Biosynthesis of Fatty Acids in Cell-Free Preparations

5. SYNTHESIS OF FATTY ACIDS FROM ACETATE IN EXTRACTS OF LACTATING-RAT MAMMARY GLAND*

By R. DILS† AND G. POPJÁK

Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W. 12

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The investigations of Folley & Popják and their colleagues demonstrated the outstanding ability of the mammary gland of lactating animals to synthesize fatty acids from acetate (for reviews see Popják, 1952; Folley & McNaught, 1961). Investigations on whole animals or on tissue slices could not establish the nature of the intermediates involved, although the overall process was shown to be analogous or equivalent to the reversal of β -oxidation of fatty acids (Popják, French, Hunter & Martin, 1951). Popják & Tietz (1954, 1955), Tietz & Popják (1955) and Hele, Popják & Laurysens (1957) made an attempt to elucidate this process in cell-free preparations of mammary gland and have shown that CoA, ATP and a reduced pyridine nucleotide were essential co-factors. Investigations on the mechanisms of fatty acid oxidation with cell-free enzyme systems and purified enzymes led to the suggestion that the biosynthesis of fatty acids occurred by the reversal of this process (Lynen & Ochoa, 1953; Seubert, Greull & Lynen, 1957; Langdon, 1957). The experimental data of Hele *et al.* (1957) obtained with fractions of soluble extracts of lactating-rabbit mammary gland were also interpreted so as to support this idea.

Meanwhile, Wakil and his colleagues (for review see Wakil, 1961) described in an elegant series of papers the purification from pigeon and chicken livers of the enzymes involved in fatty acid synthesis from acetate. This work led to the discovery of a pathway for fatty acid synthesis strikingly different from the reversal of the β -oxidation of fatty acids. The discovery came from the appreciation that carbon dioxide was an essential reactant in the process, its role being the biotin-dependent carboxylation of acetyl-CoA to malonyl-CoA (Wakil, 1958; Brady, 1958; Formica & Brady, 1959; Lynen, Knappe, Lorch, Jütting & Ringelmann, 1959). It is now known that fatty acid synthesis in the liver proceeds by the successive

condensation of malonyl-CoA with acetyl-CoA, the free carboxyl group of malonyl-CoA, which originates from carbon dioxide, being eliminated in the process.

Popják & Tietz (1955) noted that fatty acid synthesis in a soluble-enzyme system of rat mammary gland was stimulated to a very great extent by α -oxoglutarate and malonate or when the reaction was carried out in the presence of air instead of pure oxygen. In retrospect these observations strongly suggested that fatty acid synthesis in the mammary gland also proceeds by way of the acetyl-CoA + malonyl-CoA pathway and therefore a reinvestigation of the mammary-gland-enzyme system appeared necessary. A brief account of this work has been given by Dils & Popják (1961).

METHODS AND MATERIALS

Enzyme preparations. Homogenates of mammary glands of lactating rats 7–18 days after parturition were made as described by Popják & Tietz (1954, 1955) with 1.5–2.0 vol. of 0.136 M-KCl containing 0.035 M-potassium phosphate buffer, pH 7.4, and 0.03 M-nicotinamide for 1 unit wt. of minced gland. Tris-HCl buffer (0.035 M, pH 7.4) could replace the phosphate buffer in the above medium, but in either case phosphate buffer, pH 7.4 (60–200 mM), was added to the incubations. The centrifugal fractionation of the homogenates was carried out as before (Popják & Tietz, 1955). In the text, ‘mitochondria + microsomes + supernatant’ refers to the part of the homogenate not sedimented at 600g for 19 min., ‘microsomes + supernatant’ to the part not sedimented at 10 000g for 20 min., and ‘soluble supernatant’ (i.e. the ‘mammary-gland-enzyme system’, or M.G.E. of Popják & Tietz, 1955) to the part not sedimented at 110 000g for 60 min. Floating fat was removed after each centrifuging and served as a source of ‘carrier’ fatty acids during isolation of fatty acids synthesized from [14 C]acetate.

The treatment of the soluble supernatants with Dowex 1 ion-exchange resin for removal of coenzymes was carried out as described by Tietz & Popják (1955). The protein content of the enzyme preparations was determined by the method of Gornall, Bardawill & David (1949).

Incubation. This (final vol. 1.5 or 3.0 ml.) was at 37° for 2.5 hr.; unless otherwise stated, N_2 was blown through the flasks for 10–15 sec. before they were stoppered (‘anaerobic

* Part 4: Hele, Popják & Laurysens (1957).

† On leave of absence from the Department of Medical Biochemistry and Pharmacology, University of Birmingham.

incubation'). The solutions of cofactors (except KHCO_3) were brought to pH 7.4 before addition to the incubation medium. At the end of the incubations about 7 mg. of 'carrier' fatty acids (see above) and ethanolic KOH (to a final concentration of 1N) were added to the flasks, which were heated at 60° overnight. After acidification, the fatty acids were extracted three times with 20 ml. of light petroleum (b.p. 40–60°). The combined extracts were washed with an equal volume of water and then concentrated to 2.0 ml. Samples (0.1 ml.) were plated on plastic disks lined with lens paper for counting of ^{14}C in infinitely thin layers with an end-window counter (Popják, 1950). The radioactivity of individual fatty acids synthesized was determined by the gas-liquid radiochromatographic technique of Popják, Lowe, Moore, Brown & Smith (1959) after conversion of the fatty acids into their methyl esters with diazomethane. The stationary phase of the columns was either ethylene glycol adipate polyester (James, 1959), or Apiezon-L vacuum grease, supported by Celite Super-Cel (80–120 mesh; Johns-Manville Co. Ltd., London). The columns were used at 197°. The activity of the fatty acid-synthesizing system will be expressed as 'percentage conversion of acetate [into fatty acids]' or as 'μmoles of acetate converted [into fatty acids]/100 mg. of protein'.

