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Oxidative Phosphorylation accompanying Oxidation of Short-Chain Fatty Acids by Rat-Liver Mitochondria

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1. The factors concerned in the estimation of P/O ratios when fatty acids are oxidized by rat-liver mitochondria have been assessed. 2. The oxidation of butyrate, hexanoate and octanoate is accompanied by ATP synthesis. At low concentrations of the fatty acids, P/O ratios approximately 2·5 are obtained. 3. Oxidative phosphorylation is uncoupled, respiratory control ratios are lowered and respiration is inhibited when the concentration of the fatty acid in the incubating medium is raised (to 5–10mM); octanoate is a more potent uncoupler than either hexanoate or butyrate. 4. Serum albumin and carnitine, either singly or in combination, protect the mitochondria from the effect exerted by the fatty acids. 5. The rate of oxidation of short-chain fatty acids in the presence of ADP is increased in the presence of carnitine.

The oxidation of fatty acids provides a major source of energy for mammalian tissues, but the experimental evidence which directly connects this process with ATP formation is limited. Kennedy & Lehninger (1949) have shown that the oxidation of octanoate in the presence of malate by rat-liver mitochondria is accompanied by extensive incorporation of ^{32}P from inorganic phosphate into ATP. Judah & Williams-Ashman (1951) have obtained P/O ratios 0·56 during the oxidation of octanoate by rat-liver mitochondria in the presence of succinate, and Lardy & Wellman (1952) have shown that the oxidation of octanoate to acetoacetate by such mitochondria gives P/O ratios 1·1–2·0. In the presence of added succinate, rat-kidney mitochondria gave P/O ratios 1·9–2·3 (Maley & Lardy, 1953) when octanoate was oxidized.

Fatty acids have been shown to uncouple oxidative phosphorylation occurring during the oxidation of other substrates (Scholefield, 1956; Hülsmann, Elliott & Slater, 1960) and such uncoupling can be prevented by the addition of serum albumin to the system (Lehninger & Remmert, 1959; Wojtczak & Wojtczak, 1960; Björntorp, Ells & Bradford, 1964). Carnitine has been shown to increase the rate of oxidation of long-chain fatty acids but has slight or no effect on those of short-chain length (Miller & Krake, 1962; Fritz, 1963). There is no information available on the relation of this compound to ATP production during the oxidation of fatty acids.

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In the present work factors affecting the estimation of P/O ratios obtained during the oxidation of fatty acids, both manometric and amperometric methods of measuring oxygen consumption being used, have been examined. P/O ratios for the oxidation of selected short-chain fatty acids by rat-liver mitochondria have been obtained in the presence and absence of serum albumin and carnitine.

EXPERIMENTAL

Materials

ADP was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and analysed for easily hydrolysable phosphate and total phosphate (Dounce, Rothstein, Beyer, Meier & Freer, 1948). The theoretical amount of phosphate was obtained. A further check by measuring its extinction at 259mμ confirmed the phosphate analysis (Chance & Williams, 1955). ATP was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; DL-carnitine hydrochloride from Mann Research Laboratories, New York, N.Y., U.S.A.; the fatty acids (British Drug Houses Ltd., Poole, Dorset) were distilled before their conversion into the potassium salts. Crystalline bovine serum albumin was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. It was dissolved in water (20%, w/v) and dialysed for 48hr. against 10 changes of distilled water (2l.) at 2° to remove possible traces of citrate. All other reagents used were of analytical grade.

Crystalline hexokinase (EC 2.7.1.1) (170000 units/g.: one unit is the activity of enzyme which utilizes 1μmole of ATP/min. at 38° in a medium of pH 7·4) was obtained from Sigma Chemical Co. The suspension in $(NH_4)_2SO_4$ soln. (3ml.) was dialysed against 1l. of

glucose solution (0·3M) at 2° for 24hr., or until no trace of NH_4^+ remained (up to 10 changes). The hexokinase was estimated by following, with a pH-stat (Radiometer, Copenhagen, Denmark), the rate of production of protons during the conversion of glucose into glucose 6-phosphate in the presence of ATP and Mg^{2+} at 38°. Yeast inorganic pyrophosphatase was prepared from dried baker's yeast (Heppel & Hilmoe, 1951).

Methods

The formation of ATP from ADP and inorganic phosphate associated with the oxidation of glutamate and fatty acids was followed by two methods.

Method 1: uptake of inorganic phosphate and manometric estimation of oxygen. Rat-liver mitochondria were prepared according to the method described by Hird & Symons (1962). The final mitochondrial pellet was suspended for use in the tris–KCl medium of Chappell & Perry (1954) at pH7·4 (4ml. of final mitochondrial suspension was obtained from 10g. of liver fresh wt.). The absence of sucrose in this medium allowed ketone bodies to be estimated without interference. Oxygen consumption was estimated in the Warburg apparatus at 38°. The flasks contained 0·3ml. of the mitochondrial suspension in a final volume of 3·0ml. In addition to substrates (10·0mM-butyrate, 7·5mM-hexanoate and 5·0mM- or 1·0mM-octanoate) the incubation mixture consisted of: potassium phosphate buffer, pH7·4 (23·3mM); tris–HCl buffer, pH7·4 (36·7mM); $MgCl_2$ (2·5mM); EDTA (mM); KCl (30mM); ATP (mM). Bovine serum albumin in the amounts indicated in the text and inorganic pyrophosphatase (in 0·5ml.) were added to the main compartment. The side arm contained glucose (final concn. 23·3mM) and hexokinase as indicated.

