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Acetoacetate Metabolism in Infant and Adult Rat Brain *in vitro*

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1. Acetoacetate or DL-β-hydroxybutyrate increases the rate of oxygen consumption to a smaller extent than that brought about by glucose or pyruvate in adult rat brain-cortex slices but to the same extent as that in infant rat brain-cortex slices. 2. The rate of $^{14}CO_2$ evolution from [1-^{14}C]glucose considerably exceeds that from [6-^{14}C]glucose in respiring infant rat brain-cortex slices, in contrast with adult brain-cortex slices, suggesting that the hexose monophosphate shunt operates at a greater rate in the infant rat brain than in the adult rat brain. 3. The rate of $^{14}CO_2$ evolution from [3-^{14}C]acetoacetate or DL-β-hydroxy[3-^{14}C]butyrate, in the absence of glucose, is the same in infant rat brain slices as in adult rat brain slices. It exceeds that from [2-^{14}C]glucose in infant rat brain but is less than that from [2-^{14}C]glucose in adult rat brain. 4. Acetoacetate is oxidized in the brain through the operation of the citric acid cycle, as shown by the accelerating effect of glucose on acetoacetate oxidation in adult brain slices, by the inhibitory effects of malonate in both infant and adult brain slices and by its conversion into glutamate and related amino acids in both tissues. 5. Acetoacetate does not affect glucose utilization in adult or infant brain slices. It inhibits the rate of $^{14}CO_2$ formation from [2-^{14}C]glucose or [U-^{14}C]-glucose the effect not being wholly due to isotopic dilution. 6. Acetoacetate inhibits non-competitively the oxidation of [1-^{14}C]pyruvate, the effect being attributed to competition between acetyl-CoA and CoA for the pyruvate-oxidation system. 7. Acetoacetate increases the rate of aerobic formation of lactate from glucose with both adult and infant rat brain slices. 8. The presence of 0.1mM-2,4-dinitrophenol diminishes but does not abolish the rate of $^{14}CO_2$ formation from [3-^{14}C]acetoacetate in rat brain slices. This points to the participation of ATP in the process of oxidation of acetoacetate in infant or adult rat brain. 9. The presence of 5mM-D-glutamate inhibits the rate of $^{14}CO_2$ formation from [3-^{14}C]acetoacetate, in the presence or absence of glucose. 10. Labelled amino acids are formed from [3-^{14}C]acetoacetate in both adult and infant rat brain-cortex slices, but the amounts are smaller than those found with [2-^{14}C]glucose in adult rat brain and greater than those found with [2-^{14}C]glucose in infant rat brain. 11. Acetoacetate is not as effective as glucose as a precursor of acetylcholine in adult rat brain but is as effective as glucose in infant rat brain slices. 12. Acetoacetate or β-hydroxybutyrate is a more potent source of acetyl-CoA than is glucose in infant rat brain slices but is less so in adult rat brain slices.

Acetoacetate and β-hydroxybutyrate undergo oxidation in the presence of isolated brain (Quastel, 1939), but the respiration of rat brain-cortex slices in the presence of β-hydroxybutyrate is considerably less than that in the presence of glucose (Jowett & Quastel, 1935). Oxidative breakdown of acetoacetate (5mM) by adult rat brain-cortex slices accounts for about 30% of the total oxygen consumption, its utilization being increased by the addition of glucose (Itoh & Quastel, 1968). Utilization of added ketone bodies may also account for 20–40% of the oxygen consumption of guinea-pig brain slices (Rolleston & Newsholme, 1967). Although it has been reported that acetoacetate (3.3mM) and glucose are equally effective in promoting the respiration of brain slices from 5-day-old rats, in contrast with their effects on the respiration of adult rat brain slices, where glucose is more potent than acetoacetate (Drahota, Hahn, Mourek & Trojana, 1965), neither acetoacetate nor β-hydroxybutyrate is as effective as glucose, with

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either infant or adult rat brain-cortex slices, in raising the concentration of ATP (Itoh & Quastel, 1969).

We have carried out experiments to throw further light on cerebral metabolism of acetoacetate and on the interrelations between acetoacetate and glucose metabolism in the brain. The results of these experiments are described below.

MATERIALS

Animals. Male adult rats of the Wistar strain weighing 150–200g were used. Infant rats (1–3 days old) of the Wistar strain, both male and female, were obtained from the Vivarium, Department of Zoology, University of British Columbia, Vancouver, B.C., Canada.

Radioactive materials. Ethyl [3-^{14}C]acetoacetate (4.18mCi/mmol) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. The ester was hydrolysed with NaOH by the method of Ljunggren (1924) and ethanol, in the acetoacetate solution, was eliminated by the freeze-drying method (Krebs, Hems, Weidemann & Speake, 1966). DL-β-Hydroxy[3-^{14}C]-butyric acid (11.8mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. and [1-^{14}C]succinic acid (4.0mCi/mmol) was obtained from Volk Radiochemical Co., Skokie, Ill., U.S.A. Both compounds were neutralized with NaOH solution.

[U-^{14}C]Glucose (190mCi/mmol), [1-^{14}C]glucose (57.5mCi/mmol), [2-^{14}C]glucose (52.4mCi/mmol), [6-^{14}C]-glucose (36.5mCi/mmol) and sodium [2-^{14}C]pyruvate (31.0mCi/mmol) were obtained from The Radiochemical Centre. Sodium [1-^{14}C]pyruvate (3.27mCi/mmol) was obtained from New England Nuclear Corp. Sodium [1-^{14}C]acetate (10.0mCi/mmol) and sodium DL-[1-^{14}C]-lactate (5mCi/mmol) were obtained from Volk Radiochemical Co. To each of these substrates was added the corresponding unlabelled substrate, so that the specific radioactivity of each substrate became 0.033μCi/μmol.

Enzymes. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49), type VI (from yeast), and hexokinase (EC 2.7.1.1), type C-300 (from yeast), were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., and lactate dehydrogenase (EC 1.1.1.27) (from rabbit muscle) was obtained from Calbiochem, Los Angeles, Calif., U.S.A.

METHODS

Incubation procedure. Adult rat brain-cortex slices were prepared with a Stadie–Riggs tissue slicer, care being taken that the slices were not more than 0.4mm thick. One dorsal and one lateral slice ('first slices') weighing a total of 50–75mg wet wt. from the same brain were used. The slices were placed in ice-cold Krebs–Ringer phosphate medium of the following composition: 141mM-NaCl, 5mM-KCl, 2.3mM-$CaCl_2$, 1.3mM-$MgSO_4$, 10mM-Na_2HPO_4 (adjusted to pH 7.4 with HCl).

Infant rat brain-cortex slices were prepared by cutting by hand, only slices of the temporal–parietal portion of each hemisphere being used. As the infant rat brain is small, it was found expedient to use slices up to 1mm thick. Control experiments, with infant rat brain slices of different thicknesses, showed that their rates of O_2 consumption or of $^{14}CO_2$ formation from ^{14}C-labelled substrates are not significantly lower with thicknesses of 0.9–1mm than those with thicknesses of 0.4–0.5mm. This is probably largely due to the fact that the rate of respiration of infant rat brain slices is sufficiently low to allow adequate oxygenation of the brain cells even with slices 1mm thick (Warburg, 1923).

