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Lipogenesis from Ketone Bodies in Rat Brain

EVIDENCE FOR CONVERSION OF ACETOACETATE INTO ACETYL-COENZYME A IN THE CYTOSOL

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The metabolism of acetoacetate via a proposed cytosolic pathway in brain of 1-week-old rats was investigated. (−)-Hydroxycitrate, an inhibitor of ATP citrate lyase, markedly inhibited the incorporation of carbon from labelled glucose and 3-hydroxybutyrate into cerebral lipids, but had no effect on the incorporation of labelled acetate and acetoacetate into brain lipids. Similarly, *n*-butylmalonate and benzene-1,2,3-tricarboxylate inhibited the incorporation of labelled 3-hydroxybutyrate but not of acetoacetate into cerebral lipids. These inhibitors had no effect on the oxidation to $^{14}CO_2$ of the labelled substrates used. (−)-Hydroxycitrate decreased the incorporation of ^3H from ^3H_2O into cerebral lipids by slices metabolizing either glucose or 3-hydroxybutyrate, but not in the presence of acetoacetate. (−)-Hydroxycitrate also differentially inhibited the incorporation of [2-^{14}C]-leucine and [U-^{14}C]leucine into cerebral lipids. The data show that, although the acetyl moiety of acetyl-CoA generated in brain mitochondria is largely translocated as citrate from these organelles to the cytosol, a cytosolic pathway exists by which acetoacetate is converted directly into acetyl-CoA in this cellular compartment.

Ketone bodies, 3-hydroxybutyrate and acetoacetate, are excellent fuels for developing rat brain (Itoh & Quastel, 1970; Page *et al.*, 1971; Hawkins *et al.*, 1971). Since ketone bodies are degraded to acetyl-CoA, they also have the potential to serve as precursors for biosynthesis of lipids (Williamson *et al.*, 1971), in addition to their role in supplying acetyl-CoA for oxidation in the tricarboxylic acid cycle. A possible role of ketone bodies in the biosynthesis of cerebral lipids by suckling rats (Edmond, 1974; Patel & Owen, 1976) and human foetus (Patel *et al.*, 1975) has been demonstrated. It is generally accepted that the acetyl moiety of acetyl-CoA formed in the mitochondria is translocated as citrate to regenerate acetyl-CoA for lipogenesis in the cytosol (Spencer & Lowenstein, 1962; Bhaduri & Srere, 1963). Buckley & Williamson (1973) described the postnatal development of acetoacetyl-CoA synthetase (acetoacetate-CoA ligase) and acetoacetyl-CoA thiolase (EC 2.3.1.9) in the cytosol of rat brain. They suggested a cytosolic pathway for direct formation of acetyl-CoA from acetoacetate in the cytosol, in addition to a widely accepted mitochondrial pathway for the incorporation of acetoacetate carbon into brain lipids. The purpose of the present study was to investigate whether a proposed cytosolic pathway for the metabolism of acetoacetate operates in the brain. A preliminary report of this work has been presented (Patel, 1975).

Materials and Methods

Chemicals

D-3-Hydroxy[3-^{14}C]butyrate, [U-^{14}C]glucose, L-[U-^{14}C]leucine and [1-^{14}C]acetate were purchased from New England Nuclear Corp., Boston, MA, U.S.A. L-[2-^{14}C]Leucine was obtained from Schwarz/Mann, Orangeburg, NY, U.S.A. [3-^{14}C]Acetoacetate, which was prepared from ethyl [3-^{14}C]-acetoacetate (Amersham/Searle Corp., Arlington Heights, IL, U.S.A.), as previously described (Krebs *et al.*, 1966; Owen *et al.*, 1973), was kindly supplied by Dr. G. A. Reichard, Jr., of Lankenau Hospital, Philadelphia, PA, U.S.A. DL-3-Hydroxybutyrate and acetoacetate were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Benzene-1,2,3-tricarboxylate (K & K Laboratories, Plainview, NY, U.S.A.) and *n*-butylmalonate (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) were purchased. (−)-Hydroxycitrate lactone and sodium (−)-hydroxycitrate were generously supplied by Dr. Ann C. Sullivan of Hoffmann–LaRoche, Nutley, NJ, U.S.A. The lactone was hydrolysed to (−)-hydroxycitrate by heating with

3 equiv. of NaOH at 90°C for 30 min (Watson *et al.*, 1969). All other reagents were of the highest purity commercially available.