At the end of a 10min. equilibration period the contents of the side arm were added to the main compartment and the incubation was carried out with a gas phase of air. Oxygen consumption was followed until approximately 8μmoles were taken up, when the reaction was stopped by the addition of 0·3ml. of trichloroacetic acid (10%, w/v). The inorganic phosphate present before and after the addition of hexokinase was estimated by the method of Allen (1940). In each manometric experiment an estimate of glucose 6-phosphatase activity of the mitochondrial preparations was made to correct for the amount of phosphate cycled in this way. Glucose 6-phosphatase was estimated by following the production of inorganic phosphate from glucose 6-phosphate (10mM) in a medium similar to that used to determine the P/O ratios but low in phosphate (6·6mM). The K_m for liver glucose 6-phosphatase is approx. 2·5mM (Langdon & Weakley, 1957). Linearity of glucose 6-phosphate formation in the phosphorylating system can be assumed and as 30μmoles or more of glucose 6-phosphate were formed during the reaction period the enzyme was saturated for most of the time. The contribution up to saturation concentration was calculated from the K_m. The correction to the P/O ratio was never greater than 15%.

Inorganic pyrophosphatase activity in the system was followed by estimation of inorganic phosphate formed from pyrophosphate (20μmoles/3ml.), this being an excess over the amount of pyrophosphate produced in the system. The activity of the pyrophosphatase added to the incubation system was sufficient to hydrolyse 25μmoles of pyrophosphate/min. at 38°. Ketone-body production was estimated by the method of Hird & Symons (1959), with the subsequent modifications by Hird & Weidemann (1964), and fatty acid disappearance was estimated by steam-distillation and titration under CO_2-free conditions as described by Pennington (1952).

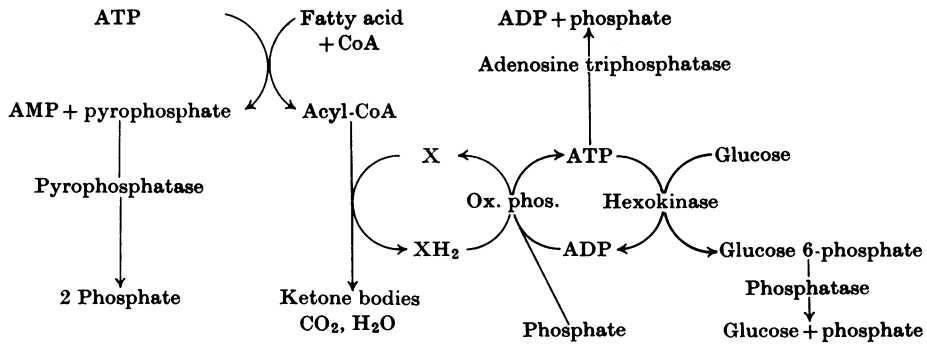
Method 2: amperometric estimation of oxygen consumption coupled to phosphorylation of ADP. Experiments were performed at 30° in a cell with capacity 1·5ml. An oscillating glass-covered electrode (Gilson Medical Electronics, Middleton, Wis., U.S.A.) with exposed platinum tip (0·6mm.), coupled to a Metrohm Polarecord type E 261 (Switzerland) amplifier and recorder, was used. The recorder was gain-controlled to give a pen deflexion of approximately 14cm. for the O_2 dissolved in the medium. The O_2 concentration in the medium was measured at −0·6v with a calomel reference electrode, and was determined by measuring the deflexion of the recorder pen with M-KCl saturated with air at 30° (*International Critical Tables*, 1928) followed by purging with O_2-free N_2, and directly comparing this deflexion with that obtained with the medium under the same conditions. Liberation of O_2 from H_2O_2 with catalase in the reaction mixture and in M-KCl (Dixon & Kleppe, 1965) indicated that with the oscillating electrode the activity of oxygen in both systems was the same within the limit of experimental error. The concentration of O_2 in the reaction mixture so determined was 0·46μg.atom/ml., which is comparable with 0·445μg.atom of oxygen in a similar system determined by Chappell (1964).

The reagents were added to the reaction cell by means of syringes. Rapid stirring of the system was accomplished by means of a small circular metal stirrer connected to a vibrator from an electric razor with a foot switch. Stirring for 1sec., after addition of reagents, was sufficient for complete mixing. The stirrer remained in the vessel during the experiment.

During the course of the incubation, contact between the platinum electrode and the incubating medium containing mitochondria led to slow poisoning of the electrode. This problem was overcome by renewing the electrode surface immediately before each run by burnishing it with fine carborundum paper.

Mitochondria were prepared from rat liver as before. In most of the experiments performed by this method, sucrose solution (0·3M) was used as the suspending medium instead of the tris–KCl medium of Chappell & Perry (1954). The mitochondria (from 5g. of liver fresh wt.) were suspended in 1·0ml. of 0·3M-sucrose solution, and 0·1ml. of the suspension was used each time; the composition of the reaction mixture was as described by Hird & Symons (1962). The final volume was 1·5ml. In some experiments, bovine serum albumin (1%, w/v) and DL-carnitine hydrochloride (in equivalent amounts to the substrate, below 1mM, and thereafter at 1mM) were also added to the incubating medium. To initiate ADP-dependent respiration, ADP (0·4μmole in 0·01ml.) was added to the reaction vessel with the aid of an Agla micro-syringe.

Mitochondria suspended in sucrose solution were used as soon as possible after preparation, and during the course of the experiment were stored in an ice bath at 0°. With each batch of mitochondria, and glutamate as substrate, it was possible to make approximately 20 estimations (approximately 2hr.) without apparent deterioration of respiratory control and P/O ratio. After this time some



Scheme 1. Ox. phos.: Oxidative phosphorylation.