The slices, from both adult and infant rat brain (average wet wt. 60–80mg), were weighed at once on a torsion balance and suspended in the incubation medium. Various substrates, either radioactive or non-radioactive, were added to the incubation medium, the final volume being 3.0ml. The incubation was carried out with the conventional Warburg manometric apparatus in an atmosphere of oxygen at 37°C for 60min. CO_2 was absorbed by small rolls of filter paper made alkaline with M-Hyamine hydroxide in methanol solution. The $^{14}CO_2$ absorbed by the Hyamine hydroxide solution was dissolved in vials in 10ml of a scintillation medium, consisting of equal volumes of toluene, dioxan and aq. 95% (v/v) ethanol containing 2,5-diphenyloxazole (5g/l), 1,4-bis-(4-methyl-5-phenoxazol-2-yl)benzene (0.05g/l) and naphthalene (80g/l). The radioactivity assay was performed with a Nuclear–Chicago mark I liquid-scintillation counter. Counting efficiency was 78%.

Hyamine hydroxide was used as absorbent for $^{14}CO_2$ (Passmann, Radin & Cooper, 1956; Snyder & Godfrey, 1961; Mayes & Felts, 1967) instead of KOH, because use of the former substance avoids the considerable quenching effect due to KOH. When allowance is made for the quenching, results obtained for rates of $^{14}CO_2$ formation are the same whether Hyamine or KOH is used for absorption.

Values of the rates of $^{14}CO_2$ formation recorded below are given as μg-atoms of ^{14}C incorporated into $^{14}CO_2$/h per 100mg wet wt. of tissue. The values of c.p.m. of $^{14}CO_2$, absorbed in the Hyamine hydroxide, were transformed into μg-atoms of ^{14}C by dividing by the values of the specific radioactivity of the ^{14}C present in the substrate (c.p.m./μg-atom). This term was corrected for the weight of tissue and expressed per 100mg wet wt. of tissue.

All experiments described below were carried out at least three times and the results are given as means ± S.D.

Determination of glucose. At the end of the incubation period the Warburg vessels were chilled in ice and the brain tissues were removed by forceps and washed twice with the Krebs–Ringer medium, homogenized with 2ml of 6% (w/v) $HClO_4$ and centrifuged. A 0.5ml sample of the incubation medium was acidified with 2ml of 6% $HClO_4$ and centrifuged and the supernatant was neutralized with 5M-K_2CO_3. When glucose was measured in the medium, it was diluted 20-fold.

Glucose was determined in 0.2ml samples of the supernatants by an enzymic method using hexokinase and glucose 6-phosphate dehydrogenase (Greengard, 1963). The reaction mixture for the enzymic assay consisted of the following: ATP, 0.2μmol; $NADP^+$, 0.05μmol; $MgCl_2$, 3μmol; EDTA (pH 7.4), 1.2μmol; triethanolamine buffer (pH 8.0), 24μmol; glucose 6-phosphate dehydrogenase, 1 unit; hexokinase, 1 unit. The total volume was 2.0ml. Incubation was carried out at room temperature for 30min. NADPH was measured with an Aminco–Bowman spectrophotofluorometer with excitation wavelength 365nm and fluorescence wavelength 460nm. One

unit of glucose 6-phosphate dehydrogenase is the amount of enzyme that catalyses reduction of 1 μmol of $NADP^+$/min at pH 7.4 at 25°C. One unit of hexokinase is the amount of enzyme catalysing the phosphorylation of 1 μmol of glucose/min at pH 8.5 at 25°C.

Determination of ATP. ATP content in brain-cortex slices was determined by the same enzymic method as that used for glucose determination, except that 220 μmol of glucose was added to the reaction mixture instead of 0.2 μmol of ATP.

Determination of lactate. The supernatants after treatment of the brain tissue or incubation medium with $HClO_4$ and neutralization with 5 M-K_2CO_3 were analysed for lactate content. The neutralized supernatant from the incubation medium was diluted fivefold with water and a 1.5 ml sample was used for enzymic assay of lactate by using the lactate dehydrogenase system (Hohorst, 1963). The neutralized supernatant from the brain-cortex slices was diluted threefold with water and a 1.5 ml sample was used for the assay. The reaction mixture for the assay was made up as follows: glycine–hydrazine buffer (glycine, 1300 μmol; hydrazine sulphate, 520 μmol), pH 9.5; NAD^+, 1.6 mg; lactate dehydrogenase, 10 units. The total volume was 3.2 ml. Incubation was carried out at room temperature for 30 min. NADH was measured in an Aminco–Bowman spectrophotofluorimeter with excitation wavelength 365 nm and fluorescence wavelength 460 nm. One unit of lactate dehydrogenase is that amount of enzyme causing an initial rate of oxidation of 1 μmol of NADH/min at pH 7.4 at 25°C.

Determination of ^{14}C-labelled amino acids. After incubation of the rat brain-cortex slices with radioactive substrates, the slices were washed with water and homogenized in a final volume of 6 ml of 80% (v/v) ethanol. The homogenate was allowed to stand at 0°C for 2 h and then, after centrifugation, the supernatant was evaporated to dryness at 30°C with a jet flow of air. The dried extract was dissolved in 0.6 ml of 80% ethanol and a 0.2 ml sample was chromatographed by using the procedure of Gonda & Quastel (1966). Measurements of the radioactivities of the spots on the paper corresponding to the amino acids were carried out with a Nuclear–Chicago mark 1 liquid-scintillation counter. Each spot corresponding to an amino acid on the paper was cut out and placed in a vial containing 10 ml of the scintillant medium described above and its radioactivity was measured. The procedure and subsequent calculation of the radioactivities of the amino acids in the filter paper were those described by Gonda & Quastel (1966), with the exception that the spots were located by the ninhydrin reaction. The values of the yields of ^{14}C-labelled amino acids given in Table 7 are approximately the total yields, as measurements of their amounts in the incubation medium show that very little leakage of the amino acids from tissue to medium takes place under the given experimental conditions (Gonda & Quastel, 1966).

Determination of [^{14}C]acetylcholine. [^{14}C]Acetylcholine content of brain-cortex slices was determined by the method of Browning & Schulman (1968), with some modification.

After incubation with radioactive substrates, in the presence or absence of 0.5 mM-eserine sulphate, the brain-cortex slices were washed twice with the Krebs–Ringer solution and homogenized in 3 ml of water containing 0.5 mM-eserine and 64 μmol of carrier (unlabelled) acetylcholine. The pH was adjusted to 4.0 by the addition of 0.1 M-HCl. The homogenate was heated in a boiling-water bath for 10 min. After cooling and centrifuging, the deposit was washed twice with 1 ml of cold water. The combined clear supernatants were chilled in ice and mixed with 0.5 ml of 20% (w/v) chloroauric acid to precipitate acetylcholine chloroaurate. The yellow precipitate was washed twice with 3 ml of cold water and finally the precipitate was dissolved in 1.5 ml of hot water. About 100 mg of zinc metal powder was added to this solution to precipitate gold metal and to release the acetylcholine. A 0.5 ml sample of the colourless clear supernatant was used for the measurement of the radioactivity of the acetylcholine liberated. The amount of [^{14}C]acetylcholine formed was expressed as ng-atoms of ^{14}C incorporated into the acetylcholine fraction/100 mg wet wt. of tissue. Control experiments were carried out at 0°C.

