same compound was also prepared in good yield by various methods from griseophenone C, and its monomethyl ether was identical with lichexanthone.

7. The sequence established for the biosynthesis of griseofulvin is: griseophenone C → B → A → griseofulvin.

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REFERENCES

Ashton, G. C. & Tootill, J. P. R. (1956). *Analyst*, **81** (961), 232.
Barton, D. H. R. & Cohen, T. (1957). *Festschrift A. Stoll*, p. 117. Basle: Birkhauser.
Barton, D. H. R. & Scott, A. I. (1958). *J. chem. Soc.* p. 1767.
Birch, A. J. & Donovan, F. W. (1953). *Aust. J. Chem.* **6**, 360.
Birch, A. J., Massy-Westropp, R. A., Rickards, R. W. & Smith, H. (1958). *J. chem. Soc.* p. 360.
Bush, M. T. & Densen, P. M. (1948). *Analyt. Chem.* **20**, 121.
Daly, C. (1961). *Analyst*, **86** (1019), 129.
Day, A. C., Nabney, J. & Scott, A. I. (1960). *Proc. chem. Soc., Lond.*, p. 285.
Di Marco, A. & Pennella, P. (1959). *Progr. industr. Microbiol.* **1**, 47.
Dutta, J. M. & Watson, E. R. (1911). *J. Soc. Chem. Ind., Lond.*, **30**, 196.
Fischer, E. & Hoesch, K. (1912). *Liebigs Ann.* **391**, 347.
Grove, J. F., Ismay, D., MacMillan, J., Mulholland, T. P. C. & Rogers, M. A. T. (1952*a*). *J. chem. Soc.* p. 3958.
Grove, J. F., MacMillan, J., Mulholland, T. P. C. & Rogers M. A. T. (1952*b*). *J. chem. Soc.* p. 3949.
Grover, P. K., Shah, G. D. & Shah, R. C. (1955). *J. chem. Soc.* p. 3982.
Grover, P. K., Shah, G. D. & Shah, R. C. (1956). *J. sci. industr. Res.* **15B**, 629.
Hassall, C. H. & McMorris, T. C. (1959). *J. chem. Soc.* p. 2831.
Hockenhull, D. J. D. & Faulds, W. F. (1955). *Chem. & Ind.* p. 1390.
McMaster, W. J., Scott, A. I. & Trippett, S. (1960). *J. chem. Soc.* p. 4628.
MacMillan, J. (1953). *J. chem. Soc.* p. 1697.
Morton, R. A. & Sawires, Z. (1940). *J. chem. Soc.* p. 1052.
Nishikawa, H. (1937). *J. agric. chem. Soc. Japan*, **13**, bull. 1–7; reported in *Chem. Abstr.* (1937), **31**, 3485.
Yates, P. & Stout, G. H. (1958). *J. Amer. chem. Soc.* **80**, 1691.

Biochem. J. (1961) **81**, 37

Studies of Fatty Acid Oxidation

7. THE EFFECTS OF FATTY ACIDS ON THE PHOSPHATE METABOLISM OF SLICE AND MITOCHONDRIAL PREPARATIONS OF RAT LIVER*

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The mechanism of fatty acid oxidation in mammalian tissues has been the subject of detailed investigation in recent years (see, for example, the review by Lynen, 1955), and it has been shown that they must undergo an initial activation step involving the participation of adenosine triphosphate to form acyl-coenzyme A derivatives. It was reasonable therefore to infer that inhibition of regeneration of adenosine triphosphate would lead to inhibition of fatty acid oxidation such as has been shown to occur in the presence of agents which uncouple oxidation from phosphorylation (Cross, Taggart, Covo & Green, 1949; Lehninger, 1951). Quastel & Wheatley (1933) observed that fatty acids when present at concentrations above an optimum value gave rise to an inhibition of respiration of guinea-pig-liver slices. Lehninger (1945, 1951) made a similar observation on the oxidation of fatty acids by liver particles, and suggested that the fatty acids might act as uncoupling agents. It was later shown that decanoate decreases the phosphate/oxygen ratio for pyruvate oxidation in rat-brain and -kidney mitochondria (Scholefield, 1956) and that fatty acids stimulate the latent adenosine-triphosphatase activity of liver mitochondria (Pressman & Lardy, 1956). Further work (Creaser & Scholefield, 1960) showed that decanoate strongly inhibits phosphate incorporation into all

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the organic phosphate fractions of Ehrlich ascites-carcinoma cells. It was decided to extend these studies on the inhibitory effects of fatty acids to determine whether the effects of several short- and medium-chain-length fatty acids on the respiratory activity of the cell could be correlated with their effects on specific aspects of phosphate metabolism.

MATERIALS AND METHODS

Animals. Adult hooded rats of a local inbred strain, weighing approx. 200 g., were used throughout this investigation.

Chemicals. All common chemicals were of reagent grade and were used without further purification. ATP and ascorbic acid were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., cytochrome *c* from Wyeth Inc., Philadelphia, Pa., U.S.A. and hexokinase from the Sigma Biochemical Co., St Louis, Mo., U.S.A. Radioactive phosphate was obtained from Charles E. Frosst and Co., Montreal, P.Q., Canada. It was boiled with N-HCl for 1 hr. in a water bath to decompose pyrophosphate and neutralized before use.

