Synthesis of Fatty Acids from [1-¹⁴C]Acetyl-Coenzyme A in Subcellular Particles of Rat Epididymal Adipose Tissue

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1. Mitochondrial and microsomal fractions of rat epididymal adipose tissue incorporated [1-¹⁴C]acetyl-CoA equally well into various fatty acids by a chain-elongation mechanism. C₁₈ and C₂₀ fatty acids were the two major products, and comprised about 80% of the total fatty acids synthesized in both particles. 2. When incubated in air, mitochondria synthesized stearic acid, octadecenoic acid and eicosamonoenoic acid in almost equal amounts (about 20% each), whereas in microsomal fractions, the synthesis of octadecenoic acid was more than fivefold the stearic acid formation. In both fractions, major components of synthesized monoenoic fatty acids were the $\Delta^{11:12}$ isomers. Hexadecenoic acid and octadecenoic acid from whole adipose tissue contained approx. 11 and 14% of the $\Delta^{11:12}$ isomer respectively. 3. When mitochondria or microsomal fractions were incubated in nitrogen, there was increased synthesis of stearic acid and palmitic acid and less of C₁₆ and C₁₈ monoenoic acids; synthesis of C₂₀ acids remained predominantly of the monoenoic acids. Determination of the position of the double bond in the monoenoic acids supported the view that the synthesis of hexadecenoic acid and octadecenoic acid involves a desaturase activity, whereas eicosamonoenoic acid and eicosadienoic acid are formed only by elongation of endogenous fatty acids. 4. Most of the radioactivity was found in free fatty acids (63%) and the phospholipid (26%) fraction. In phospholipids, phosphatidylcholine and phosphatidylethanolamine were the two major components. 5. Most of the fatty acids synthesized, including those not normally found in particle lipids (arachidic acid, eicosamonoenoic acid and eicosadienoic acid) were distributed fairly evenly in the phospholipid and free fatty acid fractions. However, stearic acid was found predominantly in the phospholipid fraction.

Since the finding that fatty acids can be synthesized by a chain-elongation mechanism (Wakil, 1961), the formation of fatty acids in particulate fractions from tissues has been studied in detail by many workers. Although both mitochondrial and microsomal fractions of various mammalian tissues possess elongation mechanisms, appreciable differences have been reported in the carbon sources and hydrogen donors used. Mitochondria utilize acetyl-CoA and NADH in rat liver (Wit-Peeters, 1969) and ox heart (Dahlen & Porter, 1968), whereas in rat liver and rat brain microsomal fractions, malonyl-CoA and NADPH were used preferentially (Nugteren, 1965; Aeberhard & Menkins, 1968). In the monkey aorta, however, acetyl-CoA is utilized equally well by both mitochondria and microsomal fractions (Howard, 1968).

Rat epididymal adipose-tissue cells are active sites of lipogenesis (Angel, 1970) and the particulate fractions of the tissue have desaturase activity for

stearic acid (Gellhorn & Benjamin, 1964) and are also the sites for glyceride synthesis (Roncari & Hollenberg, 1967; Angel & Roncari, 1967).

Detailed studies of the new synthesis of fatty acids in the supernatant fraction of adipose tissue have been reported (Martin *et al.*, 1961; Horning *et al.*, 1961), but the mechanism of fatty acid synthesis in subcellular particles has been little studied, apart from an incidental report (Alexander *et al.*, 1966). In the present work, cofactor requirements and fatty acid products of mitochondria and microsomal fractions from the adipose tissue were studied and the formation of fatty acids from [1-¹⁴C]acetyl-CoA was investigated in the two types of preparation. Evidence was obtained that both particles have an elongation system with similar cofactor requirements and that the desaturase activity of the preparations is involved in the formation of unsaturated fatty acids to an extent that depends on chain length. The esterification of the synthesized fatty acids in phospholipids was also studied. A preliminary account of some of this

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work has appeared in abstract form (Kano & Lindsay, 1971).

Materials

[1-¹⁴C]Acetyl-CoA was prepared as described by Simon & Shemin (1953) from [1-¹⁴C]acetic anhydride. Palmitoyl-CoA was a generous gift from Dr. P. K. Tubbs, Department of Biochemistry, University of Cambridge. The cofactors, ATP, NADH and NADPH, and all other chemicals were of analytical grade [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.]. Methyl esters of palmitoleic acid, arachidic acid, eicosamonoenoic acid, eicosadienoic acid and behenic acid (Sigma) were used as standards or carriers in mixtures with fatty acid methyl esters from purified rat liver phosphatidylcholine (Kano, 1969) in g.l.c. analysis. [1-¹⁴C]Acetic anhydride and [U-¹⁴C]palmitic acid were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Malonyl-CoA was prepared by the method of Trams & Brady (1960) and purified by paper chromatography in 0.1 M-potassium acetate (pH 4.5)-ethanol (1:1, v/v).

