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# Formation of Acetoacetate from 3-Hydroxy-3-methylglutarate by Rat Liver and Isolation of a Mitochondrial Coenzyme A-Transferase Activity Involved

By R. DEANA, R. MENEGHELLO, L. MANZI and C. GREGOLIN
*Department of Medical Biochemistry, University of Nairobi, Nairobi, Kenya, and Institute of Biological Chemistry, University of Padova, 'Centro per lo Studio della Fisiologia dei Mitocondri del C.N.R.', 35100 Padova, Italy*

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1. Formation of acetoacetate from 3-hydroxy-3-methylglutarate was observed in the perfused rat liver. Production of 3.5 $\mu$mol of acetoacetate/h per g of tissue was obtained. 2. Formation of acetoacetate was catalysed mainly by the mitochondrial fraction of the homogenized liver, at a rate of 62 nmol/h per mg of protein. 3. Experiments with hydroxy-[3-$^{14}$C]methylglutarate demonstrated that the acetoacetate formed was derived mainly from this compound. 4. A mitochondrial transferase activity catalysing the transfer of a CoA molecule from succinyl-CoA (3-carboxypropionyl-CoA) to hydroxymethylglutarate was shown. The $K_m$ value for hydroxymethylglutarate was $5 \times 10^{-3}$ M.

It is known that 3-hydroxy-3-methylglutaryl-CoA is an important intermediate in the biogenesis of cholesterol and steroids, in the degradation of leucine and in the formation of ketone bodies (i.e. acetoacetate, 3-hydroxybutyrate and acetone). Cleavage of hydroxymethylglutaryl-CoA by hydroxymethylglutaryl-CoA lyase, a mitochondrial enzyme particularly abundant in the liver, is the most important pathway of formation of acetoacetate (Williamson *et al.*, 1968).

In the present paper it is shown that an alternative pathway for acetoacetate formation utilizes free hydroxymethylglutarate in rat liver preparations. Since the substrate for hydroxymethylglutaryl-CoA lyase is not hydroxymethylglutarate but hydroxymethylglutaryl-CoA (Coon, 1962), the formation of acetoacetate from hydroxymethylglutarate must involve a previous activation of it to hydroxymethylglutaryl-CoA. The characteristics of the overall process are discussed here.

## Materials and Methods

Male albino rats of the Wistar strain weighing 200–300 g were used. Rat liver was isolated and perfused for 2 h at 37°C as described previously (Manzi *et al.*, 1973). Liver mitochondria were isolated by the method of Schneider & Hogeboom (1950) from rats starved for 16 h. Hydroxymethylglutaryl-CoA lyase was prepared from calf liver as described by Middleton & Apps (1969).

The cell-free preparations were incubated in a standard medium containing 35–50 mM-sucrose, 8 mM-potassium phosphate, pH 7.4, 7 mM-NaF, 10 mM NaCl, 35 mM-KCl, 4 mM-$MgCl_2$ and 5 mM-hydroxymethylglutarate (potassium salt) (basal medium). Total volume was 2 ml. Standard time of incubation was 30 min, temperature 35°C and the protein concentration 18–25 mg as determined by the method of Lowry *et al.* (1951) with albumin as standard. The reaction was stopped by addition of 0.1 ml of 70% (w/v) $HClO_4$.

Mitochondria were depleted of phosphate by preincubating them for 7 min at 37°C in a basal medium where phosphate buffer was replaced by 0.1 M-Tris-HCl buffer. The mixture contained 7 mg of protein/ml, 1 mM-glucose, 0.5 mM-ADP and 5 units/ml of hexokinase (Boehringer, Mannheim, Germany) as a trapping system. After preincubation the mitochondria were washed twice by resuspending them in 0.25 M-sucrose and centrifuging for 10 min at 7000*g*. Freeze-dried mitochondria were prepared by freezing their suspension with a mixture of acetone–$CO_2$ (−77°C) amd freeze-drying them for 3 h. Before use they were resuspended in 0.25 M-sucrose. Succinyl-CoA was synthesized as described by Wieland & Rueff (1953).

