Differential substrate oxidation by dissociated brain cells and homogenates during development

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The rates of oxidation of 3-hydroxy[3-¹⁴C]butyrate, [3-¹⁴C]acetoacetate and [6-¹⁴C]glucose were compared by using two different preparations of brain from the same animals (i.e. whole homogenates and dissociated brain cells) at various ages during development. In homogenates the rates of oxidation of 3-hydroxy[3-¹⁴C]butyrate and [3-¹⁴C]acetoacetate were high in young rats and low in adults, and were significantly higher at most ages during development than those obtained for intact cells. In contrast, rates of [6-¹⁴C]glucose oxidation by homogenates and intact cells were essentially the same at early ages; however, the rate by homogenates did not change throughout development, whereas that by intact cells increased severalfold by adulthood. In adult animals the initial glucose concentration affected the rate of glucose oxidation in homogenates, but not in intact cells. These data suggest a role for the intact cell membrane in the regulation of alternative substrate utilization by brain cells and that this process changes during development. However, the data may reflect selective differences in the cellular and subcellular components in these two preparations.

It is now well established that alternative substrates, such as ketone bodies, 3-hydroxybutyrate and acetoacetate, can provide metabolic fuel for the mammalian brain especially during periods of early development, and this subject has been reviewed by Robinson & Williamson (1980). This proposal is based in part on the observations that, during the suckling period, the activities of the enzymes 3-oxo acid CoA-transferase (EC 2.8.3.5), 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) and one of the isoenzymes of acetoacetyl-CoA thiolase (EC 2.3.1.9) are 2–3-fold higher than those found in the adult brain (Klee & Sokoloff, 1967; Page *et al.*, 1971; Tildon *et al.*, 1971; Middleton, 1973). Other studies have measured the oxidation of these substrates to CO₂ and their incorporation into structural components such as lipids (Hawkins *et al.*, 1971; Patel & Owen, 1977; Webber & Edmond, 1977; Yeh *et al.*, 1977; Roeder *et al.*, 1982).

To obtain these results, a variety of preparations have been used, including intact brain (Moore *et al.*, 1976), cortical slices (Patel & Owen, 1977; Lavau *et al.*, 1978), minces (Itoh & Quastel, 1970) and homogenates (Yeh *et al.*, 1977; Patel & Clark, 1980). Other studies have used established cell lines

derived from neuroblastoma and glioma as models for examining metabolic properties of intact brain cells (Roeder *et al.*, 1982; Patel *et al.*, 1981; deVellis & Brooker, 1973). In addition, dissociated brain-cell cultures have been used for biochemical, morphological and physiological studies (Wiesmann *et al.*, 1975; Fishbach & Nelson, 1977; Pleasure *et al.*, 1979). Dissociated brain cells as well as primary cell cultures have been used to demonstrate biochemical changes *in vitro* that resemble those that occur *in vivo* (Seeds, 1971; Schmidt, 1975; Kozak, 1977; Siegrist *et al.*, 1981).

In our initial approach to obtain information about the relative metabolic properties from two different preparations, we compared the patterns of substrate oxidation by freshly isolated intact dissociated brain cells with those obtained by using whole homogenates prepared from the contralateral hemispheres of the same brains from animals of different ages. A preliminary report of some of these data has been presented (Merrill *et al.*, 1982).

Materials and methods

Materials

Biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Reagent-grade

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chemicals were obtained from Fisher Chemical Co. (Pittsburgh, PA, U.S.A.) and liquid-scintillation fluors from National Diagnostics (Somerville, NJ, U.S.A.). Radioactive compounds {[$6\text{-}^{14}\text{C}$]glucose, D-($-$)-3-hydroxy[$3\text{-}^{14}\text{C}$]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).

Animals

Albino Wistar rats were obtained from Charles River Laboratories (Wilmington, MA, U.S.A.) and housed in our 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, and during the suckling period animals of either sex were used for these experiments. After 25 days males were used unless otherwise indicated.

