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Activity and Intracellular Distribution of Enzymes of Ketone-Body Metabolism in Rat Liver

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1. The activities of hydroxymethylglutaryl-CoA synthase and lyase in rat liver were found to be two- to 15-fold greater than those reported by other authors under similar conditions. 2. When expressed on the basis of body weight, no appreciable differences were found between the activities of hydroxymethylglutaryl-CoA synthase in whole homogenates of livers from normal and starved rats. The synthase activity increased by 70% and 140% in livers of alloxan-diabetic rats and rats fed on a high-fat diet respectively. 3. Hydroxymethylglutaryl-CoA lyase activity showed no significant increases in starvation or alloxan-diabetes, but a 40% increase was found in fat-fed rats. 4. Less than 12% of the activities of both enzymes were found in the cytoplasmic fraction of normal liver. The cytoplasmic activity doubled in alloxan-diabetes and starvation; on feeding with a high-fat diet the increase, though significant, was less marked. 6. The intracellular distribution of glutamate dehydrogenase indicated that the changes in the cytoplasmic activities observed were not due to leakage from the mitochondria. 7. Feeding with a normal or high-fat diet after 48hr. starvation caused within 24hr. a decrease in the cytoplasmic activity of hydroxymethylglutaryl-CoA synthase to values lower than those found in rats fed on a corresponding diet for a longer period of time. 8. Acetoacetyl-CoA deacylase activity in liver was about 20% of that of hydroxymethylglutaryl-CoA synthase and was primarily located in the cytoplasm. Starvation or alloxan-diabetes did not alter the acetoacetyl-CoA deacylase activity. 9. It is concluded that variations in the concentrations of enzymes involved in acetoacetate synthesis play no major role in the regulation of ketone-body formation in starvation and alloxan-diabetes. The changes in the cytoplasmic activities of hydroxymethylglutaryl-CoA synthase and lyase suggest that acetoacetate synthesis can occur in the cytoplasm. This may play a role in the disposal of surplus acetyl-CoA arising in the cytoplasm when lipogenesis is inhibited.

This paper reports measurements of the activities of the enzymes that are concerned with the synthesis of ketone bodies in rat liver. The assays of these enzymes recorded in the literature (Bucher, Overath & Lynen, 1960; Wieland, Löffler, Weiss & Neufeldt, 1960; Segal & Menon, 1961) are not suitable for direct comparisons of the activities of the key enzymes in the same tissue, because they were obtained by different authors with different methods. Therefore they do not answer the question whether the increased ketone-body formation occurring in starvation, or in diabetes, or after feeding with high-fat (low in carbohydrate) diets, is associated with increased capacities of the enzymes of ketogenesis, nor do they settle the controversy as to which of the two possible pathways of conversion of acetoacetyl-CoA into acetoacetate outlined below, i.e. reaction (2) or reactions (3) plus (4), is the major one in rat liver. There is general agreement that the first step in the formation of ketone bodies in the liver is, with minor exceptions, the condensation of two molecules of acetyl-CoA leading to acetoacetyl-CoA:

$$2\ \text{Acetyl-CoA} \underset{\text{(EC 2.3.1.9)}}{\overset{\text{Acetoacetyl-CoA thiolase}}{\rightleftharpoons}} \text{acetoacetyl-CoA} + \text{CoA} \quad (1)$$

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Acetoacetate can be formed from acetoacetyl-CoA either by direct deacylation (Stern & Miller, 1959):

$$\text{Acetoacetyl-CoA} + H_2O \xrightarrow[\text{deacylase}]{\text{Acetoacetyl-CoA}} \text{acetoacetate} + \text{CoA} \quad (2)$$

or via the HMG-CoA* pathway (Lynen, Henning, Bublitz, Sorbo & Kröplin-Rueff, 1958), involving reactions (3) and (4):

$$\text{Acetoacetyl-CoA} + H_2O + \text{acetyl-CoA} \xrightarrow[\text{(EC 4.1.3.5)}]{\text{HMG-CoA synthase}} \text{HMG-CoA} + \text{CoA} \quad (3)$$

$$\text{HMG-CoA} \xrightarrow[\text{(EC 4.1.3.4)}]{\text{HMG-CoA lyase}} \text{acetoacetate} + \text{acetyl-CoA} \quad (4)$$

The measurements of the activities of these enzymes in rat liver indicate, among other things, that the HMG-CoA pathway, i.e. reactions (3) and (4), represents the major route of acetoacetate formation in the liver and that the ketosis of starvation is not associated with an increased capacity of this pathway. This type of ketosis must therefore be the result of an increase in the flow through reactions (3) and (4) without a concomitant change in the amounts of HMG-CoA synthase and lyase. On the other hand, there is an increase of 40% in the measured activity of the rate-limiting enzyme of the pathway, i.e. HMG-CoA synthase, in alloxan-diabetes, and this may contribute to the increased hepatic ketogenesis in this condition.