Acetoacetate was measured enzymically with 3-hydroxybutyrate dehydrogenase (Boehringer) (EC 1.1.1.30) by the method of Mellanby & Williamson (1963) or by the chemical method described by Walker (1954). 3-Hydroxybutyrate was assayed by the enzymic method of Mellanby & Williamson (1963). Hydroxymethylglutarate was obtained from Fluka (Buchs, Switzerland) and hydroxy[3-$^{14}$C]methylglutarate from New England Nuclear (Boston, Mass., U.S.A.). All other products used (i.e. CoA, ATP, $NAD^+$, GTP, 2,4-dinitrophenol) were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Radioactivity was measured with a Packard liquid-scintillation counter and Insta-gel solution from Packard Instruments Co. (Downers Grove, Ill., U.S.A.) was used as scintillant

liquid. The differential centrifugation was carried out with a Spinco L50 ultracentrifuge. For the perfusion a Passoni E.C. 3A instrument (Milano, Italy) was used.

## Results

The first indication that hydroxymethylglutarate acts as precursor of acetoacetate was obtained by the use of the liver perfusion technique. In a typical experiment, a liver of about 8g was used and 6mM-hydroxymethylglutarate was added to 60ml of the recycling perfusion fluid. Acetoacetate formed at different times was compared with that obtained from a control liver, perfused without hydroxymethylglutarate. A net formation of 7$\mu$mol of acetoacetate/g of tissue was observed with the liver perfusion in the presence of hydroxymethylglutarate at the end of the 2h experiment (Table 1). The increase in acetoacetate production was constant and reproducible.

The results obtained with perfused livers were extended through experiments with rat liver homogenates and subcellular fractions. We assume that acetoacetate production from hydroxymethylglutarate is dependent on the activation of the latter to hydroxymethylglutaryl-CoA. To study this activation process we measured acetoacetate production in an incubation system which included added calf liver hydroxymethylglutaryl-CoA lyase. Direct measurement of hydroxymethylglutaryl-CoA formation was not practicable.

Whole homogenates, mitochondria and post-mitochondrial supernatants were suspended in the basal medium supplemented with CoA, ATP, $NAD^+$, succinyl-CoA and calf liver hydroxymethylglutaryl-CoA lyase.

The formation of acetoacetate was catalysed mainly by the mitochondrial fraction (Table 2). This fraction was used for subsequent experiments, to analyse requirements and characteristics of the process. The results reported in Tables 3 and 4, indicate that acetoacetate formation from hydroxymethylglutarate is substantially catalysed by intact mitochondria incubated in the minimal basal medium.

Over the first 20min of incubation the acetoacetate formation catalysed by mitochondria was proportional to time. The activity was also proportional to the protein concentration up to 15mg/ml of the incubation medium (Fig. 1). The plot of acetoacetate formed versus concentration of hydroxymethylglutarate approaches the classical Michaelis–Menten curve (Fig. 2). An apparent $K_m$ value of $5\times10^{-3}$M was obtained.

Table 1. *Acetoacetate formation from hydroxymethylglutarate in rat liver perfusion experiments*

Rat livers of about 8g and 60ml of recycling perfusion fluid [defibrinated homologous blood diluted 1:3 with Krebs & Henseleit (1932) medium containing 0.1% glucose] were used. Rats were deprived of food 3h before the experiments. Acetoacetate was determined in the neutralized 5% $HClO_4$ supernatant of samples of the perfusion fluid. The acetoacetate/3-hydroxybutyrate ratio at the end of the experiment was 1.82±0.36 for the controls and 1.61±0.28 for the hydroxymethylglutarate-perfused livers. The results are given as means±S.E.M. for three experiments.

| Addition to the perfusion fluid | Acetoacetate formed at different times ($\mu$mol/g of tissue) | | | |
|---|---|---|---|---|
| Time ... | 0 min | 30 min | 60 min | 120 min |
| None | 0.15±0.08 | 0.91±0.28 | 2.11±0.33 | 3.74±0.9 |
| Hydroxymethylglutarate (6mM) | 0.24±0.10 | 3.97±0.65 | 6.09±0.87 | 10.37±2.1 |

Table 2. *Acetoacetate formation from hydroxymethylglutarate catalysed by different rat liver subcellular fractions*

Different fractions were suspended in the basal medium in the presence of calf liver hydroxymethylglutaryl-CoA lyase (10mg/ml) with either no addition or with 0.5mM-succinyl-CoA, or with 2.5mM-ATP, 0.1mM-CoA and 0.6mM-$NAD^+$ respectively. Acetoacetate is expressed in nmol/h per mg of protein. The results are given as means±S.E.M. for three experiments.