Coenzymes and reagents. ATP, CoA (75% pure), TPN, reduced diphosphopyridine nucleotide (β -DPNH) and glucose 6-phosphate were supplied both by Sigma Chemical Co., Mo., U.S.A. and by C. F. Boehringer und Soehne, Mannheim, Germany. Biotin was obtained from Roche Products Ltd., Welwyn Garden City, Herts., and crystalline bovine plasma albumin from L. Light and Co. Ltd., Colnbrook, Bucks. Citric acid-cycle intermediates, various dicarboxylic acids, L(+)-cysteine-HCl and nicotinamide were purchased from Hopkin and Williams, Ltd., Chadwell Heath, Essex, and from British Drug Houses Ltd., Poole, Dorset. Oxaloacetic acid and tris were supplied by Sigma Chemical Co. Dowex 1 ion-exchange resin (Cl^- form; X 10; 200–400 mesh) was obtained from the Dow Chemical Co., Mich., U.S.A. [*carboxy*- ^{14}C]Acetate, diethyl [^{14}C]malonate and diethyl [^{14}C]malonate were bought from The Radiochemical Centre, Amersham, Bucks. All other reagents were of analytical grade.

Avidin (2.54×10^3 units/mg.) was prepared by Miss H. Hellig of this Unit, from egg-white by the method of Woolley & Longworth (1942); 1 antibiotic unit of avidin is equivalent to 5×10^{-4} μg. of biotin. The potency of the avidin preparations was assayed with a biotin-requiring mutant of *Aspergillus nidulans*.

Preparation of pure [^{14}C] and [^{14}C]malonate. The ^{14}C -labelled specimens of diethyl malonate, when analysed by gas-liquid radiochromatography (Popják *et al.* 1959) on ethylene glycol adipate polyester columns at 100° were found to be only 90% pure. Unlabelled diethyl malonate (Hopkin and Williams Ltd.) contained the same impurity as the labelled material. The diethyl [^{14}C]malonate specimens were therefore hydrolysed to potassium malonate and purified by paper chromatography as follows: the diethyl [^{14}C]malonate specimens (32 mg., specific activity 1 μC/mg.) were hydrolysed overnight at 70° with 4 ml. of ethanolic 0.3N-KOH; after cooling, 1 ml. of water was added and the mixture heated on a boiling-water bath for a further 5 min. followed by heating at 60° for a further 25 min. Excess of ethanol was removed *in vacuo* at 70° and the mixture neutralized to pH 7.0 with H_2SO_4 . A further 1 ml. of

water was added and the solution warmed to 70° for 10 min.; insoluble material was removed by centrifuging. Potassium malonate similarly prepared from unlabelled diethyl malonate stimulated fatty acid synthesis as effectively as commercially obtained malonate.

The labelled malonate samples were purified by paper chromatography with the propan-1-ol-water (saturated with SO_2) (70:30, v/v) solvent system of Howes (1960). The malonic acids (R_f 0.76–0.83) were eluted with dilute aq. NH_3 soln. and portions rechromatographed with propan-1-ol-aq. 2N- NH_3 soln. (70:30, v/v) as solvent system (Howes, 1960), in which malonate has R_f 0.188–0.198. Scanning of the paper strips for ^{14}C with an automatic device (Hele *et al.* 1957) showed that the first paper-chromatographic separation gave [^{14}C] and [^{14}C]malonate with 99.5 and 99.3% radiochemical purity respectively; this was considered adequate for the experiments.

RESULTS

Cofactors of fatty acid synthesis

The overall cofactor requirements of the soluble mammary-gland-enzyme system for fatty acid synthesis from acetate, shown in Table 1, are strikingly similar to those observed by Wakil and his co-workers (Porter, Wakil, Tietz, Jacobs & Gibson, 1957; Tietz, 1957; Gibson, Titchener & Wakil, 1958; Wakil, Titchener & Gibson, 1958, 1959) for fatty acid synthesis from acetate by the pigeon- and chicken-liver-enzyme systems. Added biotin had no effect on the mammary-gland-enzyme system although experiments with avidin (see below) proved that biotin was a necessary coenzyme. Both TPNH and DPNH were required,

Table 1. Cofactor requirements of rat-mammary-gland-enzyme system for fatty acid synthesis from acetate

The incubation mixture (3.0 ml.) contained sodium [*carboxy*- ^{14}C]acetate (5 μC, 10 mM), ATP (10 mM), CoA (0.07 mM), L(+)-cysteine (10 mM), TPN⁺ (0.33 mM), glucose 6-phosphate (3.3 mM), DPNH (0.33 mM), MgCl_2 (3.3 mM), MnCl_2 (3.3 mM), malonate (10 mM), KHCO_3 (10 mM), biotin (24 μg.), α-oxoglutarate (20 mM) and potassium phosphate buffer, pH 7.4 (100 mM). 'Microsomes + supernatant' (1 ml. ≡ 38.2 mg. dry wt.) or soluble supernatant (1 ml. ≡ 22.4 mg. dry wt.) was added as shown.

Omissions	Percentage conversion of acetate	
	'Microsomes + supernatant'	Soluble supernatant
None	7.0	29.1
Biotin	5.4	30.2
α-Oxoglutarate	10.3	26.7
DPNH	4.9	25.0
MgCl_2	7.4	23.1
TPN ⁺ + glucose 6-phosphate	2.3	13.0
Malonate	3.8	11.8
KHCO_3	0.8	8.6
MnCl_2	0.4	4.5

Table 2. *Effects of treatment of soluble mammary-gland-enzyme system with Dowex 1*

The incubation medium (3.0 ml.) was as described in Table 1 except that each flask contained 50 mM-KHCO₃ and 50 mM-malonate and that α -oxoglutarate and biotin were omitted. Soluble-supernatant protein (13.5 mg.) or 6.7 mg. of protein of Dowex 1-treated soluble supernatant was used. Cofactors were omitted as shown.

Enzyme system	Omissions	Percentage conversion of acetate	Acetate converted (μ moles/100 mg. of protein)
Soluble supernatant	None	12.00	26.8
Soluble supernatant treated with Dowex-1	None	11.20	50.0
	DPNH	7.65	34.4
	TPN ⁺ + glucose 6-phosphate	1.61	7.1
	TPN ⁺ + glucose 6-phosphate + DPNH	0.16	0.7
	CoA	0.98	4.4
	ATP	0.07	0.3

although DPNH alone produced only about one-half of the stimulation caused by TPNH. Mn²⁺ ions, rather than Mg²⁺ ions, were essential for fatty acid synthesis. Malonate and KHCO₃ both stimulated fatty acid synthesis.