MATERIALS AND METHODS

Chemicals. 3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) was prepared by the method of Bergmeyer, Gawehn, Klotzsch, Krebs & Williamson (1967). Phosphotransacetylase (EC 2.3.1.8), citrate synthase (EC 4.1.3.7) and hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) were obtained from Boehringer Corp. (London) Ltd., London, W. 5. Nicotinamide–adenine nucleotides, acetyl phosphate (lithium salt) and CoA were also supplied by Boehringer Corp. (London) Ltd. Acetyl-CoA was prepared by acetylation of CoA with acetic anhydride (Simon & Shemin, 1953). Excess of acetic acid was removed by acidification of the reaction mixture to pH 2 with dilute HCl and continuous extraction with diethyl ether at 4° for 2hr. Residual ether in the aqueous phase was then removed *in vacuo* and the acetyl-CoA solution was carefully adjusted to pH 7 with dilute alkali. Acetoacetyl-CoA was prepared by the interaction of diketen with CoA (Wieland & Rueff, 1953); free acetoacetic acid was removed as described for acetic acid above. The acetoacetyl-CoA was presumed to contain negligible amounts of acetoacetylglutathione, because the preparation only reacted slowly with rat liver particles (Table 6), which contain an active acetoacetylglutathione hydrolase (Sauer & Erfle, 1966). HMG-CoA was prepared from hydroxymethylglutaric anhydride and CoA (Hilz, Knappe, Ringelmann & Lynen, 1958).

Animals. Male rats of the Wistar strain weighing 120–180g. were used. Alloxan-diabetes was induced by the intravenous injection of alloxan monohydrate (70mg./kg. body wt.) under ether anaesthesia. The alloxan-diabetic rats used were maintained on 4 units of protamine–zinc–insulin/day for a week, and then killed 24–48hr. after the last insulin injection (Steiner, Rauda & Williams, 1961).

Diets. The normal diet of the rats consisted of commercial rat cubes containing approx. 15% of protein, 3% of fat and 80% of carbohydrate (Oxoid breeding diet for rats and mice; Oxoid Ltd., London, S.E. 1). The high-fat (low in carbohydrate) diet consisted of 66% of margarine, 32% of soluble casein and 2% of an inorganic salt mixture with vitamin supplement. This diet was given for 3 days before the experiments.

Fractionation of liver. The rats were killed by cervical fracture. A portion of tissue (0·5–1g.) was rapidly excised, wrapped in Parafilm and placed in crushed ice. After a few minutes the liver sample was weighed, minced finely with scissors and transferred to a Potter–Elvehjem all-glass homogenizer (capacity 8ml.). Then 4vol. of ice-cold 0·25M-sucrose in 1mM-2-mercaptoethanol–10mM-tris–HCl buffer, pH 7·4, was added and the mixture was homogenized with a glass pestle driven by a low-speed motor. All subsequent operations were carried out 0–4°. A portion of the homogenate, cooled in ice–water, was immediately exposed to ultrasonic vibration for exactly 30sec. at 15kcyc./sec. (100W model; Measuring and Scientific Equipment Ltd., London, S.W. 1). The ultrasonically treated homogenate was then centrifuged for 30min. at 30000*g*. The supernatant fluid was considered to contain the total soluble protein of the cell (i.e. cytoplasm and mitochondrial matrix) and its enzymic activity is referred to in the text as the whole-homogenate activity. Another portion of the homogenate was centrifuged for 30min. at 30000*g*. The supernatant fluid (i.e. cytoplasmic fraction) was carefully decanted and the pellet (particulate fraction) was suspended in a volume of 0·25M-sucrose equal to that of the homogenate from which the pellet was derived. This suspension was then treated ultrasonically for exactly 30sec., followed by centrifugation for 30min. at 30000*g*. The supernatant fluid was taken to represent the soluble protein of the particulate fraction and its enzymic activity is referred to in the text as the particulate activity. The cytoplasmic fraction contained some microsomes, but virtually no mitochondria, whereas the particulate fraction consisted of nuclei and microsomes as well as the mitochondria.

Determination of enzyme activities. HMG-CoA synthase activity was determined by incubation of the enzyme sample with an acetyl-CoA-generating system (Lynen *et al.* 1958) and subsequent measurement of the acetoacetate with hydroxybutyrate dehydrogenase (Williamson, Mellanby & Krebs, 1962). The incubation mixture (final volume 3·5ml.) consisted of: tris–HCl buffer, pH 8·5 (175μmoles), $MgCl_2$ (17·5μmoles), acetyl phosphate (lithium salt, 35μmoles), CoA (3μmoles) and phosphotransacetylase [12 units; suspension in $(NH_4)_2SO_4$]. Samples (1·0ml.) of the assay mixture were removed at 0, 8 and 16min. and mixed with 1·0ml. of 3% (w/v) $HClO_4$. After removal of denatured protein by centrifugation, the supernatant fluid was

* Abbreviation: HMG-CoA, hydroxymethylglutaryl-CoA.