Measurement of acid-soluble radioactivity. The acid-soluble radioactivity in the tissue slices, at the termination of the incubation experiments with labelled acetoacetate, was always measured as follows. The brain tissue was removed by forceps and washed twice with the Krebs–Ringer medium, homogenized with 5 ml of 5% (w/v) trichloroacetic acid and centrifuged. A 0.5 ml sample of the supernatant was assayed for radioactivity with the scintillation counter. This was expressed as μg-atoms of ^{14}C/h per 100 mg wet wt. incorporated into acetoacetate and its metabolic products in the tissue slice. This term was calculated by dividing the radioactive counts by the specific radioactivity of the labelled acetoacetate in the incubation medium (0.033 μCi/μmol). The radioactivity in the incubation medium, at the end of the experiment, was almost unchanged from that at the beginning owing to the presence of excess of substrate.

RESULTS

Oxygen consumption and $^{14}CO_2$ formation by adult and infant rat brain-cortex slices in the presence of various ^{14}C-labelled substrates

Oxygen consumption. The rate of oxygen consumption by adult rat brain-cortex slices in the presence of glucose is more than double that by infant (2–3 days-old) rat brain-cortex slices in the presence of glucose or sodium pyruvate on the basis of wet weight of tissue. Representative results are shown in Table 1. However, in terms of dry weight of tissue the respiration of infant rat brain approaches that of adult rat brain. This is because the dry weight of adult brain is about 20% of the wet weight, whereas that of infant brain is about 11.5% of the wet weight.

Acetoacetate or β-hydroxybutyrate (as the sodium salt) increases the rate of respiration of adult rat brain cortex, but not to the same extent as does glucose or sodium pyruvate. With infant rat brain, however, acetoacetate or β-hydroxybutyrate is as effective as glucose or pyruvate in increasing the rate of respiration. Sodium succinate has approximately the same activity as acetoacetate or β-hydroxybutyrate in increasing the rate of respiration

Table 1. *Respiration of adult and infant rat brain-cortex slices in the presence of various substrates*

Rat brain-cortex slices were incubated in the Krebs–Ringer phosphate medium described in the Methods section for 1 h in O_2 at 37°C. For full experimental details see the text. The various labelled substrates were present at the start of the experiment. The specific radioactivity of each substrate was 0.033 μCi/μmol and 15 μmol of each substrate was added to each manometric vessel, with the exceptions of succinate and acetate, of which 30 μmol was added. The total radioactivity added to each vessel was 8×10^5 c.p.m., except with succinate and acetate, for which it was 16×10^5 c.p.m. The values of rates of O_2 uptake are expressed as μmol/h per 100 mg wet wt. The values of rates of $^{14}CO_2$ formation are expressed as μg-atoms of ^{14}C incorporated into $^{14}CO_2$/h per 100 mg wet wt. All acid substrates were present as sodium salts.

	Adult		Infant	
Substrate (5 mM)	O_2 uptake	$^{14}CO_2$ formed	O_2 uptake	$^{14}CO_2$ formed
Nil	6.7 ± 0.2	—	3.7 + 0.2	—
[U-^{14}C]Glucose	12.5 ± 0.8	5.06 ± 0.20	4.7 ± 0.2	1.20 ± 0.19
[2-^{14}C]Glucose	12.5 ± 0.4	0.90 ± 0.02	5.1 ± 0.1	0.16 ± 0.01
[1-^{14}C]Glucose	11.8 ± 0.8	0.34 ± 0.02	4.6 ± 0.3	0.09 ± 0.01
[6-^{14}C]Glucose	11.8 ± 0.8	0.32 ± 0.02	4.6 ± 0.3	0.03 ± 0.006
[2-^{14}C]Pyruvate	12.8 ± 0.4	2.08 ± 0.04	5.1 ± 0.3	0.74 ± 0.02
[3-^{14}C]Acetoacetate	9.4 ± 0.2	0.58 ± 0.10	5.4 ± 0.3	0.50 ± 0.03
DL-β-Hydroxy[3-^{14}C]butyrate	8.8 ± 0.2	0.58 ± 0.06	5.1 ± 0.1	0.45 ± 0.01
[1-^{14}C]Acetate	7.0 ± 0.2	0.07 ± 0.02	4.3 ± 0.1	0.11 ± 0.02
[1-^{14}C]Succinate	8.0 ± 0.2	0.36 ± 0.01	4.1 ± 0.2	0.14 ± 0.02

of adult rat brain cortex, but is considerably less effective in increasing the rate of respiration of infant rat brain cortex.

$^{14}CO_2$ formation. Comparison of the rates of $^{14}CO_2$ formation from [U-^{14}C]glucose, [1-^{14}C]-glucose, [2-^{14}C]glucose and [6-^{14}C]glucose (Table 1) shows that the rate is greatest, as expected, with [U-^{14}C]glucose with both infant and adult rat brain-cortex slices. The rates with adult rat brain cortex with [1-^{14}C]glucose and [6-^{14}C]glucose are identical, within experimental error, but those with infant rat brain cortex with [1-^{14}C]glucose are considerably higher than those with [6-^{14}C]glucose. The latter fact suggests that the hexose monophosphate shunt operates at a greater rate in the infant brain than in the adult brain.

The rate of $^{14}CO_2$ formation from [2-^{14}C]glucose is greater than that from [1-^{14}C]glucose in either adult or infant rat brain.

Comparison of the rates of $^{14}CO_2$ formation from [3-^{14}C]acetoacetate and DL-β-hydroxy[3-^{14}C]butyrate (Table 1) shows that each yields approximately the same rate with either adult or infant brain. However, in contrast with the behaviour of glucose or pyruvate, the rate of $^{14}CO_2$ formation with infant rat brain cortex from [3-^{14}C]acetoacetate or DL-β-hydroxy[3-^{14}C]butyrate is almost the same as that with adult rat brain cortex. In fact, in infant rat brain labelled acetoacetate or β-hydroxybutyrate is a much more effective precursor of $^{14}CO_2$ than is [2-^{14}C]glucose (the position of the labelled carbon corresponding to that in the labelled acetoacetate or β-hydroxybutyrate used). The opposite is the case with adult rat brain cortex (Table 1). These results suggest that in infant rat brain cortex acetoacetate or β-hydroxybutyrate is more potent than glucose as a source of acetyl-CoA, whereas the contrary is the case with adult rat brain cortex. It is also evident that acetoacetate or β-hydroxybutyrate is more effective than acetate as a source of acetyl-CoA because, as shown in Table 1, the rate of $^{14}CO_2$ formation from [1-^{14}C]-acetate is far less than that from an equimolecular concentration of [3-^{14}C]acetoacetate or DL-β-hydroxy[3-^{14}C]butyrate.