All fatty acids were used as the potassium salts and were prepared and purified as described by Scholefield (1956). [1-^{14}C]Decanoate was purchased from Merck and Co. Ltd., Montreal. It was neutralized with $NaHCO_3$ and sufficient carrier potassium decanoate was added to give a final concentration of 20 mM. The radioactivity of this solution was approx. 4·2 μc/ml. (0·21 μc/μmole). All substrates and reagents were adjusted to pH 7·4 before use.

Tissue preparations. Rats were stunned and immediately killed by decapitation. Liver or kidneys were quickly removed from the animal and chilled in cracked ice. Tissue slices were cut by means of a Stadie–Riggs tissue slicer (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.) and immediately placed on top of a dry Petri dish containing cracked ice. The slices were then weighed on a torsion balance and placed directly in chilled Warburg vessels containing the experimental media.

Rat-liver mitochondria were isolated according to the method of Hogeboom, Schneider & Pallade (1948) in 0·25 M-sucrose.

Oxygen uptake was measured with the conventional Warburg apparatus at 37° in an atmosphere of O_2 (slices) or air (mitochondria). Experiments were repeated at least three times and mean values are quoted. The individual values did not differ from the mean by more than 10%.

Calcium was omitted from the Krebs–Ringer incubation medium, the final concentrations of the various salts being: NaCl, 145 mM; KCl, 5·8 mM; KH_2PO_4, 1·5 mM; $MgSO_4$, 1·5 mM. Sodium phosphate buffer, pH 7·4, was added to give a final concentration of 10 mM and final pH 7·3. The medium for measuring oxidative phosphorylation in mitochondria was similar to the one described by Judah & Rees (1953). In a final volume of 3 ml. it contained: $MgSO_4$, 6·7 mM; KCl, 75·0 mM; ATP, 1·67 mM; cytochrome *c*, 3·5 μM and sodium phosphate buffer, pH 7·4, 10 mM. Glucose (20 mM) and hexokinase (1 mg.) were used as the phosphate-acceptor system. Other substrates were added as indicated in the text. Inorganic phosphate was estimated by the method of Fiske & Subbarow (1929). Cytochrome *c*-oxidase activity, the associated oxidative phosphorylation and the phosphorylation occurring during the oxidation of succinate, were measured by the methods described by Lehninger, ul Hassan & Sudduth (1954), Borgstrom, Sudduth & Lehninger (1955) and Hunter & Ford (1955).

Calculation of results. Most of the results are expressed in terms of μm-moles of phosphate incorporated into the various fractions/100 mg. wet wt. of tissue, calculated from the formula

$$\mu\text{m-moles incorporated/100 mg. wet wt. of tissue} = \frac{\text{counts/min./fraction}}{\text{counts/min./}\mu\text{mole of phosphate in the incubation medium}} \times \frac{100}{\text{wet wt. of slice (mg.)}}.$$

All experiments were performed at least twice and either typical or mean values are quoted.

Study of incorporation of ^{32}P into slices. The slices were incubated as described above. ^{32}P (50–100 μc) was tipped into the experimental medium in Warburg vessels after an initial equilibration period of 10 min. At the end of the incubation, the slices were quickly removed and washed twice with 5 ml. of cold Krebs–Ringer solution. The washed slices were homogenized with 5 ml. of 5% cold trichloroacetic acid, the acid-insoluble fraction was isolated, the acid-soluble phosphate compounds were separated by paper chromatography and the radioactivity of each was measured with a thin end-window Geiger–Müller tube and Tracerlab 123 scaler as previously described (Creaser, de Leon & Scholefield, 1959), to an accuracy of within 5%.

Metabolism of [1-^{14}C]decanoate. The [1-^{14}C]decanoate was placed in the main compartments of Warburg flasks along with the other constituents of the incubation medium. The side arm contained 0·3 ml. of 2 N-H_2SO_4 and 0·5 ml. of 50%

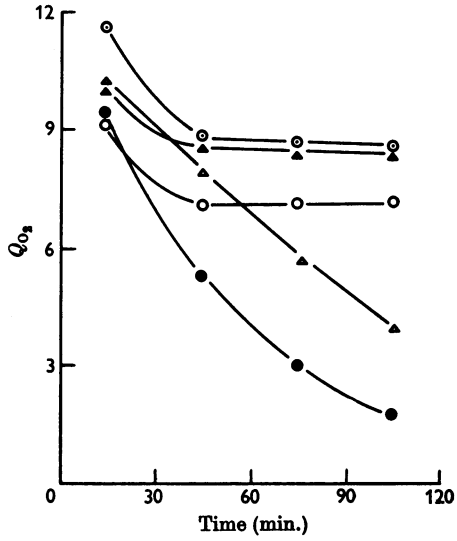

Fig. 1. Effects of potassium decanoate on the respiratory activity of rat-liver slices. ○, No decanoate added; ⊙, 0·66 mM-decanoate; ▲, 1·33 mM-decanoate; △, 2·67 mM-decanoate; ●, 4 mM-decanoate. Q_{O_2} values were calculated for the 30 min. intervals indicated.

