Competition among oxidizable substrates in brains of young and adult rats

Whole homogenates

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The rates of conversion into $^{14}\text{CO}_2$ of D-(–)-3-hydroxy[3- ^{14}C]butyrate, [3- ^{14}C]acetoacetate, [6- ^{14}C]glucose and [U- ^{14}C]glutamine were measured in the presence and absence of unlabelled alternative oxidizable substrates in whole homogenates from the brains of young and adult rats. The addition of unlabelled glutamine resulted in decreased $^{14}\text{CO}_2$ production from [6- ^{14}C]glucose in brain homogenates from both young and adult rats. In contrast, glucose had no effect on [U- ^{14}C]glutamine oxidation. In suckling animals, both 3-hydroxybutyrate and acetoacetate decreased the rate of oxidation of [6- ^{14}C]glucose, but in adults only 3-hydroxybutyrate had an effect, and to a lesser degree. The addition of unlabelled glucose markedly enhanced the rates of oxidation of both ketone bodies in adult brain tissue and had little or no effect in the young. The rate of production of $^{14}\text{CO}_2$ from [U- ^{14}C]glutamine was increased by the addition of unlabelled ketone bodies in brain homogenates from young, but not from adult, rats. In the converse situation, unlabelled glutamine added to ^{14}C -labelled ketone bodies diminished $^{14}\text{CO}_2$ production in young rats, but had no effect in adult animals. These results revealed a complex age-dependent pattern of interaction in which certain substrates apparently competed with each other, whereas an enhanced rate of $^{14}\text{CO}_2$ production was found with others.

The brain utilizes a variety of substrates for energy production and as precursors for structural components and neurotransmitters. The major compounds that the brain is capable of oxidizing are glucose, ketone bodies, glycerol, pyruvate and lactate (Cremer, 1981), and more recent studies suggest that glutamine may also provide energy for the brain (Tildon, 1983; Tildon & Roeder, 1983).

It has been pointed out that, during early development of the brain, the mechanisms regulating the utilization of ketone bodies are different from those that regulate glucose utilization (Tildon *et al.*, 1983). On the basis of the extensive cellular heterogeneity of the brain, it has been proposed that the oxidation of these different substrates may take place in separate metabolic compartments (see Balazs & Cremer, 1973; Berl *et al.*, 1975); a number of investigators have examined the relative rates of conversion of different substrates into specific products, and their findings have suggested that subcellular organelles and/or individual cell

types provide the physical separation or compartmentation of these various metabolic activities. The observed effects may be due in part to differential rates of transport of various substrates into these compartments, localization of enzymic activities, and/or differences in the sizes of their metabolic pools. It is also possible that two or more substrates may be metabolized in the same compartment, and in effect act as competitors if they share a common metabolic pathway. If the oxidation of two or more different substrates occurred in the same metabolic compartment, the rate of oxidation of one labelled substrate should be diminished by the addition of an unlabelled alternative substrate. Further studies have demonstrated that the nature of the preparation *in vitro* greatly affects the rate of substrate oxidation (Tildon *et al.*, 1983) and that transport across the plasma membrane may play a role in regulating substrate utilization (Gjedde & Crone, 1975; Hawkins & Biebuyck, 1979; Pollay & Stevens, 1980; Robinson & Williamson, 1980; Keller *et al.*, 1981; Tildon *et al.*, 1983). Furthermore, the differ-

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ence in the cellular composition of the brain of neonatal animals as compared with adults provides an additional system for probing metabolic compartmentation.

The results described in the present paper compare the rates of oxidation of several ^{14}C -labelled substrates {D-(–)-3-hydroxy[3- ^{14}C]butyrate, [3- ^{14}C]acetoacetate, [6- ^{14}C]glucose and [U- ^{14}C]glutamine} in the presence and absence of unlabelled alternative substrates by whole homogenates of brain in which the cell membranes, but not mitochondrial membranes, were disrupted. In the following paper (Roeder *et al.*, 1984) we present the results of a parallel series of experiments using intact dissociated brain cells from the contralateral hemisphere of the brains from the same animals described in the present study. A preliminary report of these data has been presented (Roeder & Tildon, 1983).