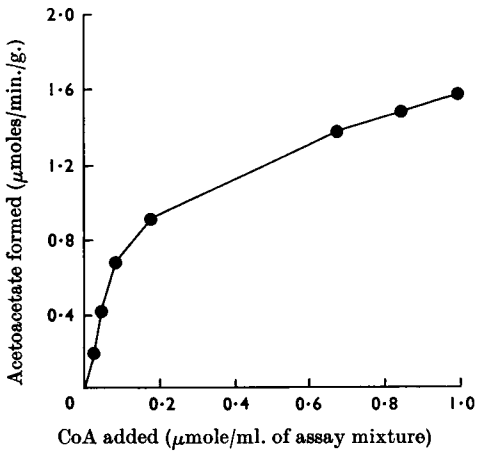

Fig. 1. Relationship between amount of CoA added and acetoacetate formed in the HMG-CoA synthase assay. The amount of CoA added was varied and the amount of enzyme (liver homogenate from fed rat) was kept constant. For other details see the Materials and Methods section.

neutralized with 10% (w/v) KOH. The precipitate of $KClO_4$ was removed by centrifugation and the supernatant fluid was analysed for acetoacetate. The production of acetoacetate was linear with time for at least 20 min. The final concentration of CoA added (about 0·8 mM; determined by the method of Michal & Bergmeyer, 1963) to the assay system was nearly optimum (Fig. 1). At least 90% of the added CoA was found to be present as acetyl-CoA during the incubation. A difficulty encountered in measuring the intracellular distribution of HMG-CoA synthase was the fact that the sum of the cytoplasmic and particulate activities of HMG-CoA synthase was usually 20% less than that of the whole homogenate. The incomplete recovery of synthase activity proved to be due to inactivation of the particulate enzyme during ultrasonic treatment. When the period of ultrasonic treatment was increased from the usual 30 sec. to 2 min., the particulate activity decreased by 90%, whereas the whole activity decreased only by 10% under the same conditions. The synthase activity of the particles could be protected during ultrasonic treatment by prior addition of whole homogenate that had been heated at 100° for 5 min. to destroy its synthase activity.

For the reason given above, though the particulate activity was determined on each sample, the values for HMG-CoA synthase activity of the particles given in the Tables were obtained by subtracting individual cytoplasmic values from those found for the whole homogenate.

HMG-CoA lyase activity was measured by incubation of the enzyme sample with HMG-CoA and subsequent determination of the acetoacetate formed. The incubation mixture (final volume 3·5 ml.) consisted of: tris–HCl buffer, pH 8·5 (175 μmoles), $MgCl_2$ (17·5 μmoles) and HMG-CoA (1·4 μmoles). Samples (1·0 ml.) were removed at 0, 4 and 8 min. and treated as for the HMG-CoA synthase assay. Suitable controls to measure acetoacetate formation in the absence of substrate were carried out for both assay systems.

Acetoacetyl-CoA thiolase activity was determined by measuring the decrease in E_{313} due to cleavage of acetoacetyl-CoA (Stern, 1956). The cuvettes (1 cm.) contained in a final volume of 2·0 ml.: tris–HCl buffer, pH 8·5 (100 μmoles), $MgCl_2$ (10 μmoles), acetoacetyl-CoA (0·1 μmole) and CoA (0·2 μmole). The sample (5–10 μl.) was mixed in and the decrease in E_{313} was followed for 2 min. The disappearance of acetoacetyl-CoA was assumed to be solely due to the thiolase activity. This assumption may not be strictly valid because other reactions could contribute to the removal of acetoacetyl-CoA (i.e. acetoacetyl-CoA deacylase or the HMG-CoA pathway enzymes); however, evidence is presented below that shows that the activities of interfering enzymes are negligible in comparison with that of the thiolase.

Glutamate dehydrogenase [L-glutamate–NAD(P) oxidoreductase (deaminating), EC 1.4.1.3] activity was determined by measuring the reduction of oxoglutarate with $NADH_2$ in the presence of NH_4^+ ions (Schmidt, 1963).

Acetoacetyl-CoA deacylase activity was measured by the rate of removal of acetoacetyl-CoA in the presence of iodoacetamide. The contents of the cuvettes were as for the assay of acetoacetyl-CoA thiolase, except for the omission of CoA and the inclusion of iodoacetamide (10 μmoles). The enzyme sample (25–100 μl.) was added to start the reaction and the decrease in E_{313} was followed for 2–3 min. The values obtained for the deacylase activity with this method agreed well with the values obtained with an indirect method in which the rate of acetoacetyl-CoA disappearance was compared with the rate of acetoacetate formation; the amount of acetyl-CoA formed was negligible. In these experiments acetoacetyl-CoA (Decker, 1963), acetyl-CoA (Pearson, 1965) and acetoacetate (Williamson *et al.* 1962) were measured by enzymic methods.

Units of enzyme activity. All measurements of enzyme activity were carried out at 25°. A unit of enzyme activity is defined as the amount of enzyme that transforms 1 μmole of substrate/min. at 25° and specific activity as units/g. fresh wt. of tissue. The ϵ_{mM} value of the acetoacetyl-CoA–Mg^{2+} complex (assay of acetoacetyl-CoA deacylase and acetoacetyl-CoA thiolase) was found to be 11·8 and this value was used for the calculations of the thiolase and deacylase activities.

