Competition of glycerol with other oxidizable substrates in rat brain

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The rate of conversion of [1,3-¹⁴C]glycerol into ¹⁴CO₂ was measured in the presence and absence of unlabelled alternative substrates in whole homogenates from the brains of young (4–6 and 18–20 days old) and adult rats. Unlabelled glucose decreased ¹⁴CO₂ production from [1,3-¹⁴C]glycerol by about 40% at all ages studied. Unlabelled 3-hydroxybutyrate significantly decreased the ¹⁴CO₂ production from both low (0.2 mM) and high (2.0 mM) concentrations of glycerol in 4–6- and 18–20-day-old rat pups. However, the addition of 3-hydroxybutyrate had no effect on the rate of ¹⁴CO₂ production from 2.0 mM-glycerol in adult rats, suggesting that the interaction of 3-hydroxybutyrate with glycerol in adult rat brain is complex and may be related to the biphasic kinetics previously reported for glycerol oxidation. Unlabelled glutamine decreased the production of ¹⁴CO₂ by brain homogenates from 18–20-day-old and adult rats, but not in 4–6-day-old rat pups. In the converse situation, the addition of unlabelled glycerol to whole brain homogenates had little effect on the rate of ¹⁴CO₂ production from [6-¹⁴C]glucose, 3-hydroxy[3-¹⁴C]butyrate and [U-¹⁴C]glutamine, although some significant differences were noted. Collectively these results suggest that glycerol and these other substrates may be metabolized in separate subcellular compartments in brain such that the products of glucose, 3-hydroxybutyrate and glutamine metabolism can dilute the oxidation of glycerol, but the converse cannot occur. The data also demonstrate that there are complex age-related changes in the interaction of glycerol with 3-hydroxybutyrate and glutamine. The fact that glycerol oxidation was only partially suppressed by the addition of 1–5 mM-glucose, -3-hydroxybutyrate or -glutamine could also suggest that glycerol may be selectively utilized as an energy substrate in some discrete brain region.

INTRODUCTION

The brain is capable of utilizing a variety of substrates for energy production, including glucose, ketone bodies, glycerol, pyruvate, lactate and glutamine (Itoh & Quastel, 1970; Patel & Owen, 1977; Yeh *et al.*, 1977; Robinson & Williamson, 1980; Cremer, 1981; Tildon, 1983; Tildon *et al.*, 1983). The increased ability of the brain to use certain substrates preferentially over other substrates during different stages of brain development for energy parallels, to a large extent, the elevated plasma concentrations of the compounds during early postnatal development (Hawkins & Biebuyck, 1979; Robinson & Williamson, 1980). We have characterized the oxidation of glycerol by developing rat brain (McKenna *et al.*, 1986), and other investigators have previously shown that adult brain tissue can oxidize glycerol for energy (Goodner *et al.*, 1973; Tildon *et al.*, 1976; Tildon & Roeder, 1980).

There is considerable evidence that the oxidation of different substrates can take place in separate subcellular organelles (Tildon & Roeder, 1980; Minn, 1982; Tildon *et al.*, 1985) and/or individual brain cell types (Ramaharobandro *et al.*, 1982; Tildon, 1983; Yu & Hertz, 1983; Yu *et al.*, 1984; Edmond *et al.*, 1985), which leads to compartmentation of the metabolism of these substrates. Differences in transport, metabolic pool size and localization of enzymes also contribute to this metabolic compartmentation of brain energy substrates. Roeder *et al.* (1984a,b) have shown, using relatively simple

preparations such as homogenates of whole brain, that the presence of a given substrate can influence the utilization of other substrates for energy by brain tissue. They also demonstrated the utility of these preparations for studying developmental changes in the influence of a substrate on the metabolism of other substrates. Similar preparations have been used by other groups (Yeh *et al.*, 1977; Crane & Morgan, 1983).

