Biochem. J. (1968) **110**, 655
Printed in Great Britain

Turnover Rates of Ketone Bodies in Normal, Starved and Alloxan-Diabetic Rats

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(*Received* 10 *June* 1968)

1. Rates of appearance and disappearance of total ketone bodies were determined in normal, starved and alloxan-diabetic rats by measuring specific radioactivities and concentrations of blood acetoacetate and 3-hydroxybutyrate at different times after injection of 3-hydroxy[^{14}C]butyrate. 2. The mean rates of appearance were 1·7, 4·2 and 10·9 μmoles/min./100g. body wt. respectively for normal, starved and alloxan-diabetic rats. The rates of disappearance were of the same order of magnitude as the rates of appearance. 3. There was a direct correlation between the rates of appearance and disappearance and the blood concentrations of the ketone bodies. 4. The results indicate that in the rat increased ketone-body production is paralleled by increased ketone-body utilization and that the raised ketone-body concentration in the blood in starvation and alloxan-diabetes is due to a slight imbalance between the rates of production and utilization. 5. The findings are discussed in relation to the concept that ketone bodies can serve as fuels of respiration when the supply of carbohydrate is limited.

It is well established that many animal tissues can oxidize ketone bodies (Snapper & Grünbaum, 1927; Wick & Drury, 1941; Williamson & Krebs, 1961) and this has led to the suggestion (see Krebs, 1961) that ketone bodies serve as a fuel of respiration when carbohydrate is in short supply, and that their raised concentration in blood in starvation and alloxan-diabetes is related to increased formation and utilization. To obtain quantitative information in support of this concept the turnover of ketone bodies was measured in normal, starved and alloxan-diabetic rats by injection of 3-hydroxy[^{14}C]-butyrate.

MATERIALS AND METHODS

Animals. Male rats of the Wistar strain weighing 160–220g. were used. Alloxan-diabetes was produced by injecting alloxan monohydrate (70mg./kg. body wt.) into the lateral tail vein of rats under ether anaesthesia. These rats were used 48hr. later, at which time the blood sugar concentration (starvation) was above 300mg./100ml. The normal rats were fed on commercial rat cubes, which contained approx. 15% of protein, 3% of fat and a residue consisting mainly of carbohydrate with mineral and vitamin supplements.

Radioactive materials. Ethyl [3-^{14}C]acetoacetate and ethyl [4-^{14}C]acetoacetate were obtained from The Radiochemical Centre, Amersham, Bucks. The ester was hydrolysed by the method of Krebs, Hems, Weidemann & Speake (1966). D-(−)-3-Hydroxy[^{14}C]butyrate was prepared by the reduction of [^{14}C]acetoacetate with NADH and 3-hydroxybutyrate dehydrogenase (Williamson, Mellanby & Krebs, 1962) in the following system: [^{14}C]acetoacetate (10–23 μmoles), sodium phosphate buffer, pH 6·5 (100 μmoles) and NADH (30 μmoles) in a final volume of 5ml. were incubated at room temperature with 3-hydroxybutyrate dehydrogenase (0·01ml.; about 0·4i.u.). Reduction of the acetoacetate, as determined by the decrease in NADH concentration, was complete after 3hr. The reaction was stopped by addition of 0·5ml. of 1N-HCl. The 3-hydroxy-[^{14}C]butyrate was separated from the incubation mixture by continuous extraction for 4hr. with diethyl ether and collected in 5ml. of 1% $NaHCO_3$. The diethyl ether was removed by distillation and the residual aqueous phase was neutralized and freeze-dried to reduce its volume to about 1ml.

The 3-hydroxy[^{14}C]butyrate was further purified by paper chromatography. The neutral solution of 3-hydroxy-[^{14}C]butyrate was applied to Whatman no. 1 chromatography paper as a narrow band. The chromatogram was developed overnight with ethanol–aq. NH_3 (sp.gr. 0·88)–water (16:1:3, by vol.) (Nordmann, Gauchery, du Ruisseau, Thomas & Nordmann, 1954). The location of the band of 3-hydroxy[^{14}C]butyrate on the chromatogram was determined by exposing an X-ray plate to the chromatogram for

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15min. The strip containing the 3-hydroxy[^{14}C]butyrate was eluted with 1ml. of water. The specific radioactivity of the 3-hydroxy[^{14}C]butyrate decreased after this treatment by about 20%. Since the determination of 3-hydroxybutyrate, which is described in the following section, was specific, chromatography resulted in the removal of radioactivity not associated with this compound. The radiochemical purity of the final product was considered to be about 98%, and it was found to contain less than 0·2% of acetoacetate by enzymic analysis.