RESULTS

Activities and intracellular distribution of HMG-CoA synthase and lyase in rat liver. Measurements of the activities and distribution of HMG-CoA synthase and lyase in the livers of normal, starved and alloxan-diabetic rats and rats fed on a high-fat diet are shown in Table 1. Starvation, alloxan-diabetes and feeding with a high-fat diet resulted in significant increases ($P < 0·01$) in the activities of both HMG-CoA synthase and lyase of whole homogenate and particles when the activities were expressed as units/g. of liver. When expressed as units/100 g. body wt. differences between the synthase activities of whole homogenate and particles of normal and starved rats disappeared. This was due to the increase in the body/liver weight ratio found in the starved rats (Table 2). When the

Table 1. *Activities and intracellular distributions of HMG-CoA synthase and lyase in livers from normal, starved, alloxan-diabetic and fat-fed rats*

The values are means ± S.D. with numbers of observations in parentheses. For full details see the Materials and Methods section. Data on body and liver weights of the rats are given in Table 2.

		Activity (units/g. of liver)			Activity (units/100g. body wt.)			Proportion of total activity in cytoplasm (%)
		Whole			Whole			
State of rats	Enzyme	homogenate	Cytoplasm	Particles	homogenate	Cytoplasm	Particles	
Normal	HMG-CoA	2·2 ± 0·3 (9)	0·25 ± 0·05 (5)	1·9 ± 0·2 (5)	10 ± 1 (5)	1·2 ± 0·3 (5)	8·9 ± 0·9 (5)	11
Starved for 48 hr.	synthase	3·7 ± 1·0 (15)†	0·73 ± 0·25 (14)†	3·0 ± 0·9 (12)*	12 ± 5 (13)	2·5 ± 1·0 (12)†	10 ± 3 (11)	20
Alloxan-diabetic		4·2 ± 1·1 (5)†	0·58 ± 0·18 (5)†	3·6 ± 1·0 (5)†	17 ± 3 (5)†	2·4 ± 0·5 (5)†	15 ± 2 (5)†	14
Fat-fed		5·3 ± 0·6 (5)†	0·43 ± 0·05 (5)†	4·9 ± 0·6 (5)†	24 ± 2 (5)†	2·0 ± 0·3 (5)†	22 ± 2 (5)†	8
Normal	HMG-CoA	8·8 ± 1·0 (11)	0·71 ± 0·19 (6)	8·0 ± 1·1 (6)	41 ± 8 (6)	3·2 ± 0·9 (6)	38 ± 9 (6)	8
Starved for 48 hr.	lyase	12·2 ± 2·4 (14)†	2·5 ± 0·8 (13)†	10·2 ± 1·8 (12)*	41 ± 9 (12)	7·8 ± 2·6 (12)†	33 ± 7 (12)	20
Alloxan-diabetic		11·9 ± 2·0 (4)†	1·7 ± 0·7 (4)†	10·2 ± 1·6 (4)†	49 ± 1 (4)	7·0 ± 1·8 (4)†	42 ± 2 (4)	14
Fat-fed		12·1 ± 1·5 (5)†	1·2 ± 0·02 (5)†	10·8 ± 1·5 (5)†	56 ± 6 (5)†	5·7 ± 0·5 (5)†	50 ± 7 (5)	10

* Significantly different from values for normal rats ($P < 0.05$).
† Significantly different from values for normal rats ($P < 0.01$).

HMG-CoA lyase activity of the whole homogenate and particles was expressed as units/100g. body wt., there was no significant difference between the values for starved, alloxan-diabetic or normal rats. The proportion of HMG-CoA synthase to lyase activity remained virtually constant regardless of treatment and location in the cell, the value for the HMG-CoA lyase activity being 2·3–4·1 times that for the synthase activity.

In starvation and alloxan-diabetes the cytoplasmic activities of HMG-CoA synthase and lyase increased twofold (Table 1) above normal values. These changes in cytoplasmic activities were significant ($P < 0.01$) when expressed both as units/g. of liver and units/100g. body wt. There was a smaller increase in cytoplasmic activities of the rats fed on a high-fat diet. In the alloxan-diabetic and fat-fed rats, the proportion of total activity in the cytoplasm remained similar to that of rats fed on the normal diet because the increase in cytoplasmic activity was accompanied by an increase in particulate activity.

Re-feeding previously starved rats with a normal or high-fat diet (Table 3) resulted within 24 hr. in a decrease of cytoplasmic HMG-CoA synthase activity to levels actually lower than those found in rats fed on the corresponding diet for a longer period of time (Table 1). After 48 hr. this difference disappeared.

Activities and intracellular distribution of acetoacetyl-CoA thiolase and glutamate dehydrogenase in rat liver. When measurements of the distribution of enzymes between cytoplasm and particles are compared it is essential to measure the activity of at least one enzyme that can act as an indicator of mitochondrial permeability or damage. Glutamate dehydrogenase is most suitable for this purpose because it is soluble and exclusively located in the mitochondrial matrix (Delbrück, Schimassek, Bartsch & Bücher, 1959). Leakage of soluble mitochondrial protein will therefore show itself by increased cytoplasmic activity of this enzyme. The activity of acetoacetyl-CoA thiolase in the cytoplasmic and particulate fractions was also measured, since the assay of HMG-CoA synthase is dependent on the presence of excess of the thiolase activity. The activities of these two enzymes were determined on the samples used for the assay of the HMG-CoA synthase and lyase.