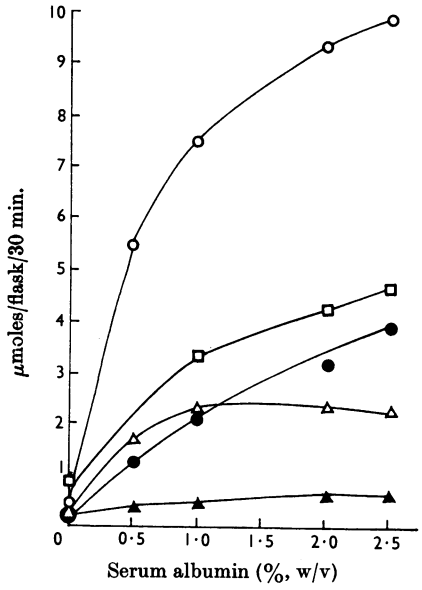


Fig. 1. Effect of serum albumin on the oxidation of butyrate by rat-liver mitochondria. Hexokinase (10 units) was added to each flask; other experimental details are given in the text. In the presence of butyrate (10mM): ○, oxygen consumption (measured manometrically); △, ketone-body production; □, butyrate disappearance. In the absence of added substrate: ●, oxygen consumption; ▲, ketone-body production.

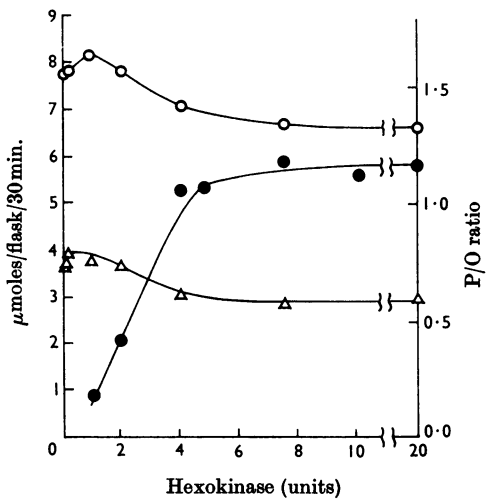


Fig. 2. Effect of hexokinase concentration on the oxidation of butyrate by rat-liver mitochondria. Butyrate (10mM) and serum albumin (1%, w/v) were added to each flask; other experimental details are given in the text. ○, Oxygen consumption (measured manometrically); △, ketone-body production; ●, P/O ratio (uncorrected).

loss of respiratory control became evident and increased progressively.

RESULTS

Method 1: uptake of phosphate and manometric estimation of oxygen

General principles of the method. The general reactions shown in Scheme 1 are involved or interfere in the determination of P/O ratios by this method.

Because the method is dependent on total inorganic phosphate disappearance in relation to oxygen consumption, the existence of processes returning phosphate to the medium will lower the P/O ratios observed. The magnitude of possible corrections to be applied must therefore be assessed. To do this it is necessary to estimate ATP consumed in the activation reaction (by measuring the disappearance of fatty acid) and to estimate pyrophosphatase, adenosine-triphosphatase and glucose 6-phosphatase activities of the system. The

ability of hexokinase to compete with the mitochondrial adenosine triphosphatase for the ATP formed, and the possible uncoupling effects of the fatty acids, have also to be assessed.

ATP utilization during the activation reaction and the status of endogenous pyrophosphatase. Determination of the fatty acid oxidized during the course of each experiment enables an estimate of the ATP consumed in the esterification reaction with coenzyme A to be made. Provided that the inorganic pyrophosphate so formed is either not hydrolysed or completely hydrolysed during the incubation, a simple correction can be applied. In the present system, pyrophosphatase activity for complete hydrolysis was sometimes found to be insufficient. To ensure that the pyrophosphate formed was completely hydrolysed, excess of inorganic pyrophosphatase prepared from yeast (Heppel & Hilmoe, 1951) was added. The amount of phosphate returned to the medium via ATP consumed by the thiokinase reaction was corrected for on the basis of 2 moles of phosphate/mole of fatty acid esterified.

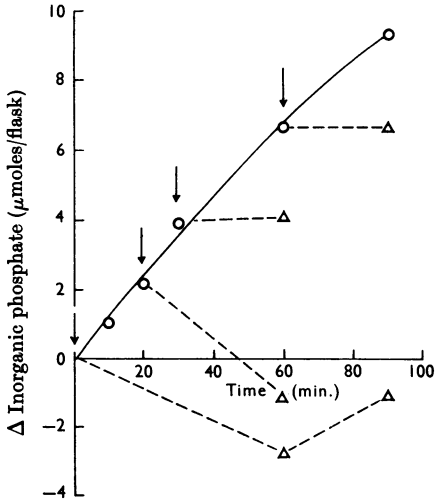


Fig. 3. Effect of hexokinase on the effective adenosinetriphosphatase activity of rat-liver mitochondria. The incubating medium contained ATP (10 μmoles) and other constituents as given in the text (including 1% serum albumin), but there was no added substrate. Inorganic phosphate liberated from ATP: ○, in the absence of added hexokinase; △, in the presence of 10 units of hexokinase added at various time-intervals as indicated by the arrows.

Correction for glucose 6-phosphatase. The glucose 6-phosphatase of rat liver is associated with the microsome fraction (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) and, even when great precautions were taken for the complete removal of the fluffy layer from the mitochondrial preparations, it was not always possible to free the mitochondria of residual activity. It was therefore necessary to estimate glucose 6-phosphatase activity in the system for each batch of mitochondria. This corresponded to a correction of 0–15% in the final P/O ratio.

Effect of bovine serum albumin. Fig. 1 shows that the rate of oxidation of butyrate in the system was markedly stimulated by serum albumin. This stimulation fell off at about 1% (w/v), especially with ketone-body production. Fig. 1 also shows that serum albumin, or its bound fatty acids, acted as a substrate in the absence of butyrate but was not a good precursor of ketone bodies. As the rate of oxidation of butyrate was inconveniently low in the absence of serum albumin, this protein was always added to the system used. A concentration of 1% (w/v) was considered appropriate. It is likely that some suppression of endogenous oxidation occurred in the presence of fatty acid substrate (Hird & Symons, 1959), but this aspect of the system has not been investigated further.