An absolute requirement for CoA, ATP and reduced pyridine nucleotides was demonstrated (Table 2) after removal of coenzymes from a soluble mammary-gland preparation with Dowex 1 ion-exchange resin. The need for both DPNH and TPNH was again confirmed, although DPNH could only partially replace TPNH.

The results of the experiments shown in Tables 1 and 2 have broadly confirmed the data of Popják & Tietz (1955) and Tietz & Popják (1955) and, in addition, brought out the need for Mn²⁺ ions, HCO₃⁻ ions and TPNH. Since α -oxoglutarate did not stimulate synthesis from acetate (Table 1) to the extent observed by Popják & Tietz (1955), the role of α -oxoglutarate in their incubation mixture, which contained neither KHCO₃ nor added reduced pyridine nucleotides, may well have been due to the production of either or both of these cofactors. The suppression of fatty acid synthesis by microsomes and the great stimulation caused by malonate were likewise confirmed.

Effects of malonate and bicarbonate ion on fatty acid synthesis

The strong stimulation of fatty acid synthesis by malonate, first observed by Popják & Tietz (1955), appeared of special interest and was investigated further together with the role of HCO₃⁻ ion.

Fig. 1 illustrates the effects of varying concentrations of malonate and HCO₃⁻ ion on fatty acid synthesis; HCO₃⁻ ion by itself had only a minor stimulatory effect, which was far surpassed by that of malonate alone. The combined effects of malonate and HCO₃⁻ ion were, however, most remarkable: the addition of malonate at a concentration of 50 mM to the system containing 25 mM-HCO₃⁻ ion

Fig. 1. Effects of malonate and KHCO₃ on the conversion of acetate into fatty acids. The incubation mixture was as described in Table 1, except that 150 mM-potassium phosphate buffer, pH 7.4, and 20.7 mg. of soluble mammary gland protein were used. Biotin and α -oxoglutarate were omitted from the system. Malonate and KHCO₃ were added as indicated. □, Increasing concentrations of KHCO₃ in the absence of malonate; Δ, increasing concentrations of malonate in the absence of KHCO₃; ○, increasing concentrations of malonate in the presence of 25 mM-KHCO₃.

caused a 50-fold increase in the conversion of acetate into fatty acids.

As it was conceivable that the malonate added to the enzyme preparation became activated with CoA and provided carbon for fatty acid synthesis, experiments were set up with [1-¹⁴C]- and [2-¹⁴C]-malonate. However, as is seen from Table 3,

Table 3. *Comparison of acetate and malonate as precursor of fatty acids*

The incubation mixture (3.0 ml.) consisted of: ATP (10 mm), CoA (0.07 mm), L(+)-cysteine (10 mm), TPN⁺ (0.33 mm); glucose 6-phosphate (3.3 mm), DPNH (0.33 mm), MgCl₂ (3.3 mm), MnCl₂ (3.3 mm), potassium phosphate buffer, pH 7.4 (30 mm), and 22.5 mg. of soluble-supernatant protein. In Expt. 1, sodium [carboxy-¹⁴C]acetate (5 μ C, 10 mm) and unlabelled potassium malonate (50 mm) were used. In Expt. 2, potassium [1-¹⁴C]- or [2-¹⁴C]-malonate (5 μ C, 7 mm) and unlabelled sodium acetate (10 mm) were used. KHCO₃ (25 mm) was added as shown.

Expt.	Labelled precursor	KHCO ₃ (mm)	Percentage conversion of labelled precursor
1	[carboxy- ¹⁴ C]Acetate	25	22.50
2	[1- ¹⁴ C]Malonate	25	0.14
	[1- ¹⁴ C]Malonate	—	0.17
	[2- ¹⁴ C]Malonate	25	0.30
	[2- ¹⁴ C]Malonate	—	0.30

Table 4. *Comparison of effect of malonate and other dicarboxylic acids on fatty acid synthesis from acetate*

The incubation mixture was as described in Table 1 except that malonate, biotin and α -oxoglutarate were omitted and 50 mm-KHCO₃ and 27.3 mg. of soluble mammary-gland protein were used. Dicarboxylic acids (50 mm) were added as shown.

Dicarboxylic acids added	Percentage conversion of acetate
Malonate	13.1
Oxalate	0.5
Succinate	7.2
Glutarate	1.1
Adipate	0.5
Pimelate	0.1

Table 5. *Inhibition of fatty acid synthesis by avidin and the reversal of inhibition by added biotin*

The incubation mixture was as described in Table 1 except that 12.4 mg. of soluble mammary-gland protein was used. Biotin (10 μ g.), avidin (0.5 mg.), malonate (50 mm) and KHCO₃ (25 mm) were added as shown. α -Oxoglutarate was left out of the system.

Additions	Percentage conversion of acetate
1 KHCO ₃	0.34
KHCO ₃ + biotin	0.35
KHCO ₃ + avidin	0.06
KHCO ₃ + avidin + biotin	0.22
2 Malonate	2.85
Malonate + biotin	3.17
Malonate + avidin	0.74
Malonate + avidin + biotin	3.41
3 KHCO ₃ + malonate	16.60
KHCO ₃ + malonate + biotin	17.40
KHCO ₃ + malonate + avidin	7.03
KHCO ₃ + malonate + avidin + biotin	16.75

under the conditions used for the incorporation of acetate into fatty acids, neither the carboxyl nor the methylene carbon atom of malonate was incorporated into fatty acids and therefore the formation of malonyl-CoA from the added malonate can be excluded.

In experiments designed to determine whether malonate could be replaced in the mammary-gland system by other dicarboxylic acids, it was found that succinate gave 55% and glutarate 16.6% of the stimulation caused by an equimolar concentration (50 mm) of malonate (Table 4).

Effect of avidin and biotin on fatty acid synthesis from acetate

The results with labelled malonate showed that this substance did not participate directly in fatty acid synthesis by forming malonyl-CoA. This conclusion was supported further by the experiments shown in Table 5 and Fig. 2. Avidin inhibited fatty acid synthesis from acetate in the presence of either HCO₃⁻ ion or malonate, but the degree of inhibition was not as great in the presence of both malonate and HCO₃⁻ ion as when either of these was added to the system alone. Although added biotin had little effect on fatty acid synthesis in an uninhibited system (cf. Table 1), the inhibition caused by avidin could be completely reversed by biotin. Fig. 2 shows that the biotin content of the soluble mammary-gland preparations may be

Fig. 2. Effect of increasing concentrations of avidin on fatty acid synthesis from acetate. The incubation mixture was as described in Table 1 except that 18.3 mg. of soluble protein, 50 mm-malonate and 25 mm-KHCO₃ were used. Biotin and α -oxoglutarate were omitted from the incubation medium. Avidin was added as shown.

