Biochem. J. (1971) **121**, 41–47
Printed in Great Britain

Activities of Enzymes involved in Acetoacetate Utilization in Adult Mammalian Tissues

By D. H. WILLIAMSON, MARGARET W. BATES,* M. ANN PAGE and H. A. KREBS
Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford OX2 6HE, U.K.

(*Received* 30 *July* 1970)

1. The activities in rat tissues of 3-oxo acid CoA-transferase (the first enzyme involved in acetoacetate utilization) were found to be highest in kidney and heart. In submaxillary and adrenal glands the activities were about one-quarter of those in kidney and heart. In brain it was about one-tenth and was less in lung, spleen, skeletal muscle and epididymal fat. No activity was detectable in liver. 2. The activities of acetoacetyl-CoA thiolase were found roughly to parallel those of the transferase except for liver and adrenal glands. The high activity in the latter two tissues may be explained by additional roles of thiolase, namely, the production of acetyl-CoA from fatty acids. 3. The activities of the two enzymes in tissues of mouse, gerbil, golden hamster, guinea pig and sheep were similar to those of rat tissues. The notable exception was the low activity of the transferase and thiolase in sheep heart and brain. 4. The activities of the transferase in rat tissues did not change appreciably in starvation, alloxan-diabetes or on fat-feeding, where the rates of ketone-body utilization are increased. Thiolase activity increased in kidney and heart on fat-feeding. 5. The activity of 3-hydroxybutyrate dehydrogenase did not change in rat brain during starvation. 6. The factors controlling the rate of ketone-body utilization are discussed. It is concluded that the activities of the relevant enzymes in the adult rat do not control the variations in the rate of ketone-body utilization that occur in starvation or alloxan-diabetes. The controlling factor in these situations is the concentration of the ketone bodies in plasma and tissues.

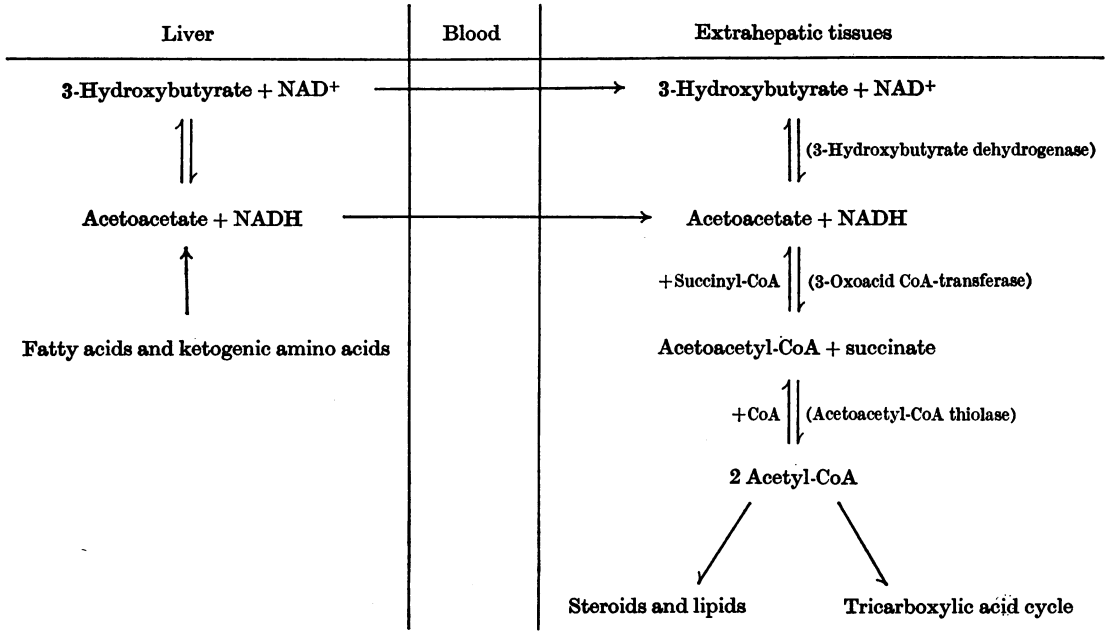
Ready utilization of acetoacetate and 3-hydroxybutyrate by various animal tissues, except the liver, has been established by experiments on tissue slices and homogenates (McCann, 1957; Krebs, 1961), isolated perfused organs (Snapper & Grünbaum, 1927; Hall, 1961; Williamson & Krebs, 1961), eviscerate preparations (Mirsky & Broh-Kahn, 1937; Söling, Garlepp & Creutzfeldt, 1965) and on the whole animal (Wick & Drury, 1941; Nelson, Grayman & Mirsky, 1941; Bates, Krebs & Williamson, 1968). The rate of ketone-body utilization in extrahepatic tissues is concentration-dependent (see review by Williamson & Hems, 1970) but otherwise little is known about the control of ketone-body utilization.