(w/v) citric acid and a filter paper soaked in 20% KOH was present in the centre well to collect the $^{14}CO_2$ produced. At the end of the incubation period the acids were tipped from the side arm to stop the reaction and to liberate any CO_2 dissolved in the medium. After a further incubation, lasting 30 min., the alkali-soaked filter papers were removed, with washings, from the centre well and extracted with carrier Na_2CO_3 ($\equiv$ 10·5 mg. of $BaCO_3$). The $^{14}CO_2$ was precipitated as $BaCO_3$ with $BaCl_2$ and the radioactivity was measured as described above, a correction for self-absorption to infinite thinness being made according to the method of Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). New alkali-soaked filter papers were placed in the centre wells and 1·0 ml. of aniline citrate (Quastel & Wheatley, 1933, as modified by Edson, 1935) was placed in the side arm. The vessels were returned to the bath and aniline citrate was tipped into the main compartment to decompose the acetoacetate present to acetone and CO_2. The $^{14}CO_2$ produced was assayed as before. The slices were then removed, washed twice with 5 ml. of cold Krebs–Ringer solution, homogenized with 5 ml. of 95% ethanol and extracted at 50° for 30 min. The residue was extracted again with 5 ml. of ethanol–ether (1:1) for 30 min. at 40°. The supernatants were mixed and the phospholipids quantitatively precipitated and purified according to the method of Sinclair & Dolan (1942). The precipitated phospholipids were dissolved in a few drops of moist ether, plated on aluminum disks and dried, and their radioactivity was counted as described before.

RESULTS

Effects of fatty acids on the respiration of tissue slices

Potassium decanoate, at concentrations of up to 1·34 mM, stimulated the oxygen uptake of rat-liver slices respiring in Krebs–Ringer solution. When the concentration of decanoate was increased to 2·67 and 4·0 mM, an initial stimulation followed by a gradual decline in the respiratory activity was observed (Fig. 1). The presence of other substrates in the medium, such as glucose, succinate, fumarate or lactate, did not alter the inhibitory effect of decanoate although the presence of the second substrate caused further stimulation of respiration when lower concentrations of fatty acid were used.

Table 1. *Effects of various fatty acids on the endogenous respiration of rat-liver slices*

All figures refer to $-Q_{O_2}$ values between the time intervals indicated. Fatty acids with chain length above C_{12} were apparently partially in suspension in the medium. All the other fatty acids were in solution at the concentration used.

Fatty acid added (4 mM) ...	None	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}
Period (min.)												
0–30	8·8	11·1	10·2	9·8	10·9	9·6	11·1	9·0	7·9	7·7	9·9	8·8
30–60	7·2	8·9	7·6	8·9	9·8	5·4	9·6	5·5	7·8	7·6	7·5	6·3
60–90	7·4	10·2	8·5	8·2	9·6	3·5	9·8	3·6	6·5	6·0	6·4	5·8
90–120	6·9	9·3	8·3	6·5	9·0	2·6	7·7	3·1	6·4	6·1	6·1	5·3

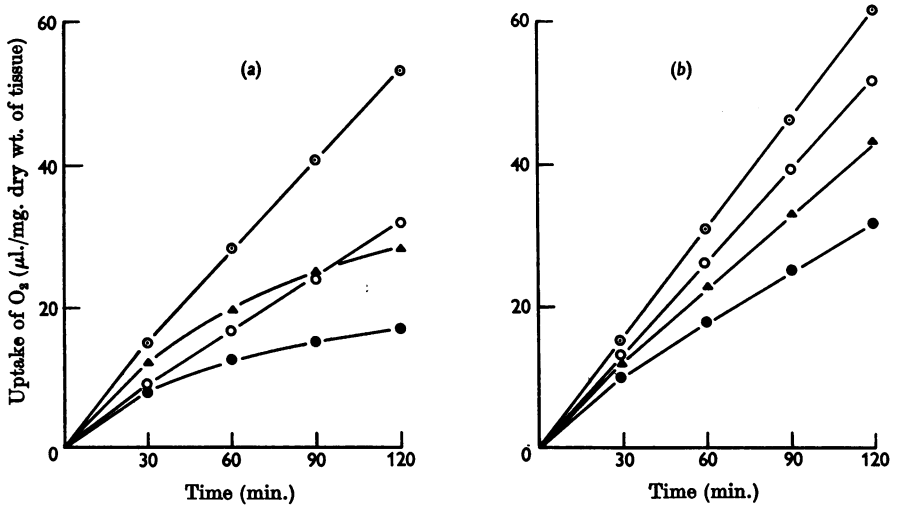

Fig. 2. Effects of potassium decanoate on the respiratory activity of rat-kidney-cortex slices (*a*) in the absence, and (*b*) in the presence, of 10 mM-sodium pyruvate. ○, No decanoate added; ⊙, 1 mM-decanoate; ▲, 1·67 mM-decanoate; ●, 2 mM-decanoate.

Fatty acids such as hexanoate, nonanoate or undecanoate, at concentrations of up to 4 mM, showed no inhibitory effect. Laurate was as effective as decanoate but higher fatty acids had little inhibitory effect (Table 1). Decanoate also inhibited the respiration of rat-kidney-cortex slices, both in the presence and absence of pyruvate, though slightly higher concentrations of the fatty acid were required to obtain inhibitions of respiration in the presence of added pyruvate (Fig. 2). The inhibitory effects on respiration increased with chain length of the fatty acid in a fashion similar to that noted above with liver slices, though with kidney slices the odd-numbered fatty acids seemed to be as effective as the even-numbered fatty acids of similar chain lengths.

