The Effect of Hexokinase and Tricarboxylic Acid-Cycle Intermediates on Fatty Acid Oxidation and Formation of Ketone Bodies by Rat-Liver Mitochondria

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1. The oxidation of butyrate, hexanoate and octanoate by rat-liver mitochondria suspended in a tris-potassium chloride medium in the presence of malate and serum albumin has been investigated. 2. The oxidation of butyrate to acetoacetate was markedly decreased by the addition of a system competitive for ATP (hexokinase-glucose). 3. Serum albumin or tricarboxylic acid-cycle intermediates prevented the inhibition by hexokinase and in their presence a greater proportion of the oxygen consumption was contributed by the tricarboxylic acid cycle. The results suggest that the energy supply for fatty acid activation is either compartmentalized in a spatial or kinetic sense or there exists a special activating mechanism not involving ATP. 4. Malate and other tricarboxylic acid-cycle intermediates caused substantial reduction (to β -hydroxybutyrate) of the acetoacetate formed during the oxidation of butyrate, hexanoate and octanoate.

The oxidation of fatty acids by mitochondria is known to be stimulated by the addition of catalytic amounts of malate and other tricarboxylic acid-cycle intermediates (Knox, Noyce & Auerbach, 1948; Kennedy & Lehninger, 1949).

The effect of tricarboxylic acid-cycle intermediates on such systems has often been interpreted as a need for oxaloacetate, to accept and initiate the oxidation of acetyl groups. It has also been suggested that the concurrent metabolism of added intermediates provides energy in some form for the initial activation of the fatty acid (Knox *et al.* 1948; Cross, Taggart, Covo & Green, 1949).

The present paper reports the result of an investigation on the effect of tricarboxylic acid-cycle intermediates and serum albumin as stimulators of fatty acid oxidation by mitochondria and of hexokinase as a competitor for available adenosine triphosphate.

fatty acids were distilled and converted into their potassium salts. All other reagents used were of analytical grade.

Methods

Rat-liver mitochondria were prepared by the method described by Hird & Symons (1962) and were finally suspended in the tris-KCl medium of Chappell & Perry (1954). The final mitochondrial suspension in 4 ml. was derived from 10 g. of liver (fresh wt.). Incubation was carried out at 38° in Warburg flasks in the medium described by Hird & Weidemann (1966), in a final volume 3.0 ml., 0.3 ml. of the mitochondrial suspension being used. Glucose and hexokinase, when added, were tipped from the side arm at the beginning of an incubation period of 30–50 min. At the end of the experiment, protein was removed from the medium by the method of Weichselbaum & Somogyi (1941). The fatty acids remaining were estimated by steam-distillation and titration under CO₂-free conditions (Pennington, 1952) and ketone bodies were estimated as described by Hird & Weidemann (1964).

EXPERIMENTAL

Materials

Crystalline hexokinase (170 000 units/g. at 38°; for definition of one unit see the preceding paper) and ATP were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and crystalline bovine serum albumin from the Commonwealth Serum Laboratories, Melbourne, Australia. The hexokinase and serum albumin were dialysed at 2° against water before use and the activity of the hexokinase was estimated as described by Hird & Weidemann (1966). The

RESULTS

Competition between hexokinase and thiokinase for ATP

Fig. 1 shows the effect of increasing the amount of hexokinase on the oxidation of butyrate. As the hexokinase was increased, oxygen consumption steadily decreased and there was a corresponding decrease in disappearance of fatty acid and production of ketone bodies. The close parallel between

fatty acid disappearance and ketone-body production suggests that under these conditions there was little oxidation through the tricarboxylic acid cycle. It was a general feature of these experiments that fatty acid oxidation could proceed without the necessity of an ADP-regenerating system (hexokinase-glucose). This could occur if a supply of ADP was made available by a mitochondrial 'adenosine triphosphatase' stimulated by the presence of the fatty acid (see also Hird & Weidemann, 1966). Because fatty acid oxidation was almost completely abolished at 10 units of hexokinase and above, the glucose-hexokinase system apparently prevented esterification of the fatty acid with coenzyme A by removal of ATP.