Effect of hexokinase concentration. Fig. 2 shows

Table 1. P/O *ratios from oxidation of fatty acids*

Rat-liver mitochondria suspended in the tris–KCl medium of Chappell & Perry (1954) were incubated in Warburg flasks for 30–40 min. at 38° in 3·0 ml. of medium as described in the text. All flasks contained hexokinase (20 units) and serum albumin (1%, w/v). The results are expressed in μmoles/0·3 ml. of mitochondrial suspension. Standard deviations are given in parentheses. Corrected P/O ratio (1) indicates correction for ATP consumed in the activation reaction; corrected P/O ratio (2) includes additional correction for glucose 6-phosphatase activity.

Additions	No. of expts.	Oxygen uptake (μmoles)	Fatty acid metabolized (μmoles)	Ketone bodies formed (μmoles)	P/O ratio: Uncorrected	P/O ratio: Corrected (1)	P/O ratio: Corrected (2)
10 mM-Butyrate	4	7·2 (±0·9)	3·4 (±0·4)	1·9 (±0·3)	1·5 (±0·1)	1·9 (±0·1)	2·1 (±0·1)
7·5 mM-Hexanoate	4	7·1 (±0·8)	2·4 (±0·3)	1·8 (±0·1)	1·5 (±0·1)	1·8 (±0·2)	2·0 (±0·1)
5 mM-Octanoate	4	6·0 (±2·6)	1·4 (±0·8)	1·4 (±0·8)	0·5 (±0·4)	0·7 (±0·5)	0·8 (±0·5)

that the addition of 6 units of hexokinase was sufficient for the determination of P/O ratios. At this concentration, however, there was a slight fall in the rate of fatty acid oxidation and ketone-body formation. The high rate of oxygen consumption in the absence of hexokinase, and the quite small stimulation in its presence, indicates uncoupling of oxidative phosphorylation by the fatty acid and/or stimulation of some type of mitochondrial adenosine-triphosphatase activity. The same stimulation occurs in the absence of a phosphate acceptor, suggesting that the high rate is not due to the presence of endogenous hexokinase and adenosine triphosphatase.

To investigate possible competition for ATP between adenosine triphosphatase and the hexokinase system, the latter was added at various time-intervals to a mitochondrial system actively hydrolysing ATP. It was necessary to perform this experiment in the absence of fatty acid and other systems regenerating ATP. Fig. 3 shows that hydrolysis of added ATP proceeded steadily with time. The addition of 10 units of hexokinase at various time-intervals rapidly removed the ATP so that adenosine-triphosphatase activity ceased. Hexokinase at this concentration was therefore an effective competitor for ATP. When hexokinase was added early there was an actual uptake of inorganic phosphate, due probably to oxidative phosphorylation at the expense of endogenous substrates. The amount of hexokinase used in the experiments to determine P/O ratios was always 20 units.

P/O *ratios as measured by phosphate uptake and manometric estimation of oxygen.* Table 1 shows that the oxidation of butyrate, hexanoate and octanoate, at equivalent concentrations of carbon atoms, was accompanied by ATP formation. The corrected P/O ratios were approximately 2, or above, with butyrate and hexanoate. Octanoate at this concentration (5mM), however, gave varying values (range 0·2–1·5). The oxygen consumption and P/O ratios with octanoate were also extremely variable, and low values for the P/O ratios invariably coincided with low oxygen consumption. It can be seen that with all three fatty acids, on a carbon atom basis, usually 50% or more of the fatty acid metabolized was converted into ketone bodies.

Because of the low P/O ratios at 5mM, the effect of concentration of octanoate was examined further. P/O ratios (1·9) obtained at mM concentration were higher than those (1·1, 0·1) obtained with the same mitochondria at 5mM, and inhibition of oxygen consumption was not apparent at the lower concentration of the fatty acid. The corrected P/O values at the lower concentration were similar to those obtained with butyrate and hexanoate at higher concentrations (Table 1).

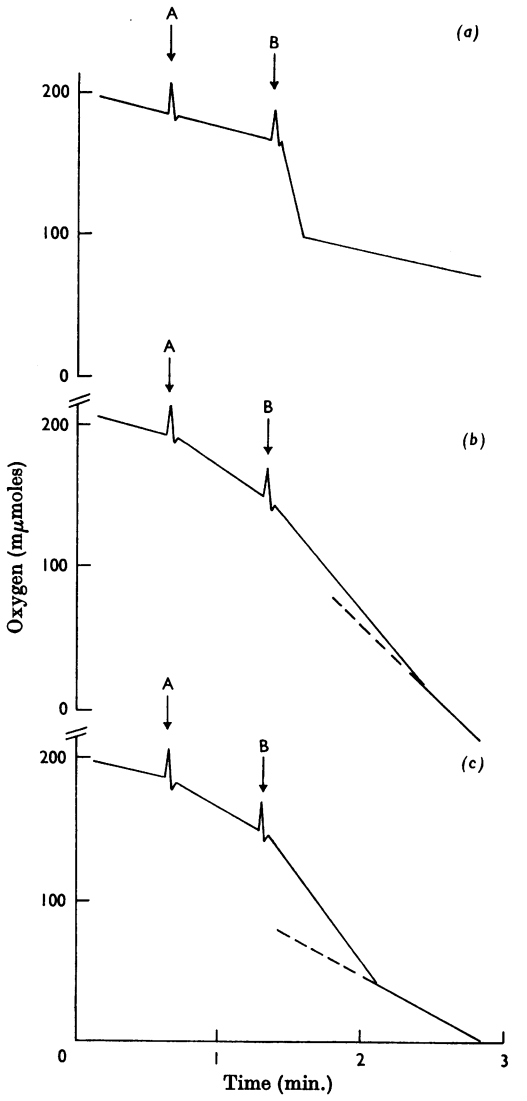


Fig. 4. Polarographic record of oxygen consumption by rat-liver mitochondria. Experimental details are given in the text. (*a*) A, glutamate added (10μmoles), followed by B, ADP (0·4μmole). (*b*) A, octanoate added (0·75μmole), followed by B, ADP (0·4μmole). (*c*) A, octanoate added (0·75μmole) to the medium containing serum albumin (1·0%, w/v), followed by B, ADP (0·4μmole).