Studies in vitro

Pups (5 days old) with nursing mothers (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) were maintained on Wayne Lab-Blok (Allied Mills, Chicago, IL, U.S.A.) and water *ad libitum*. At 7–8 days the pups were decapitated, and slices of cerebral cortex (about 0.4 mm in thickness, average wt. 75 mg) were prepared with a hand microtome (Majno & Bunker, 1957). Only the first slice was used and was immediately transferred to a 25 ml Erlenmeyer flask containing 3 ml of oxygenated high-K^+ Krebs–Ringer bicarbonate buffer, pH 7.4 (Berl *et al.*, 1968). The buffer contained labelled substrates and other compounds, as indicated in legends to Tables. The flasks were gassed with O_2+CO_2 (95:5) and sealed with a rubber serum stopper equipped with a hanging polyethylene centre well, and were placed in a shaking water bath at 37°C for 1 h. At the end of the incubation period 0.3 ml of 1 M-Hyamine 10-X was injected into the centre well, and 0.5 ml of 36% (w/v) $HClO_4$ was injected into the medium to stop the reaction and to ensure complete release of CO_2. After additional shaking for 40 min, the content of the cup was transferred to toluene scintillation fluid (Patel, 1974), and the radioactivity in $^{14}CO_2$ was counted. The tissue was removed and rinsed three times in water and placed in a tube containing 10 ml of chloroform/methanol (2:1, v/v) and left overnight. The tissue was then shaken vigorously for 1 min and the extracted lipids were separated into non-saponifiable and fatty acid fractions (Folch *et al.*, 1957); the radioactivity in each fraction was determined. For experiments in which ^3H_2O was used, the chloroform/methanol extracts were washed six to eight times with 5 ml of 0.05% $CaCl_2$ solution until the aqueous phase became free of contaminating ^3H_2O radioactivity.

Results and Discussion

It is known that ketone bodies are converted into acetyl-CoA in the mitochondria. This observation is based on the mitochondrial localization of succinyl-CoA–3-oxo acid CoA-transferase (EC 2.8.3.5) (Williamson *et al.*, 1971). This enzyme transfers the CoA moiety from succinyl-CoA to acetoacetate, thus forming acetoacetyl-CoA (Green *et al.*, 1953), which is converted into acetyl-CoA in the mitochondria (Williamson *et al.*, 1971; Itoh & Quastel, 1970). Buckley & Williamson (1973) postulated a cytosolic pathway by which acetyl-CoA could be directly generated from acetoacetate in the cytosol. Since the inner mitochondrial membrane is impermeable to acetyl-CoA, the acetyl moiety is translocated as citrate into the cytosol, and acetyl-CoA is regenerated by ATP citrate lyase (EC 4.1.3.8) (Spencer & Lowenstein, 1962; Bhaduri & Srere, 1963). By using the specific inhibitors *n*-butylmalonate (Robinson & Chappell, 1967) and benzene-1,2,3-tricarboxylate (Robinson *et al.*, 1971) for the citrate-transporter system, and (−)-hydroxycitrate for ATP citrate lyase (Watson *et al.*, 1969), it is possible to inhibit the contribution of the mitochondrial acetyl-CoA pool to the cytosolic acetyl-CoA pool. However, if acetyl-CoA is transported from mitochondria by alternative pathways not involving citrate or is formed by a cytosolic pathway(s), these compounds should not exert inhibitory effects. As seen in Table 1, (−)-hydroxycitrate had no effect on the oxidation of all four labelled substrates (glucose, acetate, 3-hydroxybutyrate and acetoacetate) studied, suggesting that this inhibitor did not alter their oxidation in the tricarboxylic acid cycle. (−)-Hydroxycitrate inhibited the incorporation of [U-^{14}C]glucose and 3-hydroxy-[3-^{14}C]butyrate into brain lipids respectively by about 65 and 25% (Table 1). In contrast, this inhibitor had no inhibitory effect on the incorporation of [1-^{14}C]-acetate and [3-^{14}C]acetoacetate into cerebral lipids. The stimulatory effect observed with [1-^{14}C]acetate may be due to either less dilution of the cytosolic acetyl-CoA pool by acetyl-CoA formed from mitochondrial citrate or a possible activation of acetyl-CoA carboxylase (EC 6.4.1.2) by (−)-hydroxycitrate (Hackenschmidt *et al.*, 1972). *n*-Butylmalonate and benzene-1,2,3-tricarboxylate, like (−)-hydroxycitrate, diminished the incorporation of 3-hydroxy[3-^{14}C]-butyrate into brain lipids by about 25%, but had no effect on lipid synthesis from [3-^{14}C]acetoacetate. The observed inhibitory effect of benzene-1,2,3-tricarboxylate on the incorporation of [3-^{14}C]acetoacetate into fatty acids but not into non-saponifiable lipids may be attributed to its inhibitory action on acetyl-CoA carboxylase (Table 1). It should be noted that both *n*-butylmalonate and benzene-1,2,3-tricarboxylate had no effect on the oxidation of ketone bodies by brain slices.