Materials and methods

Materials

Biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Reagent-grade chemicals were obtained from Fisher Chemical Co. (Pittsburgh, PA, U.S.A.). The fluor used for liquid-scintillation counting was a 3:1 (v/v) mixture of Solucint O (National Diagnostics; Somerville, NJ, U.S.A.), and methoxyethanol (Piersolve; Pierce Chemical Co., Rockford, IL, U.S.A.). Radioactive compounds {[6- ^{14}C]glucose, [U- ^{14}C]glutamine, D-(–)-3-hydroxy[3- ^{14}C]butyrate and ethyl [3- ^{14}C]acetoacetate} were purchased from New England Nuclear (Boston, MA, U.S.A.). Before use, ethyl [3- ^{14}C]acetoacetate was hydrolysed to [3- ^{14}C]acetoacetate and purified by column chromatography as described by Reed *et al.* (1981). Centre wells used to trap $^{14}\text{CO}_2$ were obtained from Kontes (Vineland, NJ, U.S.A.).

Animals

Wistar rats were obtained from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.). They were housed in a room maintained at 21°C with 12h-light/12h-dark cycles and given food (Purina Lab Chow; Ralston Purina Co., St. Louis, MO, U.S.A.) and water *ad libitum*. Three groups of rats were used for these studies, namely aged 4–6 days, 16–20 days and 90 days or older (adults). They were killed by decapitation.

Preparation of tissue

The brains were removed and placed in ice-chilled Petri dishes containing 0.9% NaCl. Two brain preparations were used as previously reported (Tildon *et al.*, 1983); one hemisphere was used to prepare a whole homogenate and the contra-

lateral hemisphere was used for preparation of dissociated cells.

The homogenates were prepared in 0.25M-sucrose by hand with four or five up-and-down strokes of an ice-jacketed Tenbroeck homogenizer (clearance 0.15 mm). It has been shown (Tildon & Roeder, 1980) that in similarly prepared homogenates the integrity of mitochondria and of synaptosomes is preserved. The weight per volume was adjusted according to the age of the animal and on the basis of preliminary studies, so that the final total reaction mixture (0.7 ml) contained between 0.75 and 1.50 mg of protein. The rate of substrate oxidation was linear with concentration within this range and with time up to 2 h. Protein contents were determined by the method of Lowry *et al.* (1951).

Oxidation of ^{14}C -labelled substrates

Portions of each homogenate were incubated in medium containing ^{14}C -labelled substrate and the $^{14}\text{CO}_2$ produced was trapped in Hyamine-hydroxide-saturated filter paper in hanging centre wells and its radioactivity counted in a liquid-scintillation spectrometer as previously described (Tildon & Roeder, 1980; Roeder *et al.*, 1982a; Tildon *et al.*, 1983). The concentrations of components of the reaction mixture were as follows: 0.21 mM-EDTA (disodium salt), 1 mM- KH_2PO_4 , 3.5 mM- MgSO_4 , 70 mM-Tris, 3.8 mM-KCl, 59 mM-NaCl, 0.16 mM- NAD^+ and 1 mM-ATP; final sucrose concentration was 150 mM. The concentrations of the labelled substrates were 2 mM for [6- ^{14}C]glucose and [U- ^{14}C]glutamine and 1.0 mM for [3- ^{14}C]acetoacetate and 3-hydroxy[3- ^{14}C]butyrate. Unlabelled alternative substrates were present at either 1.0 or 5.0 mM as indicated for the individual experiments.

Statistical analyses

The data were analysed for statistical significance by Student's *t* test.