'titrated' by measuring fatty acid synthesis in the presence of increasing concentrations of avidin. From the known potency of the avidin preparation (2540 antibiotin units/mg.) we calculated that

the 18.3 mg. of the soluble protein used in the experiment illustrated in Fig. 2 contained about 0.23 μ g. of biotin.

*Optimum conditions for fatty acid
synthesis from acetate*

From the experiments illustrated in Fig. 3 (*a-d*) it was deduced that the highest conversion of acetate into fatty acids occurred when the incubations contained 0.13 mM-CoA, 20 mM-ATP, 0.33–0.66 mM-TPN with 10–20 mM-glucose 6-phosphate and 50 mM- HCO_3^- ion in the presence of 50 mM-malonate (cf. also Fig. 1). Under such conditions a clear-cut need for DPNH in the system could not be shown, although in some experiments (Table 6) addition of DPNH (0.33 mM) to a system containing 0.5 mM-TPN and 13.3 mM-glucose 6-phosphate caused a 12% increase in the conversion of acetate into fatty acids. The mammary-gland preparations always contain adequate amounts of glucose 6-phosphate dehydrogenase for the reduction of TPN with glucose 6-phosphate. Cysteine, commonly needed in enzymic reactions dependent on CoA, did not stimulate the mammary-gland system; occasionally (cf. Table 6) it was inhibitory. The optimum concentration of acetate appeared to be 10–20 mM, as was found previously by Popják & Tietz (1954, 1955).

The essential role of Mn^{2+} ions in the mammary-gland-enzyme system has been pointed out already (cf. Table 1). Mn^{2+} ions (3.3 mM) could not be replaced by Mg^{2+} or other bivalent metal ions (Ca^{2+} , Zn^{2+} , Ni^{2+} , Fe^{2+} , Co^{2+} , Ba^{2+} , Mg^{2+} , Cu^{2+}) at this concentration.

Using the optimum concentrations of reactants described above, we found that conversion of acetate into fatty acids was proportional to the concentration of protein up to 5.5 mg./ml. (Fig. 4);

Fig. 3. Determination of optimum concentrations of cofactors required for fatty acid synthesis from acetate. (*a*), (*b*) and (*c*) For determination of optimum concentrations of CoA, ATP and TPN the incubation mixture contained: sodium [*carboxy- ^{14}C*]acetate (5 μ C, 10 mM), ATP (10 mM), CoA (0.07 mM), L(+)-cysteine (10 mM), TPN (0.33 mM), glucose 6-phosphate (3.3 mM), DPNH (0.33 mM), MgCl_2 (3.3 mM), MnCl_2 (3.3 mM), malonate (50 mM), KHCO_3 (50 mM), potassium phosphate buffer, pH 7.4 (66 mM), and 13.5 mg. of soluble protein. The concentration of the particular coenzyme being tested was altered as shown on the graphs. The final volume was 3.0 ml. (*d*) For determination of optimum concentration of glucose 6-phosphate the incubation mixture contained: sodium [*carboxy- ^{14}C*]acetate (2.5 μ C, 10 mM), ATP (20 mM), CoA (0.133 mM), L(+)-cysteine (10 mM), TPN (0.66 mM), DPNH (0.33 mM), MgCl_2 (3.3 mM), MnCl_2 (3.3 mM), malonate (50 mM), KHCO_3 (50 mM), potassium phosphate buffer, pH 7.4 (66 mM), and 31 mg. of soluble mammary-gland protein. Glucose 6-phosphate was added as shown. The final volume was 1.5 ml. (*e*) For determination of optimum concentration of HCO_3^- ion the incubation mixture contained: sodium [*carboxy- ^{14}C*]acetate (2.5 μ C, 10 mM), ATP (20 mM), CoA (0.133 mM), TPN (0.33 mM), glucose 6-phosphate (13.3 mM), MnCl_2 (3.3 mM), malonate (50 mM), potassium phosphate buffer, pH 7.4 (270 mM), and 11.6 mg. of soluble protein; KHCO_3 was added as shown. The final volume was 1.5 ml.

Table 6. Further investigations of cofactor requirements for the synthesis of fatty acids from acetate

The incubation medium (1.5 ml.) consisted of: sodium [*carboxy- ^{14}C*]acetate (2.5 μ C, 10 mM), ATP (20 mM), CoA (0.133 mM), TPN (0.33 mM), glucose 6-phosphate (13.3 mM), MnCl_2 (3.3 mM), malonate (50 mM), KHCO_3 (50 mM), potassium phosphate buffer, pH 7.4 (270 mM), and 11.3 mg. of soluble mammary-gland protein. The final molarity of the added cofactors in the medium is given below.

Additions	Percentage conversion of acetate
Control, boiled soluble supernatant	0.0
None	34.9
L(+)-Cysteine (10 mM)	20.9
TPN (0.5 mM)	36.0
TPN (0.5 mM) + DPNH (0.33 mM)	43.4
Sodium [<i>carboxy-^{14}C</i>]acetate (5 μ C, 20 mM)	38.5

beyond this concentration usually no further increase in fatty acid synthesis occurred. Fig. 5 illustrates the time-course of fatty acid synthesis, which was found to proceed linearly up to about 100 min. and stop after about 150 min.

Effects of citric acid-cycle intermediates on fatty acid synthesis

Popják & Tietz (1954, 1955) and Tietz & Popják (1955) observed a stimulating action by some members of the citric acid cycle on fatty acid synthesis in their enzyme preparations. Since the enzyme system was not fortified with HCO_3^- ion or with pure pyridine nucleotides in this earlier work, it seemed possible that the stimulating action of these acids was associated with the generation of some cofactor, e.g. CO_2 , DPNH or TPNH. This suggestion is borne out by the results of experiments shown in Table 7; under the suboptimum conditions of the 'standard' incubations, several of the citric acid-cycle intermediates stimulated fatty acid synthesis, whereas under the optimum conditions, with the exception of oxaloacetate, they

lost their stimulating action; in fact their presence interfered with the conversion of acetate into fatty acids. Similarly, glycogen, which stimulated fatty acid synthesis in 'standard' incubations, ceased to have any effect when all coenzymes were present in optimum concentrations. Bovine plasma albumin, on the other hand, in concentrations of 3–8 mg./ml. caused 50–150% increase in fatty acid synthesis even under optimum conditions.