By using the incorporation of ^3H from ^3H_2O, the rate of total lipid synthesis can be measured independently of the source of acetyl-CoA for lipogenesis (Foster & Katz, 1966; Jungas, 1968). (−)-Hydroxycitrate inhibited by about 60 or 30% the incorporation of ^3H into cerebral lipids by slices metabolizing either glucose or 3-hydroxybutyrate plus glucose respectively (Table 2). However, this inhibitor again had no effect on lipogenesis from acetoacetate. These results are consistent with findings described in Table 1 in which ^{14}C-labelled substrates instead of ^3H_2O were used.

The degradation of leucine to acetoacetate and acetyl-CoA is a mitochondrial process (Meister, 1965). When specifically labelled leucine is used, either labelled acetyl-CoA from [2-^{14}C]leucine or labelled

Table 1. *Effect of (−)-hydroxycitrate, n-butylmalonate and benzene-1,2,3-tricarboxylate on the incorporation of labelled glucose, acetate, 3-hydroxybutyrate and acetoacetate into lipids by cerebral-cortex slices from 1-week-old rats*

Cerebral-cortex slices from 1-week-old rats were incubated in high-K^+ Krebs–Ringer bicarbonate buffer, pH 7.4, containing radioactive substrate plus glucose as indicated for 1 h at 37°C. (−)-Hydroxycitrate, *n*-butylmalonate and benzene-1,2,3-tricarboxylate were also added as indicated. The collection of $^{14}CO_2$ and the separation of non-saponifiable lipids and fatty acids were carried out as described in the Materials and Methods section. The specific radioactivities of labelled glucose, acetate, 3-hydroxybutyrate and acetoacetate were about 60, 65, 40 and 25 d.p.m./nmol respectively. The results are the means ± S.E.M. for six to ten animals. *P* values are shown in parentheses. ns, Not significant ($P > 0.05$).