Method 2: P/O *ratios as measured by amperometric estimation of oxygen consumption coupled to phosphorylation of ADP*

The estimation of P/O ratios by this short-term method also involves a number of corrections of the observed value. These are considered below.

Apparent presence of adenosine triphosphatase. The results of typical experiments obtained during the oxidation of glutamate and fatty acids are given in Fig. 4. It is assumed that the increase in the rate of oxygen consumption after the addition of ADP is due to the phosphorylation of the latter to ATP and that the subsequent decrease in rate is due to the completion of such phosphorylation. With glutamate (Fig. 4*a*), after the conversion of ADP into ATP, there was little or no increase in oxygen consumption above the pre-ADP rate. In the presence of octanoate (Fig. 4*b*), however, the rate increased. This suggests that the increase in rate of oxygen consumption is due to the establishment of a steady-state concentration of ADP as the result of octanoate-stimulated adenosine-triphosphatase activity with the newly formed ATP as substrate. The magnitude of this increase was less in the presence of serum albumin (Fig. 4*c*).

Such an ADP-regenerating system operating during the phase of ADP phosphorylation to ATP would prolong the oxygen consumption and so lower the observed P/O ratio. It is possible that this apparent adenosine-triphosphatase activity is suppressed during the period of ADP-dependent oxygen consumption, but the lower ratios obtained in the presence of higher concentration of fatty acids are also consistent with the presence of an adenosine triphosphatase with high affinity for ATP. Assuming the presence of such an adenosine-triphosphatase activity during the time of ADP-dependent respiration, its contribution has been corrected for on the basis of one-half the effect observed at the final concentration of ATP obtained (0·27 mM). In most experiments the contribution of this correction to the P/O ratio was less than 10%.

ADP formation from the thiokinase reaction. This correction has been made on the basis of fatty acid utilization/mole of oxygen consumed as determined from the manometric experiments. It has been assumed that, for each fatty acid molecule esterified with coenzyme A, 2 moles of ADP have been formed for each mole of ATP utilized.

P/O *ratios as determined amperometrically.* With each batch of mitochondria the P/O ratios with glutamate were always determined. In 15 experiments, with mitochondria suspended in 0·3 M-sucrose, P/O ratios 3·0 (±0·3) and respiratory control ratios 3·7 (±1·2) were obtained. With mitochondria suspended in the tris–potassium chloride medium of Chappell & Perry (1954), both values were slightly lowered; in five experiments the P/O ratios were 2·8 (±0·1) and respiratory control ratios were 3·3 (±0·6).

Butyrate. Table 2 shows that, with butyrate as substrate, respiratory control was increased in the presence of carnitine both in the presence and absence of serum albumin. This was apparent at all concentrations of butyrate. At the higher concentrations, where respiratory control was low (or absent) and there was no definite change in rate of oxygen consumption to mark the complete phosphorylation of ADP, it was not possible to obtain an accurate estimation of the P/O ratio. The observed and corrected ratios were always highest in the presence of both serum albumin and carnitine and in all cases decreased at the higher concentrations of butyrate. The addition of carnitine usually gave a clear-cut increase in the ratio.

The effect of ADP on the oxygen consumption as related to increase in concentration of the fatty

Table 2. *Effect of concentration of butyrate on oxidative phosphorylation*

Rat-liver mitochondria suspended in sucrose were incubated at 30° in 1·5 ml. of medium in the reaction vessel in which the consumption of oxygen was followed amperometrically, as described in the text. P/O ratio A is the uncorrected ratio as measured directly; P/O ratio B has been corrected for the ADP regenerated during the activation of the fatty acid and for the small increase in apparent adenosine-triphosphatase activity occurring during ADP-dependent oxidation.

	Without serum albumin						With serum albumin					
	Butyrate			Butyrate plus carnitine			Butyrate			Butyrate plus carnitine		
Concn. of butyrate (mM)	Resp. control ratio	P/O ratio A	P/O ratio B	Resp. control ratio	P/O ratio A	P/O ratio B	Resp. control ratio	P/O ratio A	P/O ratio B	Resp. control ratio	P/O ratio A	P/O ratio B
0·1	1·5	1·4	2·0	2·4	1·7	2·3	1·4	1·7	2·3	2·3	1·9	2·5
1·0	1·1	1·4	1·9	2·5	1·7	2·2	1·4	1·1	1·9	2·6	1·6	2·3
10	1·0	1·0	1·5	2·0	1·1	1·6	1·1	1·1	1·6	2·3	1·5	2·2
100	1·0	*		1·3	1·1	1·6	1·0	*		1·2	1·4	2·1

* 'No turnover point': i.e. no change in slope on complete phosphorylation of ADP.

acid is shown in Fig. 5. It is apparent that carnitine markedly stimulated ADP-dependent oxygen consumption; this was more pronounced at lower concentrations of butyrate. A similar result was obtained in the presence of 1·0% serum albumin. Carnitine had no effect in the absence of butyrate and must mediate in some way the oxidation of butyrate and couple it to phosphorylation of ADP.