Subcellular site of fatty acid synthesis

When rat-mammary-gland homogenate was separated into its subcellular components by centrifuging and these cell fractions were tested for their ability to synthesize fatty acids from acetate, the results shown in Table 8 were obtained.

With optimum cofactor concentrations and anaerobic conditions of incubation, maximum synthesis occurred in the particle-free soluble-supernatant fraction. The presence of microsomes suppressed synthesis (cf. Table 1), and the presence of both mitochondria and microsomes suppressed synthesis even further.

Similar experiments with aerobic incubations showed that, in all cases tested, synthesis was depressed compared with those under anaerobic conditions. However, this may be due to the

Fig. 4. Relation between concentration of protein and synthesis of fatty acids from acetate. The incubation mixture (1.5 ml.) consisted of: sodium [carboxy- ^{14}C]acetate (2.5 μC , 10 mM), ATP (20 mM), CoA (0.133 mM), L(+)-cysteine (10 mM), TPN (0.33 mM), glucose 6-phosphate (13.3 mM), DPNH (0.33 mM), MgCl_2 (3.3 mM), MnCl_2 (3.3 mM), malonate (50 mM), KHCO_3 (25 mM), potassium phosphate buffer, pH 7.4 (66 mM), and soluble-supernatant protein as shown.

Fig. 5. Time-activity curve for the biosynthesis of fatty acids from acetate. Incubation media consisted of: O, that described in Fig. 4 but with 0.66 mM-TPN, 20 mM-glucose 6-phosphate, 50 mM- KHCO_3 and 31.0 mg. of protein in a final volume of 1.5 ml.; ●, that described in Fig. 4 but with 8.1 mg. of protein. The final volume was 1.5 ml. The soluble-supernatant preparations used in these two experiments were made at different times.

oxidation of added CoA or of -SH groups on the participating enzymes, since no cysteine was added to these incubation media.

It should be mentioned that the conditions used for 'anaerobic incubation' (flushing the incubation flasks with N₂ before they were stoppered) do not necessarily exclude all molecular O₂. Strictly anaerobic conditions are, in fact, often difficult to achieve.

Composition of the fatty acids synthesized

The composition of the radioactive fatty acids synthesized in most of the experiments has been determined by the gas-liquid radiochromatographic technique of Popják *et al.* (1959). The

results are expressed as percentages of the total radioactivity recovered from the adipate columns.

Usually, all the normal saturated fatty acids, containing an even number of carbon atoms from C₈ to C₁₈, were synthesized, but to different extents; the proportions of radioactivity were commonly lowest in octanoic acid and octadecanoic acid. A small amount of radioactive hexanoic acid was detected in a number of experiments (it is possible that some of the short-chain-length fatty acids may have been lost during the washing of the light-petroleum extracts with water).

A typical distribution of ¹⁴C among the fatty acids synthesized by different combinations of subcellular fractions under anaerobic and aerobic

Table 7. *Effects of various organic acids on fatty acid synthesis from acetate*

The 'standard' incubation medium was that described in Table 1 except that malonate (50 mm), KHCO₃ (25 mm) and 18.3 mg. of soluble mammary-gland protein were used and biotin and α -oxoglutarate were omitted from the system. The 'optimum' incubation medium consisted of: sodium [*carboxy*-¹⁴C]acetate (5 μ C, 10 mm), L(+)-cysteine (10 mm), ATP (20 mm), CoA (0.13 mm), DPNH (0.33 mm), TPN (0.66 mm), glucose 6-phosphate (20 mm), malonate (50 mm), KHCO₃ (50 mm), MnCl₂ (3.3 mm), MgCl₂ (3.3 mm), biotin (3.3 μ g./ml.) and potassium phosphate buffer, pH 7.4 (66 mm). Soluble mammary-gland (31 mg.) protein was used and water added to bring the final volume to 1.5 ml.

Molarity of acid added (mm) ...	Relative rate of synthesis from acetate in each medium			
	Standard medium		Optimum medium	
	17	50	17	50
Additions				
None	100	100	100	100
Oxaloacetate	154	92	125, 160	—
α -Oxoglutarate	116	116	—	—
Pyruvate	98	95	—	—
Succinate	103	105	—	—
Citrate	141	114	35	—
<i>cis</i> -Aconitate	130	156	—	13, 57
DL-Isocitrate	150	182	—	46
Fumarate	126	128	—	41, 92
DL-Malate	148	127	46, 100	—

Table 8. *Subcellular site of fatty acid biosynthesis from acetate*

The incubation mixture consisted of: sodium [*carboxy*-¹⁴C]acetate (2.5 μ C, 10 mm), ATP (20 mm), CoA (0.13 mm), TPN (0.33 mm), glucose 6-phosphate (13.3 mm), DPNH (0.33 mm), MnCl₂ (3.3 mm), malonate (50 mm), KHCO₃ (50 mm), bovine plasma albumin (8.3 mg./ml.) and potassium phosphate buffer, pH 7.4 (130 mm). Protein (18 mg.) of 'mitochondria + microsomes + supernatant', 'microsomes + supernatant' or 'supernatant' was used as shown. Water was added to bring the final volume to 1.5 ml.

Incubation conditions	Sample no.	Subcellular fractions used	Percentage conversion of acetate
Anaerobic	(1)	Mitochondria + microsomes + supernatant	12.2
	(2)	Microsomes + supernatant	22.1
	(3)	Supernatant	43.4
Aerobic	(4)	Mitochondria + microsomes + supernatant	4.1
	(5)	Microsomes + supernatant	6.0
	(6)	Supernatant	6.9

Table 9. *Composition of radioactive fatty acids formed by lactating-rat mammary gland from acetate*

Fatty acids were obtained as follows. Samples (1)–(6): from the corresponding samples shown in Table 8. Sample (7): by alkaline hydrolysis of the (unlabelled) milk fat obtained from the stomachs of 14–15-day-old rats. Sample (8): by alkaline hydrolysis of the (unlabelled) floating fat formed when homogenates of rat mammary gland were fractionated by centrifuging.

