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Fatty Acid Synthesis in Intestinal Mucosa of Guinea Pig

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1. Acetate–CoA ligase, acetyl-CoA–carbon dioxide ligase and fatty acid synthetase were shown to be present in particle-free fractions of guinea-pig intestinal mucosa. 2. Each of these enzymes was partially purified by ammonium sulphate precipitation from the particle-free supernatant. 3. The incorporation of acetate and citrate into fatty acid was measured. 4. Gas–liquid radiochromatography was used to investigate the pattern of fatty acids synthesized. 5. The rate-limiting step in fatty acid synthesis was shown to be acetyl-CoA–carbon dioxide ligase.

After fat absorption, lymph triglycerides, though reflecting the fatty acid composition of the dietary lipids (Bergström, Blomstrand & Borgström, 1954; Blomstrand & Dahlbäck, 1960), also contain endogenous fatty acids (Karmen, Whyte & Goodman, 1963; Whyte, Karmen & Goodman, 1963; Gottenbos & Thomasson, 1963). This raises the question whether these endogenous acids are synthesized in the small intestine or are transported to it from other tissues. Coniglio, McCormick & Hudson (1956) suggested that the intestine might be capable of synthesizing fatty acids, and Franks, Riley & Isselbacher (1966) have shown that jejunal rings from rats can synthesize fatty acids from [1-^{14}C]acetate. The only previous report of the enzymes of fatty acid synthesis in intestine was that of Ganguly (1960), who found the activity of fatty acid synthetase from ox intestinal mucosa to be very low. We have shown that the individual enzymes of fatty acid synthesis are present in the intestinal mucosa of guinea pig and also that a soluble fraction from this tissue can synthesize fatty acids from acetate or citrate.

MATERIALS

Chemicals. All labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks. Triton X-100 was from Lennig Chemicals Ltd., Jarrow, Co. Durham. 2,5-Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were purchased from Nuclear Enterprises (G.B.) Ltd., Edinburgh, 11. Bovine serum albumin (fat-poor) was supplied by Pentex Inc., Kankakee, Ill., U.S.A. ATP, CoA, GSH, $NADP^+$ and NADPH were supplied by Sigma Chemical Co., St Louis, Mo., U.S.A. All other chemicals were of A.R. grade.

Animals. Guinea pigs of either sex (each weighing 250–300g.) were taken from the Departmental colony of mixed strains. They were fed on cabbage and crushed oats and pellets (4:1), and had access to hay.

METHODS

Preparation of particulate-free supernatant. The full homogenate and PFS* were prepared essentially by the method of Hübscher, West & Brindley (1965), except that the washing procedures were omitted. Normally two or three animals were used for each experiment.

Preparation and assay of acetyl-CoA. Acetyl-CoA was freshly prepared for each set of assays by a modification of the method of Ochoa (1959). CoA (10mM), $KHCO_3$ (0·2M) and redistilled acetic anhydride (20mM) (final concns.) were incubated at 0° for 5min. After acidification to pH2 with HCl, the mixture was brought to room temperature for 30min. to hydrolyse any remaining acetic anhydride. The yield was estimated either by the liberation of free SH groups (Jocelyn, 1962) after mild alkaline hydrolysis or by the difference spectrum of CoA and acetyl-CoA (Stadtman, 1955). Both these methods were in agreement, showing yields to be 95–100%, based on CoA.

Preparation and assay of malonyl-CoA. This was prepared by the method of Trams & Brady (1960). The yield was determined either by measuring the liberation of SH groups after mild alkaline hydrolysis or by measuring the thio ester bonds formed. The latter were estimated by a modification of the method of Pilz (1958). In the assay, 1·0ml. of M-hydroxylamine, adjusted to pH7·6 with KOH, was added to 3·0ml. of ethanol–diethyl ether (3:1, v/v) containing the sample (0·5–3·0μmoles of thio ester bonds). This was incubated at room temperature for 15min. Then 0·5ml. of 1·35N-HCl was added, followed by 0·5ml. of 1·4M-$FeCl_3$ in 0·2N-HCl. The solution was then shaken and E_{540} measured. A standard solution of monothiophenyl malonate was used to calibrate the assay. With this modification, the results agreed with the SH determination. The yield, based on CoA, was 85–100%.

