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Incorporation of Label from D-β-Hydroxy[^{14}C]butyrate and [3-^{14}C]Acetoacetate into Amino Acids in Rat Brain *in vivo*

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The metabolism of ketone bodies by rat brain was studied *in vivo*. Rats starved for 48h were given either D-β-hydroxy[3-^{14}C]butyrate or [3-^{14}C]acetoacetate by intravenous injection and killed after 3 or 10min. Total radioactivity in the acid-soluble material of the brain and the specific radioactivities of the brain amino acids glutamate, glutamine, aspartate and γ-aminobutyrate were determined. A group of fed animals were also given D-β-hydroxy[3-^{14}C]butyrate. In the brains of all animals ^{14}C was present in the acid-soluble material and the specific radioactivity of glutamate was greater than that of glutamine.

Certain observations during recent years have indicated that under the influence of dietary changes the brain may utilize ketone bodies. Such observations were first made in obese adult humans after a period of prolonged starvation (Owen *et al.* 1967). In a recent report on rats *in vivo* it was shown that ketone bodies are removed from the blood by the brain and the amount removed was proportional to the concentration of ketone bodies in the blood (Hawkins, 1971). If ketone bodies can replace part of the usual supply of glucose to the brain as oxidizable substrates, it might be argued that the labelling of amino acids in the brain from ^{14}C-labelled ketone bodies should follow a pattern resembling that given by [^{14}C]glucose. Brain amino acids become rapidly labelled when [^{14}C]glucose is injected into rats. Within the first few minutes most of the radioactivity in the amino acid fraction of the brain is present as glutamate. In particular, the specific radioactivity of glutamate is higher than that of glutamine (Cremer, 1964, 1970; Gaitonde, Dahl & Elliott, 1965). In contrast, many other ^{14}C-labelled precursors have been tested, including bicarbonate, acetate, propionate, butyrate and glutamate (see Berle & Clarke, 1969) and all give a high radioactivity in brain glutamine. The specific radioactivity of brain glutamate is several times less than that of glutamine. The ketogenic amino acids leucine (Berle & Frigyesi, 1968; Patel & Balazs, 1970) and phenylalanine (Van den Berg, 1970) also give a high labelling of glutamine relative to that of glutamate.

In the present paper the incorporation of label into amino acids of brains of adult rats after injection of either D-β-hydroxy[3-^{14}C]butyrate or [3-^{14}C]acetoacetate is examined.

METHODS

Animals. Male rats (7–8 weeks old, 190–205g body wt.) of the Porton strain were used. Animals starved for 2 days had access to diet 41B cubes until 10.0a.m. of the morning two days before that on which a radioactive tracer was injected. Such animals lost approx. 20g body wt. No allowance was made for this loss of body weight in relation to the dose of radioactive precursor given. At 12noon animals were injected with radioactive material into a tail vein. Each animal received approx. 8μCi of either [3-^{14}C]acetoacetate or D-β-hydroxy[3-^{14}C]butyrate (specific radioactivity of both compounds was 4.69mCi/mmol). The animals were guillotined such that the heads fell directly into liquid N_2. The frozen brains were homogenized in 30ml of M-$HClO_4$, the protein was removed by centrifugation and the acid-soluble fraction neutralized to pH8.0 with KOH. The $KClO_4$ precipitate was removed by filtration, the filtrate evaporated to dryness at 35°C and the dried brain extract dissolved in water.

Blood was collected from the trunk into beakers containing heparin (approx. 50units/ml of blood).

Assay of metabolites. The concentrations of metabolites in blood and brain were determined by enzymic assay as described by Bergmeyer (1963) for L-lactate, L-glutamate and L-aspartate. D-Glucose was similarly determined by using hexokinase and glucose 6-phosphate dehydrogenase. The concentrations of acetoacetate and D-β-hydroxybutyrate in the blood were determined by the enzymic method described by Williamson, Mellanby & Krebs (1962).