Effect of decanoate on oxidative phosphorylation in rat-liver mitochondria

A concentration of 1·0–1·3 mM-decanoate was required to produce 50 % decrease of the P/O ratio for glutamate oxidation by liver mitochondria (Table 2). Scholefield (1956) observed that in brain mitochondria approximately 0·6 mM-decanoate gave 50 % decrease of the P/O ratio for pyruvate oxidation. The difference in the sensitivity of different types of mitochondria to the inhibitory effects of decanoate appeared to be related to the ability of different tissues to oxidize the fatty acid. In those tissues where the rate of oxidation of fatty acids is low, decanoate was more effective as an inhibitor.

The effect of decanoate (1·0 mM) on the oxidation of ascorbate and succinate and on the efficiency of phosphorylation was tested. The mitochondrial preparation used to measure oxidative phosphorylation with succinate as substrate was first incubated with phosphate buffer to remove endogenous diphosphopyridine nucleotide (Hunter & Ford, 1955). This treatment prevents further oxidation of the malate formed from succinate but also, in our experience, reduces the phosphorylation quotient with succinate to about one-half of that normally obtained. The addition of decanoate (1·0 mM) to this preparation had no effect on the oxygen uptake, but completely abolished the residual phosphorylation (Table 3). Similar results were obtained when ascorbate was used as substrate (Table 3). Further evidence on the similarity in action of fatty acids and dinitrophenol (DNP) was obtained by comparing their effects on the respiration of mitochondria in phosphate-deficient media. Both decanoate and DNP markedly stimulated the respiration under these conditions though the respiration in the presence of the higher concentration of decanoate gradually decreased during the incubation (Table 4).

Table 2. *Effects of decanoate on the* P/O *ratio for glutamate oxidation by rat-liver mitochondria*

Incubation was carried out for 15 min. at 37° in the medium described by Judah & Rees (1953). Glutamate (10 mM) was the substrate. Mitochondria used were equivalent to 500 mg. original wet wt. of liver. Figures in parentheses refer to percentage inhibitions of the P/O ratio.

Concn. of decanoate (mM)	Uptake of O_2 (μg.atoms)	Uptake of phosphate (μmoles)	P/O
0	10·2	25·8	2·52
0·34	11·5	23·3	2·09 (17)
0·67	13·6	19·8	1·45 (41)
1·0	13·2	18·4	1·39 (44)
1·34	13·4	7·7	0·58 (75)

Table 3. *Effects of potassium decanoate on the oxidative phosphorylation associated with cytochrome* c *oxidase and the one-step oxidation of succinate*

In Expt. 1, mitochondria were suspended in 0·075 M-sucrose and were equivalent to 500 mg. wet wt. of liver. Cytochrome *c* was reduced by 10 mM-ascorbate. 0·3 mM-Ethylenediaminetetra-acetic acid was also present. Incubation was carried out for 15 min. at 37° in the medium described in the Materials and Methods section.

In Expt. 2, 6 ml. of the standard rat-liver mitochondrial suspension in 0·25 M-sucrose was treated with 1·5 ml. of 0·1 M-phosphate buffer, pH 7·4, and allowed to stand for 10 min. at 37° as described by Hunter & Ford (1955). A portion (0·5 ml.) of this suspension was used in each vessel. This procedure depletes the mitochondria of endogenous diphosphopyridine nucleotide and thus permits succinate oxidation to proceed only as far as malate. Incubation time was 15 min. at 37° in the medium described in the Materials and Methods section.

Figures in parentheses refer to percentage inhibitions of the P/O ratio.

Expt. no.	Concn. of decanoate (mM)	Substrate	Uptake of O_2 (μg.atoms)	Uptake of phosphate (μmoles)	P/O
1	0	Ferrocytochrome *c*	12·4	6·4	0·52
	0·33		12·3	4·5	0·37 (29)
	1·0		13·0	0	0 (100)
2	0	16·7 mM-Succinate	17·6	16·8	0·96
	0·33		16·3	4·5	0·28 (72)
	1·0		14·2	0	0 (100)

Incorporation of [^{32}P]phosphate into rat-liver slices

In order to ascertain whether uncoupling of oxidation from phosphorylation occurred in intact tissues, the effects of fatty acids on [^{32}P]phosphate incorporation in various phosphate fractions of liver slices were also investigated.

Time course. When rat-liver slices were incubated with [^{32}P]phosphate, its incorporation into the inorganic phosphate fraction and the ATP plus ADP fraction was very rapid. Most of the labelling occurred within 20 min. and little increase in labelling occurred during the subsequent 60 min. of incubation (Table 5). The labelling of the total phospholipid fraction continued to increase during the entire incubation period whereas in the acid-insoluble fraction (phosphoprotein, nucleic acids etc.) an equilibrium was attained within 40 min. Glucose did not stimulate incorporation of [^{32}P]-phosphate.

Effect of potassium decanoate on incorporation of [^{32}P]phosphate. Potassium decanoate at concentrations of 0·67, 1·34 and 2·0 mM had no inhibitory effect on the respiratory activity of liver slices and little effect on the incorporation of ^{32}P into the inorganic phosphate but strongly inhibited [^{32}P]-phosphate incorporation into the ATP plus ADP, phospholipids and acid-insoluble fraction (Table 6). A time-course study of the effect of 4 mM-decanoate (which initially stimulated respiration but gradually produced an inhibition) revealed that the [^{32}P]phosphate incorporation into the organic phosphate compounds was completely inhibited (Fig. 3) and that no labelling occurred even after prolonged incubation.

