Formation and Utilization of 3-Hydroxy-3-methylglutarate in Liver Mitochondria of Starved and Streptozotocin-Diabetic Rats

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Kidney and liver mitochondria of rat, rabbit and guinea pig are able to transform 3-hydroxy-3-methylglutarate into acetoacetate, whereas ox liver mitochondria and rat mitochondria of heart, diaphragm and brain do not exhibit such an activity. Starvation and streptozotocin treatment decreases the formation of acetoacetate from 3-hydroxy-3-methylglutarate. Addition of acetoacetate and succinate to the incubation media of mitochondria results in a decrease in the transformation of 3-hydroxy-3-methylglutarate into acetoacetate. A 3-hydroxy-3-methylglutaryl-CoA hydrolase is present in rat liver mitochondria; the activity does not show appreciable changes after starvation or streptozotocin treatment.

In the early 1950s the main site of synthesis of 3-hydroxy-3-methylglutaryl-CoA was identified with the mitochondrion, and from there the 3-hydroxy 3-methylglutaryl-CoA was postulated to be transported to other cellular sites (Block *et al.*, 1954).

It is now well established that there are at least two different pathways for the synthesis of 3-hydroxy-3-methylglutaryl-CoA: one is involved in ketogenesis and is located in the mitochondrion, and the other is found in the cytoplasm and is involved in the synthesis of cholesterol (Williamson *et al.*, 1968; Lane *et al.*, 1973; Clinkenbeard *et al.*, 1973).

We have previously reported (Deana *et al.*, 1974) that 3-hydroxy-3-methylglutaryl-CoA can be formed in rat liver mitochondria through the following reaction:

To determine whether this reaction has relevance to the process of ketogenesis we have studied the activation of 3-hydroxy-3-methylglutarate in conditions of ketosis caused by either starvation or streptozotocin-induced diabetes.

The transformation of 3-hydroxy-3-methyl[3-¹⁴C]-glutarate into acetoacetate via 3-hydroxy-3-methylglutaryl-CoA by rat liver mitochondria posed the question of a possible physiological formation of 3-hydroxy-3-methylglutarate within the mitochondria. We therefore investigated the existence of a mitochondrial 3-hydroxy-3-methylglutaryl-CoA deacylase activity.

Materials and Methods

Wistar albino rats (250–300 g) of either sex were used for the experiments. For preliminary experiments livers and kidneys of guinea pig, rabbit and ox were also used.

The rats were divided into four groups. The control group received a standard laboratory pellet diet and the rats were deprived of food 16 h before being killed by decapitation. A second group was starved for 72 h. The rats termed the streptozotocin-treated group were injected intraperitoneally with a solution of 40 mg of streptozotocin/ml, corresponding to 8 mg/100 g body wt. They were fed *ad libitum* until 16 h before being killed and their food intake during the treatment was not significantly different from that of control rats. The streptozotocin (a gift of Dr. H. B. Wood, Jr., National Institutes of Health, Bethesda, MD, U.S.A.) was dissolved immediately before injection in 0.9% (w/v) NaCl slightly acidified (pH 5.0) with HCl. In this case the control group was injected with an equal amount of acidified NaCl. The concentrations of plasma acetoacetate in streptozotocin-treated rats were 6 to 10 times those of the controls, and this was taken as an indication of the diabetic state.

The experiments were carried out with rats with a lower activity of mitochondrial 3-hydroxy-3-methylglutarate utilization than those used in the previous work (Deana *et al.*, 1974). This might be due to the different animal strain and the different temperature of the environment where the animals were raised. In general we observed that the activity seems to increase with increasing environmental temperature.

Mitochondria were isolated in 0.23 M-sucrose/20 mM-Tris/HCl, pH 7.3, essentially by the method of Schneider & Hogeboom (1950). Lysed mitochondria were prepared by homogenizing them with 20 mM-Tris/HCl, pH 7.4, for 3 min in a Potter homogenizer. Microsomal fractions were prepared by centrifugation of the supernatant collected after separation of mitochondria at 100000g for 1 h.