[2-^{14}C]Pyruvate yields, with both infant and adult rat brain slices, a much higher rate of $^{14}CO_2$ formation than that from an equimolar concentration of [2-^{14}C]glucose. This may be partly due to the fact that the latter molecule will give rise to one labelled and one unlabelled molecule of pyruvate, so that isotopic dilution will occur.

Nevertheless with infant rat brain the difference is larger than can be accounted for by isotopic dilution. The relatively small yield of $^{14}CO_2$ from [2-^{14}C]glucose with infant brain compared with that from adult brain may be due partly to a lower rate of conversion of glucose into pyruvate in infant brain under our experimental conditions. This view is supported by the fact that the aerobic rate of formation of lactate from glucose is lower in infant brain than in adult brain *in vitro* (Table 5).

The rate of formation of $^{14}CO_2$ from [3-^{14}C]-acetoacetate or β-hydroxy[3-^{14}C]butyrate exceeds that from [1-^{14}C]succinate with both adult and infant rat brain cortex, the disparity being much greater with infant brain than with adult brain (Table 1). This is in accordance with the well-known

Table 2. *Glucose–acetoacetate interrelations and the respiration of adult and infant rat brain-cortex slices*

Rat brain-cortex slices were incubated in the Krebs–Ringer phosphate medium in the presence of labelled glucose or labelled acetoacetate and mixtures of these with unlabelled acetoacetate or unlabelled glucose. For full experimental details see the text and Table 1. Values of rates of O_2 uptake are expressed as μmol/h per 100mg wet wt. and those of $^{14}CO_2$ formation are expressed as μg-atoms of ^{14}C incorporated into $^{14}CO_2$/h per 100mg wet wt.

	Adult		Infant	
Substrate (5mM)	O_2 uptake	$^{14}CO_2$ formed	O_2 uptake	$^{14}CO_2$ formed
[U-^{14}C]Glucose	12.3±0.8	5.06±0.20	4.7±0.2	1.20±0.19
[U-^{14}C]Glucose + acetoacetate	13.0±0.4	3.55±0.06	5.4±0.1	0.50±0.02
[2-^{14}C]Glucose	12.6±0.4	0.90±0.02	5.2±0.1	0.16±0.01
[2-^{14}C]Glucose + acetoacetate	13.2±0.2	0.64±0.02	5.7±0.1	0.06±0.01
[3-^{14}C]Acetoacetate	9.4±0.2	0.58±0.10	5.4±0.1	0.49±0.03
[3-^{14}C]Acetoacetate + glucose	12.4±0.2	1.04±0.06	5.8±0.1	0.52±0.01

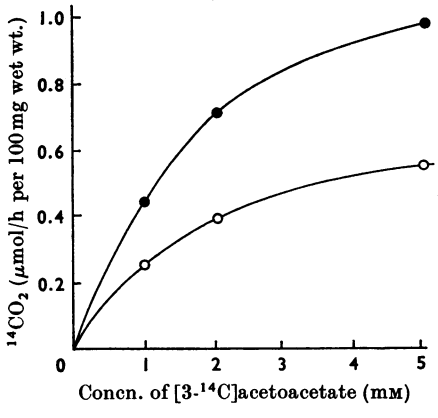


Fig. 1. Effect of glucose on the rate of $^{14}CO_2$ formation from [3-^{14}C]acetoacetate at different concentrations with adult rat brain-cortex slices incubated in O_2 at 37°C. ●, 5mM-Glucose added; ○, no added glucose.

observation that the rate of oxidation of succinate in adult rat brain cortex exceeds that in infant rat brain cortex and with the fact that the rate of turnover of the citric acid cycle is lower in the infant rat brain than in the adult rat brain (Potter, Schneider & Liebl, 1945; Tyler, 1942; Flexner, Belknap & Flexner, 1953).

The results (Table 1) showing that the rate of $^{14}CO_2$ formation from [3-^{14}C]acetoacetate in infant brain is but little less than that found in adult brain, in spite of the lower rate of turnover of the citric acid cycle in infant brain, under the usual conditions where glucose is present, suggest that in the absence of added glucose the rates of operation of the citric acid cycle in both tissues are approximately equal.

Cerebral glucose–acetoacetate interrelations. The effect of adding glucose to adult rat brain-cortex slices respiring in a medium containing [3-^{14}C]-acetoacetate is to increase markedly the rate of formation of $^{14}CO_2$. Typical results are shown in Table 2. The rate of oxygen uptake is also increased, but not to the same extent as the rate of $^{14}CO_2$ evolution. This is to be expected if the process of cerebral oxidation of acetoacetate is dependent on the operation of the citric acid cycle for the provision of succinyl-CoA, for reaction with acetoacetate to form acetoacetyl-CoA, or to ensure the increased cell concentration of ATP required if there is a direct conversion of acetoacetate into acetoacetyl-CoA by an acetoacetate kinase. The increased oxidative utilization of acetoacetate in the adult brain in the presence of glucose is probably greater than that indicated by the results given in Table 2, as doubtless some isotopic dilution takes place owing to the formation of unlabelled acetyl-CoA derived from oxidative breakdown of glucose. Results recorded in Fig. 1 indicate that the enhancing effect of 5mM-glucose on the rate of evolution of $^{14}CO_2$ from [3-^{14}C]acetoacetate reaches a maximum with approx. 2mM-acetoacetate.

With infant rat brain, however, the presence of glucose exercises little or no effect on the rate of $^{14}CO_2$ formation from [3-^{14}C]acetoacetate (Table 2), a fact in accordance with the known low rate of turnover of the citric acid cycle in the immature brain. Nevertheless acetoacetate oxidation proceeds at a relatively fast rate, indicating that the cycle is still sufficiently operative to enable acetyl-CoA, provided by acetoacetate, to be formed and oxidized.

When unlabelled acetoacetate is added to rat brain-cortex slices respiring in the presence of an equimolar concentration of [U-^{14}C]glucose there is a marked fall in the rate of $^{14}CO_2$ evolution, with both adult and infant brain, though there is little or no change in the rate of respiration (Table 2). The percentage diminution of the rate of $^{14}CO_2$ evolution by the addition of an equimolecular concentration

of acetoacetate is greater with infant rat brain than with adult rat brain (Table 2). This result may be expected if there is isotopic dilution owing to unlabelled acetyl-CoA pooling with the labelled acetyl-CoA derived from [U-^{14}C]glucose, or if acetoacetate exerts a direct inhibitory effect on the process of glucose oxidation. Similar inhibitions of the rates of $^{14}CO_2$ evolution, by unlabelled acetoacetate in the presence of either adult or infant rat brain slices are observed when [2-^{14}C]glucose is substituted for [U-^{14}C]glucose. Results given in Table 2 shows that the diminutions of the rate of $^{14}CO_2$ formation by acetoacetate from [U-^{14}C]-glucose and [2-^{14}C]glucose are 31% and 29% respectively with adult rat brain slices and 58% and 63% respectively with infant rat brain slices. The fact that acetoacetate exerts quantitatively the same inhibitory effect on the rate of $^{14}CO_2$ formation from either [U-^{14}C]glucose or [2-^{14}C]glucose under the given experimental conditions shows that there must be a direct inhibitory effect on glucose oxidation, apart from that expected from isotopic dilution. This follows from the fact that if only isotopic dilution by acetyl-CoA, derived from acetoacetate, were operating, there should be no dilution of $^{14}CO_2$ derived from C-3 or C-4 of glucose. $^{14}CO_2$ derived from [1-^{14}C]pyruvate presumably would not be affected by isotopic dilution with acetyl-CoA. Therefore isotopic dilution alone should result in a smaller percentage inhibition of the rate of $^{14}CO_2$ formation from [U-^{14}C]glucose than from [2-^{14}C]glucose. This seems not to be the case.

