no.	Chain length of fatty acid			
	C ₁₂	C ₁₄	C ₁₆	C ₁₈
	Radioactivity (% of total)			
1	6.0	49	27	14
2	2.6	50	26	21
3	—	21	58	21
4	—	24	52	24
5	0.5	56	27	16

Fig. 1. Incorporation of [1- 14 C]acetate into fatty acids by a cell-free rat-liver preparation after incubation for 3 hr. in the standard incubation mixture with increasing concentrations of acetate. Experimental details are given in the Methods section. The results shown are the means of two experiments.

Fig. 2. Time-course of incorporation of [1- 14 C]acetate into fatty acids by a cell-free rat-liver preparation in the standard incubation mixture (○) and in the standard mixture with the mitochondria removed (△). The concentrations of ATP during incubation in the standard mixture (●) and in the standard mixture with the mitochondria removed (▲) are also shown. In the flasks with the mitochondria removed, 2.0 ml. of S₀₋₇ fraction was replaced by 2.0 ml. of the supernatant obtained after centrifugation at 9000g for 20 min. Experimental details are given in the Methods section.

When mitochondrial phosphorylation was inhibited by adding 2,4-dinitrophenol (0.1 mM) to the incubation mixture, ATP disappeared rapidly during the incubation and there was a marked decrease in the amount of acetate incorporated into fatty acids (Table 2). Incorporation of acetate was partially restored by adding ATP. A similar effect was observed when the incubation was carried out with nitrogen instead of air as the gas phase.

When ATP was added to the standard incubation mixture, the initial high concentration was usually maintained throughout the incubation and there was a two- to ten-fold stimulation of acetate incorporation (Table 2). The maximal stimulatory effect of ATP occurred at a concentration of 1–2 mM;

Table 2. *Effect of 2,4-dinitrophenol, adenosine triphosphate, reduced nicotinamide-adenine dinucleotide, nicotinamide-adenine dinucleotide phosphate, avidin, malonate and bicarbonate on the incorporation of acetate into fatty acids by a cell-free rat-liver preparation*

The standard incubation mixture was used, with the additions indicated. Experimental conditions are given in the Methods section. The results shown are from five representative experiments, and are given as percentages of the values obtained in the absence of additions to the standard incubation mixture.

Expt. no.	Additions	Acetate incorporated (%)	Concn. of ATP at end of incubation (%)
1	None	100	100
	Dinitrophenol (0.1 mM)	11	0
	Dinitrophenol (0.1 mM) + ATP (2 mM)	66	83
	ATP (2 mM)	665	408
2	None	100	100
	NADH (0.5 mM)	204	181
	NADP ⁺ (0.5 mM)	177	146
	ATP (2 mM)	550	575
	ATP (2 mM) + NADP ⁺ (0.5 mM)	635	852
3	None	100	100
	Avidin (0.5 unit)*	4	51
	Avidin (0.5 unit)* + biotin (5 µg.)	64	50
	Avidin (0.5 unit)* + ATP (2 mM)	11	639
4	None	100	100
	Malonate (10 mM)	49	65
	ATP (2 mM)	642	263
	ATP (2 mM) + malonate (10 mM)	24	78
5	None	100	100
	Bicarbonate (5 mM)	49	83
	Bicarbonate (10 mM)	26	61
	Bicarbonate (20 mM)	19	—
	ATP (2 mM)	153	460
	ATP (2 mM) + bicarbonate (5 mM)	189	366

* 1 Unit of avidin is equivalent to 1 µg. of biotin.

at concentrations above 5 mM, incorporation was decreased to a level below that in the absence of added ATP (Fig. 3). The experiments with high concentrations of ATP showed that the mitochondria in the standard incubation mixture were capable of maintaining the concentration of ATP at least as high as 7 mM throughout a 3 hr. incubation period (Fig. 3). The stimulatory effect of ATP was observed only when the pH was greater than 7.0. At pH 7.0, the addition of ATP (2 mM) to the standard mixture had no effect on acetate incorporation; at pH 6.5, ATP depressed acetate incorporation to 60% of the value observed in the absence of added ATP.