Experimental procedure. In initial experiments the rats were anaesthetized with diethyl ether, but it was found that the ketone-body concentrations tended to rise throughout the experiment. More consistent results were obtained when Nembutal (9mg./rat) was injected intraperitoneally into the rats. Approx. 1·4μc of 3-hydroxy[^{14}C]butyrate (0·30μmole) was used in each experiment. In no experiment did the injected tracer amount to more than 15% of the pool size and usually it was considerably less. The tracer diluted to 0·5ml. with 0·9% NaCl was injected into the lateral tail vein.

Blood samples were taken from the tip of the tail at various intervals between 2 and 32min. after the injection. In most experiments five or six samples (0·2–0·3ml.) of blood were taken. The samples were collected in tubes containing 2ml. of 3% (w/v) $HClO_4$. The amount of blood collected was found by weighing the tubes before and after addition of the blood. After centrifugation the supernatant was neutralized with 10% (w/v) KOH. The tubes were placed in ice for 30min. and then centrifuged to remove $KClO_4$. Samples of the supernatant were taken for determination of the concentration of acetoacetate and hydroxybutyrate and radioactivity of acetoacetate and total ketone bodies.

Determination of ketone-body concentrations. The method of Williamson *et al.* (1962) was used to measure the acetoacetate concentration on a sample of the supernatant. After the determination was completed, 0·15ml. of 1·0N-HCl was added and the samples were left overnight in the cuvettes at room temperature. The samples were then neutralized with 0·15ml. of 1N-NaOH. Total ketone bodies (original hydroxybutyrate present in the sample plus hydroxybutyrate formed from acetoacetate) were determined by addition of 0·5ml. of 1M-tris, pH 9·3, and 0·1ml. of NAD^+ (4·5μmoles). The reaction was started by the addition of 5μl. of 3-hydroxybutyrate dehydrogenase (40i.u./ml.). The reduction of NAD^+ was followed spectrometrically at 340mμ until the reaction was complete (about 60min.). This modification of the original method (Williamson *et al.* 1962) was used so as to avoid trapping the acetoacetate with hydrazine, which might interfere with the determination of radioactivity in the total ketone bodies.

Measurement of radioactivity. A sample of the supernatant obtained after neutralization of the $HClO_4$ extract was used for measurement of total radioactivity. A scintillation-counting mixture (10ml.) of composition 8g. of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen (Ciba Ltd., Duxford, Cambs.), 600ml. of toluene, 400ml. of methyl-Cellosolve and 80g. of naphthalene was used and the samples were counted in a Beckman scintillation counter. The radioactivity of acetoacetate was determined by precipitating the acetoacetate as the mercury–acetone compound (Peters & Van Slyke, 1932) by the following procedure. The sample (neutralized $HClO_4$ extract) containing acetoacetate was made up to 0·5ml. with water in a 5ml. glass-stoppered centrifuge tube. Carrier acetoacetate (30μmoles) was added in 0·3ml. of water. After the addition of 0·22ml. of 18N-H_2SO_4 and 0·78ml. of $HgSO_4$ (10g. in 100ml. of 4N-H_2SO_4) the samples were stoppered, mixed and placed in a boiling-water bath for 30min. A pale-yellow precipitate of the mercury–acetone compound formed during the heating. The samples were cooled in ice and centrifuged for 15min. at 3000g in a refrigerated centrifuge. The supernatant was poured off and the precipitate washed twice with 2ml. of ice-cold water. Each washing was followed by centrifugation and removal of the supernatant. After the last washing 0·4ml. of 2N-HCl was added to the precipitate. The precipitate was dissolved by shaking the tubes in a boiling-water bath for about 1min. The solution was then transferred quantitatively to a scintillation vial, scintillation fluid (15ml.) being used for the transfer.

The contents of the cuvettes from the determination of total ketone-body concentration were used for measurement of the radioactivity in the total ketone bodies. The contents were transferred to 5ml. glass-stoppered centrifuge tubes. Acetoacetate carrier, H_2SO_4 and $HgSO_4$ were added as described above for the determination of acetoacetate radioactivity and the same procedure was used.