Effect of homologous fatty acids. The effect of fatty acids with chain lengths of from four to eight carbon atoms (used at a concentration of 4 mM) and with chain length of from eight to twelve carbon atoms (used at a concentration of 1·34 mM) on [^{32}P]phosphate incorporation was also studied (Table 7). At this concentration the fatty acids only stimulated respiration. Butyrate slightly inhibited phosphate incorporation into the various organic phosphate fractions but the effect increased with chain length and octanoate was the most inhibitory in this series, producing almost complete inhibition. The fatty acids of chain length C_8–C_{12} were much more inhibitory and 1·34 mM-dodecanoate completely inhibited phosphate incorporation into the organic phosphate compounds. Octanoate was the weakest inhibitor in this series and the odd-numbered fatty acids were less effective than either of the adjacent even-numbered fatty acids. The above results bore close resemblance to those obtained with DNP, which also inhibited phosphate incorporation into organic phosphate compounds under conditions where it had little inhibitory effect on respiratory activity of the slices (Table 8).

Table 4. *Effects of decanoate on the respiration of rat-liver mitochondria in a phosphate-deficient medium*

All values are for μl. of oxygen taken up. Experimental conditions were as described in Table 2. Incubation medium was changed by omission of glucose and hexokinase and replacement of 10 mM-phosphate by 1·67 mM-phosphate plus 67 mM-2-amino-2-hydroxymethylpropane-1:3-diol, pH 7·4.

	Additions			
	Nil	Decanoate		DNP
Time (min.)		0·34 mM	0·67 mM	0·05 mM
5	9	24	47	34
10	18	40	79	72
15	32	55	100	110
20	56	77	116	150
25	82	107	136	186

Table 5. *Time course of incorporation of [^{32}P]phosphate into various phosphate compounds in rat-liver slices*

^{32}P was tipped into the main compartment of Warburg vessels at zero time and the incubation was carried out at 37°. The basic incubation medium used was a calcium-free Krebs–Ringer phosphate solution as described in the Materials and Methods section.

		Phosphate incorporated (μm-moles/100 mg. wet wt. of tissue)			
Time (min.)	Glucose (10 mM)	P_i	ATP plus ADP	Phospholipids	Acid-insoluble fraction (phosphoproteins, RNA and DNA)
20	+	347	123	10	12
	−	329	128	8	16
40	+	366	125	13	27
	−	381	136	14	29
60	+	404	142	21	33
	−	416	152	19	33
80	+	394	111	23	27
	−	395	126	24	34

Metabolism of [1-^{14}C]decanoate

The metabolism of [1-^{14}C]decanoate, at several concentrations, to $^{14}CO_2$, to [^{14}C]acetoacetate and to ^{14}C-labelled phospholipids was studied (Table 9). The amount of $^{14}CO_2$ released by aniline citrate from the acetoacetate formed during incubation increased with increasing concentrations of [1-^{14}C]-decanoate, although less $^{14}CO_2$ was obtained when the incubation was carried out at concentrations higher than 1·33 mM. Thus the acetoacetate produced from 2·67 mM-decanoate was only slightly more than that produced in the presence of 0·33 mM.

The incorporation of the ^{14}C into phospholipids continued at an almost constant rate even in the presence of 2 mM-decanoate, but with 2·67 mM the rate of incorporation decreased slightly with time. Thus decanoate, at concentrations which strongly inhibit phosphate incorporation but do not inhibit the respiratory activity of the tissue, has little effect on its own oxidation or on its incorporation into phospholipids.

The results from respiratory studies showed that the inhibitory effects of fatty acids at higher concentrations set in gradually and only after an initial stimulatory phase. To determine if this pattern coincided with the pattern of fatty acid oxidation, a time-course study of the oxidation of 1·34 and 4 mM-decanoate was undertaken. The results presented in Fig. 4 demonstrate that with 1·34 mM-decanoate, the rates of respiration, $^{14}CO_2$ production and acetoacetate production were relatively constant during an incubation period lasting 200 min. There was an initial increase in respiratory activity in the presence of 4 mM-decanoate but it declined considerably at the end of 60 min. The initial rate of $^{14}CO_2$ production was also slightly higher than that from 1·34 mM-decanoate, but it completely ceased after 80 min. and production of $^{14}CO_2$ from acetoacetate ceased after 50 min.

Table 6. *Effect of various concentrations of decanoate on incorporation of [^{32}P]phosphate into rat-liver slices*

Decanoate was present in the main vessel and ^{32}P was tipped in at zero time. Incubation was carried out for 60 min. at 37°. No added substrate was present and the slices were respiring in a calcium-free Krebs–Ringer phosphate solution.