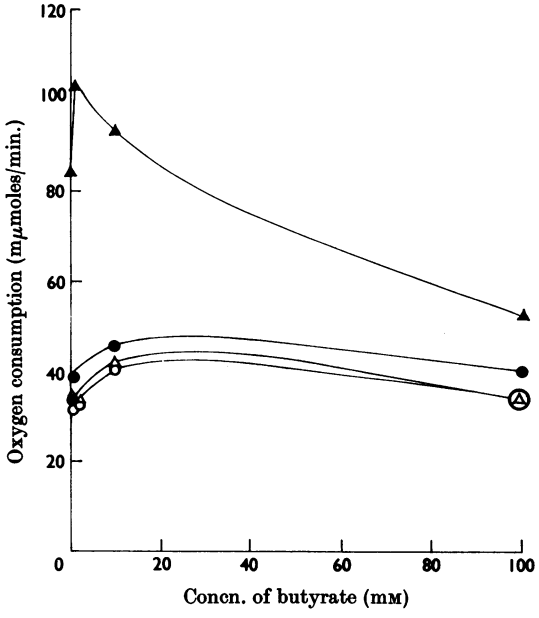


Fig. 5. Effect of butyrate concentration on the rate of oxygen consumption (measured amperometrically) by rat-liver mitochondria. Incubations were performed in the absence of serum albumin; other experimental details are given in the text. In the presence of carnitine: ●, pre-ADP rate; ▲, post-ADP rate. In the absence of carnitine: ○, pre-ADP rate; △, post-ADP rate.

Hexanoate. Table 3 shows that, with hexanoate, the respiratory control ratio and P/O ratio fell with increasing fatty acid concentration. These ratios were greater in the presence of carnitine and/or serum albumin. Figs. 6(*a*) and 6(*b*) show the response in oxygen consumption to increased concentration of hexanoate. It can be seen that the presence of carnitine markedly enhanced the response to ADP and that serum albumin enhanced it still further. The effect of both substances fell off with increase in concentration of the fatty acid.

Octanoate. The general behaviour of octanoate, as shown in Table 4, was similar to that of butyrate and hexanoate, up to a concentration of 1mM. Above this concentration, except in the presence of 1·0% serum albumin plus carnitine, the polarographic traces showed a fall in rate of oxygen consumption with time and respiratory control ratios could not be determined. Because there was no indication of the point of complete phosphorylation of ADP, P/O ratios at concentrations above 1mM could only be determined when both serum albumin and carnitine were present together in the medium. Serum albumin had a more marked coupling effect than carnitine. As with the two lower fatty acids, higher P/O ratios were obtained in the presence of serum albumin and carnitine at the lower fatty acid concentrations. The corrected ratio of 2·6 at octanoate concentration 0·1mM indicates efficient ATP formation with this fatty acid.

Figs. 7(*a*) and 7(*b*) show that at low concentrations of octanoate there was the usual marked effect of ADP and that this was further increased in the presence of carnitine and serum albumin. Increase in the concentration of octanoate up to 3mM in the absence of added ADP stimulated oxygen consumption. The addition of serum albumin shifted the curves towards a higher concentration of the fatty acid before the maximum was reached. At concentrations above 3–5mM, the actual concentration depending on the presence or

Table 3. *Effect of concentration of hexanoate on oxidative phosphorylation*

Rat-liver mitochondria suspended in sucrose were incubated as for Table 2.

Concn. of hexanoate (mM)	Without serum albumin						With serum albumin					
	Hexanoate			Hexanoate plus carnitine			Hexanoate			Hexanoate plus carnitine		
	Resp. control ratio	P/O ratio		Resp. control ratio	P/O ratio		Resp. control ratio	P/O ratio		Resp. control ratio	P/O ratio	
		A	B		A	B		A	B		A	B
1·0	1·6	1·5	2·0	2·3	1·7	2·0	2·2	1·7	2·1	3·6	1·9	2·3
10	1·3	0·9	1·3	1·4	1·3	1·7	1·5	1·4	1·8	1·8	1·5	1·9
20	1·1	0·6	1·0	1·2	0·7	1·1	1·3	1·2	1·5	1·4	1·3	1·7

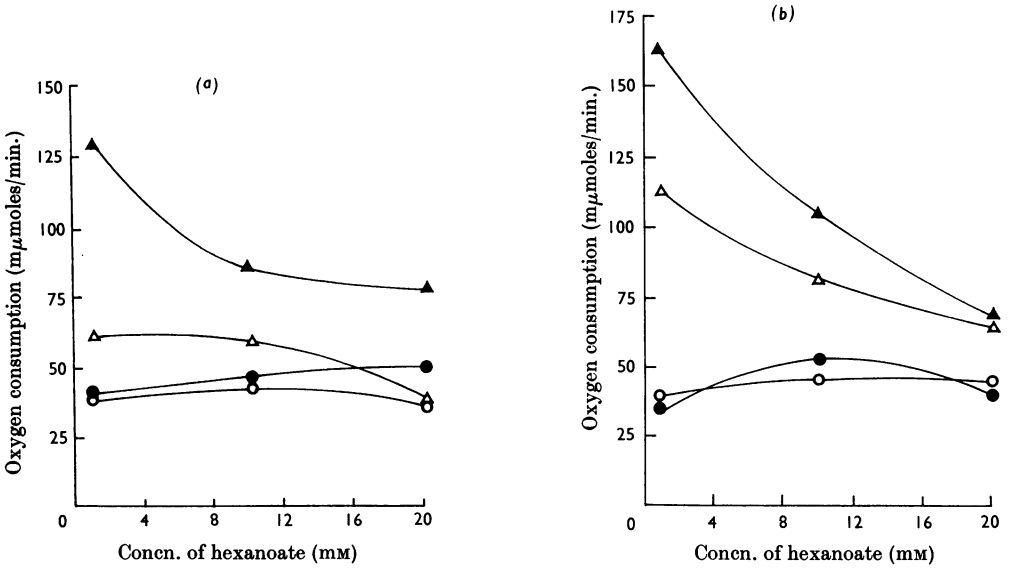


Fig. 6. Effect of hexanoate concentration on the rate of oxygen consumption (measured amperometrically) by rat-liver mitochondria; other experimental details are given in the text. Incubations were performed in the absence (*a*) and presence (*b*) of serum albumin (1%, w/v). In the presence of carnitine: ●, pre-ADP rate; ▲, post-ADP rate. In the absence of carnitine: ○, pre-ADP rate; △, post-ADP rate.