The present paper reports measurements of the activities of the three enzymes concerned in the utilization of ketone bodies, 3-oxo acid CoA-transferase (EC 2.8.3.5), acetoacetyl-CoA thiolase (EC 2.3.1.9) and 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) (see Scheme 1). The experiments show that the activities of these enzymes do not increase appreciably in starvation or alloxan-diabetes, when ketone-body utilization is much increased.

* Present address: Laboratory of Clinical Science, University of Pittsburgh, Pa. 15213, U.S.A.

MATERIALS AND METHODS

Chemicals. 3-Hydroxybutyrate dehydrogenase, 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), nicotinamide–adenine dinucleotides and CoA were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Acetoacetyl-CoA was prepared by the interaction of diketene with CoA (Wieland & Rueff, 1953); free acetoacetic acid was removed by acidification of the reaction mixture to pH 2 with dilute HCl and continuous extraction with diethyl ether at 4°C for 2h. Residual ether in the aqueous phase was then removed *in vacuo* and the acetoacetyl-CoA solution was carefully adjusted to pH 6–7 with solid $KHCO_3$. The acetoacetyl-CoA concentration of the preparation was determined by the method of Decker (1963). Succinyl-CoA was prepared from succinic anhydride and CoA (Simon & Shemin, 1953). Free succinic acid was removed by ether extraction as described above. The succinyl-CoA concentration of the ether-extracted solution was determined with 3-oxo acid CoA-transferase and 3-hydroxyacyl-CoA dehydrogenase in a coupled assay (Williamson, 1970).



Scheme 1. Ketone-body metabolism. Liver, the site of ketone-body production, discharges acetoacetate and 3-hydroxybutyrate into the blood. Extrahepatic tissues convert acetoacetate into acetyl-CoA via the 3-oxo acid CoA-transferase and acetoacetyl-CoA thiolase reactions.

Animals. Male rats of the Wistar strain weighing 160–250g were used. Alloxan-diabetes was induced by the intravenous injection of alloxan monohydrate (70mg/kg body wt.) under ether anaesthesia. The alloxan-diabetic rats were maintained on 4 units of protamine–zinc–insulin/day for a week, and then killed 24–48h after the last insulin injection (Steiner, Rauda & Williams, 1961). The blood sugar concentrations of these rats were about 30mM and those of the total ketone bodies about 2mM.

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, U.K.). 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 collection of the tissues.

Preparation of tissue extracts. The rats were killed by cervical fracture. The tissue was rapidly excised, wrapped in Parafilm and placed in crushed ice. After a few minutes the 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, pH7.4, was added and the mixture was homogenized with a glass pestle driven by a low-speed motor. All subsequent operations were carried out at 0–4°C. A portion of the homogenate, cooled in ice–water, was immediately exposed to ultrasonic vibration for exactly 30s at 15kHz (100W model; Measuring and Scientific Equipment Ltd., London S.W.1, U.K.). The ultrasonically treated homogenate was then centrifuged for 20min 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 tissue activity. In certain experiments where the intracellular distribution of the enzymes was to be studied another portion of the homogenate was centrifuged for 20min at 30000*g* before sonication. 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 exposed to ultrasonic vibration for exactly 30s, 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, microsomes and mitochondria.