Concn. of decanoate (mM)	Phosphate incorporated (μm-moles/100 mg. wet wt. of tissue) P_i	ATP plus ADP	Phospho-lipids	Acid-insoluble fraction
0	496	123	30	40
0·67	503	51	18	24
1·34	398	29	8	9
2·00	379	6	4	6

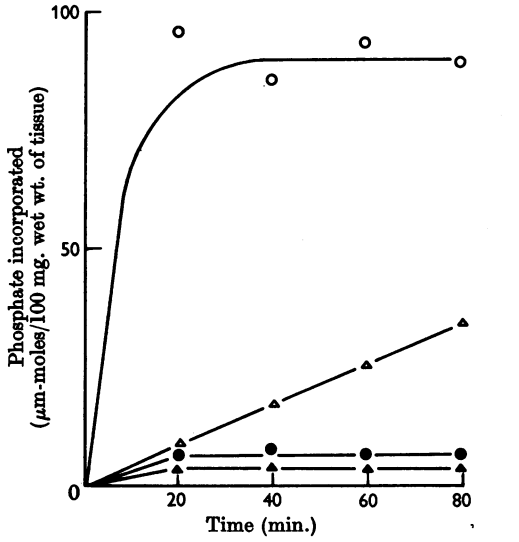

Fig. 3. Effects of 4 mM-potassium decanoate on phosphate incorporation into rat-liver slices. ○, △, No decanoate added; ●, ▲, 4 mM-decanoate added. ○, ●, Incorporation into ATP plus ADP; △, ▲, incorporation into phospholipids.

Table 7. *Effect of various fatty acids on incorporation of [^{32}P]phosphate into various fractions of rat-liver slices*

Experimental conditions were the same as described for Table 6.

Fatty acid: Chain length	Fatty acid: Concn. (mM)	$-Q_{O_2}$	Phosphate incorporated (μm-moles/100 mg. wet wt. of tissue): P_i	ATP plus ADP	Phospholipids
—	0	8·3	655	154	68
4	4·0	8·6	654	141	53
5	4·0	8·8	674	102	46
6	4·0	10·6	727	100	44
7	4·0	9·7	624	80	32
8	4·0	8·9	632	38	13
—	0	8·0	495	125	40
8	1·34	8·1	566	61	27
9	1·34	9·2	489	89	27
10	1·34	8·6	535	30	11
11	1·34	9·6	496	60	17
12	1·34	6·5	471	11	6

DISCUSSION

The present experiments on the inhibitory effects of fatty acids on the respiratory activity of rat-liver slices have confirmed earlier observations made with guinea-pig-liver slices (Quastel & Wheatley, 1933) and mitochondrial preparations (Lehninger, 1951; Scholefield, 1956). Among liver, kidney and brain tissues, liver is the least sensitive to the inhibitory effects of fatty acids on respiration and brain tissue the most sensitive (Ahmed & Scholefield, 1961), which is in keeping with the poor ability of brain tissue to oxidize fatty acids. In addition, a concentration of 1·0–1·3 mM-decanoate is required to produce a 50% decrease in the P/O ratio for glutamate oxidation in rat-liver mitochondria, whereas 0·6 mM produces a similar effect in rat-brain mitochondria (Scholefield, 1956). It may be noted that the phosphorylations associated with isolated enzyme systems such as cytochrome *c* oxidase and succinate oxidation via cytochrome *c* oxidase are even more sensitive to decanoate. These results show that the presence of fatty acids gives rise to an inhibition of the various phosphorylations associated with the respiratory chain and suggests that the inhibitory effects are due to the presence of free fatty acids themselves.

Table 8. *Effect of dinitrophenol on incorporation of* [^{32}P]*phosphate into rat-liver slices*

Experimental conditions were the same as described for Table 6.

Concn. of DNP (mM)	Phosphate incorporated (μm-moles/100 mg. wet wt. of tissue) P_i	ATP plus ADP	Phospholipids	Acid-insoluble fraction
0	422	150	37	46
0·025	513	76	23	40
0·075	378	6	10	21
0·150	284	0	6	10

Table 9. *Oxidation and incorporation of* [1-^{14}C] *decanoate by rat-liver slices*

[1-^{14}C]Decanoate had the same specific activity throughout (0·2 μc/μmole). Incubation was carried out for 60 min. at 37°. No added substrate was present in the medium, which was a calcium-free Krebs–Ringer phosphate solution.

Concn. of [1-^{14}C]decanoate (mM)	$-Q_{O_2}$	Decanoate oxidized (μm-moles/mg. dry wt./hr.)	Acetoacetate $^{14}CO_2$ (μm-moles/mg. dry wt./hr.)	[1-^{14}C]Decanoate incorporated into phospholipids (μm-moles/100 mg. wet wt. of tissue/hr.)
0	7·6	—	—	—
0·33	8·9	10·1	10·6	1·34
1·0	9·8	12·4	22·0	4·0
1·33	9·5	14·1	25·2	4·0
2·00	8·8	12·3	22·6	6·3
2·67	9·3	15·6	13·7	8·0