Tissue preparation

After decapitation of the rat, the brain was rapidly removed and placed in an ice-chilled Petri dish. In some instances when small animals were used, several hemispheres were pooled to provide enough experimental samples. In most cases, one hemisphere was used to prepare a whole homogenate and the other was used to prepare dissociated brain cells. In a few experiments results were obtained for dissociated cells or homogenates only.

Homogenates (10%, w/v) in 0.25 M-sucrose were prepared as previously described (Tildon & Roeder, 1980) by hand with an ice-jacketed Tenbroeck homogenizer. The dissociated brain cells were prepared by a modification of the method of Wiesmann *et al.* (1975) and Siegrist *et al.* (1981), both of whom described the validity of this model for biochemical studies. The tissue was immersed in 0.9% NaCl cut into small pieces (1 mm³) with surgical scissors and transferred to a 50 ml centrifuge tube. The volume was adjusted to 20 ml with a 0.2% trypsin solution. After incubation for 20 min at 37°C, the cells were washed three times with 0.9% NaCl and further dissociated by gentle trituration in a 10 ml wide-mouth pipette. The suspension was centrifuged at 100g for 5 min and the supernatant removed. The cells were resuspended in 0.9% NaCl and filtered through nylon screening cloth or lens paper (0.33 μm pore size). These freshly prepared cells were used immediately. Tests for Trypan Blue exclusion and leakage of lactate dehydrogenase revealed that cell viability was better than 90%. The protein content of the samples was determined by the method of Lowry *et al.* (1951).

Oxidation

Samples of either homogenates or dissociated cells 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 counted for radioactivity in a liquid-scintillation spectrometer. The incubations were done at 37°C; the reaction mixture contained 58 mM-KCl, 0.3 mM-EDTA, 11.2 mM-K₂HPO₄, 5 mM-MgSO₄ and 100 mM-Tris/HCl, pH 7.4. For homogenate preparations ATP (1.44 mM) and NAD⁺ (0.24 mM) were included, as described previously (Tildon & Roeder, 1980). Separate experiments showed that these additions as well as variations in the concentrations of K⁺ (5–50 mM) or Na⁺ (2.5–100 mM) did not contribute to the observed differential between homogenates and intact cells. Unless otherwise indicated, concentrations of the substrates were 1.0 mM for glucose and 3-hydroxybutyrate and 0.5 mM for acetoacetate. These conditions are similar to those in previous experiments (Tildon & Roeder, 1980; Roeder *et al.*, 1982) in which we demonstrated the capacity of slices, intact cells, homogenates and subcellular organelles to oxidize substrates by measuring the rate of $^{14}\text{CO}_2$ production. These studies also revealed that the rates of $^{14}\text{CO}_2$ production from labelled substrates were linear with time for up to 2 h and were proportional to protein concentration. In the present studies, comparing oxidation by homogenates with dissociated brain cells, protein concentrations for both systems were maintained between 0.75 and 1.5 mg/sample.

Results

The developmental profiles for the rates of oxidation of hydroxy[3- ^{14}C]butyrate by brain homogenates and dissociated brain cells obtained from the same animals are shown in Fig. 1(a). The rates of oxidation by homogenates from suckling animals were significantly higher than those for adults. A similar difference was observed with dissociated cells. However, at each time during the suckling period the rates for homogenates were much higher than those obtained for the intact cells, but the relative rates of oxidation (rate in homogenates/rate in intact cells) changed dramatically during development, such that at -2 (i.e. 19 days gestation), 5, 10, 15 and 25 days these ratios were 3.55, 3.73, 2.00, 1.98 and 1.4, respectively.