Separation of metabolites. The acid-soluble material of the original brain extract (equivalent to approx. 0.6g brain wet wt.) was placed on a column (2.5cm×1cm) of AG50 W (X4; 200–400 mesh; H^+ form) followed by 30ml of water, which removed the non-amino acids. The amino acids were removed with 20ml of 4M-NH_3.

The β-hydroxybutyrate, acetoacetate and lactate present in the water wash were separated on a column (14cm×0.9cm) of AG1 (X8; 200–400mesh; formate form)

as described by Hoberman (1965), except that a more gradual gradient of formic acid was used. The amino acid fraction was freed from NH_3 by rotary evaporation and then placed on a column (0.9cm × 25cm) of AG 1 (X8; 200–400 mesh; acetate form). The amino acids were separated by gradient elution with acetic acid as described by Minard & Mushahwar (1966). The first 40ml, which contained glutamine, was evaporated to near dryness and an equal volume of 5M-HCl was added. The solution was left at 104°C overnight to convert glutamine into glutamic acid; it was freed from HCl by repeated evaporation with additions of water and the glutamic acid was then separated on an AG 1 (X8; acetate form) column.

The separation of γ-aminobutyric acid was made on the original brain extract by a paper-electrophoretic method as described by Cremer (1970).

Measurement of radioactivity. Liquid-scintillation counting was used. The efficiency of counting was determined by the addition of standard [^{14}C]toluene and all values are corrected to d.p.m. Samples from columns of AG 1 (X8; acetate or formate form) were evaporated to near dryness, neutralized and equal amounts taken for enzymic assay (lactate, glutamate and aspartate) and measurement of radioactivity. Specific radioactivities are expressed as d.p.m./μmol.

Chemicals. Enzymes were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K., the resins AG 50W (X4) and AG1 (X8) were obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and Florisil (60–100 mesh) from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.

D-β-Hydroxy[3-^{14}C]butyrate and [3-^{14}C]acetoacetate were prepared from [3-^{14}C]ethylacetoacetate (specific radioactivity 4.69mCi/mmol, obtained from The Radiochemical Centre, Amersham, Bucks., U.K.) essentially as described by Bates, Krebs & Williamson (1968). At the end of the incubation of [3-^{14}C]acetoacetate with D-β-hydroxybutyrate dehydrogenase the D-β-hydroxy[3-^{14}C]-butyrate formed and any unchanged [3-^{14}C]acetoacetate were separated on AG 1 (X8; formate form) as described above. The fraction containing D-β-hydroxy[3-^{14}C]-butyrate also contained NAD, which was removed by passage through a column (1cm × 2.5cm) containing Florisil (60–100 mesh) and the D-β-hydroxy[3-^{14}C]butyrate was re-separated by chromatography on a column (14cm × 0.9cm) of AG 1 (X8; formate form). The samples of [3-^{14}C]acetoacetate and D-β-hydroxy[3-^{14}C]butyrate were neutralized with KOH and dissolved in 0.9% NaCl to a specific radioactivity of 40μCi/ml and these solutions were used for injection.

RESULTS AND DISCUSSION

The choice of the particular radioactive precursors used in this study was made for two main reasons. The D-stereoisomer of β-hydroxybutyrate was used because the enzyme β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) present in rat brain mitochondria has been shown by Klee & Sokoloff (1967) to be specific for this isomer. The absolute value for the enzyme activity in the brains of the rats used in the present study was 1.18μmol of substrate converted into acetoacetate/min per g of brain at 37°C. This

Table 1. *Total radioactivity in the blood and brain of rats injected with either* D-β-*hydroxy*[3-^{14}C]*butyrate or* [3-^{14}C]*acetoacetate and the concentration of blood glucose and ketone bodies*

Each rat received approx. 8μCi of either D-β-hydroxy[3-^{14}C]butyrate or [3-^{14}C]acetoacetate by intravenous injection. Total radioactivity refers to acid-soluble material. Mean values ±S.D. are given with the numbers of determination in parentheses.