Fatty acid	Shorthand designation*	Retention time relative to methyl stearate	Percentage of total radioactivity of fatty acids						Fatty acid composition	
			Sample number						Milk fat	
			(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
<i>n</i> -Octanoic	8:0	0.05†	1.3	0.9	1.2	3.0	3.2	2.0	2.4	2.0
<i>n</i> -Decanoic	10:0	0.08†	4.8	4.7	9.4	6.0	11.0	16.7	11.3	12.9
Dec-9-enoic	10:1 ⁹	0.09†	—	—	—	—	—	—	0.4	0.5
<i>n</i> -Dodecanoic	12:0	0.165	9.9	7.4	17.7	10.0	13.0	26.2	11.8	11.8
Dodec-9-enoic	12:1 ⁹	0.20	—	—	—	—	—	—	0.4	0.1
<i>n</i> -Tetradecanoic	14:0	0.302	24.7	14.5	37.2	17.0	22.4	33.1	13.7	12.9
Tetradec-9-enoic	14:1 ⁹	0.343	—	—	—	—	—	—	0.9	0.6
<i>n</i> -Hexadecanoic	16:0	0.550	16.6	17.0	29.8	20.0	16.3	14.3	26.8	23.8
Hexadec-9-enoic	16:1 ⁹	0.634	—	—	—	—	—	—	4.0	2.5
<i>n</i> -Octadecanoic	18:0	1.00†	9.9	{ 6.7 2.8 }	{ 1.0 0.4 }	10.0	12.0	{ 0.6 1.9 }	2.3	2.4
Octadec-9-enoic	18:1 ⁹	1.12†	—	—	—	—	—	—	16.2	16.7
Octadec-9,12-adienoic	18:2 ^{9,12}	1.34	—	—	—	—	—	—	9.8	13.8
Unknown	?	(1.29–1.53)	2.4	1.5	0.4	—	—	0.6	—	—
Octadeca-9,12,15-trienoic	18:3 ^{9,12,15}	1.72	—	—	—	—	—	—	—	—
Unknown	?	(1.62–1.74)	2.0	—	—	17.0	—	—	—	—
<i>n</i> -Eicosanoic	20:0	1.82	—	—	—	—	—	—	—	—
Unknown	?	(1.92–2.08)	18.2	18.4	0.9	8.5	17.0	2.9	—	—
Unknown	?	(2.36–2.42)	—	4.1	—	—	—	—	—	—
Unknown	?	(2.64–2.78)	—	6.6	0.6	—	—	0.4	—	—
Eicosa-5,8,11,14-tetraenoic	20:4 ^{5,8,11,14}	3.04	—	—	—	—	—	—	—	—
<i>n</i> -Docosanoic	22:0	3.27	—	—	—	—	—	—	—	0.9
Unknown	?	(3.45–3.77)	6.1	10.3	1.4	8.5	5.0	1.3	—	—
Unknown	?	(4.16–4.17)	4.1	5.1	—	—	—	—	—	—

* See Farquhar, Insull, Rosen, Stoffel & Ahrens (1959).

† These figures are approximate, since the methyl esters of these fatty acids were rapidly eluted from adipate columns at 197°.

conditions is shown in Table 9. In none of these cases were the acids synthesized in the proportions in which they occurred in the rat milk fat or in the mammary-gland fat represented by the 'floating' fat obtained during centrifuging of the homogenates. This was so even when the labelled fatty acids were compared with the proportions of milk and 'floating' fat representing only 'even-numbered' saturated fatty acids.

Although stearic acid and oleic acid were synthesized by the soluble-supernatant preparations, the proportion was much lower than that found in milk and 'floating' fat and was not increased by aerobic incubation. The yield of [^{14}C]hexadecanoic acid was lower in aerobic than in anaerobic incubations of the soluble supernatant; in the latter case the proportion of hexadecanoic acid synthesized reached the amount found in milk fat. Mitochondria or microsomes or both suppressed the synthesis of tetradecanoic acid.

Up to seven unidentified radioactive peaks were found by gas-liquid radiochromatography of the synthesized fatty acids. The main unidentified peaks had retention volumes of (1.62–1.74), (1.92–2.08) and (3.45–3.77) times that of methyl stearate and did not correspond to the methyl esters of octadecadienoic acid, octadecatrienoic acid, eicosanoic acid, eicosatetraenoic acid or docosanoic acid that had been added as markers. These unidentified peaks did not appear to be produced by transesterification of the labelled fatty acids with the stationary phase of the columns; when a separation of labelled fatty acids of considerable total activity was followed by the application of an unlabelled mixture of fatty acids, no radioactivity appeared in the effluent. In both aerobic and anaerobic incubations, the presence of mitochondria or of microsomes or both greatly increased the proportions of these unidentified components as compared with incubations where soluble supernatants alone had been used.

DISCUSSION

The previous work of Popják & Tietz (1955) and Tietz & Popják (1955) on fatty acid synthesis from acetate by soluble preparations of the lactating-rat mammary gland has been reinvestigated; their results have been fully confirmed and extended. The new information obtained shows that the fatty acid-synthesizing system of the mammary gland also requires Mn^{2+} ions, biotin and HCO_3^- ions in addition to CoA, ATP and a reduced pyridine nucleotide. The reduced pyridine nucleotide involved has been shown to be TPNH. The role of DPNH in this fatty acid-synthesizing system is still in doubt, since only in some experiments did it stimulate synthesis in the

presence of optimum amounts of a TPNH-generating system.

Although direct evidence for the formation of malonyl-CoA from acetyl-CoA in the mammary-gland enzyme system has not yet been obtained, the cofactors found to be necessary for optimum synthesis of fatty acids from acetate were those which are known to be required for the malonyl-CoA pathway of fatty acid synthesis. These are (1) ATP, Mn^{2+} ion and CoA for the activation of acetate to acetyl-CoA; (2) ATP, carbon dioxide, Mn^{2+} ion and biotin for the carboxylation of acetyl-CoA to malonyl-CoA; (3) TPNH for the reductive steps in the condensation of acetyl-CoA with malonyl-CoA and in subsequent 2-carbon atom elongation of the fatty acyl-CoA chain by malonyl-CoA.