The rates of oxidation of [3- ^{14}C]acetoacetate by brain homogenates and dissociated brain cells are shown in Fig. 1(b). As was found with 3-hydroxybutyrate, the rate of oxidation was higher in homogenates from suckling animals than from adults. In contrast, the rate of oxidation of acetoacetate by dissociated brain cells was essentially

Fig. 1. Rates of oxidation of 3-hydroxy[3-¹⁴C]butyrate (a), [3-¹⁴C]acetoacetate (b) and [6-¹⁴C]glucose (c) by brain homogenates (O) and dissociated cells (●) from rats of different ages

Experimental details are described in the text. Each point is the mean $\pm$ S.E.M. (indicated by the vertical bars) for the number of animals shown in parentheses. Asterisks indicate significant differences ($P \leq 0.05$) between homogenates and dissociated cells at the indicated ages. Values for adults were significantly different ($P \leq 0.05$) from those for younger animals as follows: (a) in both tissue preparations, except at -2 days; (b) for homogenates at 5, 10 and 15 days; (c) for dissociated cells, except at 10 days.

unchanged during the developmental period. However, as observed with 3-hydroxybutyrate, the relative rates of acetoacetate oxidation (rate in homogenates/rate in intact cells) also changed during development, such that at 5, 10 and 15 days the relative rates were 3.58, 3.25 and 3.59, but at 25

Fig. 2. Rates of [6-¹⁴C]glucose oxidation by brain homogenates and dissociated brain cells from adult rats as a function of initial substrate concentration

In this experiment values were normalized to 100% of the rates with 1.0 mM-glucose from means of 4.09 and 4.35 nmol/h per mg for homogenates (□) and dissociated brain cells (▨) respectively. Each bar represents the mean $\pm$ S.E.M. for four rats.

and 90 days this ratio decreased to 1.7 and 1.3 respectively.

The developmental pattern for the oxidation of [6-¹⁴C]glucose by homogenates and dissociated brain cells is shown in Fig. 1(c). In contrast with the developmental patterns for acetoacetate and 3-hydroxybutyrate, the rates of glucose oxidation were lowest in the intact cells obtained from foetal animals and increased more than 5-fold in cells obtained from 90-day-old animals. With homogenates prepared from the same animals, however, the rates of glucose oxidation were essentially unchanged during the developmental period. When the rates of glucose oxidation were expressed as relative rates (rate in homogenates/rate in intact cells), the value was almost 3.0 for preparations obtained from foetal animals. However, this value was about 1.0 for 5-, 10- and 15-day-old animals and decreased further to about 0.7 and 0.55 for 25- and 90-day-old animals respectively.

To evaluate further the difference between homogenates and dissociated brain cells, the rates of glucose oxidation by these two preparations from adult animals were compared at several different initial concentrations of this substrate. In Fig. 2 the results show that increasing the initial concentration of glucose from 0.1 to 2.5 mM resulted in more than a 6-fold increase in the rate of glucose oxidation by homogenates. In contrast, with dissociated brain cell preparations the rate of glucose oxidation was

Table 1. *Effect of initial substrate concentration on the rates of oxidation of 3-hydroxy[3-¹⁴C]butyrate and [3-¹⁴C]-acetoacetate*

Contralateral hemispheres from the same animal were used to prepare homogenates (Homog) and dissociated brain cells (Cells). The rates of oxidation were determined as described in the Materials and methods section. For the 5-day-old animals, three or four hemispheres were pooled to provide sufficient samples. The values are expressed as nmol/h per mg of protein, and all the data are the averages of triplicate measurements.

	Substrate concn. (mM)	5-day-old		25-day-old		Adult	
		Homog	Cells	Homog	Cells	Homog	Cells
3-Hydroxybutyrate	0.1	9.94	2.33	3.06	2.31	1.40	0.7
	0.5	14.63	9.16	9.74	7.13	3.25	1.95
	1.0	28.99	10.56	13.41	4.65	4.53	1.20
	5.0	23.88	8.83	13.66	6.56	3.77	1.59
Acetoacetate	0.1	9.67	0.83	3.85	2.03	1.81	1.32
	0.5	17.12	3.14	8.93	7.26	6.98	3.18
	1.0	18.36	2.48	9.64	7.07	5.50	6.51
	5.0	5.18	—	8.72	10.84	3.35	6.32

essentially the same when the concentration was increased over 10-fold, from 0.5 to 5.0 mM; even at 0.1 mM, the rate was still 70% of those obtained at higher concentrations.