Fig. 2 shows the same experiment performed in the presence of malate (mM), which is known to

have a marked effect on the oxidation of butyrate. In these experiments, oxygen consumption in the absence of hexokinase was much greater and was stimulated by the addition of up to 2 units of the enzyme before flattening off at the higher concentrations. In contrast with the system without malate, hexokinase had little inhibitory effect on the rate of oxygen consumption, even at a concentration of 50 units. It is apparent that in the presence of malate the system activating fatty acids (thiokinase reaction) represents the preferred pathway of ATP utilization, as the glucose-hexokinase seems unable to compete effectively. Fig. 2 also shows that ketone-body production fell off with increasing hexokinase, but it too was not abolished. As the hexokinase amount was increased from 0 to 2 units, the curves representing oxygen consump-

Fig. 1. Effect of hexokinase concentration on the oxidation of butyrate by rat-liver mitochondria. Experimental details are given in the text. Incubation medium contained butyrate (10mM). $\circ$, Oxygen consumption; Δ , ketone-body production; $\square$, butyrate disappearance.

Fig. 3. Effect of serum albumin on the oxidation of butyrate by rat-liver mitochondria. Experimental details are given in the text. Incubation mixture contained butyrate (10mM) and serum albumin (1%, w/v). $\circ$, Oxygen consumption; Δ , ketone-body production; $\square$, butyrate disappearance.

Fig. 2. Effect of malate on the oxidation of butyrate by rat-liver mitochondria. Experimental details are given in the text. Incubation mixture contained butyrate (10mM) and malate (1mM). $\circ$, Oxygen consumption; Δ , ketone-body production; $\square$, butyrate disappearance; $\bullet$, oxygen consumption in the presence of malate alone.

Fig. 4. Effect of malate and serum albumin on the oxidation of butyrate by rat-liver mitochondria. Experimental details are given in the text. Incubation mixture contained butyrate (10mM), malate (1mM) and serum albumin (1%, w/v). $\circ$, Oxygen consumption; Δ , ketone-body production; $\square$, butyrate disappearance; $\bullet$, oxygen consumption in the presence of malate and serum albumin.

tion and ketone-body production diverged, indicating that a progressively greater proportion of the fatty acid was metabolized through the tricarboxylic acid cycle. An overall effect was that in the presence of malate a much greater proportion of the total oxygen consumption was contributed by the tricarboxylic acid cycle.

Fig. 3 shows the effect of increasing the amount of hexokinase when serum albumin (1.0%, w/v) was also added to the system. Comparison of Figs. 1 and 3 shows that, apart from a lack of stimulation of oxygen consumption at 2 units of hexokinase, serum albumin simulated at a lower concentration the effect of malate. When both malate and serum albumin were present in the system (Fig. 4) there was no marked additive effect but a small stimulation of fatty acid removal by low concentrations of hexokinase occurred.

To test the possibility that serum albumin stimulated fatty acid oxidation by direct inhibition of hexokinase, the latter was assayed in the presence of concentrations of up to 5% (w/v) of this protein. No inhibition of hexokinase activity was observed.

Effect of tricarboxylic acid-cycle intermediates on the metabolism of fatty acids

Table 1 shows the effect of malate and related intermediates on the oxidation of butyrate in the presence and absence of serum albumin. In the presence and absence of malate the amount of

hexokinase added (2 units) caused either a marked stimulation or inhibition of butyrate oxidation. Succinate, glutamate, α -oxoglutarate and citrate as well as malate all markedly stimulated the oxidation of butyrate as measured by oxygen consumption. The oxidation of butyrate to ketone bodies was also increased, but the percentage stimulation was much less than that for oxygen consumption. The stimulation of butyrate oxidation by malate and other tricarboxylic acid-cycle intermediates therefore leads to preferential metabolism of the fatty acid through the tricarboxylic acid cycle as the ratio of moles of ketone bodies formed/moles of oxygen consumed falls (Table 1). A further effect of the addition of such intermediates was the high proportion of the total ketone bodies as β -hydroxybutyrate. The presence of serum albumin in the reaction mixture led to an increase in the oxygen consumption and this was usually accompanied by an increase in ketone-body production.