The inhibitory effect of acetoacetate on the rate of $^{14}CO_2$ evolution from 5mM-[U-^{14}C]glucose is optimum at a concentration of 2mM (Fig. 2), as increase in the concentration of acetoacetate does not affect further the rate of $^{14}CO_2$ formation. This result indicates that acetoacetate is not itself the inhibitory agent, but that a metabolic product derived from it is responsible for the inhibition. Such a product would be formed maximally with 2mM-acetoacetate, which is the concentration at which glucose exerts its optimum effect on acetoacetate breakdown (Fig. 1). This conclusion is consistent with the view that either acetyl-CoA, or acetoacetyl-CoA, derived from acetoacetate, is the responsible inhibitory agent. The fact that thiolase is active in brain (infant or adult) (Lynen, 1957) makes it likely that there is a rapid breakdown of acetoacetyl-CoA to acetyl-CoA in brain, so that the inhibitory agent, in the oxidative conversion of [U-^{14}C]glucose into $^{14}CO_2$, would be acetyl-CoA. This view is supported by the fact that malonate greatly affects the cerebral oxidative utilization of acetoacetate in the brain, indicating its dependence on the operation of the citric acid cycle in the brain.

Effects of malonate on cerebral metabolism of glucose and acetoacetate. Sodium malonate (5mM) inhibits the rate of $^{14}CO_2$ formation from [3-^{14}C]-acetoacetate approximately to the same extent with both infant and adult rat brain cortex incubated in the absence of added glucose (Table 3). This result indicates not only that the citric acid cycle operates in both infant and adult rat brain metabolism, but that it has a rate-limiting effect during the process of acetoacetate oxidation.

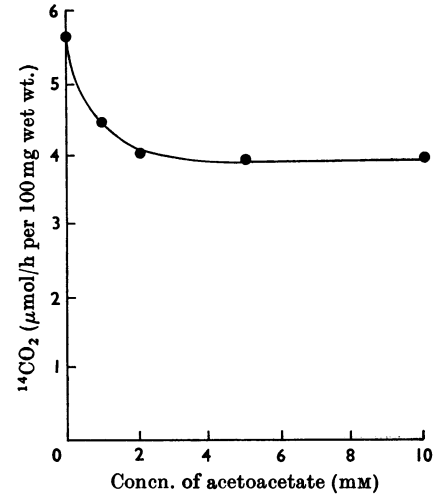


Fig. 2. Effect of sodium acetoacetate at different concentrations on the rate of $^{14}CO_2$ formation from 5mM-[U-^{14}C]glucose with adult rat brain-cortex slices incubated in O_2 at 37°C.

The acceleration of the rate of $^{14}CO_2$ formation from [3-^{14}C]acetoacetate by glucose is abolished by malonate (5mM). Results given in Table 3 show that in both adult and infant rat brain in the presence of glucose and malonate the rates of $^{14}CO_2$ formation are approximately the same, whereas in the absence of malonate the rate of $^{14}CO_2$ formation in adult brain in the presence of glucose greatly exceeds that in infant brain. These results are to be expected if the operation of the citric acid cycle is rate-limiting with regard to acetoacetate oxidation in infant brain, the addition of glucose having relatively little effect on the rate of operation of the cycle in this tissue.

Sodium malonate (5mM), although markedly inhibiting the rate of $^{14}CO_2$ formation by adult rat brain-cortex slices in the presence of [U-^{14}C]glucose, has no inhibitory effect, within the experimental error, on this rate in infant rat brain-cortex slices. The apparent lack of malonate inhibition of the rate of $^{14}CO_2$ formation in infant rat brain in the presence of [U-^{14}C]glucose may partly be because some of the $^{14}CO_2$ evolved results from operation of the hexose monophosphate pathway, which seems

Table 3. *Effects of malonate (5 mM) on [U-^{14}C]glucose and [3-^{14}C]acetoacetate metabolism in adult and infant rat brain-cortex slices*

Rat brain-cortex slices were incubated in the Krebs–Ringer phosphate medium containing mixtures of labelled glucose or labelled acetoacetate with 5 mM-sodium malonate. Full experimental details are given in the text and in Table 1. Values of rates of O_2 uptake are expressed as μmol/h per 100 mg wet wt. and those of $^{14}CO_2$ formation are expressed as μg-atoms of ^{14}C incorporated into $^{14}CO_2$/h per 100 mg wet wt.

	Adult		Infant	
Substrate (5 mM)	O_2 uptake	$^{14}CO_2$ formed	O_2 uptake	$^{14}CO_2$ formed
[U-^{14}C]Glucose	12.2±0.8	5.01±0.30	4.7±0.2	1.30±0.20
[U-^{14}C]Glucose + malonate	7.2±0.2	2.90±0.20	4.6±0.1	1.40±0.10
[3-^{14}C]Acetoacetate	9.4±0.2	0.58±0.01	5.4±0.3	0.49±0.01
[3-^{14}C]Acetoacetate + malonate	7.8±0.2	0.32±0.01	4.9±0.1	0.30±0.01
[3-^{14}C]Acetoacetate + glucose	12.4±0.2	1.04±0.06	5.9±0.1	0.52±0.01
[3-^{14}C]Acetoacetate + glucose + malonate	7.3±0.3	0.26±0.02	5.1±0.1	0.33±0.01

Table 4. *Effects of acetoacetate on the metabolism of* DL-[1-^{14}C]*lactate and* [1-^{14}C]*pyruvate in adult and infant rat brain-cortex slices*

Rat brain-cortex slices were incubated in the Krebs–Ringer phosphate medium containing labelled DL-lactate or labelled pyruvate, in the presence or absence of unlabelled acetoacetate. Full experimental details are given in the text and in Table 1. Values of the rates of O_2 uptake are expressed as μmol/h per 100 mg wet wt. and those of $^{14}CO_2$ formation are expressed as μg-atoms of ^{14}C incorporated into $^{14}CO_2$/h per 100 mg wet wt.