Table 4. *Effect of concentration of octanoate on oxidative phosphorylation*

Rat-liver mitochondria suspended in sucrose were incubated as for Table 2. Where no respiratory control ratio is given, the fatty acid caused a progressive inhibition of oxygen consumption.

Concn. of octanoate (mM)	Without serum albumin						With serum albumin					
	Octanoate			Octanoate plus carnitine			Octanoate			Octanoate plus carnitine		
	Resp. control ratio	P/O ratio		Resp. control ratio	P/O ratio		Resp. control ratio	P/O ratio		Resp. control ratio	P/O ratio	
		A	B		A	B		A	B		A	B
0·1	1·8	1·7	2·2	1·6	1·6	2·1	1·8	1·9	2·3	2·4	2·2	2·6
0·5	1·5	1·5	1·9	1·5	1·4	1·8	2·2	2·0	2·3	2·7	2·2	2·5
1·0	1·2	1·0	1·3	1·3	1·1	1·4	1·8	1·9	2·2	2·2	2·0	2·3
2·0	—	*		1·1	*		1·4	*		1·8	1·6	1·9
3·0	—	*		—	*		1·0	*		1·5	1·3	1·6
5·0	—	*		—	*		—	*		1·0	*	

* 'No turnover point': i.e. no change in slope on complete phosphorylation of ADP.

absence of carnitine or serum albumin, octanoate became inhibitory and showed no response to ADP.

Fig. 8 shows the effect of increasing the concentration of serum albumin on the rate of oxidation of octanoate and the resulting P/O ratios. At this concentration (3·5mM) of octanoate, in the absence of serum albumin, oxygen consumption is not tightly coupled with phosphorylation of ADP. It can be seen that the rate of oxygen consumption in the presence of ADP rose steeply up to 1·0% serum albumin and thereafter quite slowly. With increasing concentration of serum albumin the P/O ratio also rose and was at a maximum at about 3·0% serum albumin. The rate of oxygen consumption before the addition of ADP is also given for comparison.

Time-course of the response to carnitine. Fig. 9 shows that when butyrate was oxidized in the

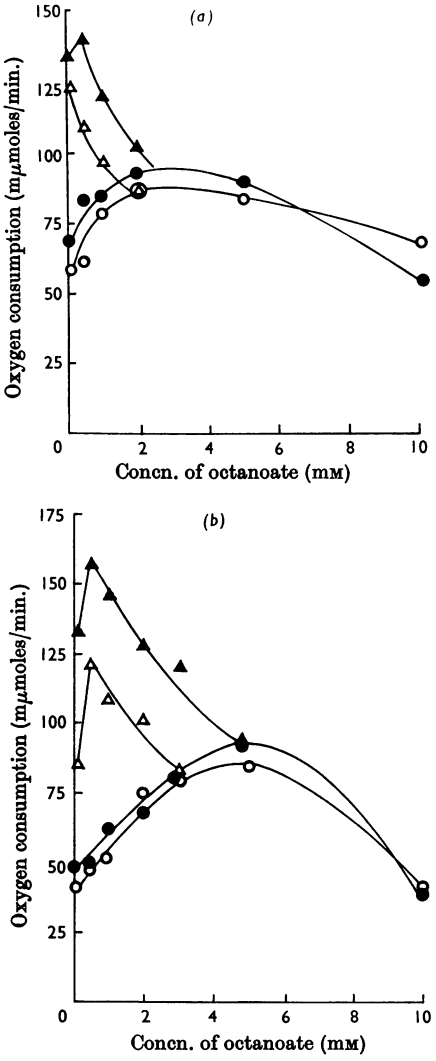


Fig. 7. Effect of octanoate concentration on the rate of oxygen concentration (measured amperometrically) by rat-liver mitochondria; other experimental details are given in the text. Incubations were performed in the absence (*a*) and presence (*b*) of serum albumin (1%, w/v). In the presence of carnitine: ●, pre-ADP rate; ▲, post-ADP rate. In the absence of carnitine: ○, pre-ADP rate; △, post-ADP rate.

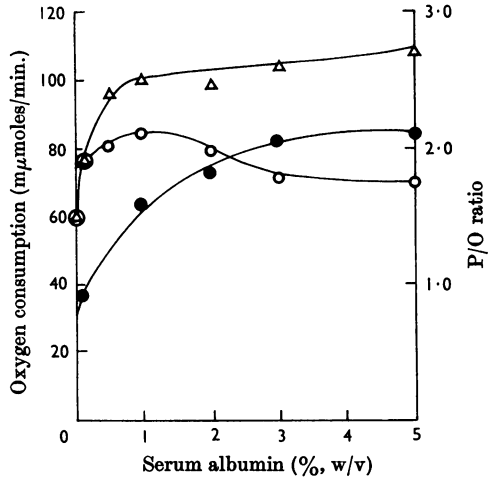


Fig. 8. Effect of serum albumin on oxidative phosphorylation and the rate of oxygen consumption (measured amperometrically) by rat-liver mitochondria in the presence of octanoate (3·5mM); other experimental details are given in the text. ○, Pre-ADP rate; △, post-ADP rate; ●, P/O ratio (corrected).