The addition of NADH or NADP⁺ to the standard mixture increased the concentration of ATP present at the end of the incubation and stimulated acetate incorporation into fatty acids (Table 2). The stimulation of acetate incorporation appeared to be secondary to the effect on ATP generation, since acetate incorporation was uninfluenced by the addition of NADP⁺ or NADH in the presence of an optimum concentration of ATP.

Influence of pH on the incorporation of acetate into fatty acids. When the pH was decreased to below 7.4, the incorporation of acetate into fatty acids in the standard incubation mixture was increased (Fig. 4). The optimum pH could not be determined accurately, owing to the weak buffering power of tris at low pH, but it was certainly not higher than 6.5. Above pH 7.5, acetate incorporation was depressed. The fall in incorporation at high pH values may have been due, at least in part,

Fig. 3. Incorporation of [1-¹⁴C]acetate into fatty acids by a cell-free rat-liver preparation after incubation for 3 hr. in the standard incubation mixture with ATP added at increasing concentrations (○). The concentrations of ATP at the end of the incubations are also shown (●). Experimental details are given in the Methods section.

to depression of oxidative phosphorylation, since there was a fall in the concentration of endogenous ATP during incubation at pH 8.0 and 8.5.

Comparison between various substrates for oxidation. Table 3 shows the results of a representative experiment in which succinate in the standard mixture was replaced by other oxidizable substrates. With citrate or isocitrate the incorporation of acetate was slightly higher than with succinate. This difference was not associated with any consistent effect on the production of ATP, as shown by the ATP concentrations at the end of the incubation. With glutamate, incorporation was decreased to less than one-fifth of that with succinate, though the ability of glutamate to maintain a high concentration of ATP throughout the incubation was equal to that of succinate. We considered the possibility that this anomalous effect might be due to removal of carbon dioxide by combination with ammonia arising from the oxidative deamination of glutamate. This, however, seems unlikely because ammonium chloride (10 mM) had no effect on acetate incorporation in the standard mixture with succinate as substrate for oxidation.

Further experiments to test the effect of citrate and isocitrate showed no stimulation of acetate incorporation comparable with that observed in particle-free preparations (Abraham, Matthes & Chaikoff, 1961; Martin & Vagelos, 1962). As shown in Table 4, isocitrate (5 mM) added to the standard mixture (containing 15 mM-succinate) was slightly stimulatory, but concentrations above 10 mM were inhibitory. Nor was there any stimulation when the isocitrate was added in two successive doses. When isocitrate was added in the presence of an optimum concentration of ATP, no stimulation was observed. At a concentration of 15 mM, citrate depressed acetate incorporation in the standard mixture.

Fig. 4. Effect of pH on the incorporation of [1-¹⁴C]acetate into fatty acids (○) by a cell-free rat-liver preparation and on the concentration of ATP at the end of the incubation (●), after incubation for 3 hr. in the standard incubation mixture. Experimental details are given in the Methods section. The results shown are the means of two experiments.

Evidence relating to the pathway of fatty acid synthesis in the standard incubation mixture:

(a) Incorporation of malonyl-CoA. In the standard incubation mixture, malonyl-CoA was incorporated into fatty acids more than 10 times as efficiently as acetate, the percentage incorporated ranging from 39 to 81 in five experiments. Incorporation was very rapid, showing no lag phase and reaching 90 % of completion within 30 min. At the highest concentration tested (0.8 mM), 525 μm-moles of malonyl-CoA were incorporated into fatty acids during the 3 hr. incubation period. The addition of ATP, or of 2,4-dinitrophenol in amounts sufficient to inhibit oxidative phosphorylation, did not significantly affect the incorporation of malonyl-CoA (Table 5), nor did removal of the mitochondria (Table 7).

(b) Effect of avidin. Avidin inhibited acetate incorporation into fatty acids and the inhibition was partially reversible by biotin (Table 2). In the presence of avidin the concentration of ATP at the

Table 3. Effect of replacing succinate with various other substrates for oxidation on the incorporation of acetate by a cell-free rat-liver preparation

The standard incubation mixture was used, with succinate replaced by other substrates (each at 15 mM) as indicated. Experimental details are given in the Methods section.