| Fraction | Acetoacetate formed | | |
|---|---|---|---|
| Additions ... | None | Succinyl-CoA | ATP+$NAD^+$+CoA |
| Whole homogenates | 10± 3.0 | 11± 4.1 | 7±3.0 |
| Homogenates without nuclei | 18± 5.5 | 16± 7.8 | 10±3.4 |
| Cytoplasmic fraction | 1± 0.7 | 2± 1.2 | 0 |
| Mitochondria | 60± 7.8 | 54±14 | 37±8.5 |
| Sonicated mitochondria | 23±12 | 34± 9.2 | 26±7.5 |

The ratio acetoacetate/β-hydroxybutyrate ranged from 8:1 to 10:1. Mitochondria incubated in the basal medium lacking phosphate or $F^-$ did not show any significant change in acetoacetate production.

Mitochondria were incubated with the basal medium in the presence of hydroxy[3-$^{14}$C]methylglutarate. Radioactive acetoacetate was determined at the end of the incubation in the reaction mixture by a modification of the method of Van Slyke (1917) (Weichselbaum & Somogyi, 1941); the total amount of acetoacetate formed was also measured. The radioactive acetoacetate was 73% of the total amount; however, when ethyl [3-$^{14}$C]acetoacetate was added to a trichloroacetic acid-treated incubation system only 75% of the radioactivity added could be measured (Table 5). This shows that in the present experiments acetoacetate was practically all formed from the added hydroxymethylglutarate and that the production from endogenous sources interferes negligibly with the process studied.

Addition of $NAD^+$ and CoA does not affect the standard activity, probably owing to a permeability barrier preventing these compounds from reaching the inner compartment of the mitochondrion.

Acetoacetate formation was inhibited in mitochondria prepared and tested in the presence of 2mM-EDTA [$Mg^{2+}$ being omitted from the assay mixture (Table 6)], but further addition of citrate and α-oxoglutarate restored, if not increased, the original activity.

In phosphate-depleted mitochondria in the presence of $Mg^{2+}$ the activity was strongly depressed and could be completely restored by the addition of α-oxoglutarate. Freeze-dried mitochondria were completely inactive and the addition of ATP, CoA and $NAD^+$ was ineffective. The activity was partially restored by the addition of succinyl-CoA (Table 7).

Table 3. *Formation of acetoacetate from hydroxymethylglutarate catalysed by rat liver mitochondria*

The results are given as means±S.E.M. for the numbers of observations in parentheses. The concentration of hydroxymethylglutarate-CoA lyase was 10mg/ml and that of oligomycin was 10μg/ml. $NAD^+$ concentration was 2.5mM and oleate 0.8mM. Citrate, α-oxoglutarate, malate, succinate and ATP concentrations were 5mM and GTP 3mM.

| Additions | Acetoacetate formed (nmol/h per mg of protein) | |
|---|---|---|
| None | 62± 6.5 | (18) |
| Hydroxymethylglutaryl-CoA lyase | 64±14 | (3) |
| Citrate | 82±11 | (4) |
| α-Oxoglutarate | 88±23 | (4) |
| Malate | 30± 6 | (4) |
| Succinate | 21± 7 | (4) |
| ATP | 28± 2 | (5) |
| ATP+oligomycin | 26± 4 | (3) |
| ATP+oligomycin+$NAD^+$ | 30± 4.5 | (3) |
| GTP | 35 | (2) |
| Pyruvate | 130±32 | (3) |
| Oleate | 111 | (2) |

## Discussion

Bloch *et al.* (1954) and Rabinowitz & Gurin (1954) have found that free hydroxymethylglutarate can be metabolized through the pathway of cholesterol biosynthesis. Burch *et al.* (1964) presented evidence that the entry of hydroxymethylglutarate into metabolic flow occurs in rat kidney mitochondria via a transferase reaction. However, they could not show a reproducible utilization of hydroxymethylglutarate by liver mitochondria and the kinase activation mechanism postulated by them appears unlikely in view of our results.