The radioactivity of the 3-hydroxybutyrate was found by subtracting the acetoacetate radioactivity from total ketone-body radioactivity. Blanks and standards of either [^{14}C]acetoacetate or 3-hydroxy[^{14}C]butyrate were run as a routine. Recoveries of radioactivity were virtually constant in the range 78–82% of the added radioactivity. The specificity of the precipitation procedure was demonstrated by converting the acetoacetate of a sample into 3-hydroxybutyrate with 3-hydroxybutyrate dehydrogenase and then adding acetoacetate carrier, H_2SO_4 and $HgSO_4$ solution and treating the sample as described above. Less than 5% of the total blood radioactivity was precipitated. However, even this amount could lead to an overestimate of the radioactivity present in acetoacetate.

Definition of terms. For the purposes of the present work the ketone-body pool (see Scheme 2) is taken to be a collection of molecules of two separate species (acetoacetate and 3-hydroxybutyrate) within the animal body that becomes rapidly and uniformly labelled after injection of radioactive molecules of one of the species (hydroxybutyrate). Any pool molecule, labelled or unlabelled, has an equal chance of being removed from the pool.

The distribution space is defined as the volume of body water in which the pool is distributed. It is generally assumed that the pool is evenly distributed in this space and that the concentration is similar to that of the blood or plasma.

Rate of appearance (production) is the rate at which molecules enter their pool.

Rate of disappearance (utilization) is the rate at which molecules leave their pool. The half-life time ($t_{\frac{1}{2}}$) is the time required to replace one-half of the molecules in the pool.

Mathematical analysis. Since in this type of tracer experiment specific radioactivities decrease with time in a logarithmic manner, log(specific radioactivity of 3-hydroxybutyrate) was plotted against time and straight lines were fitted by the least-squares method. The distribution space was found by dividing the total radioactivity injected into the animal by the radioactivity/ml. of blood at zero time. Rates of appearance and disappearance were calculated by using the slope of the log(specific radioactivity)–time curves, the distribution spaces, concentra-

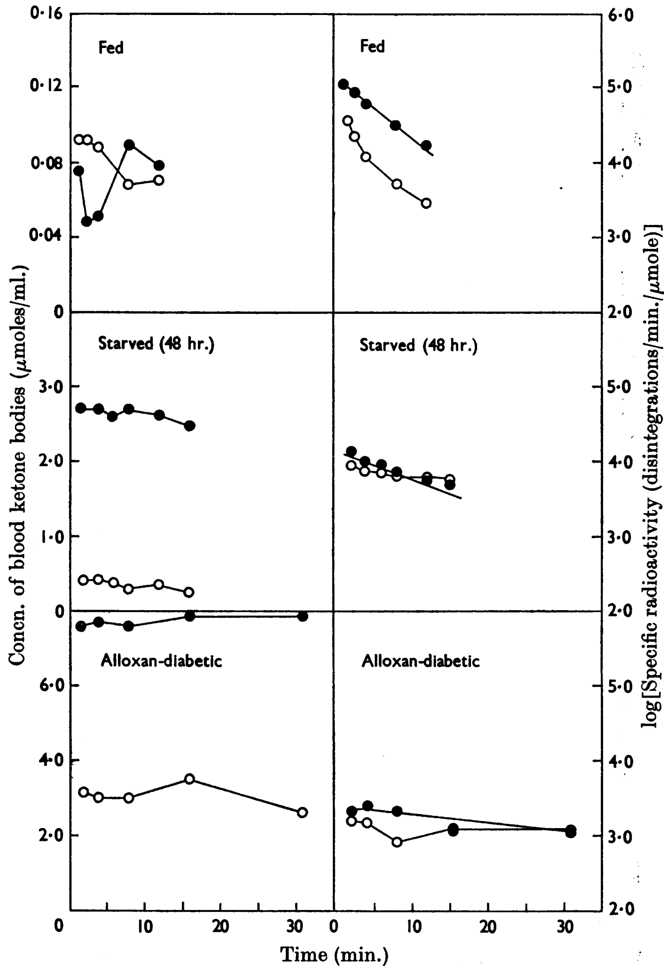


Fig. 1. Representative progress curves of the concentration and log (specific radioactivity) of acetoacetate and 3-hydroxybutyrate. For the experimental details see the Materials and Methods section. ○, Acetoacetate; ●, 3-hydroxybutyrate.

tions of the ketone bodies in the blood and the rates of change of ketone-body concentration during the experimental period (Wrenshall, 1955). The standard deviations of the slope of the log (specific radioactivity)–time curves were also calculated (Snedecor, 1946). The half-life times were calculated from the slopes of the log (specific radioactivity)–time curves (Kamen, 1957).