	No glucose added				With 5 mM-glucose			
	Acetoacetate absent		5 mM-Acetoacetate		Acetoacetate absent		5 mM-Acetoacetate	
Substrate	O_2 uptake	$^{14}CO_2$ formed	O_2 uptake	$^{14}CO_2$ formed	O_2 uptake	$^{14}CO_2$ formed	O_2 uptake	$^{14}CO_2$ formed
Adult								
DL-[1-^{14}C]Lactate (10 mM)	13.0±0.2	2.08±0.1	13.4±0.2	1.82±0.02	14.2±0.03	1.95±0.03	15.0±0.1	1.22±0.02
[1-^{14}C]Pyruvate (5 mM)	12.7±0.2	5.11±0.1	12.7±0.3	4.21±0.09	13.6±0.1	4.02±0.10	14.3±0.1	2.71±0.02
Infant								
DL-[1-^{14}C]Lactate (10 mM)	5.1±0.1	0.68±0.02	6.5±0.1	0.34±0.01	6.1±0.2	0.69±0.01	6.4±0.1	0.29±0.01
[1-^{14}C]Pyruvate (5 mM)	6.6±0.1	2.25±0.02	6.7±0.1	1.23±0.02	6.4±0.3	1.64±0.02	7.2±0.1	0.77±0.02

to be more prominent in infant rat brain than in adult brain (Table 1), and partly be due to a relatively low rate of formation of [^{14}C]acetyl-CoA from [^{14}C]glucose, which allows for its complete removal by the citric acid cycle even when this has been partially suppressed by the presence of malonate.

Effects of acetoacetate on the oxidation of lactate and pyruvate in rat brain. Experiments were carried out to observe whether acetoacetate affects the rate of oxidation of sodium lactate or sodium pyruvate in adult or infant rat brain-cortex slices. In these experiments [1-^{14}C]lactate and [1-^{14}C]pyruvate were used, to avoid the complicating factor of isotopic dilution in assessment of the effects of acetoacetate on the rates of $^{14}CO_2$ evolution from labelled lactate or pyruvate. The oxidation of [1-^{14}C]lactate or [1-^{14}C]pyruvate leads to the formation of $^{14}CO_2$ and unlabelled acetyl-CoA, so that no isotopic dilution by acetyl-CoA derived from acetoacetate may be expected.

Results given in Table 4 show that in the absence of glucose 5 mM-sodium acetoacetate has little effect on the rate of formation of $^{14}CO_2$ from 10 mM-sodium DL-[1-^{14}C]lactate or 5 mM-sodium [1-^{14}C]-pyruvate in adult rat brain-cortex slices. However, in the presence of glucose acetoacetate inhibits the rate of formation of $^{14}CO_2$ from [1-^{14}C]pyruvate by 33% and that from [1-^{14}C]lactate by 36%. This result would be expected if acetyl-CoA, derived from acetoacetate, has a direct inhibitory effect on pyruvate oxidation by brain. The yield of acetyl-CoA from acetoacetate will be increased in the presence of glucose, owing either to interaction of acetoacetate with succinyl-CoA or to an increased concentration of ATP needed for possible acetoacetate kinase activity.

Results obtained with infant rat brain (Table 4) show that, even in the absence of glucose, acetoacetate markedly decreases the rates of $^{14}CO_2$ evolution from [1-^{14}C]lactate or [1-^{14}C]pyruvate, the inhibitions being 50 and 45% respectively. In the presence of glucose the inhibitions are 58 and 53% respectively. Thus with infant rat brain the addition of glucose has a relatively small effect in enhancing the inhibition by acetoacetate of lactate or pyruvate oxidation to carbon dioxide. It seems that there is a higher rate of conversion of acetoacetate into acetyl-CoA in infant rat brain slices than

Table 5. *Effects of sodium acetoacetate on the disappearance of glucose and accumulation of lactate during the incubation of adult and infant rat brain-cortex slices*

Rat brain-cortex slices were incubated in the Krebs–Ringer phosphate medium containing glucose, or a mixture of glucose and acetoacetate, in O_2 at 37°C for 1 h. At the end of this period glucose and lactate were determined in the incubation medium and in the separated tissue. Full experimental details are given in the text. Values of glucose or of lactate are given as μmol/100 mg wet wt. Control experiments carried out with adult rat brain slices incubated in O_2 at 37°C for 1 h in the absence of any added substrate showed the presence of 0.3 μmol of lactate/100 mg wet wt. in the medium and 0.01 μmol of lactate in the tissue/100 mg wet wt.

	Glucose		Lactate	
Substrates	Disappearance from the incubation medium	Concentration in the incubated tissue	Accumulated in the incubation medium	Concentration in the incubated tissue
Adult				
Glucose 5 mM)	3.55±0.01	0.11±0.01	3.18±0.06	0.40±0.01
Glucose (5 mM) + acetoacetate (5 mM)	3.33±0.01	0.11±0.01	4.40±0.14	0.53±0.02
Infant				
Glucose (5 mM)	1.51±0.10	0.16±0.01	1.50±0.20	0.20±0.01
Glucose (5 mM) + acetoacetate (5 mM)	1.36±0.02	0.16±0.01	2.30±0.20	0.26±0.02

in adult rat brain slices in the absence of glucose. This conclusion is in accord with results already recorded (Table 2), indicating the greater inhibition by acetoacetate of $^{14}CO_2$ formation from [^{14}C]-glucose with infant rat brain slices than with adult rat brain slices.

Effect of acetoacetate on the utilization of glucose and accumulation of lactate in rat brain. When sodium acetoacetate (5 mM) is added to the glucose–saline medium in which infant or adult rat brain-cortex slices are respiring, the utilization of glucose, as judged by the rate of its disappearance from the medium, is but little affected. Typical results are shown in Table 5. Although glucose utilization is markedly less in infant rat brain slices than in adult brain slices on the basis of wet weight of tissue, the presence of acetoacetate makes very little difference (<10%) either to the rate of glucose disappearance or to the amounts of glucose accumulated in the tissues. However, the presence of acetoacetate markedly increases the rate of lactate formation from glucose with both adult and infant rat brain-cortex slices. The increase of lactate concentration in the incubated medium is 38% with adult brain slices and 53% with infant brain slices. The percentage increase in the rate of lactate formation is approximately equal to the percentage decrease in the oxidative utilization of glucose with both adult and infant brain slices.

Inhibition of the rate of $^{14}CO_2$ evolution from [1-^{14}C]lactate by acetoacetate. The inhibitory effect of acetoacetate on the rate of oxidation of 10 mM-DL-[1-^{14}C]lactate to $^{14}CO_2$ in infant rat brain slices reaches a maximum at a concentration of approx. 2 mM (Fig. 3). The fact that increase of the acetoacetate concentration leads to no further inhibition

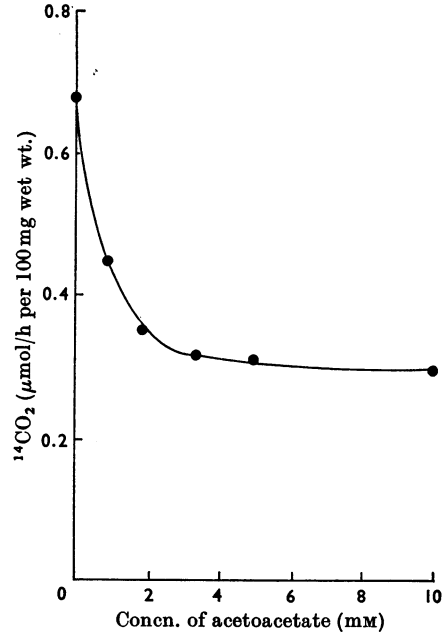


Fig. 3. Effect of sodium acetoacetate at different concentrations on the rate of $^{14}CO_2$ formation from 10 mM-DL-[1-^{14}C]lactate with infant rat brain-cortex slices incubated in O_2 at 37°C in the absence of added glucose.

suggests that acetoacetate is not itself the responsible inhibitory agent, but is in accordance with our earlier conclusion that acetyl-CoA, derived from acetoacetate, is inhibitory. Experiments to throw further light on this phenomenon were carried out with [1-^{14}C]pyruvate.