Determination of enzyme activities. 3-Oxo acid CoA-transferase activity was determined in both directions. The principle of the assays is based on the work of Stern, Coon, del Campillo & Schneider (1956). For the determination of the rate of acetoacetyl-CoA formation from succinyl-CoA and acetoacetate the silica cuvettes (1cm) contained in a final volume of 2.0ml: tris–HCl buffer, pH8.5 (100μmol), $MgCl_2$ (10μmol), iodoacetamide (10μmol) (to inhibit acetoacetyl-CoA thiolase and enzymes of the hydroxymethylglutaryl-CoA pathway) and succinyl-CoA (0.2μmol). The enzyme sample (up to

0.1ml) was added and the rate of increase in extinction at 313nm was measured for 2min. Under these assay conditions the millimolar extinction coefficient of the acetoacetyl-CoA–Mg^{2+} complex was found to be 12 (313nm). Linear reaction rates were only obtained for a short period (about 2min), presumably owing to the rapid approach to equilibrium. For determination of the rate of acetoacetyl-CoA disappearance in the presence of succinate the cuvettes (1cm) contained in a final volume of 2.0ml: tris–HCl buffer, pH8.5 (100μmol), $MgCl_2$ (20μmol), iodoacetamide (10μmol) and acetoacetyl-CoA (0.15–0.20μmol). ΔE_{303} was recorded for 2min (spontaneous hydrolysis of acetoacetyl-CoA) and then the enzyme sample (up to 0.05ml) was added; ΔE_{303} was recorded for a further 3min (spontaneous hydrolysis plus 'acetoacetyl-CoA deacylase' activity), sodium succinate (0.1ml; 100μmol) was added and ΔE_{303} recorded for a further 3min (spontaneous hydrolysis plus deacylase plus 3-oxo acid CoA-transferase activity). Except where otherwise stated, this assay was used as a routine. Under the above conditions the millimolar extinction coefficient of acetoacetyl-CoA was found to be 20.5 (303nm).

Acetoacetyl-CoA thiolase activity was determined by measuring the decrease in E_{303} due to cleavage of acetoacetyl-CoA (Stern, 1956). The cuvettes (1cm) contained in a final volume of 2.0ml: tris–HCl buffer, pH8.5 (100μmol), $MgCl_2$ (10μmol), acetoacetyl-CoA (0.15μmol) and CoA (0.2μmol). The sample (5–10μl) was added and the decrease in E_{303} was recorded for 2min. 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 hydroxymethylglutaryl-CoA pathway enzymes); however, the activities of interfering enzymes in extrahepatic tissues are negligible (McGarry & Foster, 1969) in comparison with that of thiolase.

3-Hydroxybutyrate dehydrogenase activity was determined by measurement of the acetoacetate formed on incubation of the enzyme sample with DL-3-hydroxybutyrate in the presence of NAD^+. These experiments were carried out on homogenates prepared by the method of Lehninger, Sudduth & Wise (1960). The incubation mixture (final volume 10ml) consisted of: tris–HCl buffer, pH8.5 (500μmol), NAD^+ (50μmol), sodium DL-3-hydroxybutyrate (50μmol), nicotinamide (125μmol) and enzyme sample (0.5ml). Samples (2.0ml) of the assay mixture were removed at 0, 20, 40 and 60min and mixed with 2ml of 6% (w/v) $HClO_4$. After removal of protein by centrifugation the supernatant fluid was neutralized with 10% (w/v) KOH. The precipitate of $KClO_4$ was removed by centrifugation and the supernatant was analysed for acetoacetate (Walker, 1954).

Units of enzyme activity. All measurements of enzyme activity were carried out at 25°C. A unit of enzyme activity is defined as the amount of enzyme that transforms 1μmol of substrate/min at 25°C and specific activity is defined as units/g fresh wt. of tissue.