Preparation of monothiophenyl malonate. This was prepared by the method of Trams & Brady (1960).

Assay of protein. Protein was determined by the biuret method, based on that of Weischelbaum (1946).

Assay of acetate–CoA ligase (AMP) (EC 6.2.1.1). The

* Abbreviation: PFS, particle-free supernatant.

formation of acetyl-CoA from acetate was assayed by the hydroxamate method of Lipmann & Tuttle (1950). The assay system for the PFS enzyme contained: potassium phosphate buffer, pH 8·0 (0·1 M); ATP (20 mM); $MnCl_2$ (10 mM); CoA (50 μM); sodium acetate (0·50 mM) (final concns.). The final volume was 1·0 ml. The reaction was started by the addition of about 3 mg. of enzyme protein and, after 30 min. incubation at 37°, stopped by adding 2·0 ml. of 10% (w/v) $FeCl_3$ containing 3·3% (w/v) trichloroacetic acid. In the assay system for the partially purified enzyme [50–60%-saturated $(NH_4)_2SO_4$ fraction] the concentration of CoA was increased to 0·10–0·15 mM. All other conditions remained unaltered.

Assay of acetyl-CoA–CO_2 ligase (ADP) (*EC* 6.4.1.2). This enzyme was assayed by the method of Smith, Easter & Dils (1966). The assay system for the PFS enzyme contained: potassium phosphate buffer, pH 7·0 (0·1 M); ATP (7·5 mM); $MnCl_2$ (4·0 mM); acetyl-CoA (25 μM); $KH^{14}CO_3$ (10–20 mM) (1 μC/μmole); potassium DL-citrate (20–45 mM); bovine serum albumin (fat-poor) (0·25 mg./ml.) (final concns.). The total volume was 1 ml. The reaction was started by adding 0·3 mg. of enzyme protein and, after incubation for 10 min. at 37°, stopped by adding 0·1 ml. of 5 N-$HClO_4$.

The assay system for the partially purified fraction [0–25%-saturated $(NH_4)_2SO_4$ fraction] contained: potassium phosphate buffer, pH 7·2 (0·1 M); ATP (10 mM); $MnCl_2$ (3·3 mM); acetyl-CoA (0·2 mM); $KH^{14}CO_3$ (5 mM) (1 μC/μmole); potassium DL-citrate (30 mM); bovine serum albumin (fat-poor) (1·0 mg./ml.) (final concns.). The reaction was started by the addition of 0·15 mg. of enzyme protein. Other conditions were the same as for the PFS assay system.

Assay of fatty acid synthetase. Fatty acid synthetase was assayed optically by following the oxidation of NADPH (Lynen, Hopper-Kessel & Egerer, 1964) with a Unicam SP. 700 recording spectrophotometer. The assay system for the partially purified enzyme [25–40%-saturated $(NH_4)_2SO_4$ fraction] contained: potassium phosphate buffer, pH 6·5 (0·167 M); malonyl-CoA (67 μM); NADPH (54 μM) (final concns.); 2–3 mg. of enzyme protein. The total volume was 3·0 ml. The reaction was started by the addition of malonyl-CoA and the oxidation was followed for at least 4 min. at 37°.

Assay of fatty acid synthesis from [1-^{14}C]acetate by PFS. In initial experiments, GSH had variable effects on the incorporation of acetate. In one enzyme preparation, increasing concentrations of GSH inhibited incorporation slightly (21% at 15 mM). It stimulated acetate incorporation in a second enzyme preparation by 30% at 7·5 mM, but concentrations higher than this inhibited incorporation. GSH had no effect on a third enzyme preparation. As a result, it was omitted from subsequent assay systems.