Kinetics of inhibition of pyruvate oxidation by

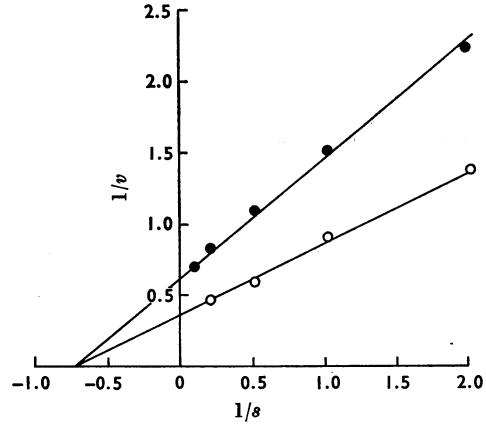


Fig. 4. Effect of sodium acetoacetate (5mM) on the rate of $^{14}CO_2$ formation from sodium [1-^{14}C]pyruvate at different concentrations with infant rat brain-cortex slices incubated in O_2 at 37°C in the absence of glucose. Values are given on double-reciprocal plots. v, $^{14}CO_2$ evolved (μmol/h per 100mg wet wt.): s, concentration (mM) of sodium [1-^{14}C]pyruvate. ●, 5mM-Sodium acetoacetate present; ○, sodium acetoacetate absent.

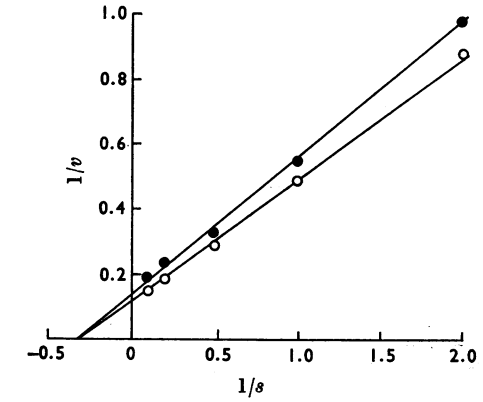


Fig. 5. Effect of sodium acetoacetate (5mM) on the rate of $^{14}CO_2$ formation from sodium [1-^{14}C]pyruvate at different concentrations with adult rat brain-cortex slices incubated in O_2 at 37°C in the absence of glucose. Values are given on double-reciprocal plots. v, $^{14}CO_2$ evolved (μmol/h per 100mg wet wt.); s, concentration (mM) of sodium [1-^{14}C]pyruvate. ●, 5mM-Sodium acetoacetate present; ○, sodium acetoacetate absent.

acetoacetate. Results recorded in Figs. 4 and 5 show that the inhibition of the rate of $^{14}CO_2$ formation from [1-^{14}C]pyruvate in infant or adult rat brain slices by acetoacetate (5mM) is non-competitive, the results being given in the form of double-reciprocal plots.

This result indicates that the inhibition of pyruvate oxidation is not due to competition between pyruvate and acetyl-CoA for the pyruvate-oxidizing system, but is more probably due to that between acetyl-CoA and CoA, as suggested by Garland & Randle (1964) for pyruvate dehydrogenase derived from heart.

Effects of 2,4-dinitrophenol on the oxidative utilization of acetoacetate by brain. Addition of 0.1mM-2,4-dinitrophenol suppresses the rate of formation of $^{14}CO_2$ from [3-^{14}C]acetoacetate with adult rat brain-cortex slices, the effect being greater in the absence of glucose than in its presence (Table 6). With glucose present the inhibition does not exceed 40%. Moreover it is clear that in the presence of 0.1mM-2,4-dinitrophenol glucose effects a considerable acceleration of the process of oxidative breakdown of acetoacetate. Since concentrations of 2,4-dinitrophenol such as we have used bring about marked falls in the concentration of ATP in brain slices (Abadom & Scholefield, 1962*a*; Gonda & Quastel, 1963), presumably by uncoupling cerebral oxidative phosphorylation (Case & McIlwain, 1951), it is unlikely that the activating effect of glucose on oxidative breakdown of acetoacetate is due solely to an increased cell concentration of ATP. It seems more likely that acetoacetate oxidation depends, at least partly, on the supply of succinyl-CoA provided by the citric acid cycle, through the operation of 3-oxo acid CoA-transferase. However, the fact that 2,4-dinitrophenol inhibits the oxidative breakdown of acetoacetate in brain slices significantly in the absence of glucose suggests the participation of ATP in the process and therefore the possible involvement of an acetoacetate kinase, which in adult brain slices may be of greater quantitative importance in the absence of glucose than in its presence. Similar considerations apply to the oxidation of [3-^{14}C]acetoacetate in the presence of infant rat brain-cortex slices.

Effects of D-*glutamate on oxidative utilization of acetoacetate.* To throw further light on the nature of the processes responsible for cerebral oxidation of acetoacetate, the effects of the addition of D-glutamate were investigated. This amino acid is considered not to be metabolized in brain slices (Tsukada, Nagata, Hirano & Matsutani, 1963; Weil-Malherbe, 1936). However, it affects brain metabolism (Krebs, 1935; Weil-Malherbe, 1938; Waelsch, 1955; Weil-Malherbe & Green, 1955; Caughey, Smiley & Hellerman, 1957), undergoes accumulation in brain against a concentration gradient (Takagaki, Hirano & Nagata, 1959; Tsukada *et al.* 1963) and promotes influx of Na^+ and K^+ into nervous tissue (Ames, Tsukada & Nesbett, 1967). It is as effective as L-glutamate in lowering the ATP concentration of brain-cortex slices when these are incubated aerobically in the presence of glucose (Abadom & Scholefield, 1962*b*).

Table 6. *Effects of* 2,4-*dinitrophenol* (0.1 mM) *and of* D-*glutamate* (5 mM) *on the oxidation of* [3-^{14}C]*acetoacetate* (5 mM) *in adult and infant rat brain-cortex slices*

Rat brain-cortex slices were incubated in the Krebs–Ringer phosphate medium containing 5 mM-[3-^{14}C]acetoacetate either alone or with 0.1 mM-2,4-dinitrophenol or 5 mM-D-glutamate. Full experimental details are given in the text and in Table 1. Values of the rates of O_2 uptake are expressed as μmol/h per 100 mg wet wt. and those of $^{14}CO_2$ formation are expressed as μg-atoms of ^{14}C incorporated in $^{14}CO_2$/h per 100 mg wet wt.