No significant differences in activities of glutamate dehydrogenase or acetoacetyl-CoA thiolase were found between the various experimental groups (Table 4). The proportion of acetoacetyl-CoA thiolase and glutamate dehydrogenase found in the cytoplasmic fraction remained relatively constant, in contrast with the changes found in the cytoplasmic activities of HMG-CoA synthase and lyase (Table 1). Since the proportion of the thiolase in the cytoplasm was about six times that of glutamate

Table 2. *Body and liver weights of rats in the different experimental groups*

The values are means ± S.D.

State of rats	No. of rats	Body wt. (g.)	Liver wt. (g).	Liver wt. (g./100g. body wt.)
Normal	6	172±14	8·2±1·1	4·8
Starved for 48hr.	11	146±13	4·8±0·5	3·3
Alloxan-diabetic	5	177±16	7·5±1·4	4·2
Fat-fed	5	155±12	7·2±0·7	4·6
Starved for 48hr., re-fed on normal diet for 24hr.	6	155±14	8·4±1·1	5·4
Starved for 48hr., re-fed on normal diet for 48hr.	4	160±18	9·1±1·6	5·7
Starved for 48hr., re-fed on high-fat diet for 24hr.	6	135±11	5·9±0·8	4·4
Starved for 48hr., re-fed on high-fat diet for 48hr.	6	141±11	6·6±1·5	4·7

Table 3. *Effects of re-feeding with a normal or high-fat diet on the activities and intracellular distribution of HMG-CoA synthase and lyase in livers of starved rats*

The values are means ± S.D. with numbers of observations in parentheses. For full details see the Materials and Methods section. Data on body and liver weights are given in Table 2.

	HMG-CoA synthase (units/100g. body wt.)		HMG-CoA lyase (units/100g. body wt.)	
State of rats	Whole homogenate	Cytoplasm	Whole homogenate	Cytoplasm
Starved for 48hr.	12±1 (13)	2·5±1·0(12)	41±9 (12)	7·8±2·6 (12)
Starved for 48hr., re-fed on normal diet for 24hr.	12±4 (6)	0·67±0·22 (6)*	35±6 (6)	2·2±1·5 (6)
Starved for 48hr., re-fed on normal diet for 48hr.	11±5 (4)	1·0±0·2 (4)	37±11 (4)	3·0±0·7 (4)
Starved for 48hr., re-fed on high-fat diet for 24hr.	18±4 (5)	1·4±0·3 (6)‡	45±7 (4)†	3·8±1·0 (4)†
Starved for 48hr., re-fed on high-fat diet for 48hr.	21±8 (5)	2·8±1·0 (6)	57±30 (3)	6·2±4·1 (6)

* Significantly different from values for normal rats given in Table 1 ($P < 0·05$).
† Significantly different from values for fat-fed rats given in Table 1 ($P < 0·05$).
‡ Significantly different from values for fat-fed rats given in Table 1 ($P < 0·01$).

Table 4. *Activities and intracellular distribution of acetoacetyl-CoA thiolase and glutamate dehydrogenase in livers of normal, starved, alloxan-diabetic and fat-fed rats*

The values are means ± S.D. with numbers of observations in parentheses. For full details see the Materials and Methods section. Data on body and liver weights are given in Table 2.

	Acetoacetyl-CoA thiolase			Glutamate dehydrogenase		
State of rats	Whole homogenate (units/100g. body wt.)	Cytoplasm (units/100g. body wt.)	Proportion of total activity in cytoplasm (%)	Whole homogenate (units/100g. body wt.)	Cytoplasm (units/100g. body wt.)	Proportion of total activity in cytoplasm (%)
Normal	110±41 (6)	26±11 (6)	24	257±78 (6)	7·2±2·3 (6)	2·8
Starved for 48hr.	94±60 (5)	21±9 (5)	22	183±49 (5)	7·4±3·8 (5)	4·0
Alloxan-diabetic	114±27 (5)	23±6 (5)	20	254±34 (5)	8·1±5·0 (5)	3·2
Fat-fed	131±45 (5)	27±5 (5)	21	213±28 (5)	5·4±1·7 (5)	2·5

dehydrogenase, it would seem that the presence of the former in the extramitochondrial compartment was not due to leakage from the particles. There was actually a greater excess of thiolase activity, relative to HMG-CoA synthase activity, in the cytoplasm than in the whole homogenate.

Re-feeding rats previously starved for 48hr. with a normal or high-fat diet caused no significant

Table 5. *Effects of re-feeding with a normal or high-fat diet on the activities and intracellular distribution of acetoacetyl-CoA thiolase and glutamate dehydrogenase in livers of rats previously starved for 48 hr.*

The values are means ± S.D. with numbers of observations in parentheses. For full details see the Materials and Methods section. Data on body and liver weights of rats are given in Table 2.