RESULTS

Representative time curves of the concentration and log (specific radioactivity) of acetoacetate and 3-hydroxybutyrate for fed, starved and alloxan-diabetic rats are shown in Fig. 1. The log (specific radioactivity)–time curves are best described by a straight line. The body weights, distribution spaces, blood ketone-body concentrations and turnover data are presented in Table 1. The mean distribution spaces were 41%, 36% and 33% of the body weight for fed, starved and alloxan-diabetic groups respectively. As the half-life times increased in the starved and alloxan-diabetic rats, the distribution spaces became more constant and smaller, though the differences between the groups were not significant. The mean distribution space for all groups was 36% of the body weight; a value of 50% was calculated by Nelson, Grayman & Mirsky (1941). In the calculation of turnover rates, the mean distribution space for the group was used to calculate the distribution spaces of the individual rats in the group. The mean distribution spaces used were obtained from a larger number of rats than those reported. This explains the small

Table 1. *Body weights, distribution spaces, blood ketone-body concentrations and turnover data of rats injected with 3-hydroxy[^{14}C]butyrate*

The distribution space, the volume of body water in which the ketone-body pool is distributed, was calculated by dividing the total radioactivity injected into the animals by the radioactivity/ml. of blood at zero time. The slope of the log(specific radioactivity)–time curve was found by fitting a line to the plotted log(specific radioactivity) found at different time-intervals during the experiment. The half-life time ($t_{\frac{1}{2}}$) is the time required for the specific radioactivity to decrease by one-half. Rates of appearance and disappearance are the rates at which ketone bodies enter or leave their pool. Each experiment represents the results from one rat. For full details see the Materials and Methods section.

				Distribution space		Mean concentration			Specific radioactivity 2 min. after injection of tracer						
State of animals	Expt. no.	Tracer used	Body wt. (g.)	Measured (ml.)	Calc. (ml.)	Acetoacetate (μmoles/ml.)	Hydroxybutyrate (μmoles/ml.)	Total ketone bodies (μmoles/ml.)	10^{-3} × Acetoacetate (disintegrations/min./μmole)	10^{-3} × Hydroxybutyrate (disintegrations/min./μmole)	Acetoacetate/Hydroxybutyrate (%)	Slope of log(specific radioactivity)–time curve ± S.D.	Half-lifetime $t_{\frac{1}{2}}$ (min.)	Rate of appearance (μmoles/min./100g. body wt.)*	Rate of disappearance (μmoles/min./100g. body wt.)*
Fed	1	3-^{14}C	190	87	78	0·095	0·040	0·135	142·1	179·8	79	0·0778 ± 0·0036	3·9	0·98	
	2	4-^{14}C	209	71	86	0·089	0·055	0·144	72·9	424·0	17	0·0884 ± 0·021	3·4	1·2	
	3	3-^{14}C	201	71	82	0·078	0·068	0·146	29·2	85·0	34	0·0767 ± 0·0053	3·9	1·1	
	4	3-^{14}C	205	94	84	0·081	0·216	0·297	21·6	36·0	60	0·128 ± 0·0112	2·3	3·6	
		Average	201	81	82	0·086	0·095	0·180					3·4	1·7	
Starved (48hr.)	6	4-^{14}C	170	67	61	0·54	1·11	1·65	30·6	43·9	70	0·0344 ± 0·0018	8·7	4·8	4·6
	7	3-^{14}C	170	—	63	0·32	1·12	1·44	6·74	9·81	69	0·0244 ± 0·0007	12·3	2·8	3·3
	8	4-^{14}C	219	48	79	0·38	1·51	1·89	27·0	31·0	87	0·0234 ± 0·0038	12·8	3·6	3·5
	9	3-^{14}C	160	62	58	0·34	1·63	1·97	9·23	12·1	76	0·0287 ± 0·0034	10·5	4·7	5·6
	10	3-^{14}C	176	66	63	0·51	1·77	2·28	8·10	9·86	82	0·0210 ± 0·0041	14·3	4·0	5·4
	11	3-^{14}C	182	—	66	0·42	1·99	2·41	8·24	9·24	87	0·0261 ± 0·0016	11·5	5·2	5·9
		Average	180	61	65	0·42	1·52	1·96					11·7	4·2	4·7
Alloxan-diabetic	12	3-^{14}C	191	66	63	0·71	2·31	3·02	6·06	8·80	69	0·0169 ± 0·0034	17·8	3·9	3·8
	13	3-^{14}C	198	71	65	1·09	3·00	4·09	5·70	6·33	90	0·0264 ± 0·0044	11·4	8·2	6·9
	14	3-^{14}C	179	74	59	0·95	3·55	4·50	4·81	5·42	89	0·0181 ± 0·0007	16·6	6·2	5·5
	15	4-^{14}C	208	69	69	2·00	5·61	7·61	8·99	6·50	138	0·0166 ± 0·0038	18·1	9·6	7·0
	16	4-^{14}C	195	55	64	3·11	7·76	10·87	1·74	2·15	81	0·0126 ± 0·0033	23·8	10·4	10·2
	17	4-^{14}C	171	57	56	2·75	11·38	14·13	3·02	4·53	66	0·0136 ± 0·0027	22·1	13·8	14·1
	18	4-^{14}C	159	55	52	4·61	16·89	21·50	1·26	2·48	51	0·0147 ± 0·0042	20·5	23·9	24·4
		Average	186	64	61	2·18	7·21	9·40					18·6	10·9	10·3