In Table 2 the oxidation of hexanoate and octanoate is shown in relation to that of butyrate. The oxidation of these fatty acids was also markedly stimulated by malate in the presence of serum albumin. In each case ketone-body production was stimulated and there was considerable reduction of acetoacetate to β -hydroxybutyrate. With malate and the longer-chain fatty acids, ketone-body production was stimulated to a greater extent than oxygen consumption.

Table 1. *Stimulation of oxidation of butyrate by malate and metabolically related compounds*

Rat-liver mitochondria suspended in the tris-KCl medium of Chappell & Perry (1954) were incubated for 30 min. at 38° in 3.0 ml. of medium containing butyrate (10 mM). Hexokinase (2 units) was added to each flask and serum albumin (0.5%, w/v) was added where indicated. Results are expressed in μ moles/0.3 ml. of mitochondrial suspension/30 min.

Expt. no.	Butyrate (10 mM) plus additions (0.3 mM)	Serum albumin (0.5%)	Oxygen consumption (μ moles)	Ketone bodies formed		Ratio moles of ketone bodies formed moles of O ₂ consumed
				β -Hydroxybutyrate (μ moles)	β -Hydroxybutyrate (%)	
1	None	—	1.9	1.5	0	0.80
		+	6.0	2.1	5	0.35
	Malate	—	11.7	2.2	41	0.19
		+	13.5	2.3	48	0.17
	Succinate	—	11.6	2.4	47	0.21
		+	13.8	3.3	63	0.24
	Glutamate	—	9.9	3.1	26	0.32
		+	12.2	3.4	33	0.28
2	None	—	2.6	2.0	10	0.78
		+	6.9	2.7	16	0.39
	Malate	—	13.0	2.8	43	0.22
		+	14.5	3.0	58	0.21
	α -Oxoglutarate	—	5.3	3.0	28	0.57
		+	9.1	3.1	26	0.35
	Citrate	—	15.5	3.6	54	0.23
		+	15.9	3.6	65	0.22

Table 2. *Stimulation of oxidation of butyrate, hexanoate and octanoate by malate*

Rat-liver mitochondria suspended in the tris-KCl medium of Chappell & Perry (1954) were incubated as described in Table 1. All flasks contained hexokinase (2 units) and serum albumin (0.5%, w/v). Results are expressed in μ moles/0.3 ml. of mitochondrial suspension/30 min.

Expt. no.	Additions	Oxygen consumption (μ moles)	Ketone bodies formed		Ratio moles of ketone bodies formed moles of O ₂ consumed
			(μ moles)	β -Hydroxy- butyrate (%)	
1	Butyrate (10 mm) plus:				
	—	8.4	4.0	10	0.48
	malate (0.3 mm)	16.5	6.4	46	0.39
2	Hexanoate (7.5 mm) plus:				
	—	6.4	2.3	0	0.35
	malate (0.3 mm)	10.4	5.4	65	0.52
3	Octanoate (5 mm) plus:				
	—	9.0	2.9	0	0.33
	malate (0.3 mm)	11.0	5.0	40	0.45

DISCUSSION

Stimulation of fatty acid oxidation. Under certain experimental conditions, the oxidation of fatty acids may be limited by the availability of ATP, especially when reactions competing for ATP are present in the system. Hexokinase has been shown to compete successfully with the activating system for fatty acid oxidation (thiokinase reaction) and in high concentration can completely inhibit the metabolism of the fatty acid. The addition of a small amount of malate prevents this inhibition. Presumably malate, by being metabolized as such and/or by acting as a source of oxaloacetate, stimulates the tricarboxylic acid cycle and provides ATP, or some equivalent energy source, at an increased rate, or in a form which is available for the activation reaction but which is unavailable to the extramitochondrial hexokinase.