Citrate also had variable effects, stimulating the reaction 3·6–30-fold (mean of five preparations, 13·7-fold). The optimum concentration of citrate was also very variable (Fig. 1). Potassium DL-citrate (40 mM) was used in subsequent experiments.

The assay system contained: potassium phosphate buffer, pH 6·8 (100 mM); ATP (10 mM); $MnCl_2$ (10 mM); sodium [1-^{14}C]acetate (50 mM) (1 μC/μmole); CoA (75 μM); $NADP^+$ (0·25 mM); potassium DL-citrate (40 mM); bovine serum albumin (fat-poor) (2·0 mg./ml.) (final concns.). The total volume was 1·0 ml. The reaction was started by adding about 2 mg. of enzyme protein and, after 60 min. at 37°, stopped by the addition of 0·2 ml. of 5 N-$HClO_4$. Then 1·0 ml. of 40% (w/v) KOH was added, followed by 0·5 ml. of ethanol. The system was then saponified for 45 min. at 80°. After cooling, the system was acidified to pH 1. Preliminary experiments established that radioactivity was incorporated into myristic acid, palmitic acid and stearic acid. Consequently, at this point these acids were added as carrier fatty acids (approx. 5 mg., made up of equal weights of each acid) in 1·0 ml. of light petroleum (b.p. 40–60°). Fatty acids were extracted three times with 4 vol. of light petroleum (b.p. 40–60°). The final volume of the light-petroleum extracts was adjusted to 10 ml., a 2·0 ml. portion of each was transferred to a counting ampoule and the solvent was removed under vacuum. Then 5 ml. of Triton X-100–phosphor (1:2, v/v) (Patterson & Green, 1965) was added and the radioactivity was determined by liquid-scintillation counting. The phosphor consisted of 6 g. of 2,5-diphenyloxazole and 0·12 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 l. of redistilled xylene. Triton X-100 was added to reduce cost without loss of efficiency. In some experiments, the remaining 8·0 ml. of the light-petroleum extract was used to determine the radioactivity of the individual fatty acids. This was done by gas–liquid chromatography of their methyl esters on a 5 ft. column of 10% (w/w) polyethylene glycol adipate on Chromosorb W (treated with hydroxydimethyldisilazine). The temperature was 175° with a flow rate of 60 ml. of argon/min. The apparatus was a Pye Radiopanchromatograph, based on the design of James & Piper (1961).

Preparation of methyl esters of fatty acids. These were prepared by the method of Metcalf & Schmitz (1961), except that light petroleum (b.p. 40–60°) replaced hexane or heptane.

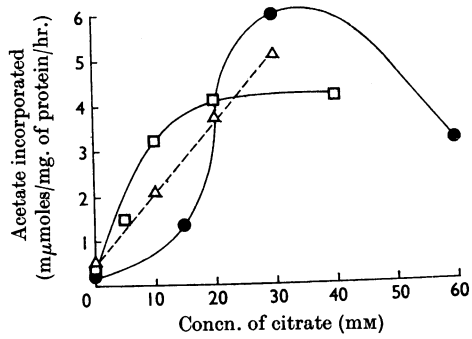

Fig. 1. Effect of citrate on acetate incorporation into fatty acids. The assay system contained: ATP (10 mM); CoA (0·15 mM); $KHCO_3$ (50 mM); NADPH (0·25 mM); glucose 6-phosphate (10 mM); GSH (10 mM). In addition, Expt. 1 (□) contained: potassium phosphate buffer, pH 7·0 (0·1 M); $MnCl_2$ (5 mM); sodium [1-^{14}C]acetate (5·7 mM) (10 μC); potassium DL-citrate (20 mM); bovine serum albumin (fat-poor) (2 mg./ml.) (final concns.); 1·93 mg. of enzyme protein. Expts. 2 (●) and 3 (△) contained: potassium phosphate buffer, pH 6·8 (0·1 M); $MnCl_2$ (10 mM); sodium [1-^{14}C]-acetate (10·7 mM) (10 μC); bovine serum albumin (1 mg./ml.) (final concns.). Expt. 2 was started with 2·35 mg. of protein and Expt. 3 with 2·47 mg. of protein.

