of heliotrine and of rats of the same strain as used in previous studies with heliotrine in this Department. We are also indebted to Dr L. M. Birt of the Department of Biochemistry, University of Melbourne, for valuable advice and discussions. The work was made possible by the financial support of the Anti-Cancer Council of Victoria.

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The Mechanism of Ketone-Body Formation from Butyrate in Rat Liver

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Two mechanisms have been proposed for the conversion of acetoacetyl-coenzyme A into acetoacetate in liver tissue. They are the direct hydrolysis of the thioester (Drummond & Stern, 1960) and the pathway via β-hydroxy-β-methylglutaryl-coenzyme A (Lynen, Henning, Bublitz, Sörbo & Kröplin-Rueff, 1958). The results obtained by Hird & Symons (1961) indicated that the β-hydroxy-β-methylglutaryl-coenzyme A pathway was operating in rumen and omasum tissue of the sheep and it was calculated that at least three-quarters of the acetoacetyl-coenzyme A formed from butyrate by these tissues was deacylated in this way.

Most of the literature on this subject has been concerned with liver tissue in which the steps in the oxidation of fatty acids are known in some detail; consequently, there are fewer assumptions in interpreting results obtained with this tissue as compared with those from the rumen and the omasum of the sheep. The experimental procedures used with sheep tissues have therefore been extended to rat-liver slices and mitochondria. The results obtained indicate a similar mechanism of ketone-body formation with each of the preparations. A preliminary account of some of this work on liver preparations has already been published (Hird & Symons, 1960).

METHODS AND MATERIALS

Chemicals. Sodium [1-^{14}C]butyrate, sodium [1-^{14}C]-acetate, sodium DL-[2-^{14}C]lactate and sodium [1-^{14}C]-octanoate were obtained from The Radiochemical Centre, Amersham, Bucks. Sodium [3-^{14}C]butyrate was obtained from Commissariat à l'Energie Atomique, France. Sodium [2-^{14}C]butyrate and sodium [4-^{14}C]butyrate were prepared as described by Hird & Symons (1961). Further supplies of sodium [2-^{14}C]butyrate were obtained from Research Specialties Co., Richmond, Calif., U.S.A.

Hexokinase (160 000 K.M. units/g.; Kunitz & McDonald, 1946) and ATP were purchased from Sigma Chemical Co.

Rat-liver slices. Fresh rat liver was chilled in ice-cold Krebs–Ringer phosphate buffer, pH 7·4, and sliced by hand. The slices were incubated in Krebs–Ringer phos-

phate buffer in a conventional Warburg apparatus with O_2 as the gas phase as previously described for rumen and omasum tissue (Hird & Symons, 1959, 1961).

Rat-liver mitochondria. All procedures were carried out at 0–2°. Chilled rat liver was homogenized in 4 vol. of 0·3M-sucrose in an all-glass Potter–Elvehjem homogenizer. Nuclei and cell debris were removed by centrifuging at 600*g* for 5 min. and the mitochondria were sedimented at 5000*g* for 15 min. The pellet was resuspended in 0·3M-sucrose and centrifuged at 10 000*g* for 10 min. After the fluffy layer was rinsed from the pellet, the mitochondria were suspended in the medium (minus EDTA) of Chappell & Perry (1954) and spun down at 5000*g* for 10 min. This final pellet was suspended in the Chappell & Perry medium for use. Washing and suspension of the mitochondria in this medium was necessary as sucrose interferes with the procedure used for the estimation of ketone bodies. The concentration of mitochondrial protein was estimated by the method of Aldridge (1957).

Incubations were carried out in a conventional Warburg apparatus. All flasks contained 0·6 ml. of mitochondrial suspension in a total volume of 3·5 ml. and the following substances at the final concentrations given: 30 mM-potassium phosphate buffer, pH 7·4; 1 mM-EDTA; 35 mM-KCl; 35 mM-tris–HCl buffer, pH 7·4; 1·6 mM-ATP; 3 mM-$MgCl_2$. When hexokinase was used, 30 μmoles of glucose and 0·1–0·5 mg. of hexokinase were added in 0·3 ml. from the side arm. Incubation procedures were as described for rat-liver slices; the gas phase was air.