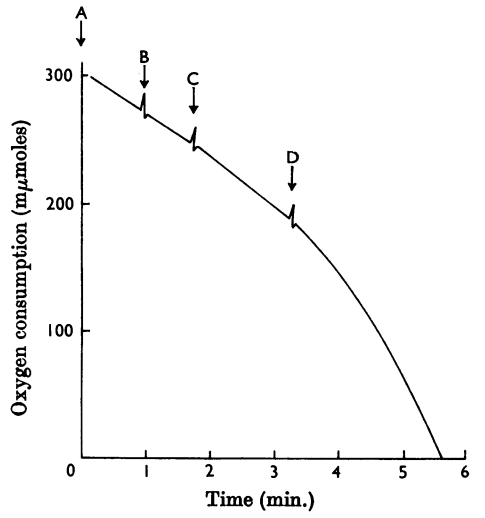


Fig. 9. Polarographic record of oxygen consumption by rat-liver mitochondria in the presence of butyrate. Incubating medium contained serum albumin (1%, w/v) and other constituents as given in the text. Additions were: A, mitochondria (0·1ml.); B, butyrate (15μmoles); C, ADP (0·8μmole); D, carnitine (1·5μmoles).

presence of ADP, the effect of carnitine on the respiration rate was not immediate but continued for up to 1·5min.

Comparison of the two suspending media. P/O ratios estimated by the manometric and amperometric procedures were carried out with different suspending media. In the manometric experiments this was done so that ketone bodies could be estimated without interference from sucrose. With octanoate (0·5 and 1·0mM) as substrate, and by using the amperometric method, there were detect-

able differences between mitochondria finally suspended in 0·3M-sucrose and in the tris–potassium chloride medium (Chappell & Perry, 1954), but these were quite small. The addition of carnitine and serum albumin, separately and together, increased the P/O ratio more markedly with the sucrose medium. Similar results were obtained with butyrate and hexanoate.

DISCUSSION

It is apparent from the results presented above that fatty acid oxidation is accompanied by ATP formation, giving P/O ratios similar to those expected for the oxidation of $FADH_2$ plus $NADH_2$. This is only the case when the concentration of the fatty acid is very low or when the reaction mixture contains sufficient serum albumin to overcome the deleterious effects of the higher concentrations of the fatty acids. Increase in the concentration of the short-chain fatty acids used causes uncoupling of oxidative phosphorylation, loss of respiratory control and, finally, inhibition of the rate of oxygen consumption. The concentration at which such effects begin is inversely related to the chain length of the fatty acid. It has been shown previously that, of the saturated series, tetradecanoic acid is the most potent uncoupler of oxidative phosphorylation, inhibitor of mitochondrial respiration (Björntorp *et al.* 1964) and of tissue respiration (Enser, 1964), and activator of adenosine triphosphatase (Pressman & Lardy, 1956). Because of the high surface activity of this fatty acid, Björntorp *et al.* (1964) have suggested the possibility of a correlation between uncoupling and surface activity.

It is possible that the fatty acids inhibit respiration by acting directly on the enzymes associated with their oxidation or on the enzymes of the electron-transport system. Succinate oxidase is inhibited in the presence of sodium oleate and other unsaturated fatty acids in such a manner (Edwards & Ball, 1954; Nakamura *et al.* 1959).

The stimulation of fatty acid oxidation by serum albumin is possibly related to the binding of fatty acids to the protein molecule. Teresi & Luck (1952) have shown that both hexanoate and octanoate can be bound to the protein in the ratio of 32 moles/mole of albumin. Removal by binding is a satisfactory explanation for the stimulation of respiration which occurs when the concentration of fatty acid is sufficient to cause respiratory inhibition in the absence of serum albumin. But the stimulation of butyrate metabolism at 10mM (Fig. 1), where there is little change in the P/O ratio on addition of serum albumin (Table 2), would require a different explanation. Catabolism of serum albumin by rat-liver mitochondrial preparations has been shown to occur (Penn, 1960) and the metabolism of glutamate and perhaps other amino acids is known to stimulate the oxidation of fatty acids (Hird, Symons & Weidemann, 1966). The effect of serum albumin in the manometric experiments, but perhaps not in the short-term amperometric determinations, is explicable in these terms.

The present work shows that the addition of carnitine to the incubation mixture increases the rate of oxidation of the short-chain fatty acids, but except with butyrate this has not previously been observed (Fritz, Kaplan & Yue, 1962). We have no explanation for the different results. The results found here are consistent with the idea that carnitine facilitates the transport of fatty acids (including those of short-chain length) to the site of oxidation within the mitochondria (Fritz, 1963). The present work shows that carnitine can also restore coupling between oxidation and phosphorylation. Similar observations with coenzyme A have been made by McMurray & Lardy (1958) and Björntorp *et al.* (1964). Björntorp *et al.* (1964) and Lehninger & Remmert (1959) explain the effect in terms of the local concentration of deleterious fatty acid being lowered by acylation. Carnitine may also act in this way.

It is possible that the increased P/O ratio observed in the presence of carnitine is only apparent, and is simply related to the short duration of the experiments by the amperometric method. An accumulation of the fatty acid as acyl-carnitine at the expense of endogenous ATP could lead to an overestimate of the P/O ratio value due to the application of a now spurious correction for ATP utilization in the activation reaction. However, as the total effect of carnitine on the system includes stimulation of oxygen consumption and increased respiratory control as well as increased P/O ratios, these speculations are inadequate as a full explanation.

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