The determination of acetoacetate, which is the end product of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA metabolism, was used to measure indirectly the activation of 3-hydroxy-3-methylglutamate. This method was used because of the presence in the mitochondrion of a high 3-hydroxy-3-methylglutaryl-CoA lyase activity (EC 4.1.3.4), which on the one hand makes the direct determination of 3-hydroxy-3-methylglutaryl-CoA unreliable, and on the other hand assures us that acetoacetate formation is a true reflection of 3-hydroxy-3-methylglutaryl-CoA produced. For this assay the incubation mixture contained 20 mM-potassium phosphate, pH 7.4, 20 mM-NaCl, 30 mM-KCl, 4 mM-MgCl₂, 2.5 mM-potassium 3-hydroxy-3-methyl[3-¹⁴C]-glutamate, 80 mM-sucrose and 15–20 mg of mitochondrial protein; the total volume was 2 ml. For the routine assay no further additions were required, since the reaction probably was initiated by the endogenous substrates and self-maintained by the acetyl-CoA produced by the lyase hydrolysing the 3-hydroxy-3-methylglutaryl-CoA. The incubation was carried out at 37°C for 15 min and the reaction was stopped by addition of 0.1 ml of 70% (w/v) HClO₄. Acetoacetate was measured in 0.7 ml samples of supernatant as described by Walker (1954).

The 3-hydroxy-3-methylglutaryl-CoA lyase activity was measured by incubation of the hypo-osmotically lysed mitochondria, prepared as described above, with synthetic 3-hydroxy-3-methylglutaryl-CoA and subsequent determination of acetoacetate formed. The incubation mixture (final volume 1 ml) consisted of 120 μmol of Tris/HCl, pH 7.6, 10 μmol of MgCl₂, 5 μmol of cysteine, 2 mg of mitochondrial protein and 0.1–0.8 μmol of 3-hydroxy-3-methylglutaryl-CoA. Samples were removed after 5 min incubation at 37°C mixed with 0.2 ml of 25% trichloroacetic acid and centrifuged 5 min at 2500g; acetoacetate concentration was determined in samples of the supernatant.

Experiments were also carried out to eliminate the possibility that an activity catalysing the transfer of CoA from 3-hydroxy-3-methylglutaryl-CoA to acetoacetate exists within the mitochondrion. The same medium used to measure 3-hydroxy-3-methylglutamate activation was used, with the addition of 10 μmol of [3-¹⁴C]acetoacetate, and incubated at 37°C for 20 min. After addition of 1 μmol of 3-hydroxy-3-methylglutamate as a carrier, the reaction was blocked with 0.2 ml of 25% trichloroacetic acid.

A 0.70 ml sample of the supernatant was strongly acidified with 0.3 ml of 1 M-HCl and free acetoacetate was removed as acetone by steam-distillation. The presence of radioactive 3-hydroxy-3-methylglutamate was then checked by paper chromatography (Whatman no. 3MM) with butan-1-ol/acetic acid/water (5:2:3, by vol.) as eluent as described by Louw *et al.* (1969) and scanning of the strip with a Packard radiochromatographic scanner. In the presence of CoA-transferring activity, first [¹⁴C]acetoacetyl-CoA should be formed, and then 3-hydroxy-3-methyl[3-¹⁴C]glutamate, by means of 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5) and 3-hydroxy-3-methylglutaryl-CoA hydrolase (EC 3.1.2.5).

The 3-hydroxy-3-methylglutaryl-CoA hydrolase activity was assayed by measuring the appearance of free thiol groups by the method of Ellman *et al.* (1961): 1 μmol of synthetic 3-hydroxy-3-methylglutaryl-CoA, 100 μmol of Tris/HCl, pH 7.8, 2.5 μmol of EDTA, 40 nmol of fluorocitrate and 1.5 mg of protein of different subcellular fractions were incubated in a final volume of 1 ml. The reaction was stopped by addition of 0.2 ml of 25% trichloroacetic acid and samples were centrifuged for 5 min at 3000g. Thiol determinations were carried out on 0.7 ml samples of the supernatant fraction, after adjustment of pH to 7.8 by adding 2 M-Tris.