Estimation procedures. The methods used for the estimation of ketone bodies in the incubation medium, the isotopic distribution between C-1 and C-3 of the ketone bodies, the specific activities of substrates and of the ketone bodies formed and the ^{14}C content of the centre well of the manometric flasks have been described previously (Hird & Symons, 1959, 1961).

RESULTS

The results in Table 1 show that in both slice and mitochondrial systems there is substantial asymmetry in the incorporation of ^{14}C, from the substrates tested, into C-1 as compared with C-3 of the ketone bodies formed. In this system the ^{14}C-labelled acetate, lactate and octanoate can be regarded as precursors of [1-^{14}C]acetyl-CoA. It is also of significance that [1-^{14}C]butyrate behaves in a similar manner even though it could conceivably be converted into ketone bodies without passing through [1-^{14}C]acetyl-CoA as an intermediate. Table 1 also shows that octanoate, in both slice and mitochondrial preparations, makes a substantial contribution of carbon atoms but that lactate does so only in the whole-cell system. Acetate, in contrast, is only poorly incorporated in both systems.

In the mitochondrial experiments in this Table, 0·5 mg. of hexokinase was added and the low production of ketone bodies is probably due to the lowering of the concentration of the ATP necessary for the activation of butyrate. However, ketone-body production is restored to 10 μmoles/flask on addition of malate. In each case the isotopic distributions were the same.

Table 2 shows that, in the process of oxidation of C-1 and C-3 of butyrate to carbon dioxide by the tricarboxylic acid cycle in both types of preparation, substantial asymmetry is also obtained. For liver slices it was necessary to make a correction (Hird & Symons, 1961) for spontaneous decomposition of acetoacetate. Asymmetry of oxidation is also shown when C-2 is compared with C-4, but it is somewhat less in magnitude. In the last-mentioned experiments there is, of course, no correction necessary for the decomposition of acetoacetate and accordingly the results are of greater accuracy than those obtained with butyrate labelled in the C-1 and C-3 positions.

To obtain further information on the change in position of carbon atoms during the conversion of

Table 1. *Incorporation of labelled precursors of* [1-^{14}C]*acetyl-coenzyme A into the ketone bodies formed in the presence of unlabelled butyrate by rat-liver slices and mitochondria*

Approx. 200 mg. wet wt. of rat-liver slices was incubated for 2 hr. at 38° in 3·0 ml. of Krebs–Ringer phosphate buffer, pH 7·4, containing substrates as shown. Results were calculated to 50 mg. dry wt. of tissue/flask. Mitochondria were incubated for 40 min. in 3·6 ml. of medium containing substrates as shown and 0·5 mg. of hexokinase. Mitochondrial protein: 5·5 mg./flask; approx. 1·2–2·3 × 10^5 counts of [^{14}C]substrate/min./flask. 10 mM-Butyrate was present in all flasks.

		Ketone bodies			
			Percentage of total ^{14}C		
System	Additions	Total (μmoles)	In C-1	In C-3	$\frac{\text{Counts/min. in ketone bodies}}{\text{Counts/min. added}} \times 100$
Liver slices	[1-^{14}C]Butyrate (3·0 μmoles)	3·0	83	17	5·9
	[1-^{14}C]Acetate (2·0 μmoles)	3·5	69	31	1·2
	DL-[2-^{14}C]Lactate (2·5 μmoles)	4·5	71	29	17
	[1-^{14}C]Octanoate (2·5 μmoles)	3·9	78	22	32
Mitochondria	[1-^{14}C]Butyrate (3·0 μmoles)	2·3	78	22	3·4
	[1-^{14}C]Acetate (2·0 μmoles)	2·6	71	29	0·3
	DL-[2-^{14}C]Lactate (2·5 μmoles)	2·9	78	22	2·8
	[1-^{14}C]Octanoate (2·5 μmoles)	4·8	67	33	26

Table 2. *Unequal oxidation of acetyl moieties of butyrate via the tricarboxylic acid cycle by rat-liver slices*

Rat-liver slices (200–350 mg. wet wt.) were incubated for 2 hr. in 3·0 ml. of Krebs–Ringer phosphate buffer, pH 7·4, containing substrates as shown. Results were calculated to 50 mg. dry wt. of tissue/flask. Mitochondria were incubated for 50 min. in 3·5 ml. of medium. Mitochondrial protein: in preparation (1), 10·2 mg.; in preparation (2), 11·6 mg. Approx. 0·3–2·5 × 10^5 counts of [^{14}C]substrate/min./flask. Substrates were in 20 mM concentration except for the two used with mitochondria preparation (2), which were 10 mM.