	Acetoacetyl-CoA thiolase			Glutamate dehydrogenase		
State of rats	Whole homogenate (units/100g. body wt.)	Cytoplasm (units/100g. body wt.)	Proportion of total activity in cytoplasm (%)	Whole homogenate (units/100g. body wt.)	Cytoplasm (units/100g. body wt.)	Proportion of total activity in cytoplasm (%)
Starved for 48hr.	94±60 (5)	21±9 (5)	22	183±49 (5)	7·4±3·8 (5)	4·0
Starved for 48hr., re-fed on normal diet for 24hr.	101±24 (6)	9·3±3·8 (6)*	9	206±30 (6)	7·1±3·1 (6)	3·4
Starved for 48hr., re-fed on normal diet for 48hr.	134 (2)	22 (2)	16	228 (2)	6·4 (1)	2·8
Starved for 48hr., re-fed on high-fat diet for 24hr.	113±31 (6)	21±4 (5)	19	206±60 (6)	6·4±1·7 (6)	3·1
Starved for 48hr., re-fed on high-fat diet for 48hr.	119±36 (4)	26±7 (4)	22	185±61 (4)	8·1±3·6 (4)	4·4

* Significantly different from values for normal rats given in Table 4 ($P < 0.01$).

Table 6. *Activities and intracellular distribution of acetoacetyl-CoA deacylase in livers from normal, starved and alloxan-diabetic rats*

The values are means ± S.D. with numbers of observations in parentheses. For full details see the Materials and Methods section. The animal and liver weights are given in Table 2.

	Acetoacetyl-CoA deacylase (unit/g. of liver)	
State of rats	Whole homogenate	Cytoplasm
Normal	0·41±0·11 (5)	0·33±0·09 (5)
Starved for 48hr.	0·41 (2)	0·36 (2)
Alloxan-diabetic	0·48 (2)	0·44 (2)

change in the activities or distribution of glutamate dehydrogenase, but the cytoplasmic activity of acetoacetyl-CoA thiolase decreased to a value 50% that of the starved animal and 34% that of animals fed on a normal diet (Table 5). After 48hr. of re-feeding with a normal diet, the value returned to the level found in rats fed on a normal diet. This lower activity of the thiolase probably had little effect on the measurement of cytoplasmic HMG-CoA synthase activity in this group of animals because the ratio of the two activities was similar to that in other experimental conditions.

Activities and intracellular distribution of acetoacetyl-CoA deacylase in rat liver. The activity of acetoacetyl-CoA deacylase in the whole homogenate of liver was about 11% of HMG-CoA synthase activity (Table 6). This activity was mainly cytoplasmic (80–92%) and was similar in magnitude to the cytoplasmic activity of HMG-CoA synthase (Table 1). Starvation and alloxan-diabetes did not significantly affect the deacylase activity of the liver when expressed on a units/g. basis, but, since starvation resulted in a lower liver weight (Table 2), total hepatic activity was decreased.

DISCUSSION

Capacity of the HMG-CoA pathway in rat liver. The highest rates of ketone-body formation (0·8 μmole/min./g. wet wt.; corrected to 25°) have been obtained on perfusion of the isolated rat liver with medium supplemented with fatty acids (Söling, Kattermann, Schmidt & Kneer, 1966; P. G. Wallace, R. Hems, R. A. Freedland & H. A. Krebs, unpublished work). Hence if the HMG-CoA pathway is responsible for the formation of ketone bodies the activity of the rate-limiting enzyme, HMG-CoA synthase, should under physiological conditions at least equal this value. The values previously reported for the activity of this enzyme (Table 7) are all below the rates postulated to be maximal for rat liver, but those calculated from the present experiments are three times the postulated rate. The calculation, however, is indicative only of the order of magnitude, because it is based on the assumption that the rate at body temperature is twice that measured at 25° and that the pH difference between the assay medium (pH 8·5) and the liver cell does not affect the reaction rate in a major way.

Table 7. *Comparison of the conditions used by various investigators for the assay of HMG-CoA synthase in liver homogenates*

The + sign indicates that no quantitative information is available.

Reference	Bucher *et al.* (1960)	Wieland *et al.* (1960)	Segal & Menon (1961)	This paper
Treatment of homogenate ...	High-speed homogenization	High-speed homogenization	Freeze-thawing	Ultrasonic treatment
Assay mixture				
Tris buffer	pH 7·9, 100mM	pH 7·85, 70mM	pH 7·9, 100mM	pH 8·5, 50mM
Acetyl phosphate	20mM	20mM	30mM	10mM
Cysteine	–	10mM	–	–
Glutathione	4·3mM	+	5mM	–
Na_2S	10mM	–	–	–
CoA	0·43mM	0·3mM	0·5mM	0·8mM
$MgCl_2$	5·7mM	–	5mM	12·5mM
EDTA	10mM	–	–	–
Phosphotransacetylase	+	+	+	+
HMG-CoA lyase added	+	–	–	–
Temperature of incubation	37°	37°	37°	25°
Method of determination of acetoacetate	Colorimetric	Colorimetric	Colorimetric	Enzymic
Activity (μmoles/min./g. at 25°)	0·25	0·12	0·95	2·2