Substrate	Acetate incorporated (μm-moles)	Concn. of ATP at end of incubation (mM)
Succinate	169	0.19
α-Oxoglutarate	82	0.16
Glutamate	29	0.19
Oxaloacetate	193	0.11
Citrate	207	0.16
Isocitrate	223	0.18

Table 4. Effect of isocitrate and citrate on the incorporation of acetate into fatty acids by a cell-free rat-liver preparation

The standard incubation mixture was used, with the additions indicated. Experimental details are given in the Methods section. The results are given as percentages of the value obtained in the absence of additions to the standard incubation mixture.

Additions	Acetate incorporated (%)
None	100
Isocitrate (5 mM)	126
Isocitrate (10 mM)	111
Isocitrate (20 mM)	44
Isocitrate (5 mM + 5 mM)*	97
ATP (2 mM)	472
ATP (2 mM) + isocitrate (20 mM)	314
Citrate (15 mM)	50

* Second addition of isocitrate after 1 hr.

end of the incubation was usually lower than in the control flasks. However, this effect of avidin on the concentration of ATP was not the cause of the inhibition of acetate incorporation, since the inhibition could not be reversed by adding ATP. Avidin had no effect on the incorporation of malonyl-CoA into fatty acids (Table 5).

(c) Effect of malonate. Malonate at all concentrations tested between 5 and 20 mM inhibited acetate incorporation into fatty acids in the standard incubation mixture (Table 2). In the presence of malonate the concentration of ATP at the end of the incubation was lower than that in the control flasks. Inhibition by malonate could not be reversed by ATP; on the contrary, ATP was inhibitory in the presence of malonate.

[2-¹⁴C]Malonate was incorporated to some extent into fatty acids in the standard incubation mixture, though the efficiency of incorporation was much

less than that of [1-¹⁴C]acetate at the same molar concentration (Table 6). Incorporation of malonate was stimulated by ATP.

(d) Effect of bicarbonate. Bicarbonate inhibited the incorporation of acetate into fatty acids at all concentrations tested between 5 and 20 mM (Table 2). There was also some depression of ATP production, as shown by the ATP concentration at the end of the incubation, but this was not sufficient to account for the observed degree of inhibition of acetate incorporation. In the presence of an optimum concentration of ATP, bicarbonate either had no effect on acetate incorporation or was slightly stimulatory.

Role of mitochondria in the standard incubation mixture. In the absence of mitochondria, the endogenous ATP in the standard incubation mixture disappeared rapidly. The incorporation of acetate into fatty acids fell to a negligible level; but that of malonyl-CoA was unaffected (Table 7). The incorporation of acetate in the absence of mitochondria could not be restored to the normal level by adding ATP, either as a single dose (Table 7), or serially at intervals frequent enough to maintain a high average concentration throughout the incubation. Very little incorporation of acetate took place in the presence of washed mitochondria incubated in the standard mixture.

Attempts to restore incorporation of acetate to the normal level in the absence of mitochondria were not wholly successful. However, provided that enough exogenous ATP was added to maintain a high concentration throughout the incubation, incorporation of acetate could be restored to 50–60 % of normal by adding bicarbonate, NADP⁺, glucose 6-phosphate and CoA (Table 7). If bicarbonate or NADP⁺ was omitted, little or no incorporation took place, even in the presence of a high concentration of ATP.

Table 5. *Effect of adenosine triphosphate, 2,4-dinitrophenol and avidin on the incorporation of malonyl-coenzyme A by a cell-free rat-liver preparation*

The standard incubation mixture was used, with malonyl-CoA (0.1 mM) added, and other additions as indicated. Experimental details are given in the Methods section.

Additions	Malonyl-CoA incorporated into fatty acids (% of that added)	Concn. of ATP at end of incubation (mM)
None	51.0	0.10
ATP (2 mM)	58.7	0.82
Dinitrophenol (0.1 mM)	50.8	0
Dinitrophenol (0.1 mM) + ATP (2 mM)	61.2	0.04
Avidin (0.1 unit)*	45.7	0.03
Avidin (0.5 unit)*	44.3	0.04

* 1 unit of avidin is equivalent to 1 μ g. of biotin.