Assay of avidin. This was assayed according to the method of Wei & Wright (1964).

All assays were done in duplicate.

RESULTS

In initial experiments, optimum conditions were established for both PFS and partially purified acetate–CoA ligase and acetyl-CoA–carbon dioxide ligase, but only for the partially purified fatty acid synthetase. The optimum conditions were determined for fatty acid synthesis from [1-^{14}C]acetate in the PFS. In all cases, rate determinations with respect to time of incubation and protein concentration ensured that assays were done under zero-order conditions. The results were used to determine the final assay conditions described in the Methods section.

Acetate–CoA ligase. The specific activity obtained for the PFS enzyme was 310mμmoles of acetohydroxamate formed/mg. of protein/hr. This enzyme was partially purified from the PFS by precipitation with ammonium sulphate. All the detectable activity precipitated at 50–60% saturation with ammonium sulphate (Table 1). The specific activity obtained for this enzyme under optimum conditions was 550mμmoles of acetohydroxamate formed/mg. of protein/hr.

Acetyl-CoA–carbon dioxide ligase. This enzyme was demonstrated in the PFS, and in four out of the five preparations over 75% of the activity in the full-homogenate activity was recovered in the PFS. A striking feature of the enzyme is the low requirement for acetyl-CoA. The specific activity under optimum conditions was 30–40mμmoles of malonyl-CoA formed/mg. of protein/hr. (mean of four preparations, 39·5). On partial purification, most of the activity precipitated at 0–25% saturation with ammonium sulphate (Table 2). Some activity also precipitated at 25–40% saturation. The effects of citrate, isocitrate and malonate on this fraction are given in Table 3. GSH had no effect on two separate enzyme preparations and consequently was not included in the optimum assay system. Under optimum conditions, the specific activities obtained were 87–161mμmoles of malonyl-CoA formed/mg. of protein/hr. (mean of four preparations, 111).

The partially purified enzyme was reasonably stable when stored for 8 days at −20° (3·8mg. of protein/ml. in 0·3M-sucrose). It was thawed and refrozen twice during this period and the activity fell by 21%. Several workers (Wakil, 1958; Brady, 1958) have identified the product of acetyl-CoA–carbon dioxide ligase reaction as malonyl-CoA. In the present work the product was identified, after hydrolysis, as [^{14}C]malonic acid. A portion of the incubation system was subjected to alkaline hydrolysis, and the malonic acid was extracted with water-saturated diethyl ether and chromatographed according to Lugg & Overell (1948). All the radioactivity was in the malonic acid spot.

Fatty acid synthetase. An attempt was made to assay the PFS enzyme, with four separate enzyme preparations, without success. The assay was tried

Table 1. *Partial purification of acetate–CoA ligase (AMP) from PFS*

The units of specific activity are mμmoles of acetohydroxamate formed/mg. of protein/hr. The assay system was that described for the PFS (see the Methods section).

$(NH_4)_2SO_4$ fraction (% satd.)	Protein in assay (mg.)	Specific activity
45–50	1·76	<13
50–60	3·96	172
60–65	2·40	<22
65–70	2·37	<23

Table 2. *Partial purification of acetyl-CoA–carbon dioxide ligase (ADP) from PFS*

The units of specific activity are mμmoles of malonyl-CoA formed/mg. of protein/hr. The assay conditions used were those given by Smith *et al.* (1966).