Liver contains high hydroxymethylglutaryl-CoA lyase activity (Williamson *et al.*, 1968), but since this enzyme does not act on hydroxymethylglutarate (Coon, 1962) we can conclude from our data that hydroxymethylglutarate is activated by rat liver

Table 4. *Formation of acetoacetate from hydroxymethylglutarate catalysed by rat liver mitochondria in the presence of 2,4-dinitrophenol, sodium arsenate and malonate*

The results are given as means±S.E.M. for the numbers of experiments in parentheses. Dinitrophenol concentration was 0.1mM, sodium arsenate 5mM and malonate 5mM.

| Additions | Acetoacetate formed (nmol/h per mg of protein) | |
|---|---|---|
| Dinitrophenol | 7± 3 | (5) |
| Dinitrophenol+oligomycin | 12± 2.5 | (3) |
| Dinitrophenol+α-oxoglutarate | 50±12 | (3) |
| Dinitrophenol+oligomycin+α-oxoglutarate | 52 | (2) |
| Sodium arsenate | 28± 6 | (3) |
| Sodium arsenate (in the absence of phosphate) | 0 | (3) |
| Sodium arsenate + α-oxoglutarate (in the absence of phosphate) | 10 | (2) |
| Malonate | 13± 9 | (3) |

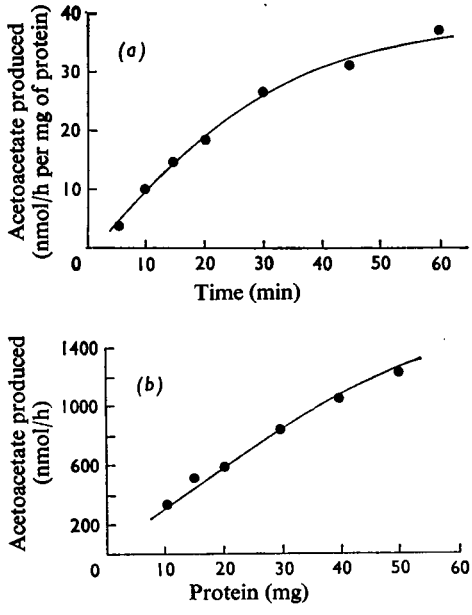


Fig. 1. *Acetoacetate production from hydroxymethylglutarate by rat liver mitochondria as function of time (a) and protein concentration (b)*

Experimental details are given in the Materials and Methods section. Hydroxymethylglutarate concentration was 5mM. (*a*) Protein concentration 10mg/ml; (*b*) incubation time 30min.

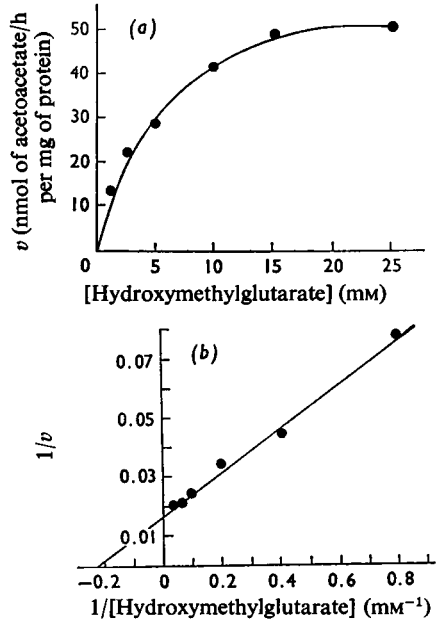


Fig. 2. *Acetoacetate production by rat liver mitochondria with different concentrations of hydroxymethylgutarate*

The reaction mixture was the same as used for the standard assay indicated in the Materials and Methods section. Protein concentration was 10mg of protein/ml; the substrate concentration was as indicated on the abscissa. (*a*) Saturation curve; (*b*) Lineweaver–Burk plot.

Table 5. *Formation of* [3-$^{14}$C]*acetoacetate from hydroxy*[3-$^{14}$C]*methylglutarate*

Incubation was carried out for 30min in the basal medium as indicated in the Materials and Methods section. Protein concentration was 10mg/ml. Final concentration of hydroxymethylglutarate was 5mM, obtained by adding to the radioactive hydroxymethylglutarate, as given below, the required amount of carrier. The [3-$^{14}$C]acetoacetate formed was calculated from the measured radioactivity by assuming that from 1mol of radioactive hydroxymethylglutarate originates 1mol of radioactive acetoacetate. The blank value was obtained from experiments where the reaction was stopped just after the addition of hydroxy[3-$^{14}$C]methylglutarate. The values, from which a blank was subtracted, are averages from two experiments. The counting efficiency, measured by the channels-ratio technique, was between 65 and 70%.

| Sample | Total radioactivity of sample (d.p.m.) | Actual yield (d.p.m.) | [3-$^{14}$C]Acetoacetate formed ($\mu$mol) | Total acetoacetate formed ($\mu$mol) | [3-$^{14}$C]Acetoacetate as percentage of total acetoacetate |
|---|---|---|---|---|---|
| 1 | 1160000 | 34900 | 0.308 | 0.44 | 70.0 |
| 2 | 1740000 | 55000 | 0.316 | 0.44 | 71.8 |
| 3 | 2320000 | 77400 | 0.334 | 0.44 | 75.7 |

through a mitochondrial reaction before acetoacetate production.