* Calculated from values for total ketone body pool.

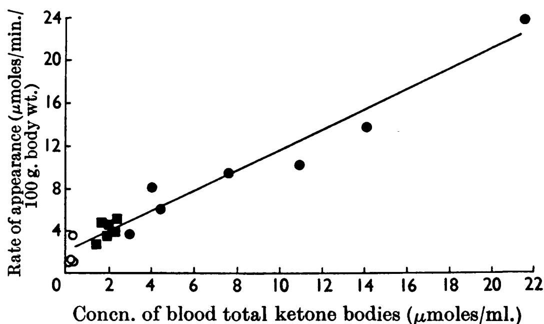


Fig. 2. Relationship between rate of appearance of ketone bodies and total ketone-body concentration in blood. For the experimental details see the Materials and Methods section. ○, Fed rats; ●, alloxan-diabetic rats; ■, starved rats (48hr.)

discrepancies found between measured and calculated group averages (Table 1).

The fed rats had very low blood ketone-body concentrations. Changes in concentration with time therefore could not be determined accurately. The individual values found during an experiment were therefore averaged and this average was used to calculate specific radioactivities. The starved animals had a mean total ketone-body concentration in the blood about 11 times that of the fed animals. A considerable range of concentrations was found in the alloxan-diabetic group, but the average was about 50 times that of the fed group.

The specific radioactivities of acetoacetate in the starved and alloxan-diabetic rats were, on average, 77% that of the hydroxybutyrate and were unrelated to the blood concentration of ketone bodies. Considerable variation was found in the fed group. The specific radioactivities of acetoacetate decreased with time suggesting that maximum specific radioactivities were reached before the first samples were taken at 2min.

Turnover rates were calculated from the values for the total ketone-body pool; the reason for this is discussed in the next section. The rates of appearance were greater in the starved and alloxan-diabetic rats than in the fed rats, but overlapping of values occurred (Table 1). A good correlation, however, between blood ketone-body concentrations and rates of appearance was found (Fig. 2).

Rates of disappearance were calculated for the starved and alloxan-diabetic rats and in general were similar to the rates of appearance (Table 1). This could not be done reliably for the normal group since, as mentioned above, the blood ketone-body concentrations were too low to be measured with sufficient accuracy.

The 3-hydroxy[^{14}C]butyrate was labelled in either position 3 or 4. The position of the label used in each experiment is shown in Table 1. The rates obtained were similar regardless of the position of the label.