Table 6. *Incorporation of [2-¹⁴C]malonate into fatty acids, in the presence and absence of adenosine triphosphate, by a cell-free rat-liver preparation*

The standard incubation mixture was used, with the additions indicated. Experimental details are given in the Methods section. The incorporation of [1-¹⁴C]acetate by the same preparation is also shown for comparison.

Radioactive precursor	Malonate (or acetate) incorporated (μ m-moles)	Concn. of ATP at end of incubation (mM)
[1- ¹⁴ C]Acetate (2.5 mM)	285	0.39
[2- ¹⁴ C]Malonate (0.1 mM)	4	0.32
[2- ¹⁴ C]Malonate (2.5 mM)	19	—
[2- ¹⁴ C]Malonate (5.0 mM)	13	0.31
[2- ¹⁴ C]Malonate (2.5 mM) + ATP (2 mM)	59	2.40
[2- ¹⁴ C]Malonate (5.0 mM) + ATP (2 mM)	36	2.42

DISCUSSION

In the absence of any added cofactors, up to 500 μ m-moles of acetate/g. wet wt. of liver were incorporated into fatty acids in the standard mixture during a 3 hr. incubation period. This compares favourably with the rate of about 200 μ m-moles of acetate incorporated/hr./g. wet wt. of rat-liver slices, observed by Chernick & Chaikoff (1951). The finding that malonyl-CoA may be incorporated into fatty acids with a net efficiency of at least 80 % suggests that there is little breakdown of newly synthesized fatty acids in our system, despite the presence of mitochondria.

Judged by the extent to which fatty acid synthesis can be reversibly inhibited by avidin (an anti-biotin factor), synthesis via the malonyl-CoA pathway accounts for almost all the long-chain

Table 7. *Effect of removing mitochondria from the standard incubation mixture on the incorporation of acetate and malonyl-coenzyme A into fatty acids, and the effect of replacing mitochondria by adenosine triphosphate, bicarbonate, nicotinamide-adenine dinucleotide phosphate, glucose 6-phosphate and coenzyme A on the incorporation of acetate into fatty acids, by a cell-free rat-liver preparation*

The standard incubation mixture with an acetate concentration of 2.5 mM was used, with the modifications indicated (the concentration of malonyl-CoA, when present, was 0.1 mM). In the flasks with the mitochondria removed, 2.0 ml. of $S_{0.7}$ fraction in the standard mixture was replaced by 2.0 ml. of supernatant obtained from centrifugation of $S_{0.7}$ fraction at 9000g for 20 min. In the flasks with washed mitochondria, 2.0 ml. of $S_{0.7}$ fraction was replaced by 2.0 ml. of mitochondrial suspension. Experimental details are given in the Methods section. The results shown are the means of two experiments, and are given as percentages of the values obtained in the absence of modifications to the standard incubation mixture.

Modifications to standard incubation mixture	Acetate incorporated (%)	Malonyl-CoA incorporated (%)	Concn. of ATP at end of incubation (%)
None	100	100	100
Mitochondria removed	0	106	0
Mitochondria removed; ATP (10 mM) added	0	—	610
Mitochondria removed; ATP (10 mM) + NADP ⁺ (0.5 mM) added	0	—	687
Mitochondria removed; ATP (15 mM) + bicarbonate (5 mM) added	14	—	108
Mitochondria removed; ATP (15 mM) + bicarbonate (5 mM) + NADP ⁺ (0.5 mM) added	32	—	420
Mitochondria removed; ATP (15 mM) + bicarbonate (5 mM) + NADP ⁺ (0.5 mM) + glucose 6-phosphate (0.5 mM) added	36	—	421
Mitochondria removed; ATP (15 mM) + bicarbonate (5 mM) + NADP ⁺ (0.5 mM) + glucose 6-phosphate (0.5 mM) + CoA (0.05 mM) added	56	—	321
Mitochondria replaced by washed mitochondria; ATP (2 mM) added	7	—	212