	Recovery of full-homogenate activity (%)				Specific activity				
		$(NH_4)_2SO_4$ fractions				$(NH_4)_2SO_4$ fractions			
Enzyme prep. no.	PFS	0–25% satd.	25–40% satd.	Full homogenate	PFS	0–25% satd.	25–40% satd.	40–60% satd.	60–100% satd.
1	106	29	18	14	25	121	30	3·2	1·4
2	55	14	8	23	32	122	35	—	—
3	76	40	16	8·5	23	87	18	0	0
4	88	52	10	3	13	88	9	—	—
5	77	48	8	—	13	48	5	—	—

Table 3. *Effect of citrate, isocitrate and malonate on partially purified acetyl-CoA–carbon dioxide ligase activity*

The units of specific activity are mμmoles of malonyl-CoA formed/mg. of protein/hr. The optimum assay system was used (see the Methods section). One enzyme preparation was used throughout.

Concn. of added substance (mM)	Specific activity		
Addition.....	DL-Citrate	DL-Isocitrate	Malonate
0	7	14	7
3·25	55	42	31
7·5	74	61	43
15	88	81	55
30	76	97	66
45	74	88	62
75	38	81	64

Table 4. *Location of fatty acid synthetase in ammonium sulphate fractions of guinea-pig intestinal mucosa PFS*

The units of specific activity are mμmoles of NADPH oxidized/mg. of protein/hr. The incubation system contained: potassium phosphate buffer, pH 6·7 (0·2M); malonyl-CoA (0·1mM); acetyl-CoA (50μM); NADPH (89μM) (final concns.). Full details are in the Methods section.

Preparation 1			Preparation 2		
$(NH_4)_2SO_4$ fraction (% satd.)	Protein in assay (mg.)	Specific activity	$(NH_4)_2SO_4$ fraction (% satd.)	Protein in assay (mg.)	Specific activity
0–30	2·5	402	0–25	4·7	26
30–40	2·2	416	25–40	3·5	263
40–50	5·6	22	40–50	10·1	12
50–60	4·8	26	50–100	9·8	12
60–100	4·8	26			

at pH 6·8, 6·6 and 6·3. Malonyl-CoA concentrations up to 293μM were used in the absence and presence of various amounts of acetyl-CoA (up to 67μM). Protein concentrations of up to 1·27mg./ml. were used. Cysteine at a concentration of approx. 65mM had no effect (this assay was at pH 6·8 in the presence of 293μM-malonyl-CoA; no acetyl-CoA was added).

Lactating-rabbit mammary-gland PFS (1·59mg. of protein) (Smith *et al.* 1966) was assayed for fatty acid synthetase activity, under conditions optimum for the partially purified enzyme from guinea-pig PFS. The specific activity was 636mμmoles of NADPH oxidized/mg. of protein/hr. The addition of 2·58mg. of guinea-pig PFS protein to this did not inhibit it but, in fact, doubled the rate of oxidation of NADPH. However, it was found that PFS fatty acid synthetase activity was precipitated at 25–40% saturation with ammonium sulphate (Table 4). No activity could be detected in any other fraction. Under optimum conditions, only one enzyme preparation showed a small requirement for acetyl-CoA, three preparations showed no requirement, and all preparations were inhibited by increasing concentrations of acetyl-CoA; 50% inhibition was achieved at 100μM with two separate enzyme preparations, and the inhibition was almost linear with increasing acetyl-CoA concentration up to 133μM. Lynen *et al.* (1964) also found no requirement for acetyl-CoA with purified fatty acid synthetase from yeast. They reported that malonyl-CoA carboxy-lyase (EC 4.1.1.9) is purified together with fatty acid synthetase in yeast. Such a decarboxylase could account for there being no acetyl-CoA requirement in the experiment reported above.