We have investigated the interaction of glycerol with other oxidizable substrates in the developing rat brain by the methods of Roeder *et al.* (1984a). The results described in the present paper compare the rates of oxidation of several ¹⁴C-labelled substrates ([6-¹⁴C]glucose, 3-hydroxy[3-¹⁴C]butyrate and [U-¹⁴C]-glutamine) in the presence and absence of unlabelled glycerol by homogenates of whole brains from rats of different ages. The converse situation was also examined by measuring the rate of oxidation of [1,3-¹⁴C]glycerol in the presence and absence of unlabelled glucose, 3-hydroxybutyrate and glutamine. A preliminary report of these data has been presented (McKenna *et al.*, 1985).

METHODS

Materials

Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All reagent-grade chemicals were obtained from Fisher Chemical Co. (Pittsburgh, PA, U.S.A.) and liquid-scintillation fluors from National

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Table 1. Rate of conversion of ^{14}C -labelled substrates into $^{14}\text{CO}_2$ by homogenates from whole brains of young and adult rats

Values are means $\pm$ S.E.M., for the numbers of experiments shown in parentheses. Data were analysed by using analysis of variance and Scheffé's multiple comparisons test: *significantly different from adult values ($P < 0.05$).

Substrate	Substrate oxidized (nmol of $^{14}\text{CO}_2$ produced/h per mg of protein)		
	Young (4–6 days)	Young (18–20 days)	Adult (≥ 90 days)
[1,3- ^{14}C]Glycerol (0.2 mM)	0.58 $\pm$ 0.04* (8)	0.74 $\pm$ 0.02 (6)	0.82 $\pm$ 0.04 (9)
[1,3- ^{14}C]Glycerol (2.0 mM)	5.10 $\pm$ 0.36* (7)	6.40 $\pm$ 0.20 (6)	6.04 $\pm$ 0.34 (9)
[6- ^{14}C]Glucose (2.0 mM)	1.94 $\pm$ 0.11* (7)	3.94 $\pm$ 0.42* (7)	2.91 $\pm$ 0.21 (10)
3-Hydroxy[3- ^{14}C]butyrate (1.0 mM)	28.05 $\pm$ 2.17* (10)	33.80 $\pm$ 2.03* (9)	7.81 $\pm$ 0.38 (10)
[U- ^{14}C]Glutamine (2.0 mM)	27.74 $\pm$ 1.77* (10)	29.90 $\pm$ 1.55 (9)	34.10 $\pm$ 0.87 (10)

Diagnostics (Somerville, NJ, U.S.A.). Radiolabelled [1,3- ^{14}C]glycerol, [6- ^{14}C]glucose, 3-hydroxy[3- ^{14}C]butyrate and [U- ^{14}C]glutamine were purchased from New England Nuclear (Boston, MA, U.S.A.) and used without further purification.

Animals

The animals used in this study were offspring born to breeding pairs of Albino Wistar rats obtained from Charles River Laboratories (Wilmington, MA, U.S.A.). Some adult animals were obtained directly from the supplier. Animals were housed in the University of Maryland School of Medicine animal facility in a room maintained at 22 °C with a 14 h-light/10 h-dark cycle. The birth dates of all litters were recorded. Both male and female animals were used for the studies on 4–6- and 18–20-day-old rat pups; only male animals were used for the studies with ≥ 90 -day-old rats.

Tissue preparation

Animals were decapitated and the brain was rapidly removed and placed in an ice-chilled Petri dish. The homogenates were prepared in 0.25 M-sucrose at low speed with three up-and-down strokes of an ice-jacketed Potter–Elvehjem homogenizer. With 4–6-day-old rat pups three brains were pooled, and with 18–20-day-old rats two brains were pooled, to provide enough sample for the assays. The volume of sucrose added per g of brain was adjusted according to the age of the rats, so that the final total reaction mixture (0.7 ml) contained 0.75–1.50 mg of protein. The protein content of the samples was determined by the method of Lowry *et al.* (1951).