System	Substrate	Oxidation to $^{14}CO_2$ (%)
Liver slices	[1-^{14}C]Butyrate	2·0
	[3-^{14}C]Butyrate	0·5
	[2-^{14}C]Butyrate	0·55
	[4-^{14}C]Butyrate	0·37
Mitochondria (1)	[1-^{14}C]Butyrate	2·1
	[3-^{14}C]Butyrate	0·5
Mitochondria (2)	[2-^{14}C]Butyrate	0·76
	[4-^{14}C]Butyrate	0·32

butyrate into ketone bodies, experiments were done to compare the movement of C-3 of butyrate into C-1 of ketone bodies as well as the reverse. Table 3 shows that similar percentage changes of position occur in with liver slices. However, with mitochondria the agreement is not as close, there being a greater movement of C-3 to C-1 than the reverse. This observation has also been made with tissue preparations of rumen and omasum of the sheep (Hird & Symons, 1961). This behaviour is correlated with a greater rate of synthesis of ketone bodies. Perhaps under these conditions there is a preferential oxidation of the C-1 moiety or a preferential utilization of the C-3 moiety for ketone-body synthesis, but so far no satisfactory explanation has been found.

The results given in Table 4 show that butyrate, hexanoate and octanoate, but not acetate, are potent sources of ketone bodies when these substances are oxidized by mitochondria. As in the other experiments there is asymmetry of in-

Table 3. *Isotopic distribution in ketone bodies formed in the presence of* [1-^{14}C]*butyrate and* [3-^{14}C]*butyrate by rat-liver slices*

Rat-liver slices (200–350 mg. wet wt.) were incubated for 2 hr. at 38° in 3·0 ml. of Krebs–Ringer phosphate buffer, pH 7·4, containing substrates as shown. Results were calculated to 50 mg. dry wt. of tissue/flask. Mitochondria were incubated for 50 min. in 3·5 ml. of medium. Mitochondrial protein: 10·2 mg./flask. Approx. 0·6–1·7 × 10^5 counts of [^{14}C]substrate/min./flask.

System	Substrate	Ketone bodies: Total (µmoles)	Percentage of total ^{14}C: In C-1	In C-3
Liver slices	5 mM-[1-^{14}C]Butyrate	3·5	77	23
	20 mM-[1-^{14}C]Butyrate	3·7	79	21
	5 mM-[3-^{14}C]Butyrate	3·7	24	76
	20 mM-[3-^{14}C]Butyrate	4·0	22	78
Mitochondria	5 mM-[1-^{14}C]Butyrate	19	82	18
	20 mM-[1-^{14}C]Butyrate	20	80	20
	5 mM-[3-^{14}C]Butyrate	19	30	70
	20 mM-[3-^{14}C]Butyrate	21	32	68

Table 4. *Incorporation of labelled precursors of* [1-^{14}C]*acetyl-coenzyme A into the ketone bodies formed in the presence and absence of unlabelled butyrate by rat-liver mitochondria*

Rat-liver mitochondria were incubated for 60 min. at 38° in 3·5 ml. of medium, as described in the text, containing substrates as shown; 11·6 mg. of mitochondrial protein/flask. Specific activity = counts/min./µg. atom of C. Approx. 1·1–2·3 × 10^5 counts of [^{14}C]substrate/min./flask.