Results

Table 1 shows the rates of oxidation of each of the ^{14}C -labelled substrates by whole homogenates of brain from rats in the different age groups. In the youngest (4–6-day-old animals) the rate of substrate oxidation was lowest for [6- ^{14}C]glucose, considerably higher for [U- ^{14}C]glutamine and 3-hydroxy[3- ^{14}C]butyrate and highest for [3- ^{14}C]acetoacetate. Both [6- ^{14}C]glucose and [U- ^{14}C]glutamine showed increased rates of oxidation with age, and the two ketone bodies showed decreased rates of oxidation between 4–6 days and adulthood.

Table 2 shows the effect of glutamine on the rates of oxidation of [6- ^{14}C]glucose by brain homogenates from animals at three different ages. When

Table 1. Rates of conversion of ^{14}C -labelled substrates into $^{14}\text{CO}_2$ by whole homogenates from brains of young and adult rats. Results are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses. Asterisks indicate significant differences from adult values ($P \leq 0.05$).

Substrate	Substrate oxidized (nmol/h per mg of protein)		
	Young (4–6 days)	Young (16–20 days)	Adult (90 days or older)
[6- ^{14}C]Glucose (2 mM)	2.27 $\pm$ 0.16* (13)	3.52 $\pm$ 0.40 (5)	4.80 $\pm$ 0.54 (10)
[U- ^{14}C]Glutamine (2 mM)	17.19 $\pm$ 1.29* (13)	24.73 $\pm$ 2.27* (6)	29.72 $\pm$ 2.03 (11)
3-Hydroxy[3- ^{14}C]butyrate (1 mM)	18.25 $\pm$ 1.25* (13)		4.68 $\pm$ 0.36 (9)
[3- ^{14}C]Acetoacetate (1 mM)	25.77 $\pm$ 2.07* (13)		4.34 $\pm$ 0.25 (7)

Table 2. Effects of added unlabelled substrate on rates of $^{14}\text{CO}_2$ production from ^{14}C -labelled glucose and glutamine in whole homogenates from brains of young and adult rats.

Results are means $\pm$ S.E.M.; number of rats per group = 5–13 for controls and 5–9 for groups with added unlabelled substrate. Asterisks indicate statistically significant ($P \leq 0.05$) differences from rates with labelled substrate alone.

Labelled substrate	Unlabelled substrate	Change from rate of $^{14}\text{CO}_2$ production with labelled substrate alone (%)		
		Young		Adult
		(4–6 days)	(16–20 days)	(90 days or older)
[6- ^{14}C]Glucose	Glutamine (1 mM)	–14 $\pm$ 6.7	–27 $\pm$ 5.8*	–32 $\pm$ 2.1*
	(5 mM)	–44 $\pm$ 6.0*	–51 $\pm$ 6.1*	–47 $\pm$ 2.5*
[U- ^{14}C]Glutamine	Glucose (1 mM)	+1 $\pm$ 2.1	–6 $\pm$ 16.7	+8 $\pm$ 5.5
	(5 mM)	0 $\pm$ 4.0	0 $\pm$ 11.3	–1 $\pm$ 3.4

Table 3. Effects of added unlabelled substrate on rates of $^{14}\text{CO}_2$ production from ^{14}C -labelled glucose and ketone bodies in whole homogenates from brains of young and adult rats.

Results are means $\pm$ S.E.M.; number of rats per group = 3–13 for controls and 3–7 for groups with added unlabelled substrates. Asterisks indicate statistically significant ($P \leq 0.05$) differences from rates with labelled substrate alone.

Labelled substrate	Unlabelled substrate	Change from rate of $^{14}\text{CO}_2$ production with labelled substrate alone (%)	
		Young	Adult
		(5–6 days)	(90 days or older)
[6- ^{14}C]Glucose	3-Hydroxybutyrate (1 mM)	–30 $\pm$ 3.5*	–15 $\pm$ 8.0
	(5 mM)	–37 $\pm$ 8.1*	–18 $\pm$ 2.7*
[6- ^{14}C]Glucose	Acetoacetate (1 mM)	–35 $\pm$ 5.0*	–16 $\pm$ 10.4
	(5 mM)	–32 $\pm$ 10.0*	+8 $\pm$ 6.3
3-Hydroxy[3- ^{14}C]butyrate	Glucose (1 mM)	–1 $\pm$ 3.4	+60 $\pm$ 7.5*
	(5 mM)	+8 $\pm$ 3.6	+52 $\pm$ 9.6*
[3- ^{14}C]Acetoacetate	Glucose (1 mM)	–10 $\pm$ 2.3	+42 $\pm$ 10.2*
	(5 mM)	–13 $\pm$ 4.6	+59 $\pm$ 6.4*