Though malonate strongly stimulated fatty acid synthesis from acetate, it was not itself incorporated into fatty acids, indicating that the mammary-gland system cannot form malonyl-CoA from free malonate. The stimulation by malonate must therefore be an indirect effect; it is unlikely to be due to the formation of additional carbon dioxide by decarboxylation of the malonate, since malonate produces a far greater effect on synthesis than does an equimolar concentration of HCO_3^- ion (Fig. 1). Its role may be the suppression of deacylation of malonyl-CoA formed from acetyl-CoA or inhibition of a reversible decarboxylation of malonyl-CoA. Ganguly (1960) has shown that the carboxylation of acetyl-CoA to malonyl-CoA is a rate-limiting step in fatty acid synthesis. The striking stimulation of fatty acid synthesis by malonate has also been noted in rat-liver systems by Abraham, Matthes & Chaikoff (1960) and by Fletcher & Myant (1961).

The conclusion which can be drawn from Fig. 2 is that when fatty acid synthesis by the malonyl-CoA pathway is blocked by avidin, virtually no synthesis by the reversal of β -oxidation occurs, since avidin should have no effect on this latter pathway. In the experiments described in Fig. 2, 98.6% inhibition of synthesis was finally produced; the small amount of labelled fatty acids which was synthesized at this level of inhibition consisted of fatty acids of the chain-length range C_{10} – C_{16} . No attempts have been made, however, to determine the optimum conditions for synthesis from acetate by reversal of β -oxidation.

The increased synthesis of fatty acids from acetate brought about by some of the intermediates of the citric acid cycle (Popják & Tietz, 1955) or by glycogen has been shown to be due to the increase in concentration of one or more cofactors required for maximum synthesis. The stimulatory role of oxaloacetate is not clear at the moment, but the stimulation of synthesis by

added albumin might be due either to the protection of the mammary-gland enzymes from hydrolytic attack or to the removal of the products of the reaction which are probably free fatty acids rather than fatty acyl-CoA compounds (palmitoyl-CoA deacylase has been reported in a variety of tissues, e.g. by Porter & Tietz, 1957; Porter & Long, 1958; Srere, Seubert & Lynen, 1959). In the presence of optimum concentrations of TPN, glucose 6-phosphate and DPNH, citrate did not stimulate fatty acid synthesis from acetate (Table 7).

Cell-fractionation studies have shown that mitochondria or microsomes or both depress fatty acid synthesis from acetate in the soluble-supernatant system. This depression of synthesis by microsomes confirms the earlier results of Popják & Tietz (1955), though Ganguly (1960) has shown that the enzymes involved in fatty acid synthesis by the malonyl-CoA pathway occur in both the microsomes and soluble-enzyme fractions of a wide variety of tissues, including ox mammary gland. In liver preparations, however, fatty acid synthesis requires the presence of both microsomes and the particle-free soluble supernatant (Matthes, Abraham & Chaikoff, 1960; Fletcher & Myant, 1961), maximum synthesis occurring at a ratio of microsomal to supernatant protein approximating to that found in the intact liver cell. Similarly, studies on lactating-rabbit mammary gland have shown that maximum synthesis of fatty acids from acetate via the malonyl-CoA pathway requires the presence of both microsomes and the particulate-free soluble supernatant (R. Dils & G. Popják, unpublished work). It appears therefore that the subcellular site of synthesis can vary both with tissue and with species. The possibility also exists that enzymes present in cell particles of various tissues of different species may be extracted into solution to a varying extent.

When optimum conditions for fatty acid synthesis were used (see sample 3, Tables 8 and 9), all the 'even-numbered' straight-chain fatty acids from C_8 to C_{18} were synthesized, as well as a small amount of oleic acid. The major components were in the chain-length range C_{10} – C_{16} , though compared with the milk fatty acids excess of C_{12} and C_{14} were being made. However, this is nearer to the composition of the milk fatty acids than that obtained previously with soluble supernatants of mammary gland in the presence of ATP and oxaloacetate (Popják & Tietz, 1955), where 76% of the synthesized fatty acids were in the chain-length range up to and including C_{10} .

When mitochondria or microsomes or both were present in this system (samples 1 and 2, Tables 8 and 9) considerable suppression of the synthesis of C_{10} – C_{16} fatty acids was observed, and the proportion of the unidentified components was strikingly

raised. The increase in the amount of synthesis of oleic acid did not raise it to the amount found in milk, however. This is in contrast with the earlier findings of Popják & Tietz (1954) that in the presence of ATP and oxaloacetate, 'mitochondria + microsomes + supernatant' synthesized a mixture of fatty acids of which 17.6% was oleic acid. Wakil (1960) has recently shown that liver mitochondria can elongate existing fatty acids by a mechanism involving the enzymes of β -oxidation with a TPN-linked $\alpha\beta$ -unsaturated fatty acyl-CoA reductase. With our soluble mammary-gland-enzyme system, however, the main effect of the presence of subcellular particles was to increase greatly the proportion of unidentified radioactive components. The large retention volumes of these unidentified components in gas-liquid chromatography suggest substances of high molecular weight or of high polarity due to the presence of oxygen functions beyond the ester group. They may represent intermediates formed during fatty acid synthesis, or they may be intermediates of fatty acid oxidation.

Aerobic incubation of the soluble-enzyme system did not cause any striking increase in the synthesis of oleic acid or of the unknown components.

Since this work has been completed, Abraham, Matthes & Chaikoff (1961*a*) have confirmed the inhibition by avidin of fatty acid synthesis from acetate by lactating-rat mammary glands and the reversal of this inhibition by biotin. Malonate was not added to their incubation media.