Oxidation

Samples of the brain homogenates from animals of different ages were incubated at 37 °C for 1 h in medium containing the appropriate labelled substrate and the following final concentrations of other components: 4.0 mM-KCl, 0.21 mM-EDTA, 2.0 mM-KH₂PO₄, 3.5 mM-MgSO₄, 59 mM-NaCl, 70 mM-Tris/HCl, pH 7.4, 1.2 mM-ATP and 0.16 mM-NAD⁺. The concentrations of labelled substrates were 0.2 and 2.0 mM for [1,3- ^{14}C]glycerol, 2.0 mM for [6- ^{14}C]glucose and [U- ^{14}C]glutamine, and 1.0 mM for 3-hydroxy[3- ^{14}C]butyrate. To examine substrate competition, unlabelled glucose, 3-hydroxy-

butyrate and glutamine were present at 1.0 or 5.0 mM, and unlabelled glycerol was present at 1.0, 5.0 or 10.0 mM. The reactions were stopped by acidification, and the $^{14}\text{CO}_2$ produced was trapped in filter paper saturated with Hyamine hydroxide in hanging centre wells and counted for radioactivity in a liquid-scintillation spectrometer. These conditions used were similar to those used in earlier competition experiments (Roeder *et al.*, 1984a,b). Previous studies (Tildon & Roeder, 1980; Tildon *et al.*, 1983) revealed that the rate of $^{14}\text{CO}_2$ production from labelled substrates was linear with time up to 2 h and was proportional to protein concentration.

Statistical analysis

Data from the competition experiments were analysed by using a paired Student's *t* test (Zar, 1974) comparing the oxidation of substrates with added unlabelled substrates with the oxidation of labelled substrates alone without unlabelled substrate. Data comparing the rates of oxidation of the various substrates by homogenates of brain tissue from rats of different ages were analysed by analysis of variance and by Scheffé's multiple-comparisons test (Zar, 1974).

RESULTS

The rates of oxidation of the ^{14}C -labelled substrates by homogenates of whole brains from young (4–6 and 18–20 days old) and adult rats are shown in Table 1. The rates of oxidation of [6- ^{14}C]glucose and 0.2 mM-[1,3- ^{14}C]glycerol were lower in the brain tissue from 4–6-day-old rats than in 18–20-day-old and adult rats, as found in previous studies (Roeder *et al.*, 1984a; McKenna *et al.*, 1986). The rate of oxidation of 2.0 mM-glycerol was lower in brain tissue from 4–6-day-old rats than in 18–20-day-old rats, but was not significantly different from that in adult rat brain. In the 4–6- and 18–20-day-old rats the rates of oxidation of 3-hydroxy[3- ^{14}C]butyrate and [U- ^{14}C]glutamine were much higher than the rates of oxidation of glucose and of both concentrations of glycerol. Consistent with previous data reported by our laboratory (Tildon *et al.*, 1983; Roeder *et al.*, 1984a; Tildon & Roeder, 1984a), the rate of oxidation of glutamine remained high in adult rats, whereas the rate of oxidation of 3-hydroxybutyrate

Table 2. Effect of adding unlabelled glucose on the rate of $^{14}\text{CO}_2$ production from [1,3- ^{14}C]glycerol by homogenates of whole brain from rats of different ages

Experimental details are described in the text. Values are means $\pm$ S.E.M., and are expressed as per cent of oxidation of labelled glycerol with no unlabelled substrate added. The rate of glycerol oxidation was measured at low and high concentrations (0.2 and 2.0 mM), since glycerol oxidation by brain tissue has been shown to exhibit biphasic kinetics. The numbers of experiments are in parentheses after each value. Data were analysed by using a paired Student's *t* test (Zar, 1974): ***significantly different from controls ($P < 0.001$).

[1,3- ^{14}C]Glycerol concn.	Unlabelled glucose concn.	$^{14}\text{CO}_2$ production rate (% of control without added unlabelled substrate)		
		Young (4–6 days)	Young (18–20 days)	Adult (≥ 90 days)
0.2 mM	1.0 mM	58.1 $\pm$ 1.9*** (7)	66.1 $\pm$ 2.9*** (6)	60.5 $\pm$ 2.7*** (9)
0.2 mM	5.0 mM	57.1 $\pm$ 2.6*** (8)	64.3 $\pm$ 2.1*** (6)	57.7 $\pm$ 3.6*** (9)
2.0 mM	1.0 mM	58.2 $\pm$ 3.3*** (8)	61.8 $\pm$ 3.1*** (6)	58.7 $\pm$ 3.9*** (9)
2.0 mM	5.0 mM	57.7 $\pm$ 3.3*** (8)	62.6 $\pm$ 2.1*** (6)	57.8 $\pm$ 2.8*** (9)