fatty acids formed from acetate in mitochondrion-free homogenates of rat liver supplemented with ATP and other cofactors at optimum concentrations (Abraham, Matthes & Chaikoff, 1960; Fletcher & Myant, 1961a). In the present work, the inhibitory effect of avidin and the high efficiency with which malonyl-CoA is incorporated into fatty acids show that the malonyl-CoA pathway is active in the standard incubation mixture. However, it is difficult to be sure whether or not this pathway accounts for all the acetate incorporated into fatty acids. Our failure to obtain an inhibition by avidin that was complete and, at the same time, fully reversible by biotin may be due to the fact that avidin, in addition to inhibiting the carboxylation of acetyl-CoA, also interferes irreversibly with the generation of ATP (Table 2). On the other hand, it is possible that in the presence of mitochondria and of suboptimum concentrations of cofactors, other pathways for the formation of fatty acids, or of malonyl-CoA, become operative. There is some evidence, as yet unconfirmed, for the presence in mitochondria of an avidin-insensitive pathway through which long-chain fatty acids are formed by elongation of already existing fatty acyl-CoA units of moderate chain length (Wakil, 1961). In our system, however, isolated mitochondria failed to bring about incorporation of acetate into long-chain fatty acids from endogenous fatty acyl-CoA units.

In cell-free systems in which synthesis of fatty acids is now known to proceed via malonyl-CoA, stimulation of fatty acid synthesis from acetate has

been observed in the presence of citrate and isocitrate (Brady & Gurin, 1952) and of malonate (Popják & Tietz, 1955). Under certain conditions stimulation may be very marked; up to 60-fold stimulation has been observed with citrate (Abraham *et al.* 1961; Martin & Vagelos, 1962) and up to 50-fold with malonate (Dils & Popják, 1962). Work with partially purified enzymes has shown that citrate (Vagelos, Alberts & Martin, 1963) and malonate (Waite & Wakil, 1962) act by stimulating the carboxylation of acetyl-CoA, possibly by activating or stabilizing acetyl-CoA carboxylase.

In view of this, it is perhaps surprising that citrate and isocitrate had little or no stimulatory effect in the standard incubation mixture. One possibility is that in the presence of mitochondria the tricarboxylic acids are metabolized before they have time to activate the acetyl-CoA carboxylase. Against this, however, hardly any stimulation was observed when isocitrate was added in two successive doses (Table 4). Another possibility is that the stimulatory effect of tricarboxylic acids becomes apparent only if the carboxylase has been altered in some way during the preparation of cell-free systems. It may be that in our preparation, which requires only a single centrifugation, the enzyme is not altered and so does not become sensitive to the action of citrate and isocitrate. The point is of some importance because it has been suggested that citrate (Martin & Vagelos, 1962) and isocitrate (Lynen, Matsushashi, Numa & Schweizer, 1963) exert a controlling influence on fatty acid synthesis

in the whole cell by altering the activity of acetyl-CoA carboxylase. The inhibitory effect of citrate on acetate incorporation may be due to the formation of non-radioactive acetyl-CoA by the action of the citrate cleavage enzyme (Srere & Bhaduri, 1962; Spencer & Lowenstein, 1962). This would lower the specific activity of the pool of $[1-^{14}\text{C}]$ -acetyl-CoA formed from the $[1-^{14}\text{C}]$ acetate added as precursor.

Malonate did not stimulate acetate incorporation into fatty acids in the standard incubation mixture. This was not due to masking of a stimulatory effect by interference with the generation of ATP in the mitochondria, since no stimulation was observed when the ATP concentration was maintained by adding ATP. Abraham *et al.* (1961) found that malonate did not stimulate fatty acid synthesis from acetate in mammary-gland slices.