[¹⁴C]Acetoacetate was measured by the procedure proposed by Weichselbaum & Somogyi (1941) and modified by Bates *et al.* (1968).

Total protein content was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Radioactivity measurements were carried out with Insta-Gel (Packard, Downers Grove, IL, U.S.A.) as scintillant liquid.

All chemicals were of analytical grade. 3-Hydroxy-3-methylglutaryl-CoA was synthesized by the method of Hilz *et al.* (1958) modified by Louw *et al.* (1969). Synthetic trisodium fluorocitrate was a gift from Professor P. Buffa (University of Modena, Italy) and was prepared by the method of Peters *et al.* (1953) and contained about 4% of the inhibitory isomer.

Results

In preliminary experiments the formation of acetoacetate from 3-hydroxy-3-methylglutamate was investigated in organs of different animal species as shown in Table 1. Rat liver appears to be the organ with the highest activity of acetoacetate formation from 3-hydroxy-3-methylglutamate. Ox liver and kidney mitochondria did not show any detectable activity, nor did mitochondria of rat heart, brain and diaphragm.

On this basis rat liver mitochondria were chosen for subsequent experiments. The transformation of

Table 1. Formation of [¹⁴C]acetoacetate from 3-hydroxy-3-methyl[3-¹⁴C]glutarate in mitochondria of different animal organs

The results are given as means ±s.d. for the numbers of experiments in parentheses. Experimental details are given in the text.

	Acetoacetate formed (nmol/h per mg of protein)			
	Rat	Guinea pig	Rabbit	Ox
Liver	24.6 ± 4.2 (12)	11.5 ± 2.7 (6)	7.9 ± 0.6 (6)	0.0 (3)
Kidney	2.7 ± 0.6 (6)	8.6 ± 1.5 (6)	5.4 ± 1.3 (6)	0.0 (3)

Fig. 1. Formation of acetoacetate in liver mitochondria of starved rats

Mitochondria were incubated with 3-hydroxy-3-methyl[3-¹⁴C]glutarate and [¹⁴C]acetoacetate formed (▲) was measured. In parallel experiments, endogenous acetoacetate (●) was measured without any addition to the incubation medium. Further details are given in the Materials and Methods section.

3-hydroxy-3-methyl[3-¹⁴C]glutarate into [¹⁴C]acetoacetate was assayed in starved and streptozotocin-diabetic rats.

Fig. 1 illustrates acetoacetate formation by rat liver mitochondria as a function of the duration of the period of starvation. The production of [¹⁴C]acetoacetate synthesized by mitochondria decreased with the length of starvation, whereas the production of endogenous unlabelled acetoacetate increased.

Liver mitochondria of streptozotocin-treated rats showed a marked increase in production of unlabelled acetoacetate, associated with a net decrease of [¹⁴C]acetoacetate formation (Table 2).

To rule out the possibility that the decreased [¹⁴C]acetoacetate production in the above conditions depends on a shortage of 3-hydroxy-3-methylglutaryl-CoA lyase, this activity was measured by incubating lysed rat liver mitochondria in the presence of different 3-hydroxy-3-methylglutaryl-CoA concentrations. The maximum 3-hydroxy-3-methylglutaryl-CoA lyase activity obtained from a double-reciprocal plot of acetoacetate formed against substrate concentration was 30nmol of acetoacetate/min per mg of protein. Such a value is much higher than that necessary to produce the acetoacetate found in our experiments, and it is even increased in starved and diabetic rats (Williamson *et al.*, 1968). We can therefore exclude the possibility that the lower

Table 2. Acetoacetate formed by rat liver mitochondria of streptozotocin-treated animals incubated with 3-hydroxy-3-methyl[3-¹⁴C]glutarate

Results are the means ±s.d. for six animals. Experimental details are given in the text.