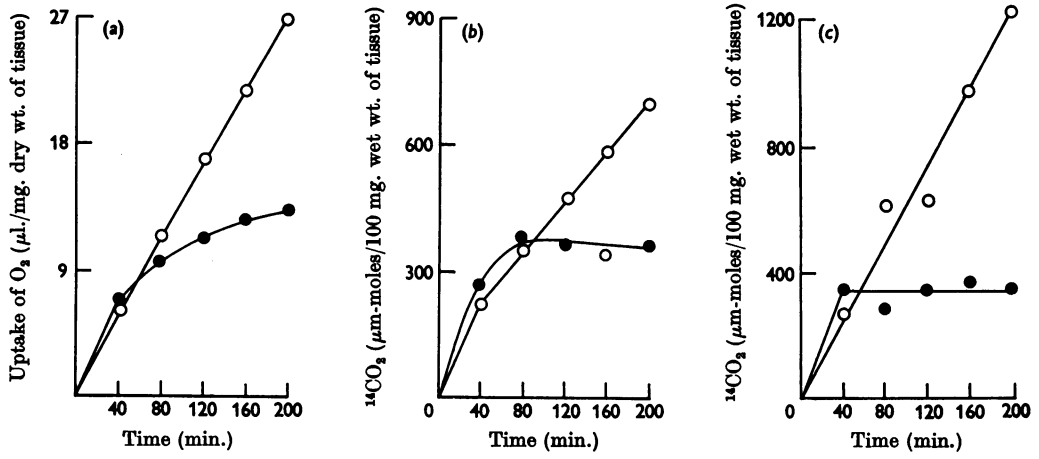

Fig. 4. Effects of potassium decanoate on (*a*) respiratory activity of rat-liver slices, (*b*) oxidation of [1-^{14}C]decanoate to $^{14}CO_2$ and (*c*) production of $^{14}CO_2$ from decarboxylation of acetoacetate formed during incubation. ○, 1·34 mM-[1-^{14}C]Decanoate present; ●, 4 mM-[1-^{14}C]decanoate present.

The results obtained in the study of the metabolism of [1-^{14}C]decanoate leave no doubt that it is oxidized and also incorporated into lipids during an incubation period of 1 hr. Under these conditions, enough ATP must be available for continued activation of the fatty acid since 1·34 mM-[1-^{14}C]-decanoate continued to be oxidized at a steady rate even after 3 hr. However, the oxidation of 4 mM-[1-^{14}C]decanoate to $^{14}CO_2$ stops at the end of about 80 min., which suggests that all the ATP in the cell may have then been depleted and that the process of oxidative phosphorylation may have been completely inhibited. The respiratory activity of the slice also falls to a low level. With 4 mM-[1-^{14}C]-decanoate a small amount of the acid may initially be transported into the cell, where some of it is activated and oxidized, but as it accumulates inside the cell it eventually causes an inhibition of the ATP-regenerating mechanism.

On the other hand, incorporation of [^{32}P]phosphate into liver slices is strongly inhibited by fatty acids at concentrations at which their own oxidation and incorporation into lipids proceeds at a high rate and at which they are not inhibitory to respiration. A satisfactory explanation of this effect may be offered in terms of the ATP–$^{32}P_i$ exchange reaction in liver mitochondria (Ahmed & Scholefield, 1960). This exchange proceeds very rapidly in isolated liver mitochondria and is sensitive to fatty acids (0·2 mM-decanoate inhibited the exchange reaction by about 50%, compared with the concentration of 1·0–1·3 mM required for a similar inhibition of oxidative phosphorylation in rat-liver mitochondria). A large part of the labelling of ATP with ^{32}P (and hence of other organic phosphates whose synthesis is dependent upon ATP) in intact slices must therefore take place through the participation of this reaction rather than by true synthesis of ATP, and it must be the exchange reaction which is inhibited on addition of relatively low concentrations of decanoate (and other fatty acids) to rat-liver slices. It is probable that the exchange reaction is also of importance *in vivo*, for Kaplan & Greenberg (1944) have reported a rapid equilibration of $^{32}P_i$ with the ATP of liver *in vivo*.

It was observed by Dianzani (1954) that mitochondria prepared from fatty livers showed a low P/O ratio. He suggested that the loss of phosphorylative ability in fatty livers may be a consequence of morphological changes which had led to degenerative processes *in vivo*. It is possible that the development of fatty livers leading to steatosis, or complete liver degeneration, is a manifestation of the uncoupling action of fatty acids *in vivo*. Similar considerations might apply to the deposition of excess of lipids in other tissues, such as occurs, for example, in arteries during arteriosclerosis.

Lehninger & Remmert (1959) have shown that 3 μM-oleate produces swelling of mitochondria. In addition to inhibitory effects on ATP–$^{32}P_i$ exchange, fatty acids at still lower concentrations considerably stimulate the exchange reaction, an effect which may be considered to be a consequence of changed permeability of mitochondria in the presence of added fatty acids (Ahmed & Scholefield, 1960). This increased permeability of mitochondria might cause a loss of cofactors, such as DPN, and eventually lead to the observed loss of respiratory activity. These considerations suggest that the mechanism of action of fatty acids is somewhat different from that of DNP, which generally does not cause swelling of mitochondria (Chappell & Greville, 1958; Lehninger, 1959).

Several workers (Pullman & Racker, 1956; Polis & Schmukler, 1957; Lehninger & Remmert, 1959) have reported the presence of endogenous uncoupling factors in mitochondria and it is likely that some of these factors resemble fatty acids such as oleic acid (Lehninger & Remmert, 1959). Of the saturated fatty acids, tridecanoate had the greatest inhibitory effect on the ATP–^{32}P exchange in mitochondria (Ahmed & Scholefield, 1960) and dodecanoate the greatest effect on respiration of slices, but oleic acid was more effective than any of the saturated fatty acids in inhibiting the ATP–^{32}P exchange, e.g. 2 μM-oleate inhibited this reaction by about 50% (K. Ahmed & P. G. Scholefield, unpublished data). The unsaturated fatty acids and the medium-chain-length saturated fatty acids may thus have some role as normal intracellular or endogenous swelling and uncoupling agents.

SUMMARY

1. Fatty acids increase the respiratory activity of rat-liver and -kidney slices. At higher concentrations of the fatty acids, the initial stimulation of the rate of oxygen uptake is followed by a gradual decrease.