Methods

Preparation of subcellular particles

Male Wistar rats (180–250 g) fed *ad libitum* were decapitated. Epididymal fat-pads were collected from three to five animals and were homogenized by hand in a glass homogenizer with 5 vol. of 0.25 M-sucrose–1 mM-EDTA (pH 7.0) at 35°C. Fractionation of the homogenates was performed essentially as described by Roncari & Hollenberg (1967). The homogenates were chilled in an ice bath and centrifuged at 1000g for 10 min. Overlying lipid was carefully removed and the supernatant (defatted homogenate) was obtained relatively free from fat. All subsequent operations were carried out at 4°C. The defatted homogenate was centrifuged at 10000g for 20 min in a Servall refrigerated centrifuge to obtain the mitochondrial fraction. The microsomal fraction was obtained from the 10000g supernatant by centrifuging at 100000g for 1 h. The particles obtained were washed once with the sucrose–EDTA solution and were finally suspended at a concentration of approx. 2 mg of protein/ml in the same solution.

Incubation

The standard incubation mixture contained 80 mM-potassium phosphate buffer (pH 7.0), 2 mM-NADH, 2 mM-NADPH, 10 mM-ATP, 0.2 mM-EDTA, 50–70 nmol of [1-¹⁴C]acetyl-CoA (sp. radioactivity 1.30×10^3 – 10.0×10^3 d.p.m./nmol) and tissue fraction (0.15–0.30 mg of protein) in a final volume of 0.5 ml. The mixture was incubated in air at 37°C for 30 min

or 1 h. In anaerobic incubations, the reaction mixture was bubbled with nitrogen in an ice bath for 15 min and incubated in Thunberg tubes in an atmosphere of nitrogen.

Total incorporation of substrate was similar in aerobic or anaerobic conditions. Under these conditions, incorporation of substrate was linear with incubation time and amount of tissue fraction.

Determination of fatty acid synthesis

For the determination of total fatty acid synthesis, the reaction was stopped by adding 1.0 ml of methanolic 10% (w/v) KOH. The tubes were flushed with nitrogen, tightly stoppered and heated in a water bath at 80°C for 1 h. The non-saponifiable materials were extracted once with 3 ml of light petroleum (b.p. 40–60°C) and the aqueous phase was acidified with 1.0 ml of 5 M-H₂SO₄. Radioactivity in the non-saponifiable fraction was negligible. Free fatty acids were extracted with three successive 3 ml portions of light petroleum. The combined extracts were washed once with 1 M-acetic acid and evaporated to dryness under nitrogen. The samples were dissolved in appropriate amounts of toluene and samples were taken to determine radioactivity in a Nuclear-Chicago scintillation counter in 10 ml of toluene scintillation mixture, containing 0.4% 2,5-diphenyl-oxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)-benzene. Counting efficiency was determined by the external-standard channels-ratio method. In some experiments, the reaction was stopped with 0.5 ml of 10% (w/v) trichloroacetic acid and acid-insoluble materials were collected by centrifugation. The sediment was washed once with water and the lipids were extracted twice with 5 ml portions of chloroform–methanol (1:1, v/v). The lipid extracts were washed successively with 0.9% NaCl and 0.9% NaCl in aq. 50% methanol. The chloroform layer was examined for radioactivity and its lipid components were analysed.

The two methods described above gave similar results for fatty acid formation.

Thin-layer chromatography

Lipids obtained from the reaction mixture were fractionated on thin layers of Kieselgel F (Merck A.G., Darmstadt, Federal German Republic) with carrier triolein, dioleins, mono-oleins, oleic acid and cholesteryl oleate. The plates were first developed with diethyl ether for about 5 cm from the origin and then fully developed with light petroleum (b.p. 40–60°C)–diethyl ether–acetic acid (84:15:1, by vol.).

Spots were detected by brief exposure to I₂ vapour and scraped off carefully into counting vials. Radioactivity was measured in a dioxan solution (Bray, 1960). In some experiments, free fatty acids and

phospholipids were eluted from the gel quantitatively with diethyl ether and methanol respectively.

The phospholipid fraction obtained was further fractionated by t.l.c. on Kieselgel F with two developing systems: (1) chloroform-methanol-aq. NH_3 (sp.gr. 0.88) - water (110:50:3:5, by vol.) and (2) chloroform-methanol-water-acetic acid (65:50:4:1, by vol.). Standard phospholipids (phosphatidic acid, phosphatidylinositol, sphingomyelin, phosphatidylcholine and phosphatidylethanolamine, supplied by Dr. R. M. C. Dawson) were co-chromatographed with the samples. Radioactivity content of each spot was determined as described above.

T.l.c. on 5% AgNO_3 -impregnated Kieselgel G plates was used to subfractionate fatty acid methyl esters according to the degree of unsaturation as described by Morris (1964). The bands of methyl esters were detected by spraying with 0.05% 1,2-dichlorofluorescein in acetone and by exposure to u.v. light. Methyl esters of fatty acids were recovered from the gel by elution with ether. Methyl palmitate, methyl oleate and methyl linoleate were used as standards.