The lower values obtained by previous investigators may have been due to one or a combination of the following factors. Segal & Menon (1961) have shown that complete disruption of the mitochondria is essential for obtaining maximum activity. In the present work this was achieved by the ultrasonic treatment. Another critical factor is the concentration of acetoacetyl-CoA, which in the present experiments depended on the phosphotransacetylase generating system (Lynen *et al.* 1958). If the activity of this enzyme is high and if acetyl phosphate is present in large excess added CoA is rapidly acetylated and the acetoacetyl-CoA concentration is governed by the equilibrium constant of the thiolase reaction (Goldman, 1954):

$$K = \frac{[\text{acetoacetyl-CoA}][\text{CoA}]}{[\text{acetyl-CoA}]^2} = 6{\cdot}0 \times 10^{-5} \text{ at pH } 8{\cdot}5$$

The amount of CoA added to the assay system is important for it controls the concentration of acetyl-CoA. Previous workers have used lower concentrations of CoA, which would have resulted in lower concentrations of acetoacetyl-CoA (see Fig. 1). Finally, incomplete recovery of the acetoacetate formed can be responsible for apparently low rates of enzyme activity. The colorimetric method of Walker (1954) used by the previous workers is now known to give incomplete yields in the presence of glutathione and other thiol compounds (Allred, 1965). It is relevant in this context that the assay mixtures used previously contained glutathione in relatively high concentrations. The enzymic method for the determination of acetoacetate eliminates this source of error.

Table 8. *Comparison of hepatic HMG-CoA synthase activity and blood ketone-body concentrations in various experimental situations*

The values for HMG-CoA synthase activities are taken from Table 1, and the sources of the values for the blood ketone-body concentrations are as indicated. The values are means ± S.D. with the numbers of animals in parentheses.

State of rats	HMG-CoA synthase activity (units/100g. body wt.)	Total ketone-body concn. (μmoles/ml.)
Normal	10 ± 1 (5)	0·20 ± 0·04 (7)*
Starved for 48hr.	12 ± 5 (13)	2·3 ± 0·7 (23)*
Fat-fed for 3 days	24 ± 2 (5)	1·0 ± 0·4 (10)†
Alloxan-diabetic, insulin withdrawal	17 ± 3 (5)	2·0 ± 1·6 (10)‡

* Williamson, Lund & Krebs (1967)
† Present work.
‡ Williamson, Lopes-Vieira & Walker (1967).

Relationship between ketosis and the particulate activity of the enzymes of the HMG-CoA pathway. Ketone bodies are formed mainly in the mitochondria because the degradation of their precursors occurs almost exclusively in this compartment, the only extramitochondrial precursors of acetyl-CoA being acetate and citrate transferred from the mitochondria (Kornacker & Lowenstein, 1965).

Hence any correlation between the rate of ketone-body formation and enzyme activity would in the first instance refer to the enzyme activity in the mitochondria. There were no differences in the activities of the rate-limiting enzyme, HMG-CoA synthase, between particles from starved and normal rats when calculated in terms of units/100g. body wt. But significant increases occurred in the alloxan-diabetic livers, which is qualitatively in accordance with the findings of Segal & Menon (1961) and Wieland *et al.* (1960). However, there was no obvious correlation between the concentrations of the ketone bodies in the blood and the activities of HMG-CoA synthase in the various experimental groups, with the possible exception of the alloxan-diabetic rats (Table 8). The HMG-CoA synthase activities of the livers from normal and starved rats were similar, though there was a tenfold difference in the concentrations of the blood ketone bodies, whereas in the fat-fed rats the concentrations of ketone bodies in the blood were half those of starved rats and the synthase activity was twice as high. On the assumption that the concentrations of blood ketone bodies are indications of the rate of hepatic ketogenesis, it follows that the activity of HMG-CoA synthase can only be of minor importance in determining the extent of ketogenesis. The main factors regulating hepatic ketogenesis are probably the concentrations of free fatty acids and the balance between fatty acids and other substrates that can serve as fuels of respiration (Fredrickson & Gordon, 1958).

Activity of the HMG-CoA pathway in rat liver cytoplasm. The slight activity of the enzymes of the HMG-CoA pathway found in the supernatant fraction has been attributed by Bucher *et al.* (1960) to mitochondrial leakage or mitochondrial damage during the preparation of liver homogenates. Sauer & Erfle (1966) found definite activity in the cytoplasm of normal guinea-pig liver and that this rose two- to four-fold in ketotic liver. The present results confirm their findings for rat liver. There was a significant increase in the cytoplasmic activity in the livers from alloxan-diabetic and starved rats (Table 1), and since there was no change in the intracellular distribution of glutamate dehydrogenase, an enzyme exclusively located in the mitochondrial matrix, it cannot be argued that the increased activities are due to increased fragility or leakage from the mitochondria. The physiological role of the cytoplasmic enzymes may lie in the disposal of extramitochondrial acetyl-CoA not required for fatty acid synthesis or other cytoplasmic reactions. This situation may arise in starvation and alloxan-diabetes, where lipogenesis is depressed (Masoro, 1962) and the hepatic acetyl-CoA concentration is raised (Tubbs & Garland, 1964; Wieland & Weiss, 1963). Further support for the postulated role of the cytoplasmic enzymes is the immediate decrease in their activity on re-feeding, when lipogenesis is increased. The disposal of acetyl-CoA formed in the cytoplasm is essential for the maintenance of the concentration of free CoA. Another reaction that may bring about the disposal is the acetyl-CoA deacylase that Hepp, Prüsse, Weiss & Wieland (1966) have found in mouse liver.