The stimulation of fatty acid oxidation by tricarboxylic acid-cycle intermediates has been shown by Cross *et al.* (1949) to be inhibited by uncoupling agents. It is known, also, that the substitution of NADH₂ for such intermediates promotes the activation of octanoate and its subsequent oxidation to acetoacetate (Kennedy & Lehninger, 1951). In each case the increased metabolism of the fatty acid can be attributed to an increased supply of ATP for the activation of the fatty acid. In the presence of malonate the addition of intermediates of the tricarboxylic acid cycle stimulates the conversion of fatty acid into acetoacetate, the stimulation in this case being apparently unrelated to the availability of oxaloacetate (Cheldelin & Beinert, 1952).

A simple explanation of the accumulated facts is that, in the presence of malate and serum albumin,

newly formed ATP is either (a) compartmentalized, i.e. it is available to the activating enzymes and substantially unavailable to hexokinase and similar enzymes utilizing ATP which are exterior to the mitochondrion, or (b) in the presence of malate it is produced at a faster rate with more being available for each competing reaction. The existence of a barrier to exogenous ATP at the level of the mitochondrial membrane is suggested by the work of Judah & Rees (1953). It is also possible (Bode & Klingenberg, 1964) that intermediates other than ATP, i.e. intermediates of high group transfer potential produced by the cytochrome system during the oxidation of tricarboxylic acid intermediates, or perhaps other nucleotide triphosphates, may serve for the activation of fatty acids. In this connexion a GTP-specific thiokinase has recently been identified in ox-liver mitochondria (Rossi & Gibson, 1964). There is also the possibility of thiophorase reactions leading to formation of fatty acid-CoA esters, i.e. derived from acetyl-CoA and succinyl-CoA.

The stimulation of oxidation by serum albumin is not readily explicable. Perhaps by stabilizing mitochondrial permeability in some way it raises the internal energy supply or restricts the availability of ATP to the glucose-hexokinase system. There is the possibility, however, that it may, by its catabolism (Penn, 1960), contribute precursors of tricarboxylic acid-cycle intermediates by metabolism of related amino acids.

Direction of fatty acid oxidation. The preferential direction of acetyl-CoA into the tricarboxylic acid cycle by malate and other intermediates is a separate effect from that which causes the overall increase in metabolism of the fatty acid. The oxidation of fatty acids to acetoacetate does not require

the presence of endogenous oxaloacetate or its precursors (Lehninger, 1946; Cheldelin & Beinert, 1952), but the addition of these, in the absence of malonate, does, however, lower the proportion of fatty acid converted into acetoacetate (Lehninger, 1946). This effect has also been shown with carnitine esters of fatty acids when succinate is added to the system (Bremer, 1962). The most likely explanation is that the provision of oxaloacetate initiates metabolism through the tricarboxylic acid cycle. There is, however, the possibility that metabolic direction in this case is mediated by the effect of saturation on the kinetics of the two pathways.

Formation of ketone bodies. Substantial reduction of the acetoacetate formed during fatty acid oxidation has been shown to occur when malate or some other tricarboxylic acid-cycle intermediate is added to the medium. The reduction of added acetoacetate by succinate (Ernster, 1962; Kulka, Krebs & Eggleston, 1961; Azzone, Ernster & Weinbach, 1963), and of newly synthesized acetoacetate from butyrate, by rumen epithelium in the presence of glucose has been previously established (Hird & Symons, 1959). From the present results it is apparent that the power to reduce acetoacetate can come from a number of sources, but the reduced coenzymes formed during the oxidation of fatty acids are not by themselves very effective sources of hydrogen.

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