Table 3. Effect of adding unlabelled 3-hydroxybutyrate on the rate of $^{14}\text{CO}_2$ production from [1,3- ^{14}C]glycerol by homogenates of whole brain from rats of different ages

Values are means $\pm$ S.E.M., and are expressed as per cent of oxidation of labelled glycerol with no unlabelled substrate added. The numbers of experiments are in parentheses after each value. Data were analysed by using a paired Student's *t* test: ** $P < 0.01$ and *** $P < 0.001$ for significance of difference from control.

[1,3- ^{14}C]Glycerol concn.	Unlabelled 3-hydroxybutyrate concn.	$^{14}\text{CO}_2$ production rate (% of control without added unlabelled substrate)		
		Young (4–6 days)	Young (18–20 days)	Adult (≥ 90 days)
0.2 mM	1.0 mM	73.5 $\pm$ 1.0*** (8)	71.8 $\pm$ 4.7** (6)	86.0 $\pm$ 3.9** (9)
0.2 mM	5.0 mM	74.1 $\pm$ 4.5** (8)	68.6 $\pm$ 4.6*** (6)	84.0 $\pm$ 3.7** (9)
2.0 mM	1.0 mM	76.7 $\pm$ 2.8*** (7)	73.0 $\pm$ 5.4** (6)	94.9 $\pm$ 4.6 (9)
2.0 mM	5.0 mM	77.3 $\pm$ 3.9** (7)	71.1 $\pm$ 3.5*** (6)	91.4 $\pm$ 4.8 (9)

Table 4. Effect of adding unlabelled glutamine on the rate of $^{14}\text{CO}_2$ production from [1,3- ^{14}C]glycerol by homogenates of whole brain from rats of different ages

Values are means $\pm$ S.E.M., and are expressed as per cent of oxidation of labelled glycerol with no unlabelled substrate added. The numbers of experiments are in parentheses after each value. Data were analysed by using a paired Student's *t* test: * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ for significance of difference from control.

[1,3- ^{14}C]Glycerol concn.	Unlabelled glutamine concn.	$^{14}\text{CO}_2$ production rate (% of control without added unlabelled substrate)		
		Young (4–6 days)	Young (18–20 days)	Adult (≥ 90 days)
0.2 mM	1.0 mM	95.8 $\pm$ 2.9 (8)	86.2 $\pm$ 3.8** (6)	85.9 $\pm$ 4.4** (9)
0.2 mM	5.0 mM	95.8 $\pm$ 4.1 (8)	76.9 $\pm$ 4.6** (6)	82.2 $\pm$ 5.4** (8)
2.0 mM	1.0 mM	99.8 $\pm$ 3.2 (9)	84.5 $\pm$ 3.0** (6)	89.1 $\pm$ 3.4** (9)
2.0 mM	5.0 mM	100.9 $\pm$ 3.1 (9)	81.3 $\pm$ 4.9*** (6)	89.0 $\pm$ 4.4* (9)

decreased in adult rats to about 25% of the rate during the suckling period.

The addition of unlabelled glucose to the incubation mixture decreased by approx. 40% ($P < 0.001$) the rate of oxidation of [1,3- ^{14}C]glycerol by brain tissue from rats of all ages (Table 2). Unlabelled glucose decreased the

rate of oxidation of both 0.2 mM- and 2.0 mM-glycerol, but there was no difference in the magnitude of the effect with 1.0 mM- or 5.0 mM-glucose. In contrast with the pattern seen with glucose, the addition of unlabelled 3-hydroxybutyrate had a greater effect on the rate of oxidation of glycerol by brain tissue from 4–6- and

Table 5. Effect of added unlabelled glycerol on the rate of $^{14}\text{CO}_2$ production from ^{14}C -labelled precursors by homogenates of whole brain from rats of different ages

Values are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses, and are expressed as per cent of oxidation of labelled substrates with no unlabelled glycerol added. Data were analysed by using a paired Student's *t* test: *significantly different from control ($P < 0.05$).