Preparation of fatty acid methyl esters

The hexane extracts of free fatty acids were evaporated to dryness under nitrogen. Methyl esters were prepared by heating at 100°C with 14% (w/v) BF_3 in methanol for 3 min under nitrogen.

In some experiments, fatty acid methyl esters were prepared from phospholipids obtained from the incubation mixture or from total lipid extracts of epididymal fat-pads by the method of Morrison & Smith (1964). Methyl esters were extracted three times with hexane, and the solution was then washed successively with 4% (w/v) Na_2CO_3 solution and water.

Gas-liquid chromatography

Analytical g.l.c. Subcellular fractions from the adipose tissue were directly saponified as described above and the free fatty acids were recovered in hexane. Methyl esters of fatty acids from mitochondria and microsomal fractions were analysed with a Pye 104 analytical gas-liquid chromatograph with a flame ionization detector.

A column (2.5 mm $\times$ 6 mm) of 10% diethylene glycol succinate on Phase B (Phase Separations Ltd., Queensferry, Flintshire, U.K.), 60-85 mesh, was operated at 175°C with a flow rate of 27 ml of argon/min. Fatty acid peaks were identified by co-chromatography of standard fatty acid methyl esters. Peaks were quantitatively measured by triangulation and the results presented as percentages of area.

Preparative g.l.c. A column (2.5 mm $\times$ 6 mm) of 10%

polyethyleneglycol adipate on Diatomite CAW (Pye Unicam, Cambridge, U.K.), 100-120 mesh, was used at 185°C with a flow rate of 50 ml of argon/min. Collection of the samples was performed with a Pye 105 preparative gas-liquid chromatograph equipped with a 4-to-1 splitter, by which 80% of the applied samples could be collected in glass U-tubes loosely packed at the entrance with an absorbent filter. Fatty acid methyl esters from the incubation mixture were chromatographed with a carrier mixture containing methyl esters of myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, arachidic acid, eicosamonoenoic acid, arachidonic acid and behenic acid. After each fraction had been trapped manually, the U-tubes were rinsed with 10 ml of toluene scintillation fluid to determine radioactivity. In some experiments methyl esters were recovered from the tubes with hexane for further analysis.

Purification of monoenoic fatty acids and periodate-permanganate oxidation

Methyl esters of monoenoic acids from the reaction mixture or from the total lipids of whole adipose tissue were first separated on AgNO_3 -impregnated t.l.c. plates. Hexadecenoic acid, octadecenoic acid and eicosamonoenoic acid were purified by preparative g.l.c. as described above.

Radioactive monoenoic acids were oxidized by von Rudloff's (1956) method with 1 mg of methyl oleate as a carrier. Dicarboxylic acids formed were methylated by diazomethane and analysed by radio-g.l.c. Hexadecenoic acid and octadecenoic acid obtained from the total lipids of the adipose tissue were oxidized without carrier and analysed in the same condition.

Radio-g.l.c.

Radioactive dimethyl esters of dicarboxylic acids obtained by the oxidation of the acids were analysed on a 10% polyethyleneglycol adipate column as described above, but with CO_2 +argon (5:95) as carrier gas in a Pye 104 analytical gas-liquid chromatograph combined with a combustion chamber and proportional counter (Panax Ltd., Holmethorpe Industrial Estate, Redhill, Surrey, U.K.). Radioactive peaks were identified by comparison of the retention times with those of standard methyl esters of dicarboxylic acids (kindly supplied by Dr. P. Kemp).

Enzyme assays

ATPase* was determined by Pennington's (1961) method in the presence of 5 mM- MgCl_2 . Glutamate dehydrogenase was assayed as described by Beaufay

* Abbreviation: ATPase, adenosine triphosphatase.

et al. (1959). NADH- and NADPH-cytochrome *c* reductase activity was measured by the method of Green & Ziegler (1963).

Other procedures

Acetyl-CoA and CoA were determined by Chase's (1967) method, malonyl-CoA by the method of Lipmann & Tuttle (1945) and P_i by Allen's (1940) method. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. The Schmidt degradation was done to determine carboxyl-¹⁴C radioactivity in synthesized fatty acids by Phares' (1951) method as modified by Dahlen & Porter (1968). [U-¹⁴C]Palmitic acid was used as standard and about 5.95–6.10% of the radioactivity was recovered as ¹⁴CO₂. Results are expressed as mean value ± S.E.M., with the number of experiments in parentheses.

Results

Subfraction of the adipose tissue

Yield of the particles was small [mitochondria: 1.33 ± 0.07(10); microsomal fraction: 0.75 ± 0.05(10) mg of protein/g wet wt. of tissue], but the total particulate fraction represented approx. 20% of protein present in a defatted homogenate. The results of various enzyme assays are shown in Table 1. From the specific activity of glutamate dehydrogenase, mitochondrial contamination in microsomal fractions was less than 10%, and less than 20% of

mitochondrial protein was microsomal, as judged by the specific activity of NADPH-cytochrome *c* reductase. The results of the assay of cytochrome *c* reductases are consistent with those reported by Oshino *et al.* (1971). It should be noted that microsomal ATPase was extremely active in the presence of 5 mM-MgCl₂. Without Mg²⁺, ATPase activity of both particles was very low.