If malonate stimulates fatty acid synthesis in cell-free homogenates by activating acetyl-CoA carboxylase (Waite & Wakil, 1962), its failure to stimulate in our preparation may be due to the same cause that was suggested above for the failure of citrate and isocitrate to stimulate. The finding that ATP becomes inhibitory in the presence of malonate is difficult to understand. Malonate is incorporated into fatty acids in our preparation, possibly by direct activation to malonyl-CoA, the priming units of acetyl-CoA arising from the activation of the acetate present in the incubation mixture. The incorporation of malonate is stimulated by ATP. ATP would therefore tend to favour the formation of non-radioactive malonyl-CoA in the presence of non-radioactive malonate, and thus to diminish the specific activity of the radioactive malonyl-CoA formed from $[1-^{14}\text{C}]$ acetate. However, this effect is not sufficient to explain why 10 mM-malonate changes the effect of exogenous ATP from one of marked stimulation to one of inhibition. In the presence of ATP a maximum of about 60 μm -moles of malonate are incorporated into fatty acids (Table 6). This would not be sufficient to diminish the incorporation of $[1-^{14}\text{C}]$ acetate, in the presence of ATP, from 642 to 24 μm -moles (Table 2).

In particle-free systems there is an absolute requirement for carbon dioxide or bicarbonate for the synthesis of long-chain fatty acids from acetate (Wakil, 1961). Presumably, the carbon dioxide required for the carboxylation of acetyl-CoA in the standard incubation mixture is derived from the oxidation of intermediates of the tricarboxylic acid cycle in the mitochondria. This would explain why bicarbonate does not stimulate acetate incorporation in our system, except in the presence of optimum concentrations of ATP.

In the standard incubation mixture the maintenance of an adequate concentration of ATP

depends on the presence of phosphorylating mitochondria. If the mitochondria are removed, or if phosphorylation is inhibited, the endogenous ATP present at the beginning of the incubation rapidly disappears and acetate incorporation into fatty acids does not take place. The experiments in which we attempted to replace mitochondria show that they are also essential for supplying the bicarbonate required for the formation of malonyl-CoA from acetyl-CoA.

The effect of thyroid hormone on the incorporation of acetate into fatty acids must be due, however indirectly, to an action on a rate-limiting step in the sequence of reactions by which fatty acids are formed from acetate. Ganguly (1960) has shown that the activity of malonyl-CoA synthetase in liver is several times as high as that of acetyl-CoA carboxylase. Numa, Matsushashi & Lynen (1961) have also shown that there is a fall in the activity of acetyl-CoA carboxylase in the liver after starvation, a condition in which fatty acid synthesis is depressed. From these findings it has been concluded that the carboxylation of acetyl-CoA is the rate-limiting step in fatty acid synthesis. But this conclusion is only justified if it can be assumed that the rate at which the carboxylation reaction takes place is determined by the activity of the carboxylase, an assumption that may not be true for the intact cell and that certainly cannot be true for systems, such as that of Dils & Popják (1962), in which fatty acid synthesis can be increased several-fold by raising the concentrations of cofactors. Nevertheless, the rate-limiting step in our system does appear to be the carboxylation reaction, since the maximal capacity of the standard mixture for incorporating malonyl-CoA into fatty acids is several times as great as that for incorporating acetyl-CoA or acetate. However, the factor that determines the rate of this reaction in our system is not enzyme activity but ATP concentration, since the incorporation of acetate is increased by adding ATP or by stimulating endogenous ATP production with NADP^+ and NADH , and is depressed when mitochondrial phosphorylation is inhibited.

This raises the question as to how far it is possible to deduce what is rate-limiting for fatty acid synthesis in the intact cell from observations on cell-free preparations. In experiments with cell-free preparations it is usual to retain only those components of the cell that are directly concerned in fatty acid synthesis, and to add cofactors in optimum concentrations rather than to rely on endogenous cofactors generated by the system itself. For some purposes this has obvious advantages, but it may be misleading if a change in the rate of fatty acid synthesis in the whole cell is caused by a change in the supply of cofactors. Thus

the experiments of Numa *et al.* (1961) do not exclude the possibility that the fall in the activity of acetyl-CoA carboxylase, demonstrated in the presence of optimum concentrations of cofactors, is a secondary adaptation to a fall in the supply of a cofactor which is rate-limiting for fatty acid synthesis in the intact liver cell. If it is true that the intracellular concentration of citrate or isocitrate influences the activity of acetyl-CoA carboxylase in the living cell, this would add a further difficulty to the interpretation of results obtained with cell-free preparations in which tricarboxylic acids are not generated physiologically. Despite its complexity, the system we have described above may be more suitable for studying the way in which hormones regulate fatty acid synthesis *in vivo*, since all the necessary cofactors are generated endogenously by mechanisms similar to those in the intact cell.