Labelled substrate	Unlabelled glycerol concn.	$^{14}\text{CO}_2$ production rate (% of control without added unlabelled substrate)		
		Young (4–6 days)	Young (18–20 days)	Adult (≥ 90 days)
[6- ^{14}C]Glucose (2.0 mM)	1 mM	95.0 $\pm$ 4.8 (7)	91.6 $\pm$ 2.6* (7)	100.1 $\pm$ 7.9 (9)
	5 mM	91.6 $\pm$ 5.0 (7)	97.5 $\pm$ 2.4 (7)	95.2 $\pm$ 3.8 (10)
	10 mM	99.3 $\pm$ 5.1 (7)	96.7 $\pm$ 2.4 (7)	96.6 $\pm$ 5.0 (10)
3-Hydroxy[3- ^{14}C]-butyrate (1.0 mM)	1 mM	98.2 $\pm$ 3.6 (10)	95.5 $\pm$ 3.0 (9)	97.8 $\pm$ 2.3 (10)
	5 mM	94.7 $\pm$ 2.0* (10)	95.5 $\pm$ 2.7 (9)	97.7 $\pm$ 2.2 (10)
	10 mM	100.7 $\pm$ 2.5 (10)	90.6 $\pm$ 3.4* (9)	98.3 $\pm$ 1.8 (10)
[U- ^{14}C]Glutamine (2.0 mM)	1 mM	99.6 $\pm$ 3.8 (10)	93.7 $\pm$ 3.2 (9)	96.3 $\pm$ 2.1 (10)
	5 mM	99.0 $\pm$ 5.7 (10)	93.4 $\pm$ 3.6 (9)	94.2 $\pm$ 2.0* (10)
	10 mM	98.6 $\pm$ 5.4 (10)	92.4 $\pm$ 3.2* (9)	94.3 $\pm$ 3.4 (10)

18–20-day-old animals than from adult rats (Table 3). In adult rats the effect of 3-hydroxybutyrate appeared to be related to the biphasic kinetics of glycerol oxidation in brain tissue, since the addition of unlabelled 3-hydroxybutyrate decreased the rate of oxidation of low (0.2 mM), but not high (2.0 mM), concentrations of glycerol. At all ages studied, the addition of 5.0 mM-3-hydroxybutyrate did not have any greater inhibitory effect on glycerol oxidation than did the addition of 1.0 mM-3-hydroxybutyrate. In contrast with the competition found with glucose and 3-hydroxybutyrate in young rats, the addition of 1.0 mM- or 5.0 mM-glutamine had no effect on the rate of oxidation of glycerol by brain tissue from 4–6-day-old rat pups (Table 4). However, the addition of unlabelled glutamine significantly decreased the rate of oxidation of glycerol by brain tissue from both 18–20-day-old and adult rats.

In the converse situation, unlabelled 1.0 mM-, 5.0 mM- or 10.0 mM-glycerol was added to compete with labelled glucose, 3-hydroxybutyrate and glutamine. In contrast with the profound decrease of glycerol oxidation with added glucose, there was little effect on the rate of [6- ^{14}C]glucose oxidation when unlabelled glycerol was added (Table 5). Although some significant differences were noted, in general the addition of unlabelled glycerol to the reaction mixture had little effect on the rates of oxidation of 3-hydroxy[3- ^{14}C]butyrate and [U- ^{14}C]glutamine by homogenates of whole brain (Table 5). When unlabelled glycerol was added to the other oxidative substrates, no consistent pattern was found with any of the oxidative substrates or concentrations of unlabelled glycerol.

DISCUSSION

The present studies are an extension of earlier investigations by our group that were carried out with homogenates of whole brain tissue (Roeder *et al.*, 1984a). This relatively simple brain tissue preparation was chosen to circumvent the effects caused by differences in uptake and transport of the individual substrates into brain cells.

For example, it has been shown that the uptake of exogenous glucose (Roeder *et al.*, 1985) and 3-hydroxybutyrate (Tildon & Roeder, 1984b) into brain cells differs with the age of the rat.