Table 2 shows the results of g.l.c. analysis of fatty acids of total lipids of the particles obtained. Microsomal fractions contained almost equal amounts of stearic acid and octadecenoic acid, whereas in mitochondria the content of stearic acid was half that of the corresponding monoenoic acids.

Cofactor requirements and effect of various additions on acetyl-CoA incorporation

As shown in Table 3, even under standard conditions of incubation fatty acid-synthesizing activity of the particles in different experiments, with different tissue preparations, was extremely variable. The average rate of incorporation of acetyl-CoA was 4.12 ± 0.66 (10) in mitochondria and 5.40 ± 1.07 (10) nmol/30min per mg of protein in microsomal fractions. Freezing and thawing the preparations three times did not affect the incorporation appreciably. Because of this variable synthesizing activity from different tissue preparations it was only possible to assess the effect of various additions on incorporation by comparison with the value for standard conditions measured with the same preparation.

Table 1. Enzyme activities of the subcellular particles of rat adipose tissue

Results are means ± S.E.M., expressed as nmol of substrate converted/min per mg of protein. The numbers of observations are given in parentheses.

	Glutamate dehydrogenase	NADH-cytochrome <i>c</i> reductase	NADPH-cytochrome <i>c</i> reductase	ATPase*
Mitochondria (3)	19.8 ± 0.00	96.3 ± 14.0	5.20 ± 0.00	110.0 ± 33.0
Microsomal fraction (3)	1.7 ± 0.00	347.0 ± 0.00	26.8 ± 0.00	500.0 ± 10.0

* Measured in the presence of 5 mM-MgCl₂.

Table 2. Fatty acid composition of the lipids of subcellular fractions

The particle lipids were saponified directly and the fatty acids obtained were analysed by analytical g.l.c. (see the Methods section). The headings represent chain length and number of double bonds. The results are given in area percentages of the peaks. The numbers of observations are given in parentheses.

	C _{14:0}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{20:4}
Mitochondria (4)	1.08 ± 0.36	26.6 ± 2.41	6.8 ± 0.65	13.1 ± 0.51	27.6 ± 1.2	17.4 ± 2.2	6.8 ± 2.2
Microsomal fraction (3)	Trace	27.7 ± 0.7	4.7 ± 0.15	22.0 ± 1.3	23.1 ± 0.77	14.7 ± 0.96	7.8 ± 1.9

Table 3. *Cofactor requirements and effects of various additions on [1-¹⁴C]acetyl-CoA incorporation into fatty acids*

Details of the standard incubation conditions are described in the Methods section. The amounts of acetyl-CoA incorporated in standard conditions are expressed as nmol/30min per mg of protein and as percentages of these values when conditions varied. Each value is the mean of duplicate incubations. Duplicate values within one experiment never differed by more than 0.2nmol/30min per mg of protein and generally by much less than this.

	Incorporation	
	Mitochondria	Microsomal fraction
Expt. 1		
Standard incubation	4.72	4.78
–ATP	5%	1%
–NADPH	67%	91%
–NADH	19%	24%
Expt. 2		
Standard incubation	3.61	2.46
+10 μ mol of NaHCO ₃	97%	139%
+20 μ mol of tripotassium citrate	85%	100%
+0.1 μ mol of sodium palmitate and 1 mg of bovine serum albumin*	79%	115%
Expt. 3		
Standard incubation	7.9	6.9
+10 μ mol of mercaptoethanol	60%	103%
+Carboxylase system†	100%	125%
Expt. 4		
Standard incubation	2.87	5.15
+100 μ g of avidin	102%	105%
+1 μ mol of KCN	95%	86%
Expt. 5		
Standard incubation	4.35	9.5
+60nmol of malonyl-CoA	56%	55%
+120nmol of malonyl-CoA	31%	28%

* Bovine serum albumin was not defatted.

† The carboxylase-stimulating system contained 20 μ mol of potassium isocitrate, 15 μ mol of NaHCO₃ and 0.3 μ mol of MnCl₂.

Table 4. *Effect of addition of palmitoyl-CoA on fatty acid synthesis in the presence or absence of ATP*

Details of the standard incubation conditions are as described in the Methods section, except that ATP was omitted where indicated. The amounts of acetyl-CoA incorporated were expressed as nmol/30min per mg of protein. Each value is the average of duplicate incubations. These never differed by more than 0.2nmol/30min per mg of protein, and generally by much less than this.