SUMMARY

1. A cell-free preparation of rat liver is described in which long-chain fatty acids are synthesized from acetate by way of malonyl-CoA.

2. All the cofactors required for fatty acid synthesis from acetate are derived endogenously from the preparation itself; the ATP and bicarbonate required for the conversion of acetate into malonyl-CoA are derived entirely from phosphorylating mitochondria.

3. Citrate, isocitrate and malonate do not stimulate fatty acid synthesis in this preparation.

4. The rate-limiting step for fatty acid synthesis in this preparation is the conversion of acetyl-CoA into malonyl-CoA, and the rate at which this takes place is limited by the supply of ATP generated by the mitochondria.

5. For the study of hormonal regulation of fatty acid synthesis in the whole cell this preparation has some advantages over more purified cell-free systems in which exogenous cofactors are added in optimum concentrations.

REFERENCES

- Abraham, S., Matthes, K. J. & Chaikoff, I. L. (1960). *J. biol. Chem.* **235**, 2551.
 Abraham, S., Matthes, K. J. & Chaikoff, I. L. (1961). *Biochim. biophys. Acta*, **49**, 268.
 Brady, R. O. & Gurin, S. (1952). *J. biol. Chem.* **199**, 421.
 Bruce, H. M. & Parkes, A. S. (1946). *J. Hyg., Camb.*, **44**, 501.
 Chernick, S. S. & Chaikoff, I. L. (1951). *J. biol. Chem.* **188**, 389.
 Cleland, K. W. & Slater, E. C. (1953). *Biochem. J.* **53**, 547.
 Dils, R. & Popják, G. (1962). *Biochem. J.* **83**, 41.
 Fletcher, K. & Myant, N. B. (1960). *J. Physiol.* **154**, 145.
 Fletcher, K. & Myant, N. B. (1961a). *J. Physiol.* **155**, 498.
 Fletcher, K. & Myant, N. B. (1961b). *J. Physiol.* **157**, 542.
 Ganguly, J. (1960). *Biochim. biophys. Acta*, **40**, 110.
 Lynen, F., Matsuhashi, M., Numa, S. & Schweizer, E. (1963). *Symp. biochem. Soc.* **24**, 43.
 Martin, D. B. & Vagelos, P. R. (1962). *J. biol. Chem.* **237**, 1787.
 Numa, S., Matsuhashi, M. & Lynen, F. (1961). *Biochem. Z.* **334**, 203.
 Popják, G., Lowe, A. E., Moore, D., Brown, L. & Smith, F. A. (1959). *J. Lipid Res.* **1**, 29.
 Popják, G. & Tietz, A. (1955). *Biochem. J.* **60**, 147.
 Seale, J. F. & Migicovsky, B. B. (1957). *Canad. J. Biochem.* **35**, 15.
 Spencer, A. F. & Lowenstein, J. M. (1962). *J. biol. Chem.* **237**, 3640.
 Srere, P. A. & Bhaduri, A. (1962). *Biochim. biophys. Acta*, **59**, 487.
 Strehler, B. L. & Totter, J. R. (1954). *Meth. biochem. Anal.* **1**, 341.
 Trams, E. G. & Brady, R. O. (1960). *J. Amer. chem. Soc.* **82**, 2972.
 Vagelos, P. R., Alberts, A. W. & Martin, D. B. (1963). *J. biol. Chem.* **238**, 533.
 Waite, M. & Wakil, S. J. (1962). *J. biol. Chem.* **237**, 2750.
 Wakil, S. J. (1961). In *Proc. 5th int. Congr. Biochem., Moscow*, vol. 7, p. 3. Oxford: Pergamon Press Ltd.
 Woolley, D. W. & Longworth, L. G. (1942). *J. biol. Chem.* **142**, 285.