The fact that addition of 1.0 mM- or 5.0 mM-glucose significantly decreased the rate of oxidation of labelled glycerol by about 40% at all ages is consistent with the observations in the literature (Yeh *et al.*, 1977; Tildon *et al.*, 1983) that rat brain tissue utilizes glucose as a fuel from birth to adulthood. This is not unexpected, since the conversion of dihydroxyacetone 3-phosphate, from glucose metabolism, into glycerol 3-phosphate via glycerol phosphate dehydrogenase should dilute the pool of [^{14}C]glycerol 3-phosphate and lead to the decreased $^{14}\text{CO}_2$ production observed. The lack of inhibition of glucose oxidation by added unlabelled glycerol suggests that dihydroxyacetone 3-phosphate formed from glycerol did not enter the same metabolic pool as that formed from glucose, hence no measurable dilution occurred. Additional support for the compartmentation of these two substrates is provided by the report by Binaglia *et al.* (1977), who demonstrated that phosphatidic acid formed from glucose in brain is located in a different metabolic pool from the phosphatidic acid synthesized from glycerol.

Unlike glucose, which decreased glycerol oxidation to the same extent at all ages, the competition of 3-hydroxybutyrate with glycerol showed age-related changes. There is considerable evidence in the literature that 3-hydroxybutyrate and acetoacetate are the preferred substrates for energy by brain tissue during the suckling period in rats (Itoh & Quastel, 1970; Patel & Owen, 1977; Webber & Edmond, 1977; Yeh *et al.*, 1977; Robinson & Williamson, 1980; Tildon *et al.*, 1983). Therefore it is not surprising that 3-hydroxybutyrate decreased glycerol oxidation more in young rats than in adults. The fact that the addition of 3-hydroxybutyrate had an effect on the rate of $^{14}\text{CO}_2$ production from 0.2 mM-, but not 2.0 mM-, glycerol suggests that the interaction of 3-hydroxybutyrate with glycerol in adult rat brain is complex and may be related to the biphasic kinetics previously reported for

glycerol oxidation (Tildon & Roeder, 1980). It is curious that 3-hydroxybutyrate decreased the rate of glycerol oxidation in developing rat brain to a lesser extent than did glucose, since 3-hydroxybutyrate is oxidized to a much greater extent than glucose by developing brain. The fact that glycerol had little effect on the rate of oxidation of 3-hydroxybutyrate suggests that there are also differences in the subcellular compartmentation of the metabolism of these two substrates.

In contrast with the effects of glucose and 3-hydroxybutyrate, unlabelled glutamine did not decrease the rate of glycerol oxidation by brain tissue from 4–6-day-old rat pups, but it did decrease the oxidation in older pups and adult rats. The lack of effect of added unlabelled glutamine on glycerol oxidation in 4–6-day-old rat pups may be related to differences in the activities of several of the enzymes of brain glutamine metabolism, which have been shown to be substantially lower in young rat pups than at older ages (Ozand *et al.*, 1975). However, it should be noted that there is little difference in the rate of glutamine oxidation during development (Table 1; Tildon & Roeder, 1984a). The lack of competition of added glycerol suggests that these two substrates may be metabolized in different subcellular compartments, such that the products of glutamine metabolism can dilute the oxidation of glycerol, but the converse cannot occur.

Overall, our data suggest that differences in the age-dependency and relative utilization of brain energy substrates are not solely due to differential transport across the blood/brain barrier, as has been suggested (Hawkins & Biebuyck, 1979). The data also show that complex age-dependent interactions between glycerol and other substrates can be shown even in relatively simple preparations such as homogenates of whole brain. Much of the complexity of these interactions may be related to differences in subcellular compartmentation of the metabolism of these substrates. Each of the substrates studied can be oxidized by synaptosomes and free mitochondria, the organelles present in the homogenate (Bradford *et al.*, 1978; Tildon & Roeder, 1980; Minn, 1982; Tildon *et al.*, 1985). In adult rat brain the rates of oxidation of glucose and 3-hydroxybutyrate are the same in both organelles, whereas the rates of oxidation of glutamine and glycerol are much higher in mitochondria (Tildon & Roeder, 1980; Tildon *et al.*, 1985). The results suggest the need for further investigation of the interactions of glycerol with other substrates in subcellular organelles such as isolated mitochondria and synaptosomes.

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