Labelled substrate + other addition	Inhibitor (−)-Hydroxycitrate (2.5 mM)	Labelled substrate converted into products (nmol/h per g)		
		CO_2	Non-saponifiable lipids	Fatty acids
[U-^{14}C]Glucose (10 mM)	−	5872 ± 180	92 ± 7	265 ± 17
	+	6072 ± 87	29 ± 1	97 ± 8
		(ns)	(<0.001)	(<0.001)
[1-^{14}C]Acetate (10 mM) + glucose (5 mM)	−	1131 ± 53	126 ± 7	513 ± 23
	+	1286 ± 145	127 ± 8	627 ± 45
		(ns)	(ns)	(<0.05)
D-3-Hydroxy[3-^{14}C]butyrate (2.5 mM) + glucose (5 mM)	−	4916 ± 222	163 ± 10	371 ± 22
	+	5573 ± 261	122 ± 7	280 ± 13
		(ns)	(<0.01)	(<0.01)
[3-^{14}C]Acetoacetate (2.5 mM) + glucose (5 mM)	−	4939 ± 189	183 ± 9	456 ± 26
	+	5461 ± 213	185 ± 11	484 ± 29
		(ns)	(ns)	(ns)
	n-Butylmalonate (5 mM)			
[3-^{14}C]Acetoacetate (2.5 mM) + glucose (5 mM)	−	5321 ± 316	167 ± 13	386 ± 27
	+	5696 ± 303	179 ± 19	434 ± 49
		(ns)	(ns)	(ns)
D-3-Hydroxy[3-^{14}C]butyrate (2.5 mM) + glucose (5 mM)	−	5331 ± 343	132 ± 6	292 ± 14
	+	5048 ± 111	106 ± 8	220 ± 14
		(ns)	(<0.05)	(<0.025)
	Benzene-1,2,3-tricarboxylate (10 mM)			
[3-^{14}C]Acetoacetate (2.5 mM) + glucose (5 mM)	−	5321 ± 316	167 ± 13	386 ± 27
	+	4814 ± 344	154 ± 23	252 ± 20
		(ns)	(ns)	(<0.005)
D-3-Hydroxy[3-^{14}C]butyrate (2.5 mM) + glucose (5 mM)	−	4504 ± 88	201 ± 7	374 ± 7
	+	4107 ± 165	179 ± 3	202 ± 11
		(ns)	(<0.05)	(<0.001)

acetyl-CoA as well as labelled acetoacetate from [U-^{14}C]leucine can be formed in the mitochondria (Rous & Favarger, 1973). The further metabolism of these intermediates of leucine degradation in the presence of (−)-hydroxycitrate was investigated. (−)-Hydroxycitrate had no effect on the oxidation to $^{14}CO_2$ of either [2-^{14}C]leucine or [U-^{14}C]leucine by brain slices; however, it inhibited the incorporation of carbon atoms from both labelled leucine molecules into cerebral lipids (Table 3). It should be noted again that (−)-hydroxycitrate-induced inhibition was only about 28% with [U-^{14}C]leucine, compared with about 68% with [2-^{14}C]leucine. Additional support for the operation of a cytosolic pathway in rat brain is given by these experiments. The oxidation of [2-^{14}C]leucine results in the formation of [^{14}C]-acetyl-CoA in the mitochondria, and the incorporation of the latter into cerebral lipids is inhibited by (−)-hydroxycitrate to a degree similar to that of [U-^{14}C]glucose (inhibition is about 65% with both substrates; Tables 1 and 3). The metabolism of [U-^{14}C]-leucine results in labelled acetyl-CoA as well as labelled acetoacetate in the mitochondria, and the latter can be converted into acetyl-CoA in the mitochondria. However, the incorporation of carbon from [U-^{14}C]leucine into cerebral lipids in the presence of (−)-hydroxycitrate is not similar to that observed with [2-^{14}C]leucine. On the other hand, the data for [U-^{14}C]leucine are very similar to those observed for 3-hydroxy[3-^{14}C]butyrate in the presence of (−)-

Table 2. *Effect of (−)-hydroxycitrate on the incorporation of ^3H from ^3H_2O into lipids by cerebral-cortex slices from 1-week-old rats*

Cerebral-cortex slices from 1-week-old rats were incubated in high-K^+ Krebs–Ringer bicarbonate buffer, pH 7.4, containing substrate(s) as indicated for 1 h at 37°C. (−)-Hydroxycitrate was also added as indicated. Each flask contained about 0.6 mCi of ^3H_2O. The separation of non-saponifiable lipids and fatty acids were carried out as described in the Materials and Methods section. The results are the means ± S.E.M. for six animals. *P* values are shown in parentheses. ns, Not significant ($P > 0.05$).