Substrate	Ketone bodies: Total (µmoles)	Percentage of total ^{14}C: In C-1	In C-3	$\frac{\text{Counts/min. in ketone bodies}}{\text{Counts/min. added}} \times 100$	$\frac{\text{Sp. activity of ketone bodies}}{\text{Sp. activity of substrate}} \times 100$
15 mM-Butyrate plus 3 µmoles of [1-^{14}C]butyrate	22·6	75	25	34	85
15 mM-Butyrate plus 1 µmole of [1-^{14}C]acetate	22·3	73	27	6	0·1
15 mM-Butyrate plus 1 µmole of [1-^{14}C]hexanoate	22·4	72	28	84	5
15 mM-Butyrate plus 1 µmole of [1-^{14}C]octanoate	21·8	66	34	77	7
20 mM-[1-^{14}C]Acetate	2·3	56	44	4	71
10 mM-[1-^{14}C]Hexanoate	18·7	67	33	32	92
5 mM-[1-^{14}C]Octanoate	22·3	62	38	51	83

corporation of [1-^{14}C]acetyl groups into the ketone bodies and this asymmetry occurs in the absence of butyrate as a source of the carbon skeleton of the ketone bodies. However, when acetate was the only source of ketone bodies added, the incorporation of ^{14}C into C-3 is higher than when it is in the presence of unlabelled butyrate. The same is possibly true of octanoate but here the results are equivocal, as the different concentration used in the experiments with and without butyrate would make the accuracy of comparisons uncertain. The proportion of ^{14}C incorporated into ketone bodies from [1-^{14}C]-hexanoate and -octanoate is considerable and in the presence of unlabelled butyrate is even greater. Acetate is again a poor precursor of ketone bodies. In all experiments replication gave similar results.

DISCUSSION

It is now accepted that acetoacetyl-CoA is the precursor of the ketone bodies (acetoacetate and β-hydroxybutyrate), and there are two main hypotheses about its pathway to acetoacetate. Drummond & Stern (1960), from work with a partly purified system, suggest that direct hydrolytic deacylation of acetoacetyl-CoA is the important pathway (see also Segal & Menon, 1960). Caldwell & Drummond (1961) have re-examined the earlier system of Drummond & Stern (1960) and conclude that the pathway is by β-hydroxy-β-methylglutaryl-CoA, as proposed by Lynen *et al.* (1958).

Each of these hypotheses arises from results obtained from a selection of conditions which include the use of partly purified extracts and an arbitrary choice of pH and substrate concentration. Our results allow a qualitative and a partly quantitative answer to the question of the mechanism of ketone-body formation in whole cells.

The details of the two pathways proposed for acetoacetate synthesis have been discussed by Hird & Symons (1960, 1961). It was concluded that a greater conversion of the first acetyl moiety (C-1 and C-2) into carbon dioxide as compared with the second acetyl moiety (C-3 and C-4) would indicate the operation of the β-hydroxy-β-methylglutaryl-CoA pathway, and that a greater incorporation of C-1 of acetyl-CoA into C-1 of acetoacetate would constitute good evidence for the operation of this pathway. Such results have been obtained in the present investigation and also by Zabin & Bloch (1951) who found that when [1-^{13}C]- and [3-^{14}C]-butyrate were oxidized by liver slices there was a much greater dilution of C-1 than of C-3 of acetoacetate by endogenously produced acetyl groups.

The existence of an active thiolase in the preparations used can be inferred from the metabolism of C-2, C-3 and C-4 of butyrate to carbon dioxide and also from the extent of the appearance of C-1 and C-3 of butyrate in C-3 and C-1 respectively of the ketone bodies formed. The relative activities of thiolase and the ketone-body-forming system operating through β-hydroxy-β-methylglutaryl-CoA will determine the degree of asymmetry in metabolism of the acetyl moieties of butyrate and the incorporation of acetyl groups into ketone bodies. Therefore, in the presence of thiolase, complete asymmetry will not be obtained even if the β-hydroxy-β-methylglutaryl pathway were the sole mechanism of ketone-body formation. The proportion of the total ketone bodies that have 'passed through' the thiolase step can be calculated (Hird & Symons, 1961) from the amount of incorporation of C-1 of butyrate into C-3 of acetoacetate, and from the results presented in this paper mean values of 25 % (range 20–30 %) for liver slices and 23 % (range 14–33 %) for liver mitochondria are obtained. These figures are similar to those obtained for the rumen and omasum of the sheep: mean value 26 % (range 19–32 %) (Hird & Symons, 1961).