These workers (Abraham *et al.* 1961*b*) have also obtained results similar to ours, although their conditions for optimum synthesis of fatty acids from acetate were substantially different from those we have found necessary. These differences may also explain the fact that the best preparation of Abraham *et al.* (1961*b*) converted only about 112 μ moles of acetate into fatty acids/mg. of protein in contrast with the conversion of 1–2 μ moles/mg. of protein under our optimum conditions. Their fatty acid-synthesizing system showed an absolute requirement for citrate (11.4–21.4 mM), which could not be replaced by glucose 6-phosphate (5.7–22.8 mM). Table 7 shows that, in our system, citrate did not stimulate fatty acid synthesis from acetate in the presence of optimum concentrations of TPN, glucose 6-phosphate and DPNH. Malonate (50 mM) caused a 50-fold increase in the conversion of acetate into fatty acids (Fig. 1) in our system, whereas Abraham *et al.* (1961*b*) reported that malonate (11.4 mM) approximately doubled synthesis in the presence of citrate (14.3 mM), but had no effect in its absence. The fatty acids synthesized by their enzyme system consist predominantly of C_8 – C_{12} chain length, with some tetradecanoic acid, hexadecanoic acid, stearic acid and oleic acid; in general, the proportion of fatty acids of

chain length C_8 – C_{10} is much higher than that reported here. The small amounts of linolenic acid, arachidic acid, arachidonic acid and higher chain-length acids they described may, in fact, be similar to the unidentified compounds we have reported. (These higher acids have retention volumes in gas chromatography close to but not identical with those of our unknown components.)

SUMMARY

1. The major route for the biosynthesis of fatty acids from acetate in lactating-rat mammary gland is the malonyl-coenzyme A pathway, the intracellular site of the synthesis being the particle-free extracts of this gland. Virtually no synthesis from acetate takes place when the biotin-mediated carboxylation of acetyl-coenzyme A to malonyl-coenzyme A is inhibited by avidin.

2. The cofactors required for this synthesis from acetate are adenosine triphosphate, coenzyme A, reduced triphosphopyridine nucleotide, HCO_3^- ion, Mn^{2+} ion and biotin. The requirement for reduced diphosphopyridine nucleotide is less clearly marked.

3. Malonate strongly stimulated fatty acid synthesis in the presence of these cofactors, but was not incorporated into the synthesized fatty acids. Therefore this mammary-gland-enzyme system cannot form malonyl-coenzyme A from malonate.

4. Optimum conditions for synthesis are described. With these conditions, a number of citric acid-cycle intermediates that had previously been found to stimulate synthesis as well as glycogen, were no longer required.

5. The main products of synthesis were all of the even-numbered saturated fatty acids from C_8 to C_{18} as well as oleic acid and a number of unidentified compounds. Quantitative analyses are given.

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REFERENCES

- Abraham, S., Matthes, K. J. & Chaikoff, I. L. (1960). *J. biol. Chem.* **235**, 2551.
 Abraham, S., Matthes, K. J. & Chaikoff, I. L. (1961a). *Biochim. biophys. Acta*, **46**, 197.
 Abraham, S., Matthes, K. J. & Chaikoff, I. L. (1961b). *Biochim. biophys. Acta*, **49**, 268.

- Brady, R. O. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 993.
 Dils, R. & Popják, G. (1961). *Biochem. J.* **80**, 47p.
 Farquhar, J. W., Insull, W., Rosen, P., Stoffel, W. & Ahrens, E. H. (1959). *Nutritional Reviews* (Suppl. 8, Part n).
 Fletcher, K. & Myant, N. B. (1961). *J. Physiol.* **155**, 498.
 Folley, S. J. & McNaught, M. L. (1961). In *Milk: the Mammary Gland and its Secretion*, vol. 1, p. 441. Ed. by Kon, S. K. & Cowie, A. T. New York and London: Academic Press Inc.
 Formica, J. V. & Brady, R. O. (1959). *J. Amer. chem. Soc.* **81**, 752.
 Ganguly, J. (1960). *Biochim. biophys. Acta*, **40**, 110.
 Gibson, D. M., Titchener, E. B. & Wakil, S. J. (1958). *Biochim. biophys. Acta*, **30**, 376.
 Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
 Hele, P., Popják, G. & Laurysse, M. (1957). *Biochem. J.* **65**, 348.
 Howes, J. R. (1960). *J. Chromat.* **3**, 389.
 James, A. T. (1959). *J. Chromat.* **2**, 552.
 Langdon, R. G. (1957). *J. biol. Chem.* **226**, 615.
 Lynen, F., Knappe, T. H., Lorch, E., Jütting, G. & Ringelmann, E. (1959). *Angew. Chem.* **71**, 481.
 Lynen, F. & Ochoa, S. (1953). *Biochim. biophys. Acta*, **12**, 299.
 Matthes, K. J., Abraham, S. & Chaikoff, I. L. (1960). *J. biol. Chem.* **235**, 2560.
 Popják, G. (1950). *Biochem. J.* **46**, 560.
 Popják, G. (1952). *Symp. Biochem. Soc.* no. 9, p. 37.
 Popják, G., French, T. H., Hunter, G. D. & Martin, A. J. P. (1951). *Biochem. J.* **48**, 612.
 Popják, G., Lowe, A. E., Moore, D., Brown, L. & Smith, F. A. (1959). *J. Lipid Res.* **1**, 29.
 Popják, G. & Tietz, A. (1954). *Biochem. J.* **56**, 46.
 Popják, G. & Tietz, A. (1955). *Biochem. J.* **60**, 147.
 Porter, J. W. & Long, R. W. (1958). *J. biol. Chem.* **233**, 20.
 Porter, J. W. & Tietz, A. (1957). *Biochim. biophys. Acta*, **25**, 41.
 Porter, J. W., Wakil, S. J., Tietz, A., Jacobs, M. I. & Gibson, D. M. (1957). *Biochim. biophys. Acta*, **25**, 35.
 Seubert, W., Greull, G. & Lynen, F. (1957). *Angew. Chem.* **69**, 359.
 Srere, P. A., Seubert, W. & Lynen, F. (1959). *Biochim. biophys. Acta*, **33**, 313.
 Tietz, A. (1957). *Biochem. biophys. Acta*, **25**, 303.
 Tietz, A. & Popják, G. (1955). *Biochem. J.* **60**, 155.
 Wakil, S. J. (1958). *J. Amer. chem. Soc.* **80**, 6465.
 Wakil, S. J. (1960). *Amer. J. clin. Nutr.* **8**, 630.
 Wakil, S. J. (1961). *J. Lipid Res.* **2**, 1.
 Wakil, S. J., Titchener, E. B. & Gibson, D. M. (1958). *Biochim. biophys. Acta*, **29**, 225.
 Wakil, S. J., Titchener, E. B. & Gibson, D. M. (1959). *Biochim. biophys. Acta*, **34**, 227.
 Woolley, D. W. & Longworth, L. G. (1942). *J. biol. Chem.* **235**, 2551.