	Incorporation	
	Mitochondria	Microsomal fraction
ATP omitted		
Standard incubation	0.168	0.02
+25nmol of palmitoyl-CoA	1.96	0.73
+50nmol of palmitoyl-CoA	1.63	0.60
ATP present		
Standard incubation	3.32	4.44
+25nmol of palmitoyl-CoA	3.16	2.18
+50nmol of palmitoyl-CoA	2.56	2.08

As Table 3 shows, the incorporation was dependent on ATP, and NADH was a preferred hydrogen donor in both particles. Additions of NaHCO_3 , potassium citrate and palmitic acid plus albumin did not affect the incorporation appreciably, although microsomal uptake was stimulated slightly by NaHCO_3 . Mercaptoethanol had some inhibitory effect on the incorporation into fatty acid by mitochondria; avidin and KCN had no appreciable effect with either particle. The carboxylase-stimulating system, which is required for the maximum formation of malonyl-CoA from acetyl-CoA (Waite & Wakil, 1962), caused only a slight stimulation in microsomal fractions, although a dilution effect was observed on the addition of non-labelled malonyl-CoA in both particles. Throughout these experiments, the ratio of total radioactivity of synthesized fatty acids to *carboxyl*- ^{14}C radioactivity remained between 1.10 and 1.50, as judged by the Schmidt degradation. The results are consistent with synthesis of fatty acids in the particles by means of chain elongation of the endogenous fatty acids. To test whether ATP was needed only for the initial

activation of primer fatty acids, various amounts of palmitoyl-CoA were incubated with the particles under standard conditions (Table 4). In the absence of ATP, palmitoyl-CoA did increase incorporation into fatty acid, although it could not entirely replace it. This is probably because palmitoyl-CoA itself inhibits the elongation reaction in the presence of ATP (Table 4).

Cofactors for esterification

Fatty acid synthesis could be limited by the extent of esterification of the fatty acids synthesized. The effect of cofactors that stimulate esterification (Angel & Roncari, 1967; Roncari & Hollenberg, 1967) on the incorporation of acetyl-CoA into fatty acids was therefore studied (Table 5). Addition of bivalent cations had no effect in mitochondria but Mg^{2+} was strongly inhibitory in microsomal fractions. This inhibition was much less with increased ATP concentration and was negligible when ATP was replaced by an ATP-generating system (Table 5). This suggests that inhibition by Mg^{2+} is closely related to stimula-

Table 5. *Effects of cations and cofactors that stimulate esterification of fatty acids on the incorporation of [1- ^{14}C]acetyl-CoA into fatty acids*

Details of the standard incubation procedure are described in the Methods section. Amounts of acetyl-CoA incorporated are expressed as nmol/30 min per mg of protein for standard incubations and as percentages of these values when conditions were varied. Each value is the mean of duplicate incubations, which never differed by more than 0.2 nmol/30 min per mg of protein.

	Incorporation	
	Mitochondria	Microsomal fraction
Expt. 1		
Standard incubation	3.42	3.90
+2.5 μmol of MgCl_2	89%	32%
+2.5 μmol of MnCl_2	116%	73%
+2.5 μmol of CaCl_2	86%	75%
+1.0 μmol of EDTA	111%	106%
Expt. 2		
Standard incubation	3.62	3.82
+40 nmol of CoA	59%	48%
+3.0 μmol of DL-glycerol 3-phosphate	65%	80%
+2.5 μmol of MgCl_2 , 40 nmol of CoA and 3.0 μmol of DL-glycerol 3-phosphate	26%	13%
Expt. 3		
Standard incubation	4.10	4.65
+10 nmol of CoA	99%	115%
+40 nmol of CoA	60%	53%
+5 μmol of MgCl_2	85%	22%
+5 μmol of MgCl_2 + 10 μmol of ATP*		71%
+5 μmol of MgCl_2 + ATP-generating system†		91%

* 10 μmol of ATP in place of normal 5 μmol of ATP.

† ATP-generating system contains 0.6 μmol of ADP, 7 μmol of creatine phosphate and 0.9 (EC) units of creatine phosphokinase.

Table 6. *Percentage distribution of radioactivity in fatty acids synthesized from [1-¹⁴C]acetyl CoA*

The percentages do not sum to 100 because the values have been omitted for the radioactivity collected in fractions that did not correspond with the fatty acids shown in the Table. Each value is the mean $\pm$ S.E.M. of four analyses (except where indicated in parentheses), the material from at least three incubations being used for each analysis.

Incubation conditions	Fatty acid	Distribution of radioactivity (%)											
		C _{14:0}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{20:0}	C _{20:1}	C _{20:2}	C _{20:4}	C _{22:0}	
Air	Mitochondria	2.8 $\pm$ 0.4	4.6 $\pm$ 0.3	3.5 $\pm$ 0.3	15.1 $\pm$ 1.9	24.1 $\pm$ 1.2	2.5 $\pm$ 0.3	3.5 $\pm$ 0.4	20.5 $\pm$ 1.0	10.6 $\pm$ 1.2	2.3 $\pm$ 0.3	3.8 $\pm$ 0.8	
	Microsomal fraction	0	2.6 $\pm$ 0.3	5.0 $\pm$ 0.5	7.4 $\pm$ 1.2	39.6 $\pm$ 1.3	0	2.6 $\pm$ 0.5	22.6 $\pm$ 0.3	8.1 $\pm$ 0.6	0	2.5 $\pm$ 0	
Nitrogen	Mitochondria	2.1 $\pm$ 0.3	3.8 $\pm$ 0.1	1.1 $\pm$ 0.2	25.7 $\pm$ 1.9	15.4 $\pm$ 1.5	2.0 $\pm$ 0.4	5.0 $\pm$ 0.5	21.3 $\pm$ 0.3	11.8 $\pm$ 0.8	1.7 $\pm$ 0.2	6.6 $\pm$ 0.1	(2)
	Microsomal fraction	0	6.6 $\pm$ 0.1	1.3 $\pm$ 0.1	30.3 $\pm$ 0.5	15.8 $\pm$ 0.4	0	3.8 $\pm$ 0.1	20.6 $\pm$ 0.5	10.9 $\pm$ 0.6	0	4.5 $\pm$ 0.3	(2)