increasing amounts of unlabelled glutamine were added to this system, decreased amounts of $^{14}\text{CO}_2$ were produced in each age group, and this effect was related to the concentration of added glutamine. In contrast, when the reverse situation was tested, i.e. the effect of glucose on [U- ^{14}C]glutamine oxidation, there was little or no decrease in the rate of $^{14}\text{CO}_2$ production at any age.

The effects of adding ketone bodies as alternative substrates to homogenates oxidizing [6-

^{14}C]glucose are shown in Table 3. When unlabelled 3-hydroxybutyrate or acetoacetate was added, there were marked decreases in the rates of $^{14}\text{CO}_2$ production from [6- ^{14}C]glucose by homogenates from the young animals. In the adult, although there was a tendency for similar effects, the magnitude of change was considerably less and not always significant.

When the effect of adding unlabelled glucose on the rates of oxidation of the two ketone bodies was

Table 4. Effects of added unlabelled substrates on rates of $^{14}\text{CO}_2$ production from ^{14}C -labelled ketone bodies and glutamine in whole homogenates from brains of young and adult rats

Results are means $\pm$ S.E.M.; number of rats per group = 6–13 for controls and 3–7 for groups with added unlabelled substrate. Asterisks indicate statistically significant ($P \leq 0.05$) differences from rates with labelled substrate alone.

Labelled substrate	Unlabelled substrate	Change from rate of $^{14}\text{CO}_2$ production with labelled substrate alone (%)	
		Young (4–6 days)	Adult (90 days or older)
3-Hydroxy[3- ^{14}C]butyrate (1 mM)	Glutamine (1 mM)	$-19 \pm 1^*$	-6 ± 3
	(5 mM)	$-28 \pm 2^*$	-15 ± 9
[3- ^{14}C]Acetoacetate (1 mM)	Glutamine (1 mM)	$-18 \pm 3^*$	-12 ± 11
	(5 mM)	$-19 \pm 4^*$	$+6 \pm 16$
[U- ^{14}C]Glutamine (2 mM)	3-Hydroxybutyrate (1 mM)	$+27 \pm 11^*$	$+10 \pm 6$
	(5 mM)	$+42 \pm 18^*$	$+3 \pm 6$
[U- ^{14}C]Glutamine (2 mM)	Acetoacetate (1 mM)	$+43 \pm 15^*$	$+12 \pm 4$
	(5 mM)	$+76 \pm 17^*$	$+18 \pm 12$

examined, there were marked differences in the results, depending on the age of the animals. With either 3-hydroxy[3- ^{14}C]butyrate or [3- ^{14}C]acetoacetate the addition of unlabelled glucose increased the rate of $^{14}\text{CO}_2$ production by homogenates from adult animals, in contrast with the tendency for decreased $^{14}\text{CO}_2$ production when unlabelled ketone bodies were added to [6- ^{14}C]glucose. On the other hand, in young rats there was little or no effect on the rate of oxidation of [3- ^{14}C]hydroxybutyrate by unlabelled glucose, whereas [3- ^{14}C]acetoacetate oxidation was actually diminished, although the magnitude of the effect was not large.

The effects of added unlabelled substrate on $^{14}\text{CO}_2$ production from the two labelled ketone bodies or labelled glutamine are shown in Table 4. The addition of unlabelled glutamine decreased the rates of oxidation of both 3-hydroxy[3- ^{14}C]butyrate and [3- ^{14}C]acetoacetate by homogenates from young animals, but was without effect in adults.