NADPH can only partially be replaced by NADH as shown in Table 5. As citrate and albumin are included in the assay of [1-^{14}C]acetate incorporation into fatty acids, their effect on fatty acid synthetase was investigated. Albumin, at concentrations up to 5mg./ml., had no effect, but 50mM-DL-citrate inhibited slightly, decreasing the specific activity from 339 to 283mμmoles of NADPH oxidized/mg. of protein/hr. An explanation could be that ATP–citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating) may be

precipitated in the same fraction, thus cleaving citrate to give acetyl-CoA. This would then inhibit fatty acid synthetase. The specific activities of the partially purified enzyme were 272–415mμmoles of NADPH oxidized/mg. of protein/hr. (mean of six preparations, 363).

Incorporation of [1-^{14}C]*acetate into fatty acids.* In initial experiments with PFS, NADPH was used with glucose 6-phosphate to act as a generating system. However, NADPH could be replaced by $NADP^+$ with an increase in the specific activity (Table 6). The reason for this was that, even in the absence of added glucose 6-phosphate, the $NADP^+$ was reduced to NADPH. Indeed, in two separate preparations, the rate of $NADP^+$ reduction was 900 and 950mμmoles of NADPH formed/mg. of protein/hr. As this rate was 68-fold faster than the rate required for acetate incorporation (2moles of NADPH are required/mole of acetate incorporated into fatty acids), no glucose 6-phosphate was required in the assay system. The rate of acetate incorporation was 5·1–7·9mμmoles/mg. of PFS protein/hr. (mean of eight preparations, 6·8). Avidin inhibited acetate incorporation completely (Fig. 2).

The pattern of fatty acids synthesized by the PFS from [1-^{14}C]*acetate.* This was found to be unusually simple, in that only two fatty acids were synthesized. The proportion of each acid, in terms of percentage of the total radioactivity incorporated, was 20–36% of myristic acid and 64–80% of palmitic acid. However, in two out of seven preparations, there was a small amount (up to 10%) of radioactivity in stearic acid.

Determination of the rate-limiting step in fatty acid synthesis. The rate-limiting step is taken to be the step that limits the amount of product formed. This was determined by adding back separately to the PFS each of the three ammonium sulphate fractions described. Two separate experiments showed that acetyl-CoA–carbon dioxide ligase was the rate-limiting step. The results of the second experiment are given in Table 7. The increase in incorporation of acetate into fatty acids after the addition of the 25–40% fraction is probably due to the presence of a small amount of acetyl-CoA–carbon dioxide ligase in this fraction (see Table 2). The reason for the decrease in rate after the addition of 0·37mg. of the 50–60% fraction is not known.

Incorporation of citrate into fatty acids. It has been shown in other tissues that acetyl-CoA–carbon dioxide ligase is rate-limiting (Ganguly, 1960; Numa, Matsuhashi & Lynen, 1961). Though we have confirmed this in guinea-pig intestinal mucosa, the rate of fatty acid synthesis from acetate was lower than the rate-limiting formation

Table 5. *Effect of NADPH and NADH on partially purified fatty acid synthetase*

The units of specific activity are mμmoles of nucleotide oxidized/mg. of protein/hr. Optimum assay conditions were used (see the Methods section). One enzyme preparation was used throughout.

Nucleotide	Concn. of nucleotide (mM)	Specific activity
NADPH	0·023	315
NADPH	0·046	272
NADPH	0·091	305
NADH	0·109	36
NADH	0·215	63
NADH	0·546	63

Table 6. *Effect of NADPH and $NADP^+$ on acetate incorporation into fatty acids by PFS*

The assay system contained: potassium phosphate buffer, pH 7·0 (0·1M); ATP (10mM); $MnCl_2$ (10mM); CoA (0·15mM); sodium [1-^{14}C]acetate (10·7mM) (1μc/μmole); $KHCO_3$ (50mM); glucose 6-phosphate (10mM); potassium DL-citrate (40mM); bovine serum albumin (fat-poor) (2mg./ml.) (final concns.); 2·34mg. of PFS enzyme protein. The final volume was 1·0ml.