The results in the present paper offer circumstantial evidence that this activation occurs via a transferase mechanism. Hydroxymethylglutaryl-CoA formation is presumed to depend on an efficient system of intramitochondrial generation of succinyl-CoA (3-carboxyproprionyl-CoA).

Acetoacetate production could thus be summarized by the following reaction:

Hydroxymethylglutarate + succinyl-CoA ⇌ hydroxymethylglutaryl-CoA + succinate (1)

Hydroxymethylglutaryl-CoA → acetoacetate + + acetyl-CoA (2)

Table 6. *Formation of acetoacetate from hydroxymethylglutarate catalysed by rat liver mitochondria depleted of $Mg^{2+}$ or phosphate*

The results are given as means±S.E.M. for the numbers of observations in parentheses. α-Oxoglutarate and citrate concentrations were 5mM.

| | Additions | Acetoacetate formed (nmol/h per mg of protein) | |
|---|---|---|---|
| Control mitochondria | None | 62± 6.6 | (20) |
| Mitochondria depleted of $Mg^{2+}$ | None | 28±13 | (4) |
| | Citrate | 72±32 | (4) |
| | α-Oxoglutarate | 64±11 | (4) |
| Mitochondria depleted of phosphate | None | 8± 2.1 | (3) |
| | α-Oxoglutarate | 79±24 | (3) |

Table 7. *Acetoacetate formed by freeze-dried mitochondria incubated with hydroxymethylglutarate*

Incubation time was 30min, temperature 35°C. ATP, CoA, succinyl-CoA and GTP were added progressively with an addition every 5min within the first 20min. Final ATP concentration was 10mM, CoA 0.5mM, $NAD^+$ 3mM, GTP 10mM, succinyl-CoA 0.5mM, GSH 5mM and oligomycin 10μg/ml. The results are given as means±S.E.M. for three experiments.

| Additions | Acetoacetate formed (nmol/h per mg of protein) |
|---|---|
| None | 0 |
| ATP+oligomycin+CoA+$NAD^+$ | 0 |
| GTP+CoA+$NAD^+$ | 0 |
| Succinyl-CoA | 11±0.5 |
| Succinyl-CoA+GSH | 12±1.1 |

The oxidation of endogenous substrates presumably assures sufficient succinyl-CoA to drive hydroxymethylglutarate activation. The stimulating effect of exogenous citrate and α-oxoglutarate is presumably due to conversion of these compounds into succinyl-CoA. The effect of added pyruvate and oleate may well also result from increased tricarboxylic acid-cycle activity.

Inhibition by exogenous ATP (or GTP) might be associated with inhibition of citrate synthase with a consequent slowing of the tricarboxylic acid cycle and decrease of the rate of formation of succinyl-CoA. The inhibitory effect shown by dinitrophenol, EDTA and arsenate may be due to the requirement for small amounts of ATP to produce the acetyl-CoA necessary to maintain citrate production. This inhibition is removed by the addition of α-oxoglutarate or citrate, which would indicate that under conditions where thiokinases are inactive, hydroxymethylglutaryl-CoA formation is restored by addition of substrates that can act as precursors of succinyl-CoA.

The high production of acetoacetate observed after addition of α-oxoglutarate to mitochondria depleted of phosphate supports the above idea, and so does the succinyl-CoA dependence of acetoacetate production by freeze-dried mitochondria.

If we consider reaction (1) to be reversible, the inhibition by succinate, shown to be related to its concentration, can be explained as a single mass-action effect. Inhibition by malate might result from increased succinate concentrations, particularly since fumarate has a similar effect.

The metabolic function of this transferase activity is at present obscure, but it would appear that rat liver possesses a means of utilizing free hydroxymethylglutarate. This compound could derive from the deacylase activity described by Dekker *et al.* (1958).

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