	Time after streptozotocin injection (h)	Mitochondrial [¹⁴ C]acetoacetate (nmol produced/h per mg of mitochondrial protein)	Total mitochondrial acetoacetate (nmol produced/h per mg of mitochondrial protein)	Plasma acetoacetate (μmol of acetoacetate/100 ml)
Controls	—	21.8 ± 3.0	34 ± 4	8 ± 2.7
Diabetic rats	24	19.0 ± 3.2	51 ± 8	34 ± 3.2
	48	9.7 ± 1.8	77 ± 12	63 ± 8.6
	72	8.9 ± 2.2	87 ± 16	78 ± 3.5
	96	8.6 ± 2.4	90 ± 17	73 ± 9.9

Table 3. *Acetoacetate produced by rat liver mitochondria incubated with 3-hydroxy-3-methyl[3-¹⁴C]glutarate and palmitoylcarnitine with and without fluorocitrate*

The results (means $\pm$ s.d. of six experiments) express the amount of acetoacetate produced: 2 mM-ADP was added to the incubation medium. Where added, fluorocitrate concentration was 10 μ M. Experimental details are given in the text.

Additions	[¹⁴ C]Acetoacetate (nmol/h per mg of protein)	Total acetoacetate (nmol/h per mg of protein)
None	20.9 $\pm$ 3.2	33.6 $\pm$ 4.0
Palmitoylcarnitine (25 μ M)	25.7 $\pm$ 5.5	34.5 $\pm$ 3.8
Palmitoylcarnitine (100 μ M)	14.0 $\pm$ 2.7	49.3 $\pm$ 5.9
Palmitoylcarnitine (200 μ M)	7.1 $\pm$ 1.6	78.0 $\pm$ 12.0
Palmitoylcarnitine (200 μ M) + fluorocitrate	2.3 $\pm$ 0.8	96.8 $\pm$ 17.0
Fluorocitrate	1.0 $\pm$ 0.6	3.5 $\pm$ 0.7

Table 4. [¹⁴C]Acetoacetate formed by normal rat liver mitochondria incubated with 3-hydroxy-3-methyl[3-¹⁴C]glutarate in the presence of different metabolites

The results are given as means $\pm$ s.d. of four experiments. Experimental details are given in the text.

Additions (mM)	[¹⁴ C]Acetoacetate formed (nmol/h per mg of protein)
None	24.7 $\pm$ 4.0
Acetoacetate 0.5	21.0 $\pm$ 3.2
1.0	17.2 $\pm$ 3.8
2.0	11.4 $\pm$ 2.9
Succinate 1.0	20.2 $\pm$ 2.7
2.5	12.5 $\pm$ 2.8
5.0	5.8 $\pm$ 1.1

[¹⁴C]acetoacetate formation shown by ketotic animals is due to a lyase deficiency.

Radioactive acetoacetate formation by mitochondria was also measured after incubation with different amounts of palmitoylcarnitine as shown in Table 3. Low concentrations of palmitoylcarnitine induced a slight increase in radioactive acetoacetate, whereas high concentrations of palmitoylcarnitine decreased the radioactive acetoacetate formation with an increase of non-radioactive acetoacetate. The addition of fluorocitrate made this phenomenon more evident. Under such experimental conditions the mitochondrial acetyl-CoA concentrations are most probably very high, therefore the lower formation of labelled acetoacetate should exclude a transfer reaction of a CoA molecule from acetyl-CoA to 3-hydroxy-3-methyl[¹⁴C]glutarate.

The existence of an activity able to transport CoA from 3-hydroxy-3-methylglutaryl-CoA to acetoacetate was also excluded (see the Materials and Methods section).

Table 4 shows the production of labelled acetoacetate by incubating rat liver mitochondria with different amounts of non-radioactive acetoacetate. A marked decrease in [¹⁴C]acetoacetate was found,

and a similar decrease was also observed when succinate was added to the incubation medium.

Hence the presence of a 3-hydroxy-3-methylglutaryl-CoA deacylase activity in rat liver mitochondria was investigated. To avoid any permeability problem of 3-hydroxy-3-methylglutaryl-CoA, lysed mitochondria were used. It was observed (Table 5) that mitochondrial membranes are the subcellular fraction showing the highest deacylase activity.