RESULTS

Activities of 3-oxo acid CoA-transferase and acetoacetyl-CoA thiolase in rat tissues. The relative activities in various rat tissues of the two enzymes involved in acetoacetate utilization are shown in Table 1. The activity of the 3-oxo acid CoA-transferase was measured in both directions in some tissues. In all tissues examined it was, under the test conditions, about five times higher in the direction of acetoacetate synthesis. Heart and kidney cortex were the two most active tissues followed by submaxillary gland and adrenal glands. The activity of the latter two tissues was about one-quarter of that of heart and kidney. The activity

Table 1. *Relative activities of 3-oxo acid CoA-transferase and acetoacetyl-CoA thiolase in rat tissues*

The values were calculated from the mean of at least three observations taking the activity of kidney to be 100%; the actual activity of kidney expressed as μmol of acetoacetyl-CoA removed or formed/min per g fresh wt. is shown in parentheses. For other details see the Materials and Methods section.

	Relative activity (as % of kidney)			
	3-Oxo acid CoA-transferase		Acetoacetyl-CoA thiolase	
Tissue	(*a*) Acetoacetate ↓ Acetoacetyl-CoA	(*b*) Acetoacetyl-CoA ↓ Acetoacetate	Present work	Wieland, Reinwein & Lynen (1956)
Kidney	100 (4.0)	100 (25.8)	100 (14.4)	100
Heart	130	100	62	145
Submaxillary gland	—	27	33	—
Adrenal glands	—	22	216	—
Brain	10	9	14	11
Lung	—	6	4	—
Spleen	—	5	10	—
Muscle (hind limb)	6	3	4	10
Epididymal fat-pad	—	1	1	—
Liver	<0.5	<0.5	140	—

Table 2. *Activities of 3-oxo acid CoA-transferase and acetoacetyl-CoA thiolase in kidney, heart and brain of mammalian species*

The values are expressed as μmol of acetoacetyl-CoA removed/min per g fresh wt. of tissue and are the means of at least two observations. For other details see the Materials and Methods section.

	Tissue					
	Kidney		Heart		Brain	
Species	3-Oxo acid CoA-transferase	Acetoacetyl-CoA thiolase	3-Oxo acid CoA-transferase	Acetoacetyl-CoA thiolase	3-Oxo acid CoA-transferase	Acetoacetyl-CoA thiolase
Mouse	30.9	20.7	29.8	27.5	4.3	2.0
Gerbil (*Meriones lybicus*)	47.5	20.9	44.0	19.0	1.5	1.4
Golden hamster	16.7	16.3	29.1	6.6	0.7	0.4
Rat	25.5	14.4	24.8	8.9	2.1	2.1
Guinea pig	5.2	9.1	14.0	3.1	0.3	1.5
Sheep	35.4	13.2	2.7	1.3	0.2	0.4

Table 3. *Activities of 3-oxo acid CoA-transferase in tissues of normal, starved alloxan-diabetic and fat-fed rats*

The values are expressed as μmol of acetoacetyl-CoA removed/min per g fresh wt. of tissue and are means± S.D. with the numbers of observations in parentheses. Values that are statistically significantly different from normal values are indicated by: *($P<0.01$). For full details see the Materials and Methods section.

	Tissue				
State of rats	Kidney	Heart	Brain	Muscle (hind limb)	Epididymal fat-pad
Normal	25.5±6.1 (14)	24.8±4.8 (7)	2.1±0.6 (21)	0.47±0.25 (10)	0.19±0.06 (8)
Starved for 48h	22.5±5.6 (20)	27.3±5.8 (8)	2.3±0.4 (13)	0.80±0.24 (10)*	0.13±0.02 (7)
Alloxan-diabetic	20.8±3.7 (6)	32.2±2.6 (6)	2.6±0.4 (6)	—	—
Fat-fed	29.3±5.9 (11)	28.1±4.4 (11)	2.7±1.1 (5)	—	—

Table 4. *Activities of acetoacetyl-CoA thiolase in tissues of normal, starved alloxan-diabetic and fat-fed rats*

The values are expressed as μmol of acetoacetyl-CoA removed/min per g fresh wt. of tissue and are means± S.D. with the numbers of observations in parentheses. Values that are statistically significantly different from normal values are indicated by: *($P<0.001$) and †($P<0.01$). For full details see the Materials and Methods section.