tion of microsomal ATPase activity. CoA below 10 μ M had little effect, but at higher concentrations it was somewhat inhibitory. DL-Glycerol 3-phosphate (6mM) slightly inhibited incorporation in mitochondria and had little effect in microsomal fractions, although it has been reported to be necessary for desaturase activity of subcellular particles of adipose tissue (Gellhorn & Benjamin, 1964). Addition of all factors known to stimulate esterification strongly inhibited the incorporation of [1-¹⁴C]acetyl-CoA into total fatty acids (Table 5).

Analysis of synthesized fatty acids

Incubations were performed in air or nitrogen for 1 h and the radioactivity distribution among the synthesized fatty acids was determined by a combination of t.l.c. on AgNO₃-impregnated plates and preparative g.l.c. Under both incubation conditions, about 45 and 35% of total radioactivity was in C₁₈ and C₂₀ fatty acids respectively, in both particles (Table 6). When incubations were in air, more radioactivity was incorporated into the monoenoic acids than into the corresponding saturated acids. This is seen to some extent with both particles, but is much more marked in microsomal fractions. When incubations were in nitrogen, the pattern of incorporation was unchanged for C₂₀ acids, but for C₁₈ (and to some extent for C₁₆) acids, there was increased incorporation into saturated acids and decreased incorporation into the corresponding monoenoic acids, although the total incorporation into the fatty acids of a given chain length was almost unchanged. Since it is well established that desaturation of stearic acid is an oxygen-requiring reaction in adipose tissue (Gellhorn & Benjamin, 1964), these results can be explained by supposing that synthesis of hexadecenoic acid and octadecenoic acid involves desaturase activity, whereas the formation of eicosamonoenoic acid and eicosadienoic acid is exclusively due to the elongation of endogenous fatty acids.

To obtain further information about the elongation mechanism, monoenoic fatty acids from the incubation mixture as well as from the total lipids of the whole adipose tissue were purified and oxidized by the periodate-permanganate method. The results of the analysis of the dicarboxylic acids formed are shown in Table 7.

The naturally occurring hexadecenoic fatty acids and octadecenoic fatty acids of adipose tissue contain appreciable amounts of the $\Delta^{11:12}$ isomer, although the major components are palmitoleic acid and oleic acid ($\Delta^{9:10}$ isomers). In the octadecenoic acid fraction synthesized by both mitochondria and microsomal fractions, the content of oleic acid was similar and the major product (65%) was the $\Delta^{11:12}$ isomer, presumably formed from endogenous palmitoleic acid (Holloway & Wakil, 1964). This would

Table 7. *Isomers of monoenoic acid present in and formed by adipose tissue*

Subcellular particles were incubated with [$1\text{-}^{14}\text{C}$]acetyl-CoA for 1 h under standard conditions. Ten or more incubations were pooled for analysis. Radioactive monoenoic acids formed were isolated and oxidized. Distribution of radioactivity in the dicarboxylic acids formed was determined as described in the Methods section. For fatty acids from the microsomal fraction the measurement was repeated on a further set of 10 incubations. Two samples of adipose tissue were used for the determination of the proportions by weight of the isomers of the monoenoic acids in the bulk lipid, and the values shown are the means $\pm$ S.E.M.

Distribution of radioactivity in dicarboxylic acids (%)				
	C ₇	C ₉	C ₁₁	C ₁₃
Mitochondria				
C _{18:1}	0.0	33.0	67.0	0.0
C _{20:1}	0.0	0.0	74.2	25.8
Microsomal fraction				
C _{18:1}	0.0	33.2 $\pm$ 3.6	66.8 $\pm$ 3.5	0.0
C _{20:1}	0.0	0.0	79.0	21.0
Distribution of mass in dicarboxylic acids (%)				
	C ₇	C ₉	C ₁₁	C ₁₃
Lipids of whole adipose tissue				
C _{16:1}	0.0	91.1 $\pm$ 2.0	9.1 $\pm$ 2.0	0.0
C _{18:1}	0.0	86.5 $\pm$ 1.0	13.5 $\pm$ 0.9	0.0

Table 8. *Percentage distribution of radioactivity in lipid classes*

Subcellular fractions were incubated for 1 h with [$1\text{-}^{14}\text{C}$]acetyl-CoA under standard conditions. Lipids were extracted from the reaction mixture and fractionated by t.l.c. Percentage of radioactivity in each lipid fraction is presented as the mean value $\pm$ S.E.M. (number of analyses in parentheses, each being based on the pooled sample from three or more incubations).