This proportion of ketone bodies could be formed by direct deacylation and be undetected, but there is no formal evidence for such a pathway in the present work. However, some uncertainty remains in the quantitative assessment of the importance of these two pathways. It follows that there should be general agreement between the asymmetry of the labelling in ketone bodies beginning with [1-^{14}C]- and [3-^{14}C]-butyrate, of the incorporation of acetyl groups into ketone bodies in the presence of acetoacetyl-CoA, and also of the metabolism of the two acetyl moieties to carbon dioxide. There is in fact good general agreement. The results obtained on the location of radioactivity in ketone bodies formed from [1-^{14}C]-butyrate are in agreement with those obtained with [1-^{14}C]- and [1-^{13}C]-butyrate in liver slices (Medes, Weinhouse & Floyd, 1945; Zabin & Bloch, 1951), liver mitochondria (Cheldelin & Beinert, 1952) and soluble extracts of liver mitochondria (Drysdale & Lardy, 1953). In the live goat Plaut & Smith (1951) have found that in the metabolism of [1-^{14}C]hexanoate to acetoacetate the isotope is preferentially incorporated into C-1. In the absence of preformed acetoacetyl-CoA, [1-^{14}C]-acetate might be expected to produce ketone bodies symmetrically labelled in C-1 and C-3, i.e. when the thiolase step would be obligatory in the conversion of acetate into acetoacetyl-CoA. Symmetry of labelling (see also Weinhouse, Medes & Floyd, 1945) was not fully obtained, because of the presence of endogenous sources of acetoacetyl-CoA. With slices of rat liver, the ratio of the specific activities of the ketone bodies to that of the butyrate added was 66 % (range, 58–76 %). Medes *et al.*

(1945), Weinhouse *et al.* (1945), Weinhouse, Millington & Friedman (1949) and Zabin & Bloch (1951) report similar dilutions from endogenous substrates during the metabolism of fatty acids to ketone bodies. Even with mitochondria up to 30% of the ketone bodies may be derived from endogenous sources. Complete symmetry is therefore not to be expected with such preparations.

The increase in the proportion of ^{14}C in C-3 of ketone bodies as the chain length of the 1-^{14}C-labelled fatty acid is lengthened (Table 4) has been reported by Geyer, Cunningham & Pendergast (1950) and Cheldelin & Beinert (1952). This increase may be a reflexion of the greater quantitative significance of the reverse thiolase reaction that is due to a higher relative concentration of acetyl-CoA to acetoacetyl-CoA when the longer-chain acids are metabolized.

The similar results obtained with mitochondria and liver slices are consistent with the observation of Bucher, Overath & Lynen (1960) that the ketone-body-forming system is mainly located on the mitochondria. By the methods used the mechanism of ketone-body formation in the liver of the rat is shown to be the same as that in the rumen and omasum of the sheep.

SUMMARY

1. The pathway for the conversion of acetate, lactate, butyrate, hexanoate and octanoate into ketone bodies by mitochondria and slices of rat liver has been investigated.

2. [1-^{14}C]Acetate as such, and also from its precursors [2-^{14}C]lactate, [1-^{14}C]hexanoate and [1-^{14}C]-octanoate, was found to be preferentially incorporated into C-1 rather than C-3 of the ketone bodies. However, in the absence of unlabelled butyrate the incorporation of [1-^{14}C]acetate into these carbon atoms approaches symmetry.

3. The first acetyl moiety of butyrate is preferentially metabolized to carbon dioxide by the tricarboxylic acid cycle when compared with the second acetyl moiety.

4. In the metabolism of [1-^{14}C]butyrate to ketone bodies, the contribution of the reverse thiolase reaction to acetoacetyl-CoA formation accounts for 14–33% of this compound passing to ketone bodies.

5. It is concluded that, as in the tissue from the rumen and omasum of the sheep, the bulk of ketone-body formation in rat liver proceeds through β-hydroxy-β-methylglutaryl-CoA as an intermediate and does not result from direct hydrolytic deacylation of acetoacetyl-CoA.

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