Treatment of animal	Radioactive compound injected	Time killed (min)	Blood				Brain		
			Glucose (μmol/ml)	β-Hydroxybutyrate (μmol/ml)	Acetoacetate (μmol/ml)	Total radioactivity (d.p.m./ml)	Total radioactivity (d.p.m./g)	Non-amino acids (d.p.m./g)	Amino acids (d.p.m./g)
Fed	D-β-Hydroxy-[3-^{14}C]butyrate	3	5.70±0.17 (3)	0.10±0.02 (3)	—	71590±12580 (3)	14310±2840 (3)	6060±2650 (3)	7320±1390 (3)
Starved 48h	D-β-Hydroxy-[3-^{14}C]butyrate	3	4.06±0.71 (6)	1.32±0.56 (6)	0.25±0.56 (6)	148720±23510 (6)	17640±3470 (6)	7530±1490 (6)	10830±1840 (6)
Starved 48h	D-β-Hydroxy-[3-^{14}C]butyrate	10	3.70±0.70 (3)	0.81±0.34 (3)	0.43±0.14 (3)	51850±5450 (3)	18040±1560 (3)	5230±1540 (3)	11560±1920 (3)
Starved 48h	[3-^{14}C]Aceto-acetate	3	4.60 (2)	0.98±0.20 (3)	0.34 (1)	59610±5600 (3)	11930±4050 (3)	3180±1000 (3)	8930±2450 (3)

rate was higher than that found by Klee & Sokoloff (1967) and was not changed by a 48h period of starvation. These findings are quoted as a personal communication in a report by Pull & McIlwain (1971).

Labelling in C-3 of acetoacetate and D-β-hydroxybutyrate was chosen so that a direct comparison could be made with previous studies in which glucose labelled in C-2 and acetate in C-1 were used (O'Neal & Koeppe, 1966; Van den Berg, Kržalíc, Mela & Waelsch, 1969; Cremer, 1970). All of these compounds will give rise to [1-^{14}C]acetyl-CoA.

Table 1 shows values for the concentrations of blood glucose and ketone bodies and the total amount of radioactivity in the acid-soluble material of blood and brain at the time of death. The animals starved for 48h had raised concentrations of ketone bodies in the blood and lowered glucose.

After an intravenous injection into a tail vein of either [3-^{14}C]acetoacetate or D-β-hydroxy[3-^{14}C]-butyrate there was a rapid removal of ^{14}C from the blood. The results were in fairly close agreement with those of Bates *et al.* (1968). Although there was considerable variation between animals in the concentration of D-β-hydroxybutyrate in the blood, the amount of radioactivity present at the time of death, expressed at d.p.m./ml, was similar within the various groups.

The values given for the total radioactivity in the brain have not been corrected for the contribution expected to be made by blood. The amount of blood/g of frozen rat brain is about 0.035ml (Thompson, Robertson & Bauer, 1968), so that in no instance could the blood present in the brain at the time of death account for all the radioactivity found in the acid-soluble material of the brain, although it would appear to account for a considerable proportion of the non-amino acid fraction.

In Table 2 are given the specific radioactivities (d.p.m./μmol) of amino acids and lactate in the brain. In every animal the specific radioactivity of brain glutamate was higher than that of the other amino acids. The mean specific radioactivity of glutamine relative to that of glutamate was 0.37 at 3min and 0.63 at 10min in starved rats given D-β-hydroxy[3-^{14}C]butyrate. The values are virtually identical with those obtained with [2-^{14}C]-glucose (Cremer, 1970).

Where measured the specific radioactivity of brain lactate was low. This is taken to indicate that conversion in the body of either D-β-hydroxy[3-^{14}C]-butyrate or [3-^{14}C]acetoacetate into [^{14}C]glucose was low. The amount of radioactive glucose present in the blood has not been determined. If any [^{14}C]glucose was formed from the injected ^{14}C-labelled ketone bodies it would be labelled in C-3 and C-4. These would be lost as $^{14}CO_2$ on entry of pyruvate into the tricarboxylate cycle in the brain, so that ^{14}C from glucose should not contribute to the labelling found in the amino acids in the brain. It is therefore very noteworthy that the brain amino acids were labelled with ^{14}C in a way similar to that obtained with [2-^{14}C]glucose (O'Neal & Koeppe, 1966; Van den Berg, *et al.* 1969; Cremer, 1970).