	Tissue				
State of rats	Kidney	Heart	Brain	Muscle (hind limb)	Epididymal fat-pad
Normal	14.4±3.1 (14)	8.9±2.5 (6)	2.1±0.6 (14)	0.61±0.24 (18)	0.13±0.07 (9)†
Starved for 48h	16.0±3.2 (15)	10.2±2.2 (8)	1.9±0.4 (8)	0.61±0.27 (4)	0.06±0.02 (9)
Alloxan-diabetic	14.4±0.9 (7)	13.2±2.4 (6)†	1.8±0.4 (6)	—	—
Fat-fed	23.0±6.1 (11)*	15.7±3.7 (11)*	—	—	—

of brain was about three times higher than that of skeletal muscle. In confirmation of the finding of Mahler (1953) no activity of the 3-oxo acid CoA-transferase could be detected in the liver. The distribution of acetoacetyl-CoA thiolase was similar to that of the transferase except that liver and adrenal glands had higher activities than heart and kidney. This reflects the role of the thiolase in processes other than ketone-body utilization (fatty acid oxidation and cholesterol synthesis).

Fractionation of the tissue homogenates indicated that at least 98% of the transferase activity and about 80% of the thiolase activity in kidney and brain were associated with the particulate fraction.

Table 5. *Activities of 3-hydroxybutyrate dehydrogenase in rat brain, kidney, heart and liver*

The values are expressed as μmol of acetoacetate formed/min per g fresh wt. of tissue and are means±S.D. with the numbers of observations in parentheses. For other details see the Materials and Methods section.

	Tissue			
State of rats	Kidney	Heart	Brain	Liver
Normal	1.47±0.16 (4)	0.89±0.18 (4)	0.57±0.08 (26)	17.4±3.0 (9)
Starved (48h)	—	—	0.53±0.07 (5)	18.5 (2)

Activities of 3-oxo acid CoA-transferase and acetoacetyl-CoA thiolase in tissues of various species. The activity of the transferase in kidney was about three times lower in the guinea pig than in the other species tested (Table 2). Transferase activity was also lower in heart and brain of guinea pig and sheep. The ratio of activities of transferase to thiolase was generally between 1 and 3 in most tissues. Table 2 shows that the enzymes required for acetoacetate utilization occur in many mammalian tissues.

Effects of starvation, alloxan-diabetes and fat-feeding on the activities of enzymes concerned with acetoacetate utilization. Since starvation, alloxan-diabetes and fat-feeding are associated with an increased turnover of ketone bodies (Bates *et al.* 1968) experiments were carried out to test whether enzymes involved in acetoacetate utilization are increased in these situations (Table 3). There was no significant change in the transferase activity of kidney, heart, brain, skeletal muscle and epididymal fat-pad. By contrast, the thiolase activity was higher in kidney (50%) and in heart (75%) after fat-feeding (Table 4). In heart, the activity was also raised in alloxan-diabetes (50%).

3-Hydroxybutyrate dehydrogenase activity in rat tissues. The activity of 3-hydroxybutyrate dehydrogenase was about the same in the brain from fed and starved (48h) rats (Table 5), in contradiction to the report by Smith, Satterthwaite & Sokoloff (1969) who found a six- to seven-fold increase after starvation. The values found in kidney, heart and liver for fed rats are similar to those reported by Lehninger *et al.* (1960). In agreement with the findings of Ohe, Morris & Weinhouse (1967) there was no change of activity in the liver on starvation.

DISCUSSION

As tissues other than liver are known to utilize acetoacetate it was to be expected qualitatively that the enzymes responsible for its utilization, 3-oxo acid CoA-transferase and acetoacetyl-CoA thiolase, are present in these tissues. The measurements reported in this paper, together with already available results on the activity of 3-hydroxybutyrate dehydrogenase (Lehninger *et al.* 1960), provide quantitative information on the activity of the enzymes of ketone-body utilization in various tissues. The virtual absence of the transferase from liver is the factor directing ketone bodies formed in the liver to the peripheral tissues. As shown in Table 6, the enzyme activities found in the various tissues are more than sufficient to account for the rates of ketone-body oxidation observed in slices and perfused organs. Thus there is no need to postulate additional enzymes of ketone-body utilization, such as acetoacetate-CoA kinase (Stern & Ochoa, 1951) or 3-hydroxybutyrate-CoA kinase. It has been suggested (Alexandre, Siliprandi & Siliprandi, 1969) that acetoacetyl-CoA can be formed from acetoacetate in rat heart and kidney mitochondria by other reactions, but we failed to detect such enzymes in extracts of rat brain and kidney.