No significant differences were found between the mitochondrial activity of starved or streptozotocin-treated animals and the control animals.

Discussion

The data reported in this paper show a decreased transformation of 3-hydroxy-3-methylglutarate into acetoacetate, via 3-hydroxy-3-methylglutaryl-CoA, in conditions characterized by a pronounced ketosis. Such a transformation might be influenced by the 3-hydroxy-3-methylglutaryl-CoA deacylase activity that we found to be present in rat liver mitochondria. As described by Saleemuddin & Siddiqi (1972) the cytoplasmic deacylase activity markedly increases under conditions of prolonged starvation. However, no significant changes in activity in liver mitochondria of starved or streptozotocin-treated rats emerge from our results: this allows us to exclude the suggestion that changes of 3-hydroxy-3-methylglutaryl-CoA deacylase activity are responsible for the decreased formation of acetoacetate from 3-hydroxy-3-methylglutarate observed in ketotic animals.

The activity of 3-hydroxy-3-methylglutaryl-CoA lyase evaluated on the basis of the apparent V_{max} value largely exceeds the activity required for the formation of the acetoacetate found in our experiments. The decreased radioactive acetoacetate formation observed in experiments with mitochondria of starved or diabetic rats and in those with normal mitochondria incubated with palmitoylcarnitine should not depend therefore on 3-hydroxy-3-methylglutaryl-CoA lyase deficiency (owing to the increased amounts of unlabelled 3-hydroxy-3-methylglutaryl-

Table 5. 3-Hydroxy-3-methylglutaryl-CoA hydrolase in liver subcellular fractions of starved and diabetic rats

The results are given as means $\pm$ s.d. for six experiments. Experimental details are given in the Materials and Methods section.

	CoA formed (nmol/h per mg of protein)		
	Controls	Starved rats	Diabetic rats
Lysed mitochondria	117 $\pm$ 23	132 $\pm$ 19	142 $\pm$ 26
Mitochondrial membranes	168 $\pm$ 31	189 $\pm$ 22	183 $\pm$ 22
Mitochondrial matrix	76 $\pm$ 18	85 $\pm$ 14	91 $\pm$ 28
Cytosol	108 $\pm$ 16	—	—
Microsomal fraction	41 $\pm$ 11	—	—

CoA). Most probably the effect observed in the above cases might be the consequence of a deficiency of succinyl-CoA (owing to a decreased availability of free CoA or tricarboxylic acid-cycle activity).

Acetoacetate has no effect on 3-hydroxy-3-methylglutaryl-CoA lyase (Coon, 1962) and in mammalian liver it is not transformed into acetoacetyl-CoA. When unlabelled acetoacetate was added to the incubation medium, rat liver mitochondria appeared less efficient in producing [14 C]acetoacetate from 3-hydroxy-3-methyl[3- 14 C]glutarate.

In addition, our data show that in all conditions where there is a high endogenous acetoacetate production the amount of 3-hydroxy-3-methylglutarate transformed into acetoacetate decreases. Therefore it is quite conceivable that acetoacetate could exert a feedback inhibition on 3-hydroxy-3-methylglutarate activation to 3-hydroxy-3-methylglutaryl-CoA.

The inhibitory effect of succinate on [14 C]acetoacetate formation, which is roughly proportional to the former's concentration, might be explained as an effect of succinate on the 3-hydroxy-3-methylglutaryl-CoA formation by shifting the equilibrium of eqn. (1) towards the left-hand side.

It has been found that 3-hydroxy-3-methylglutarate is involved in the regulation of the synthesis of several lipids, and it decreases cholesterologenesis (Beg & Siddiqi, 1967; Lupien *et al.*, 1973; Yousufzai & Siddiqi, 1976). The decreased transformation of 3-hydroxy-3-methylglutarate into acetoacetate in conditions of pronounced ketosis should cause an intracellular accumulation of free 3-hydroxy-3-methylglutarate, whose metabolic fate is still a matter of investigation and it might indicate that this compound is involved also in the regulation of ketone-body synthesis.

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