Fractions	Mitochondria (5)	Microsomal fraction (4)
Cholesterol ester	0.2 $\pm$ 0.3	0.2 $\pm$ 0.1
Triglyceride	1.4 $\pm$ 1.0	3.0 $\pm$ 1.0
Cholesterol	0.2 $\pm$ 0.2	—
Free fatty acids	68.8 $\pm$ 3.1	66.1 $\pm$ 3.8
Diglycerides	2.2 $\pm$ 0.6	1.9 $\pm$ 0.6
Monoglycerides	0.8 $\pm$ 0.2	1.6 $\pm$ 0.9
Phospholipids	24.8 $\pm$ 1.3	24.8 $\pm$ 0.9
Phospholipid fractions	(3)	(3)
Sphingomyelin	—	—
Phosphatidylcholine	31.2 $\pm$ 1.1	34.6 $\pm$ 3.7
Phosphatidylinositol	4.2 $\pm$ 2.1	5.9 $\pm$ 0.7
Phosphatidic acid	8.9 $\pm$ 0.5	8.8 $\pm$ 0.8
Phosphatidylethanolamine	38.3 $\pm$ 1.1	36.0 $\pm$ 2.5
Unidentified	15.6 $\pm$ 0.5	14.8 $\pm$ 0.8

suggest that one-third of the octadecenoic acid synthesized was derived by desaturation of stearic acid and the rest by elongation of palmitoleic acid. For mitochondria, this result fits well with the results in Table 6, since incorporation into octadecenoic acid is decreased by about one-third on incubation under

nitrogen. It does not, however, explain the result in the microsomal fraction, where there is a fall in incorporation in anaerobic conditions to less than half that in air (Table 6). Incorporation into eicosamonoenoic acid was similar in the two particles; more than 70% of radioactivity was in the $\Delta^{11:12}$

Table 9. Percentage distribution of radioactivity in fatty acids in the phospholipid and free fatty acid fractions of subcellular preparations

Subcellular fractions were incubated for 1 h with [1-¹⁴C]acetyl-CoA under standard conditions. Lipids were extracted from the reaction mixture, fractionated by t.l.c. and methyl esters of fatty acids in each fraction separated by preparative g.l.c. Behenic acid (C_{22:0}) contains unidentified fractions of similar retention time. Each result is the average of two or three estimations (numbers in parentheses) $\pm$ s.e.m., each estimation being derived from pooled samples of five to ten incubations.

Fatty acid	Distribution of radioactivity (%)									
	C _{14:0}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{20:0}	C _{20:1}	C _{20:2}	C _{22:0}
Mitochondria (2)										
Free fatty acids	2.0 $\pm$ 0.1	5.3 $\pm$ 0.3	3.0 $\pm$ 0.3	7.0 $\pm$ 1.4	27.0 $\pm$ 1.0	3.5 $\pm$ 2.5	3.5 $\pm$ 2.5	29.0 $\pm$ 1.6	15.0 $\pm$ 0.2	3.0 $\pm$ 1.3
Phospholipids	2.0 $\pm$ 0.1	3.0 $\pm$ 0.3	3.0 $\pm$ 0.3	42.0 $\pm$ 0.3	20.0 $\pm$ 1.1	3.1 $\pm$ 0	1.0 $\pm$ 0.2	7.0 $\pm$ 0.3	5.0 $\pm$ 2.8	4.0 $\pm$ 1.7
Microsomal fraction (3)										
Free fatty acids	1.7 $\pm$ 0.2	2.5 $\pm$ 0.3	7.8 $\pm$ 0.5	0.7 $\pm$ 0.4	43.0 $\pm$ 2.6	1.1 $\pm$ 0.5	1.4 $\pm$ 0.6	21.0 $\pm$ 0.8	14.1 $\pm$ 2.5	4.7 $\pm$ 0.2
Phospholipids	0.7 $\pm$ 0.7	3.9 $\pm$ 0.9	3.3 $\pm$ 0.7	16.9 $\pm$ 1.3	36.8 $\pm$ 2.8	0.7 $\pm$ 0.9	0.6 $\pm$ 0.6	10.7 $\pm$ 0.3	19.5 $\pm$ 3.2	3.2 $\pm$ 0.9

fraction and the remaining radioactivity was in the $\Delta^{13:14}$ isomer. This result can reasonably be explained if both isomers of octadecenoic acid ($\Delta^{9:10}$ and $\Delta^{11:12}$) can be elongated. It seems clear that eicosamonoenoic acid and probably eicosadienoic acid were formed by the elongation of endogenous octadecenoic acid and linoleic acid respectively, without participation of desaturation.

Lipid analysis

Lipids were extracted from the reaction mixture and the radioactivity distribution among lipid classes was determined by t.l.c.