Acetoacetyl-CoA deacylase. In connexion with the interpretation of the significance of the deacylase activity it has to be borne in mind that the assay was carried out with 0·05mM-acetoacetyl-CoA, a concentration at least 2500 times that calculated for intact liver from the concentrations of acetyl-CoA, free CoA and the equilibrium constant of acetoacetyl-CoA thiolase. Though there are uncertainties in the calculation, for example whether the CoA determined is actually accessible to the acetoacetyl-CoA thiolase and whether the thiolase system is in equilibrium, the order of magnitude is unlikely to be wrong. Preliminary measurements of the K_m of the deacylase for acetoacetyl-CoA give a value of 0·01mM. In view of this, and of the low activity found under the test conditions, it is unlikely that direct deacylation plays a significant role in hepatic ketogenesis.

The finding that most of the deacylase activity is located in the cytoplasm supports the view that the mitochondrial deacylase activity reported by Stern & Miller (1959) was due to an acetoacetylglutathione hydrolase (Sauer & Erfle, 1966).

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REFERENCES

Allred, J. B. (1965). *Analyt. Biochem.* **11**, 138.
Bergmeyer, H. U., Gawehn, K., Klotzsch, H., Krebs, H. A. & Williamson, D. H. (1967). *Biochem. J.* **102**, 423.
Bucher, N. L. R., Overath, P. & Lynen, F. (1960). *Biochim. biophys. Acta*, **40**, 491.
Decker, K. (1963). In *Methods of Enzymatic Analysis*, p. 425. Ed. by Bergmeyer, H. U. Weinheim: Verlag Chemie.
Delbrück, A., Schimassek, H., Bartsch, K. & Bücher, Th. (1959). *Biochem. Z.* **331**, 297.
Fredrickson, D. S. & Gordon, R. S., jun. (1958). *Physiol. Rev.* **38**, 585.
Goldman, D. S. (1954). *J. biol. Chem.* **208**, 345.
Hepp, D., Prüsse, E., Weiss, L. & Wieland, O. (1966). *Biochem. Z.* **344**, 87.
Hilz, H., Knappe, J., Ringelmann, E. & Lynen, F. (1958). *Biochem. Z.* **329**, 476.
Kornacker, M. S. & Lowenstein, J. M. (1965). *Biochem. J.* **94**, 209.
Lynen, F., Henning, V., Bublitz, C., Sorbo, B. & Kröplin-Rueff, L. (1958). *Biochem. Z.* **330**, 269.
Masoro, E. J. (1962). *J. Lipid Res.* **3**, 149.

Michal, G. & Bergmeyer, H. U. (1963). In *Methods of Enzymatic Analysis*, p. 517. Ed. by Bergmeyer, H. U. Weinheim: Verlag Chemie.
Pearson, D. J. (1965). *Biochem. J.* **95**, 23c.
Sauer, F. & Erfle, J. D. (1966). *J. biol. Chem.* **241**, 30.
Schmidt, E. (1963). In *Methods of Enzymatic Analysis*, p. 752. Ed. by Bergmeyer, H. U. Weinheim: Verlag Chemie.
Segal, H. L. & Menon, G. K. K. (1961). *J. biol. Chem.* **236**, 2872.
Simon, E. J. & Shemin, D. (1953). *J. Amer. chem. Soc.* **75**, 2520.
Söling, H. D., Kattermann, R., Schmidt, H. & Kneer, P. (1966). *Biochim. biophys. Acta*, **115**, 1.
Steiner, D. F., Rauda, V. & Williams, R. H. (1961). *Endocrinology*, **68**, 809.
Stern, J. R. (1956). *J. biol. Chem.* **221**, 33.
Stern, J. R. & Miller, G. E. (1959). *Biochim. biophys. Acta*, **35**, 576.
Tubbs, P. K. & Garland, P. B. (1964). *Biochem. J.* **93**, 550.
Walker, P. G. (1954). *Biochem. J.* **58**, 699.
Wieland, O., Löffler, G., Weiss, L. & Neufeldt, I. (1960). *Biochem. Z.* **333**, 10.
Wieland, T. & Rueff, L. (1953). *Angew. Chem.* **65**, 186.
Wieland, O. & Weiss, L. (1963). *Biochem. biophys. Res. Commun.* **10**, 333.
Williamson, D. H., Lopes-Vieira, O. & Walker, B. (1967). *Biochem. J.* **104**, 497.
Williamson, D. H., Lund, P. & Krebs, H. A. (1967). *Biochem. J.* **103**, 514.
Williamson, D. H., Mellanby, J. & Krebs, H. A. (1962). *Biochem. J.* **82**, 90.