Substrates	(−)-Hydroxycitrate (2.5 mM)	^3H incorporated into products (ng-atoms/h per g)	
		Non-saponifiable lipids	Fatty acids
None	−	134 ± 21	190 ± 24
	+	109 ± 12	193 ± 24
		(ns)	(ns)
Glucose (5 mM)	−	1022 ± 32	2312 ± 137
	+	440 ± 19	857 ± 25
		(<0.001)	(<0.001)
DL-3-Hydroxybutyrate (5 mM) + glucose (5 mM)	−	815 ± 29	1918 ± 139
	+	580 ± 29	1337 ± 95
		(<0.001)	(<0.01)
Acetoacetate (2.5 mM) + glucose (5 mM)	−	1007 ± 77	2102 ± 198
	+	909 ± 83	2044 ± 79
		(ns)	(ns)

Table 3. *Effect of (−)-hydroxycitrate on the oxidation and the incorporation of [U-^{14}C]leucine and [2-^{14}C]leucine into cerebral lipids by cortex slices from 1-week-old rat brain*

Cerebral-cortex slices from 1-week-old rats were prepared and incubated for 1 h at 37°C in high-K^+ Krebs–Ringer bicarbonate buffer containing specifically labelled leucine and glucose as indicated. (−)-Hydroxycitrate (2.5 mM) was also added as indicated. The collection of $^{14}CO_2$ and the separation of non-saponifiable lipids and fatty acids were carried out as described in the Materials and Methods section. The specific radioactivities of [U-^{14}C]leucine and [2-^{14}C]leucine were about 65 and 120 d.p.m./nmol respectively. The results are the means ± S.E.M. for six animals. *P* values are shown in parentheses. ns, Not significant ($P > 0.05$).

Labelled substrate + addition	(−)-Hydroxycitrate (2.5 mM)	Labelled substrate converted into products (nmol/h per g)		
		CO_2	Non-saponifiable lipids	Fatty acids
[U-^{14}C]Leucine (5 mM) + glucose (5 mM)	−	225 ± 18	27 ± 1.0	44 ± 1.0
	+	248 ± 20	19 ± 0.9	33 ± 1.5
		(ns)	(<0.001)	(<0.001)
[2-^{14}C]Leucine (5 mM) + glucose (5 mM)	−	340 ± 25	18 ± 2.0	52 ± 5.5
	+	374 ± 12	5 ± 0.3	18 ± 1.4
		(ns)	(<0.001)	(<0.001)

hydroxycitrate (about 30% inhibition in both cases; Tables 1 and 3). These findings strongly suggest that when [^{14}C]acetoacetate is formed from either 3-hydroxy[3-^{14}C]butyrate or [U-^{14}C]leucine in the mitochondria, it partially diffuses into the cytosol, where it is converted into acetyl-CoA via a cytosolic pathway.

Although it is well accepted that citrate is a carrier of the acetyl moiety from the mitochondria to the cytosol in rat liver (Spencer & Lowenstein, 1962; Bhaduri & Srere, 1963) and adipose tissue (Wise & Ball, 1964), the route(s) by which acteyl-CoA is translocated in the brain is less certain. In addition to citrate, several other compounds have been suggested to carry out a similar function (D'Adamo & D'Adamo, 1968; D'Adamo & Yatsu, 1966). (−)-Hydroxycitrate-induced inhibition (about 65%) of the incorporation of [U-^{14}C]glucose into cerebral lipids (Table 1) shows that citrate transport is also a major pathway for translocating the mitochondrial acetyl-CoA into the cytosol of the brain. Additionally, acetoacetate either formed in the mitochondria (from either 3-hydroxybutyrate or degradation of leucine) or taken up from the plasma also effectively serves as

a precursor of acetyl-CoA in the cytosol. Although studies *in vitro* with cerebral-cortex slices do not allow accurate quantitative assessment of the pathway, studies reported here strongly suggest that the cytosolic pathway may operate in the intact brain.

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