The results are in complete contrast with those obtained by using the ketogenic amino acids phenylalanine and leucine. Van den Berg (1970) found that after an injection of L-[U-^{14}C]phenylalanine into adult mice the specific radioactivity of brain glutamate was severalfold greater than that of glutamate. Glutamine/glutamate ratios of between 2 and 4 were observed. Patel & Balazs (1970) found similar ratios in the brains of mature rats injected with [U-^{14}C]leucine. It seems likely that these amino acids are metabolized to acetoacetate by the brain itself. If the subsequent labelling of glutamate and glutamine in the brain had been derived from acetoacetate formed from phenylalanine or leucine elsewhere in the body,

Table 2. *Specific radioactivity of brain amino acids and lactate of rats given either* D-β-*hydroxy*[3-^{14}C]*butyrate or* [3-^{14}C]*acetoacetate by intravenous injection*

Each rat received approx. 8μCi of either radioactive compound. Mean values±S.D. are given with the number of determinations in parentheses.

Treatment of animal	Radioactive compound injected	Time killed (min)	Sp. radioactivity (d.p.m./μmol)				
			Glutamate	Glutamine	Aspartate	γ-Aminobutyrate	Lactate
Fed	D-β-Hydroxy-[3-^{14}C]butyrate	3	1080±115 (3)	360±30 (3)	555 (2)	—	78 (2)
Starved 48h	D-β-Hydroxy-[3-^{14}C]butyrate	3	1100±330 (6)	390±48 (6)	454±206 (6)	223±38 (4)	166±83 (5)
Starved 48h	D-β-Hydroxy-[3-^{14}C]butyrate	10	1290±110 (3)	816±101 (3)	—	—	246±61 (3)
Starved 48h	[3-^{14}C]Acetoacetate	3	1020±280 (3)	365±140 (3)	405±111 (3)	—	178±56 (3)

there seems no obvious reason why their specific radioactivities should not be the same as that found after an injection of [^{14}C]acetoacetate into the bloodstream (Table 2). The results indicate that ^{14}C-labelled intermediates common to the ketogenic amino acids and the ketone bodies enter different 'metabolite pools' in the brain, but there is no direct evidence of this.

Hawkins (1971) showed by simultaneous sampling of femoral-artery and cerebral-sinus blood that the rat brain is capable of a net removal of ketone bodies from the blood. The metabolism by the brain of the ketone bodies was not studied by Hawkins. In the present work it has been shown that within 3min of an intravenous injection into rats of ^{14}C-labelled ketone bodies there is ^{14}C in the amino acid fraction of the brain. It would seem likely though there is not conclusive proof that ^{14}C-labelled ketone bodies *per se* entered the brain and were metabolized.

If ketone bodies entering the brain from the blood were converted into acetoacetyl CoA by the enzyme acid–CoA ligase (EC 6.2.1.2) they might be expected to give the same labelling pattern of amino acids as that found with propionate (O'Neal, Koeppe & Williams, 1966) or butyrate (O'Neal & Koeppe, 1966) but they do not. These fatty acids give rise to brain glutamine with a specific radioactivity greater than that of glutamate. An alternative pathway of metabolism of the ketone bodies is via 3-oxoacid CoA transferase (EC 2.8.3.5) and acetoacetyl-CoA thiolase (EC 2.3.1.9) both of which enzymes are present in adult rat brain (Page & Williamson, 1971).

It is suggested that because ^{14}C-labelled ketone bodies have been shown to give a distribution pattern of ^{14}C in the brain amino acids similar to that found with [^{14}C]glucose, ketone bodies could partially replace glucose as oxidizable substrates. For a more precise quantitative measure of the rate of utilization of acetoacetate and D-β-hydroxybutyrate by the brain further work is required.

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