Nucleotide	Concn. of nucleotide (mM)	Acetate incorporated (mμmoles/mg. of protein/hr.)
NADPH	0	0
NADPH	0·25	2·2
NADPH	0·50	3·2
NADPH	1·0	3·4
$NADP^+$	0	0
$NADP^+$	0·25	6·5
$NADP^+$	0·50	6·6
$NADP^+$	1·0	7·2

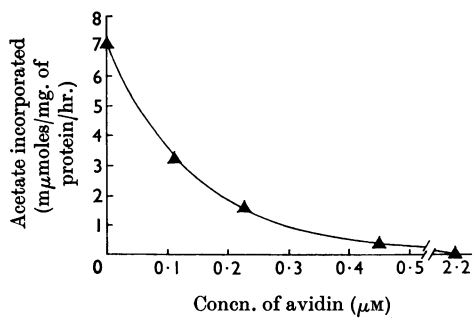

Fig. 2. Effect of avidin on acetate incorporation into fatty acids. The rate of acetate incorporation was measured under the optimum conditions described in the Methods section.

Table 7. *Determination of rate-limiting step in fatty acid synthesis from acetate by PFS*

In all cases, 2·5mg. of PFS protein was used under optimum assay conditions for acetate incorporation (see the Methods section).

Enzyme added	$(NH_4)_2SO_4$ fraction added (% satd.)	Concn. of protein (mg./ml.)	[1-^{14}C]Acetate incorporated (mμmoles/mg. of PFS protein/hr.)
None	None	0	5·8
Acetyl-CoA–CO_2 ligase	0–25	0·5	10·5
	0–25	1·0	12·7
	0–25	2·0	17·3
Fatty acid synthetase	25–40	0·58	8·7
	25–40	1·15	9·2
Acetate–CoA ligase	50–60	0·37	4·4
	50–60	0·73	5·8

Table 8. *Comparison of acetate and citrate as precursors for fatty acid biosynthesis in PFS*

Incorporation was measured under optimum conditions for acetate incorporation (see the Methods section) with one enzyme preparation. The specific activities assume that only [1-^{14}C]acetyl-CoA is incorporated into fatty acids. The substrates used had specific radioactivities of 1 μc/μmole (acetate) and 0·1 μc/μmole (citrate). The units of specific enzyme activity are mμmoles of substrate incorporated/mg. of protein/hr.

Substrate	Specific activity
[1-^{14}C]Acetate + citrate	5·8
DL-[1,5-$^{14}C_2$]Citrate + acetate	23·0
DL-[1,5-$^{14}C_2$]Citrate	31·3

Table 9. *Effect of unlabelled acetyl-CoA on the incorporation of* [1,5-$^{14}C_2$]*citrate into fatty acids in PFS*

Incorporation was measured under optimum conditions for acetate incorporation (see the Methods section); 5 μc of [1,5-$^{14}C_2$]citrate (40mM) was used. The specific activities assume that only [1-^{14}C]acetyl-CoA was incorporated into fatty acids.

Concn. of acetyl-CoA (μM)	[1,5-$^{14}C_2$]Citrate incorporated (mμmoles/mg. of protein/hr.)
0	16·5
5	13·7
10	12·7
20	11·7
50	7·4
520	1·4

of malonyl-CoA, when it would be expected to be the same. The assay of acetyl-CoA–carbon dioxide ligase measures the incorporation of $KH^{14}CO_3$ into added and endogenous acetyl-CoA. The assay of fatty acid synthesis from acetate only measures the incorporation of radioactive acetate. The difference between these two rates indicates that, in addition to radioactive acetate, an endogenous but unlabelled substrate is being incorporated into fatty acids. Such a substrate could be acetyl-CoA, provided by cleavage of citrate. Consequently, the incorporation of [1,5-$^{14}C_2$]citrate into fatty acids was investigated. Two separate enzyme preparations showed that citrate was a better precursor for fatty acid synthesis than acetate. The results from the second enzyme preparation are given in Table 8. If labelled citrate was used as a precursor in the absence of acetate, the rate of incorporation was increased to the order expected. The addition of unlabelled acetate apparently lowered the incorporation of citrate, presumably due to an isotope-dilution effect. Support for this is given in Table 9, which shows that unlabelled acetyl-CoA gave this effect with [1,5-$^{14}C_2$]citrate. In the optimum assay system for acetate incorporation, the unlabelled citrate would have an isotope-dilution effect and thus account for the low incorporation of labelled acetate. These results indicate that citrate was able to supply acetyl-CoA at a rate at least equal to the rate of malonyl-CoA production.