In the reverse combination, i.e. ketone bodies added to [U- ^{14}C]glutamine, again only brain preparations from young animals were affected. However, in this situation, there was a striking reversal of effect, i.e. when either of the two ketone bodies was added, glutamine oxidation was markedly enhanced.

Discussion

Many studies examining metabolic compartmentation in the brain have been carried out by determining the appearance of the label in end products in brain after the injection of a specific radioactive compound (see Balazs & Cremer, 1973; Berl *et al.*, 1975). In the present paper we measured the rate of production of $^{14}\text{CO}_2$ from labelled sub-

strates and evaluated the effect of adding alternative substrates on these rates of oxidation in homogenates of brain from young and adult rats. Many laboratories have used one tissue preparation *in vitro* for studies on metabolic compartmentation, such as slices (Benjamin & Quastel, 1974; Nicklas *et al.*, 1979), synaptosomes (Jope, 1981; Bradford *et al.*, 1978) or cultured cells of established neural cell lines (Nicklas & Browning, 1978, 1983); others have examined mitochondria from different origins in the brain, i.e. synaptosomal and non-synaptosomal (Van den Berg *et al.*, 1975; Dienel *et al.*, 1977; Lai *et al.*, 1977). We have chosen to examine the effects of alternative substrates on rates of oxidation in two quite different brain preparations. The present paper focused on results obtained with whole homogenates, and the following paper reports studies on these interactions in dissociated cells in which the plasma membrane is intact (Roeder *et al.*, 1984); both series have included preparations from young as well as adult animals, and are an extension of other studies (Tildon *et al.*, 1983; Tildon, 1983).

Many investigators have measured the rates of oxygen consumption resulting from addition of substrates to the incubation medium (Lai *et al.*, 1977; Bradford *et al.*, 1978). It is usually assumed that the increased oxygen uptake reflects the rate of oxidation of that particular added substrate. However, the data in the present study indicate that this interpretation may not be valid. For example, when glucose was added to medium containing ^{14}C -labelled ketone bodies and brain tissue from adult rats, the enhanced rate of oxidation was due to increased production of CO_2 originating from the labelled acetoacetate or 3-hydroxybutyrate. Similarly, the addition of unlabelled ketone bodies stimulated the conversion of [U- ^{14}C]glutamine into $^{14}\text{CO}_2$. The data from these

studies show the complexity of substrate utilization in the brain and the need to reconcile the results of oxygen uptake with CO_2 production.

If two alternative substrates are oxidized in the same tricarboxylic acid cycle in the same compartment, one would expect a reciprocal dilution of the $^{14}\text{CO}_2$ produced when the unlabelled substrate was added to the labelled compound. However, in the present results, unlabelled glutamine was effective in competing with glucose, but the addition of unlabelled glucose, even at 5 mM, had no effect on glutamine oxidation. These results may suggest that glutamine or one of its metabolites may inhibit a step in the pathway for utilizing glucose in the brain. In liver, glutamine inhibits pyruvate dehydrogenase activity (Häussinger *et al.*, 1982). Other amino acids have also been shown to regulate glycolytic enzymes, including the inhibitory action of phenylalanine on pyruvate kinase and hexokinase of brain (Weber, 1969) and the inhibition of hepatic pyruvate kinase by alanine (Weber *et al.*, 1968). On the other hand, the lack of reciprocal effects by glutamine and glucose may reflect the higher rates of oxidation of glutamine as compared with that of glucose. However, examination of these rates suggests that some degree of dilution of $^{14}\text{CO}_2$ from 1 mM-glutamine by added glucose (5 mM) should have been detectable. Thus the findings suggest there is not simply a common pathway in the same compartment by which these two substrates are oxidized. Since these experiments were carried out in whole homogenates, separate compartments may be provided by subcellular organelles such as synaptosomes or 'free' mitochondria.