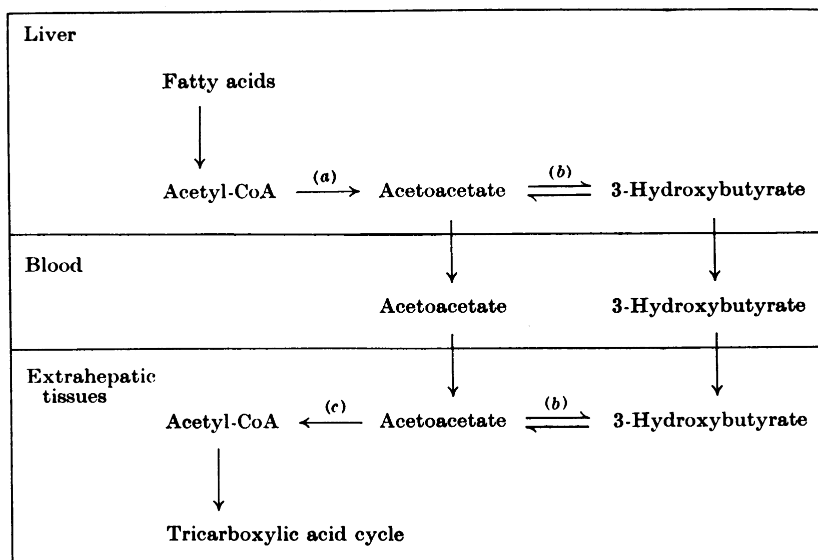
Similar experiments have been carried out with [^{14}C]acetoacetate, but are not reported here because of the uncertainties as to the radiochemical purity of the acetoacetate, and also the failure of the two ketone bodies to equilibrate rapidly. If it is assumed that no equilibration occurred between acetoacetate and 3-hydroxybutyrate during the experimental period (5–6min.) then the calculated turnover rates are comparable with those reported with 3-hydroxy-[^{14}C]butyrate (Table 1).

DISCUSSION

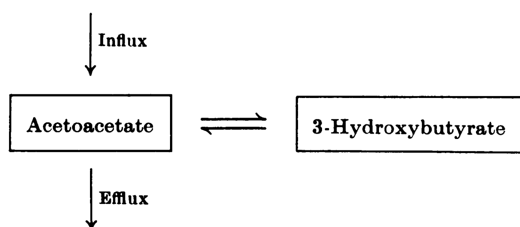
Isotopic equilibrium between acetoacetate and 3-hydroxybutyrate. The liver is the major site of ketogenesis in the rat and the extrahepatic tissues are responsible for the further metabolism of ketone bodies. Any study of ketone-body metabolism is complicated by the existence of two forms of ketone bodies, acetoacetate and 3-hydroxybutyrate, which are readily interconverted in certain tissues by 3-hydroxybutyrate dehydrogenase. An outline of ketone-body metabolism is shown in Scheme 1. For the purposes of the present work, this scheme has been further simplified to give the model pool system shown in Scheme 2.

In calculating rates of appearance and disappearance from results obtained by using 3-hydroxy[^{14}C]butyrate, it was assumed that the rate of equilibration between acetoacetate and 3-hydroxybutyrate was so fast that the rate of decrease in the 3-hydroxybutyrate specific radioactivity actually reflected the rate at which acetoacetate entered the total ketone-body pool. In practice, the specific radioactivities of acetoacetate were similar to those of 3-hydroxybutyrate within 2min. of the injection of 3-hydroxy[^{14}C]butyrate. It therefore seemed justifiable to calculate the rates of appearance and disappearance from the values for the total ketone-body pool.

Comparison with rates of appearance and disappearance obtained by other methods. Data bearing on the comparison between the rates of turnover reported here and the rates of utilization measured by infusing ketone bodies into intact animals and rates of production measured in liver perfusion experiments are given in Table 2. The rates of disappearance calculated from results obtained by using 3-hydroxy[^{14}C]butyrate (Table 1) agree with the rates of utilization reported for normal and diabetic rats in which the ketone-body concentration was increased by injection of either 3-hydroxybutyrate or acetoacetate. The values reported for diabetic (pancreatectomized) rats by Scow &



Scheme 1. Outline of ketone-body metabolism in the rat. Enzymes involved in reactions: (*a*) acetoacetyl-CoA thiolase, hydroxymethylglutaryl-CoA synthase and hydroxymethylglutaryl-CoA lyase; (*b*) 3-hydroxybutyrate dehydrogenase; (*c*) 3-oxo acid-CoA transferase and acetoacetyl-CoA thiolase.