2. The inhibitory effects increase with chain length, dodecanoate having the greatest effect. The inhibitions produced by higher fatty acids were not as great. Fatty acids containing an odd number of carbon atoms were less effective inhibitors than those with one more or one less carbon atom.

3. Decanoate (1 mM) inhibited the oxidative phosphorylation associated with glutamate oxidation in rat-liver mitochondria by approximately 50%. It also decreased the P/O ratio associated with the oxidation of succinate and reduced cytochrome *c*.

4. Decanoate and other fatty acids inhibited incorporation of [^{32}P]phosphate into rat-liver slices at a concentration (1·34 mM) which caused a stimulation of respiratory activity. [1-^{14}C]Decano-

ate was oxidized to $^{14}CO_2$ and incorporated into lipids at these concentrations.

5. The inhibitory effects of fatty acids are explained in terms of their effects on the enzyme systems involved in oxidative phosphorylation.

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REFERENCES

Ahmed, K. & Scholefield, P. G. (1960). *Nature, Lond.*, **186**, 1046.
Ahmed, K. & Scholefield, P. G. (1961). *Biochem. J.* **60**, 432.
Borgstrom, B., Sudduth, H. C. & Lehninger, A. L. (1955). *J. biol. Chem.* **215**, 571.
Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. E. (1949). *Isotopic Carbon*, p. 317. New York: John Wiley and Sons Inc.
Chappell, J. B. & Greville, G. D. (1958). *J. biol. Chem.* **182**, 813.
Creaser, E. H., de Leon, R. P. & Scholefield, P. G. (1959). *Cancer Res.* **19**, 705.
Creaser, E. H. & Scholefield, P. G. (1960). *Cancer Res.* **20**, 257.
Cross, R. J., Taggart, J. V., Covo, G. A. & Green, D. E. (1949). *J. biol. Chem.* **177**, 655.
Dianzani, M. U. (1954). *Biochim. biophys. acta*, **14**, 514.
Edson, N. L. (1935). *Biochem. J.* **29**, 2082.
Fiske, C. H. & Subbarow, Y. (1929). *J. biol. Chem.* **81**, 629.
Hogeboom, G. H., Schneider, W. C. & Pallade, G. E. (1948). *J. biol. Chem.* **172**, 619.
Hunter, F. E. & Ford, L. (1955). *J. biol. Chem.* **216**, 357.
Judah, J. D. & Rees, K. R. (1953). *Biochem. J.* **55**, 664.
Kaplan, N. O. & Greenberg, D. M. (1944). *J. biol. Chem.* **156**, 511.
Lehninger, A. L. (1945). *J. biol. Chem.* **157**, 363.
Lehninger, A. L. (1951). In *Phosphorus Metabolism*, vol. 1, p. 350. Ed. by McElroy, W. D. & Glass, B. Baltimore, The Johns Hopkins Press.
Lehninger, A. L. (1959). *J. biol. Chem.* **234**, 2465.
Lehninger, A. L. & Remmert, L. F. (1959). *J. biol. Chem.* **234**, 2459.
Lehninger, A. L., ul Hassan, M. & Sudduth, H. C. (1954). *J. biol. Chem.* **210**, 910.
Lynen, F. (1955). *Annu. Rev. Biochem.* **24**, 653.
Polis, B. D. & Schmukler, H. W. (1957). *J. biol. Chem.* **227**, 419.
Pressman, B. C. & Lardy, H. A. (1956). *Biochim. biophys. acta*, **21**, 458.
Pullman, M. E. & Racker, E. (1956). *Science*, **123**, 1105.
Quastel, J. H. & Wheatley, A. H. M. (1933). *Biochem. J.* **27**, 1756.
Scholefield, P. G. (1956). *Canad. J. Biochem. Physiol.* **34**, 1211, 1229.
Scholefield, P. G. (1958). *Cancer Res.* **18**, 1026.
Sinclair, R. G. & Dolan, M. (1942). *J. biol. Chem.* **142**, 659.

Biochem. J. (1961) **81**, 45

Studies on Fatty Acid Oxidation

8. THE EFFECTS OF FATTY ACIDS ON METABOLISM OF RAT-BRAIN CORTEX *IN VITRO*

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In the preceding paper (Ahmed & Scholefield, 1961) it was shown that fatty acids uncouple oxidation from phosphorylation in rat liver. The present studies were undertaken to survey the effects of fatty acids on various aspects of brain metabolism *in vitro*. These studies were of interest in view of the observation by Samson & Dahl (1955) that injection of short-chain fatty acids produces narcotic-like effects in normal animals.

It was previously demonstrated that the rapid exchange reaction between adenosine triphosphate and ^{32}P-labelled phosphate brought about by rat-liver mitochondria is a significant factor in the labelling of adenosine triphosphate and other organic phosphates in rat-liver slices (Ahmed & Scholefield, 1960). In the present paper the conditions necessary for measurement of the rate of the adenosine triphosphate–inorganic [^{32}P]phosphate (ATP–$^{32}P_i$) exchange reaction in rat-brain mitochondria have been examined. Findlay, Rossiter & Strickland (1953) observed that glucose and oxygen were essential for the incorporation of ^{32}P into the nucleic acid fraction of cat-brain slices. This labelling was markedly decreased when the members of the tricarboxylic acid cycle were used

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