Ketone bodies and glucose

The enhancement of ketone-body oxidation by added glucose in homogenates from adult animals is similar to observations by other investigators using brain slices (Itoh & Quastel, 1970; Patel & Owen, 1977). This result may reflect a stimulation of tricarboxylic acid-cycle activity owing to the availability of increased amounts of oxaloacetic acid from glucose via pyruvate carboxylation. There was no similar enhancement in homogenates from young rats, and this may reflect the low rate of glucose oxidation by brain at this age (see Table 1). In the converse situation, when either 3-hydroxybutyrate or acetoacetate was added in the presence of $[6\text{-}^{14}\text{C}]\text{glucose}$, tissue from young rats showed a markedly decreased production of $^{14}\text{CO}_2$. In the adult, there was a diminution of glucose oxidation only when 3-hydroxybutyrate was added, and the magnitude of the effect was less than in young rats. The age difference in this effect is due probably to more effective utilization of ketone bodies than of glucose at the younger age as

a result of the high activities of the enzymes involved (Page *et al.*, 1971; Tildon *et al.*, 1971). The differential effect between the two ketone bodies in adults serves to emphasize that the two compounds must be considered as two separate substrates, with different pathways available for their metabolism and with different consequences for the cell. Of particular relevance are the data of Pleasure *et al.* (1979), evaluating the significance of the non-mitochondrial pathway available for the metabolism of acetoacetate, but not of 3-hydroxybutyrate, and showing that a specific cell type (oligodendroglia) is enriched in acetoacetyl-CoA synthetase, the first enzyme in this pathway. The data of the present study suggest that in adults acetoacetate may be preferentially used for lipid synthesis via this cytosolic pathway rather than for oxidation in mitochondria. It should also be noted that in liver the addition of acetoacetate stimulated and the addition of 3-hydroxybutyrate inhibited the synthesis of ketone bodies from fatty acid precursors, probably via shifts in the relative amounts of NAD^+ and NADH (Roeder *et al.*, 1982b). It would not be unreasonable to propose that in tissues that utilize ketone bodies, such as brain, a similar type of regulation may be operative.

Glutamine and ketone bodies

Both 3-hydroxybutyrate and acetoacetate enhanced the production of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]\text{glutamine}$ in homogenates from young rats. The mechanism accounting for this enhancement may be related to a release of inhibition of the activity of α -oxoglutarate dehydrogenase. It has been shown (Garland, 1964; Smith *et al.*, 1974) that succinyl-CoA is an effective inhibitor of this enzyme, and the addition of either ketone body would decrease the amount of succinyl-CoA by using it for the synthesis of acetoacetyl-CoA (yielding free succinate). As a consequence the oxidation of glutamine via the tricarboxylic acid cycle would be less inhibited. The finding that the enhancement of glutamine oxidation by ketone bodies occurred in young rats, but not in adults, is consistent with the established high activities of ketone-body-metabolizing enzymes in young animals (Page *et al.*, 1971; Tildon *et al.*, 1971) and with the higher rates of oxidation of both 3-hydroxybutyrate and acetoacetate in 5–6-day-old animals than in adults in the present study. The results of the converse situation, i.e. a decrease in $^{14}\text{CO}_2$ production from labelled ketone bodies with the addition of unlabelled glutamine, suggests a synergistic action between these particular substrates. The interactions between glutamine and the two ketone bodies in brain tissue of young animals appear to support greater use of glutamine as an energy source; it is

intriguing to hypothesize that the ketone bodies may be simultaneously diverted for use in lipid synthesis.

The results of the present studies include the rates of oxidation of multiple substrates in homogenates of brain from rats of different ages and the effects of the presence of alternative oxidizable compounds. The findings emphasize that compartmentation of metabolic activities in subcellular organelles may be a significant factor in the interactions among these alternative energy sources. In addition, they suggest some possible mechanisms by which certain substrates may regulate the utilization of other compounds, and emphasize that developmental changes in brain play a role in these processes. Together with the results obtained on intact dissociated brain cells (Roeder *et al.*, 1984), the data also illustrate the value of examining a variety of tissue preparations *in vitro* to further our understanding of these complex phenomena.

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