Scheme 2. Model pool system for ketone bodies (simplified from Scheme 1).

Chernick (1960) were for rats that were exposed also to the stress of starvation and underfeeding. Rates of production for perfused livers from normal rats varied from 0·5 to 9·3 μmoles/min./100g. body wt. (Table 2). Here again variable results have been obtained with diabetic rats, because Söling, Kattermann, Schmidt & Kneer (1966) found no increase in ketone-body production whereas Heimberg, Dunkerley & Brown (1966) reported a three- to four-fold increase.

Measurements of the hepatic activity of hydroxymethylglutaryl-CoA synthase, considered to be the rate-limiting enzyme in acetoacetate synthesis (Williamson, Bates & Krebs, 1968), gave a mean value for normal and starved rats of 23 μmoles/min./100g. body wt. (corrected to 37°). This activity is well in excess of the rates of appearance calculated for these groups (Table 1). The highest rate of appearance found for alloxan-diabetic rats was 24 μmoles/min./100g. body wt. and the corresponding hydroxymethylglutaryl-CoA synthase activity was 36 μmoles/min./100g. body wt. Thus it would appear that the capacity of the hydroxymethylglutaryl-CoA pathway is sufficient in all cases to account for the rates of ketogenesis measured *in vivo*. In contrast, it can be calculated from recent measurements of mitochondrial acetoacetyl-CoA deacylase (Burch & Triantafillou, 1968) that the activity of this enzyme is only 0·6 μmole/min./100g. body wt.

Physiological implications. The results are in accordance with the concept that it is a physiological function of the ketone bodies (Wick & Drury, 1941; Krebs, 1961) to serve as fuels of respiration when glucose is in short supply (as in starvation), or is not readily available (as in diabetes). The experiments show that increased production is closely matched by increased utilization. The raised concentration of ketone bodies in the blood in certain states is caused by a relatively slight 'imbalance' between production and utilization. Utilization, over a wide range, is approximately proportional to the concentration of ketone bodies in the blood (Nelson *et al.* 1941; this paper), which suggests that a rise in the blood ketone-body concentration is a prerequisite for higher rates of utilization. Thus the opposing views held by earlier workers as to whether ketosis is due to overproduction or underutilization

Table 2. *Comparison of rates of production and utilization of ketones bodies calculated from available data*

Rates measured	Animal preparation used	Substrate used	Method of administration	Concn. of blood ketone-bodies or substrate (μmoles/ml.)	Rate of production or utilization (μmoles/min./100g. body wt.) Normal rats	Diabetic rats	Reference
Utilization	Starved and underfed rats	D-3-Hydroxybutyrate	Single injection	11–13	8	2·4	Scow & Chernick (1960)
		Acetoacetate	Single injection	10–11	9	5	Scow & Chernick (1960)
	Eviscerated rats	Acetoacetate	Constant infusion	4–6	4·6	4·9	Söling, Garlepp & Creutzfeldt (1965)
	Nephrectomized rats	DL-3-Hydroxybutyrate	Single injection	5	12	—	Nelson *et al.* (1941)
Production	Perfused rat liver	Oleate	Infusion	2	9·0	6·5	Krebs (1967)
	Perfused rat liver	Palmitate	Infusion	0·6	0·7	2·7	Heimberg *et al.* (1966)
				1·2	0·5	3·0	
				2·4	1·0	3·0	
	Perfused rat liver	Free fatty acids	Infusion	2·7–3·0	9·3	8·4	Söling *et al.* (1966)

of ketone bodies no longer seem appropriate. The sequence of events leading to 'physiological ketosis' may be visualized as follows. As a consequence of hormonal interrelationships a low blood sugar concentration (starvation) causes an increase in adipose-tissue lipolysis and a rise in the concentration of free fatty acids in the plasma. This in turn results in an increased rate of ketogenesis in the liver, which is followed by a rise in blood ketone-body concentrations and an increased rate of peripheral utilization. Only in the more severe stages of diabetes does there appear to be a real overproduction of ketone bodies beyond physiological needs. The high blood concentrations reached in these situations result in large losses of ketone bodies and alkali via urine.

We thank Miss B. Walker for skilled technical assistance. This work was supported by U.S. Public Health Service Grant no. AM08715.

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