DISCUSSION

The factors found here to be necessary for [1-^{14}C]-acetate incorporation into fatty acids are those known to be required for the malonyl-CoA pathway (Wakil, 1961). The existence of this pathway in guinea-pig intestinal mucosa is supported by the presence of acetate–CoA ligase, acetyl-CoA–carbon dioxide ligase and fatty acid synthetase activities. Further support is given by the fact that avidin inhibited fatty acid synthesis (Numa, Matsuhashi & Lynen, 1964). However, this does not necessarily

demonstrate a synthesis *de novo* of fatty acids, since elongation may take place. Two pathways of fatty acid elongation are known. One, which has been demonstrated in pigeon liver (Harlan & Wakil, 1963), is dependent on endogenous fatty acids and acetyl-CoA. The other, demonstrated in rat liver microsomes, is dependent on endogenous fatty acids and malonyl-CoA (Nugteren, 1965). Avidin would be expected to inhibit the latter but not the former.

The effect of citrate shows some unusual features in intestinal mucosa compared with other tissues. Martin & Vagelos (1962), found a 40-fold stimulation of purified acetyl-CoA–carbon dioxide ligase from liver with 6mM-citrate. Matsuhashi, Matsuhashi & Lynen (1964), with rat liver, showed that optimum stimulation of purified acetyl-CoA–carbon dioxide ligase was obtained with 2·5mM-citrate. Smith & Dils (1966) found a threefold stimulation of fatty acid synthesis from acetate in lactating-rabbit mammary-gland PFS with 8mM-citrate. All three groups found that higher concentrations strongly inhibited fatty acid synthesis. However, with PFS from guinea-pig intestinal mucosa, a much higher and rather variable concentration was required for optimum stimulation, which was usually about 10–12-fold. Higher than optimum concentrations did not inhibit nearly as strongly as in other tissues. The high concentration of citrate required may partially be explained by its incorporation into fatty acids. The results presented here show that citrate can supply the major portion of the carbon incorporated into fatty acids (Table 8). They also indicate that citrate is incorporated via acetyl-CoA, since both acetate (Table 8) and acetyl-CoA (Tables 8 and 9) give an isotope dilution.

Another unusual feature of this system is the simple pattern of fatty acids synthesized from acetate. Under optimum conditions, only myristic acid, palmitic acid and occasionally stearic acid contained radioactivity. Of the total radioactivity incorporated, 20–36% was in myristic acid and 64–80% in palmitic acid. When radioactivity did occur in stearic acid, it accounted for 10% or less of the total. Franks *et al.* (1966), with jejunal rings from rat, found that, of the radioactivity incorporated from acetate, 5% was in myristic acid, 30% in palmitic acid, 44% in stearic acid and 4% in oleic acid. The system used by these authors represents more nearly what might occur *in vivo*. Smith & Dils (1966), with lactating-rabbit mammary gland, demonstrated that by altering the concentration of various cofactors the pattern of incorporation of radioactivity into fatty acids can be altered. It remains to be seen whether the pattern of incorporation of radioactivity into fatty acids synthesized by PFS from intestinal mucosa can be altered in the same way, and whether the pattern would become more like that described by Franks *et al.* (1966).

The occurrence of fatty acid synthesis in the intestinal mucosa raises questions as to whether it is concerned primarily with supplying the structural needs of the cell, or whether it plays a part in supplying specific fatty acids required for glyceride synthesis.

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