Incorporation by both types of preparation was similar: more than 60% of the radioactivity was in free fatty acids and most of the remaining radioactivity was in the phospholipid fraction. In phospholipids, phosphatidylcholine and phosphatidylethanolamine were the two major radioactive components (Table 8).

Methyl esters of fatty acids were obtained from free fatty acid and phospholipid fractions after aerobic incubation for 1 h and were analysed by preparative g.l.c. Table 9 shows the radioactivity distribution among the fatty acids of each fraction. Synthesized fatty acids, which are not normally present in particle lipids, such as eicosamonoenoic acid and eicosadienoic acid, were incorporated in phospholipids as well as free fatty acids. However, preferential incorporation of stearic acid in phospholipids relative to its appearance in free fatty acids was clearly observed.

Discussion

Only minor differences were found between the chain-elongation systems of the mitochondrial and microsomal fractions of rat epididymal adipose tissue. Both utilized NADH, acetyl-CoA and endogenous fatty acids. The inhibition by Mg²⁺ observed in microsomal fractions could be explained by a potent microsomal ATPase activity. It is improbable that malonyl-CoA is an intermediate in the elongation reaction in mitochondria. No stimulation of incorporation was observed with NaHCO₃ or a carboxylase-stimulating system, and avidin had no inhibitory effect. Decreased incorporation of [1-¹⁴C]acetyl-CoA on addition of unlabelled malonyl-CoA can be explained by the presence of malonyl-CoA decarboxylase in mitochondria (Landriscina *et al.*, 1971). In microsomal fractions it is known that carboxylation of acetyl-CoA to malonyl-CoA can occur, and the slight stimulation of incorporation of acetyl-CoA by NaHCO₃ or the carboxylase-stimulating system suggest that malonyl-CoA could be used for the chain-elongation system. However, the absence of any inhibitory effect of avidin shows that acetyl-CoA can be used directly, as in mitochondria.

With both mitochondria and microsomal fractions, the addition of cofactors known to promote esterification of fatty acids resulted in a marked inhibition of the elongation reaction. It has been found that esterification of the synthesized fatty acids could occur subsequent to elongation, to form phosphatidic acid (H. Kanoh, unpublished work), but the physiological significance of this temporal separation of synthesis and esterification is unclear. Significant incorporation into diglycerides and triglycerides was not observed, but this is not unexpected, since according to Roncari & Hollenberg (1967) it is necessary to add the cytosol fraction to subcellular particles to obtain glyceride formation.

In both mitochondria and microsomal fractions there was a predominance of monoenoic fatty acids in the products of chain elongation, as has been reported by many investigators with other tissue preparations (e.g. Harlan & Wakil, 1963; Alexander *et al.*, 1966). It appears from the present work that in both subcellular fractions eicosamonoenoic acid is formed only by elongation of endogenous octadecenoic acid, whereas octadecenoic acid is derived both from palmitoleic acid by elongation and from palmitic acid by elongation and desaturation. Although Gellhorn & Benjamin (1964) localized stearoyl-CoA desaturase in the microsomal fraction of adipose tissue, the content of oleic acid in formed octadecenoic acid and the decreased incorporation in anaerobic incubations suggest that mitochondria may have considerable desaturase activity. Dahlen & Porter (1968) have described the synthesis of polyunsaturated fatty acids in ox heart mitochondria.

The indication that eicosamonoenoic acid is formed only by elongation of oleic acid is not unexpected, since Brett *et al.* (1971) showed a very poor utilization of arachidic acid by desaturases of various tissues. The small extent of formation of arachidic acid and very high formation of octadecenoic acid ($\Delta^{11:12}$ isomer) can also be explained by substrate specificity, since Nugteren (1965) reported a very low utilization of stearic acid in the elongation system of rat liver microsomal fractions whereas palmitoleic acid was readily utilized. Holloway & Wakil (1964) reported the wide occurrence of *cis*-vaccenic acid ($\Delta^{11:12}$ isomer) in animal tissues and its biosynthesis from palmitoleic acid by a chain elongation. In the present study fatty acids that are not normally present in adipose tissue ($C_{20:0}$, $C_{20:1}$, $C_{20:2}$) were formed in appreciable quantity, whereas the synthesis of arachidonic acid was negligible. Both particles, however, normally contain linoleic acid and arachidonic acid (Table 2). It is possible that the system for synthesizing polyunsaturated fatty acids was damaged during cell fractionation. Stearic acid was esterified preferentially to phospholipids in both particles. This is probably due to selectivity of acyltransferase of the particles (Van Golde *et al.*, 1969).

It is not possible at present to assess the physiological significance of the particulate synthesis of fatty acids in adipose tissue. In rat adipose tissue it is probably quantitatively small in comparison with the rate of new synthesis in the cytoplasm. In adipose tissue of other species (e.g. man), however, where there are indications that cytoplasmic synthesis is much smaller (Shrago *et al.*, 1969), the particulate system may be more important and may account for much of the reported incorporation of labelled glucose into adipose-tissue fatty acids.

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