In a similar group of experiments, the effects of increasing the initial concentrations on the rates of oxidation of both 3-hydroxybutyrate and acetoacetate were also evaluated. The data in Table 1 reveal that the pattern of oxidation as a function of substrate concentration was similar in dissociated brain cells and homogenates and that in most instances the optimum substrate concentration was either 0.5 or 1.0 mM for both of these preparations. Furthermore, this seemed to be the case for 5- and 25-day-old animals as well as for adults.

Discussion

Several different preparations of mammalian brain have been used to study the comparative utilization of different substrates, including whole animals (Hawkins *et al.*, 1971), brain slices (Itoh & Quastel, 1970) and whole homogenates of brain (Yeh *et al.*, 1977; Patel & Clark, 1980). The results described in the present paper demonstrate striking differences between the rates of substrate oxidation by dissociated brain cells and those by whole homogenates prepared from the same animals. With either 3-hydroxybutyrate or acetoacetate this difference was most pronounced during the suckling period.

With homogenates, the age-associated pattern in the rates of oxidation of [3-¹⁴C]acetoacetate and 3-hydroxy[3-¹⁴C]butyrate were similar to those obtained by Yeh *et al.* (1977). These changes are probably related to the increased activities of ketone-body-utilizing enzymes during this period, as

has been demonstrated for 3-hydroxybutyrate dehydrogenase (Klee & Sokoloff, 1967; Page *et al.*, 1971), 3-oxo acid CoA-transferase (Page *et al.*, 1971; Tildon *et al.*, 1971) and acetoacetyl-CoA thiolase (Middleton, 1973). However, the rates of oxidation by dissociated brain cells from suckling animals were significantly less than those by the homogenates and remained essentially unchanged during development, so that the difference between the two preparations was decreased in the older animal.

The age-related pattern of oxidation of [6-¹⁴C]-glucose by whole brain homogenates differed markedly from those for ketone bodies; the rate was essentially the same at all times during development, as also observed by Yeh *et al.* (1977). On the other hand, the rate of oxidation by dissociated brain cells increased more than 5-fold over the same period. This resulted in a shift in the relative rates of oxidation, i.e. rate in homogenates/rate in intact cells, from more than 2.9 with foetal animals to less than 0.5 for preparations from adults.

Collectively, these data suggest that the mechanism(s) regulating the utilization of ketone bodies by the brain during neonatal development are different from those mechanisms that regulate glucose utilization. These findings suggest that membrane transport plays an important role in regulating the utilization of these substrates, and a variety of other studies support this proposal. Using whole animals, Moore *et al.* (1976) demonstrated an age-dependence of cerebral uptake of 3-hydroxybutyrate, and Cremer *et al.* (1976) provided evidence for a monocarboxylic acid transport system in suckling rats that was 6 times more active than that present in adult animals. Furthermore, Pollay & Stevens (1980) suggested that the kinetics

for the affinity and uptake of 3-hydroxybutyrate into rat brain were altered by starvation. The differential effect of initial glucose concentration on oxidation rates in homogenates as opposed to dissociated brain cells in the present study adds additional weight to the proposal that membrane transport plays an important role in the regulation of substrate utilization by brain cells. Thus the concept of selective uptake (the blood/brain barrier) should be broadened to include possible involvement of the individual cell membrane. Although the absolute rates were different for dissociated brain cells and homogenates when 3-hydroxybutyrate or acetoacetate was the substrate, the effect of increasing the initial concentration was essentially the same with either preparation. This suggests that the transport of ketone bodies across the cell membrane may be rate-limiting in brain cells, especially in suckling animals.

Variations in rates of respiration by different brain preparations, including perfused brain, brain slices, homogenates and isolated neurons and glia, has been summarized by Hertz (1973). It is therefore not unexpected that preparations of isolated cells and brain whole homogenates gave different developmental profiles. The most attractive explanation for this difference is the regulatory contribution of an intact membrane. However, it may be that the decreased rate of oxidation of ketone bodies by dissociated brain cells reflects a regulation by the presence of higher concentrations of inhibitors or metabolites within the cells that become diluted in homogenates. It is also possible that the method of preparing brain cells and homogenates could result in the differential selection of a unique population of cells and/or subcellular organelles with different substrate-oxidation profiles.

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