The fact that neither starvation nor alloxan-diabetes alters the activities of the enzymes of ketone-body utilization, whereas the rate of utilization increases in these conditions (Bates *et al.* 1968), shows that changes in ketone-body utilization in a given tissue are not controlled by variations in the amount of the enzymes, but by the concentrations of ketone bodies in the blood, i.e. the rate at which the ketone bodies are made available by the liver.

The step controlling the extent to which ketone bodies serve as a fuel is the transferase reaction, which under steady-state conditions depends on the concentration of acetoacetate. The ready reversibility of the transferase and thiolase reactions in peripheral tissues (see Weidemann & Krebs, 1969; Williamson & Hems, 1970) indicates that these two enzyme systems are at near-equilibrium. The balance of the two reactions is:

$$\text{Acetoacetate} + \text{succinyl-CoA} + \text{CoA} \rightleftharpoons 2\ \text{acetyl-CoA} + \text{succinate}$$

The flux through this system depends on the rate at which acetyl-CoA disappears and on the concentration of acetoacetate.

Thus it is not the concentration of total ketone bodies but that of acetoacetate which, in the first instance, determines the rate of ketone-body

Table 6. *Enzyme activities of the ketone-body utilization pathway and rates of oxidation of ketone bodies in various rat tissues*

The values for enzyme activities have been taken from Tables 1, 4 and 5 and the rates of ketone-body utilization have been calculated from results in the cited references. Values are expressed as μmol of substrate oxidized/min per g fresh wt.

Tissues	Preparation	Substrate	Rate of utilization	Enzyme activity			Reference
				3-Hydroxybutyrate dehydrogenase	3-Oxo acid CoA-transferase*	Acetoacetyl-CoA thiolase	
Kidney	Cortex slices	Acetoacetate, 10mM	0.65	—	4.0	14.4	Weidemann & Krebs (1969)
Heart	Perfused	Acetoacetate, 10mM	1.03	—	5.2	8.9	Williamson & Krebs (1961)
Brain	Cortex slices	DL-Hydroxybutyrate, 17mM	0.03	0.57	0.42	2.1	Ide, Steinke & Cahill (1969)
Brain	Cortex slices	Acetoacetate, 5mM	0.02	—	0.42	2.1	Itoh & Quastel (1970)
Brain	Mince	Acetoacetate, 10mM	0.04	—	0.42	2.1	Openshaw & Bortz (1968)

* Measured in the direction of acetoacetate removal.

removal. If the activity of 3-hydroxybutyrate dehydrogenase were high the reaction catalysed by this enzyme may be included in the above equilibrium reaction:

$$\text{3-Hydroxybutyrate} + \text{NAD}^+ + \text{CoA} + \text{succinyl-CoA} \rightleftharpoons 2\ \text{acetyl-CoA} + \text{NADH} + \text{succinate}$$

However, in all tissues tested the activity of 3-hydroxybutyrate dehydrogenase is considerably lower than that of the transferase, the dehydrogenase/transferase activity ratio being 1:4 in brain, 1:28 in heart and 1:17 in kidney under test conditions. The relations may be different in physiological situations when the enzymes are not saturated with substrates and cofactors, and it is therefore not possible to decide whether the activity of 3-hydroxybutyrate dehydrogenase can be rate-limiting.

Smith *et al.* (1969) reported an approximately sixfold increase of the activity of 3-hydroxybutyrate dehydrogenase in rat brain on starvation. The present experiments do not confirm this, and show that the activities of the other enzymes of ketone-body metabolism in brain also do not change in response to starvation. The utilization of ketone bodies by the human brain during prolonged starvation (Owen *et al.* 1967) may therefore be attributed to the hyperketonaemia of starvation, rather than to a change in enzyme concentrations.

This work was supported by grants from the Medical Research Council and U.S. Public Health Service (Grant no. AM11748). D.H.W. is a member of the External Staff of the Medical Research Council.

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