Substances added to incubation medium	Adult				Infant			
	Glucose absent		5 mM-Glucose		Glucose absent		5 mM-Glucose	
	O_2 uptake	$^{14}CO_2$ formed	O_2 uptake	$^{14}CO_2$ formed	O_2 uptake	$^{14}CO_2$ formed	O_2 uptake	$^{14}CO_2$ formed
[3-^{14}C]Acetoacetate	9.2±0.4	0.46±0.01	12.8±0.2	0.90±0.05	5.8±0.1	0.44±0.02	6.0±0.2	0.55±0.02
[3-^{14}C]Acetoacetate+2,4-dinitrophenol	5.2±0.1	0.23±0.01	13.8±0.1	0.61±0.04	3.5±0.1	0.17±0.01	5.5±0.1	0.33±0.01
[3-^{14}C]Acetoacetate+D-glutamate	6.5±0.4	0.28±0.01	12.2±0.1	0.50±0.04	4.2±0.2	0.24±0.01	5.5±0.1	0.32±0.03

The results in Table 6 show that the rates of oxygen consumption by both infant and adult rat brain-cortex slices in the absence of glucose are diminished in the presence of D-glutamate, but those in the presence of glucose are but little affected by this amino acid.

The presence of 5 mM-D-glutamate lowers the rate of $^{14}CO_2$ formation from [3-^{14}C]acetoacetate with both infant and adult rat brain slices (Table 6), the fall (in the presence of glucose) being considerably larger than the fall in the rate of respiration. As D-glutamate is not oxidized in rat brain slices the fall in the rate of $^{14}CO_2$ formation cannot be due to isotopic dilution, but may be due to the diminished brain-cell concentration of ATP, indicating the possible participation of ATP in acetoacetate activation and utilization.

Formation of labelled amino acids from [2-^{14}C]*glucose and* [3-^{14}C]*acetoacetate in infant and adult rat brain-cortex slices*

Labelled glucose is converted into labelled amino acids in infant rat brain-cortex slices as it is in adult rat brain-cortex slices, but the total yield of ^{14}C in amino acids formed from [2-^{14}C]glucose in infant rat brain is less than half that in adult brain on the basis of wet weight (Table 7). Notable differences are the lowered yields of labelled glutamate, glutamine and γ-aminobutyrate, but the yields of labelled alanine and aspartate in infant brain are not diminished to the same extent. It is evident that glutamine synthetase and glutamate decarboxylase are present in immature brain (Sperry, 1962). The lower rate of turnover of the citric acid cycle in the infant brain may account for the lessened yield of glutamate.

Labelled amino acids are formed from [3-^{14}C]-acetoacetate in both adult and infant rat brain-cortex slices. The amounts of ^{14}C incorporated into amino acids are smaller than those found with [2-^{14}C]glucose in the adult rat brain, but are higher than those found with [2-^{14}C]glucose in the infant rat brain. The yields of ^{14}C in amino acids, mostly in the form of glutamate and aspartate, from [3-^{14}C]acetoacetate are a little higher (<20%) in infant than in adult rat brain cortex in terms of wet wt. of tissue. This result accords with our earlier conclusion that the rate of operation of the citric acid cycle in adult rat brain in the absence of glucose is about the same as that in infant rat brain (either with or without glucose).

The addition of glucose to [3-^{14}C]acetoacetate raises the yields of ^{14}C in amino acids to about the same values in both infant and adult rat brain slices, the increases being due to enhanced rates of formation of glutamate, with some diminution of those of aspartate.

The addition of unlabelled acetoacetate to [2-^{14}C]glucose leads to marked diminution in the rates of ^{14}C incorporation into amino acids in both adult and infant rat brain slices (Table 6). The diminution by acetoacetate in the presence of [2-^{14}C]glucose is 32% with adult brain and 55% with infant brain. These values are about the same as the percentage inhibitions of the rates of $^{14}CO_2$ evolution found with adult and infant rat brain slices. The direct inhibition of cerebral pyruvate oxidation by acetoacetate probably accounts for the observation (Table 7) that the yield of ^{14}C incorporated into alanine from labelled glucose in brain slices is less affected by acetoacetate than that into glutamate.

Acetoacetate uptake by rat brain-cortex slices

Measurements of the acid-soluble radioactivities accumulated in rat brain-cortex slices (adult or infant), in the presence of [3-^{14}C]acetoacetate, show that the ratio of the concentrations of ^{14}C in the tissue water and in the incubation medium is approx. 1 in the presence of 0.1 mM-2,4-dinitrophenol or of 5 mM-D-glutamate. There is no indication of a diminished rate of diffusion of aceto-

Table 7. *Formation of labelled amino acids from* [2-^{14}C]*glucose and* [3-^{14}C]*acetoacetate in adult and infant rat brain-cortex slices* in vitro

Rat brain-cortex slices were incubated in the Krebs–Ringer phosphate medium containing labelled glucose or labelled acetoacetate or mixtures of these with unlabelled substrates in O_2 at 37°C for 1h. The radioactivity of the amino acids formed was measured as described in the text. Other details are given in Table 1. The values for the ^{14}C-labelled amino acids refer to those found in the tissue at the end of the experiment. Results are means of four independent observations, the S.D. not exceeding ±7%. Values are given as μg-atoms of ^{14}C incorporated into each amino acid.

Substrates (5mM)	Adult						Infant					
	Alanine	Glutamate	Glutamine	γ-Aminobutyrate	Aspartate	Total amino acids	Alanine	Glutamate	Glutamine	γ-Aminobutyrate	Aspartate	Total amino acids
[2-^{14}C]Glucose	0.018	0.382	0.072	0.042	0.054	0.568	0.020	0.109	0.018	0.012	0.047	0.206
[2-^{14}C]Glucose+acetoacetate	0.016	0.202	0.048	0.044	0.055	0.365	0.032	0.015	0.015	0.005	0.023	0.090
[3-^{14}C]Acetoacetate	0.002	0.110	0.004	0.012	0.106	0.234	0.002	0.184	0.010	0.013	0.069	0.278
[3-^{14}C]Acetoacetate+glucose	0.002	0.314	0.050	0.026	0.026	0.418	0.003	0.327	0.018	0.011	0.039	0.398

acetate into brain slices in the presence of the metabolic inhibitors.

Relative effects of glucose and acetoacetate in promoting biosynthesis of acetylcholine in rat brain-cortex slices in the presence of eserine

It is known that acetoacetate cannot effectively replace glucose for the synthesis of acetylcholine in rat brain preparations incubated aerobically in a physiological saline medium (Mann, Tennenbaum & Quastel, 1938).

Experiments were carried out to compare the effects of [2-^{14}C]glucose and [3-^{14}C]acetoacetate in bringing about synthesis of acetylcholine in rat brain slices. Each of these molecules will give rise, on breakdown in the brain, to 1 molecule of [1-^{14}C]-acetyl-CoA and one molecule of unlabelled acetyl-CoA. As shown by the results in Table 8, glucose is more effective than acetoacetate for the synthesis of labelled acetylcholine in adult rat brain-cortex slices incubated aerobically at 37°C. Glucose and acetoacetate are equally effective with infant rat brain slices. When similar experiments were carried out in the absence of eserine, values of labelled acetylcholine formed were approx. 70% of the values obtained when eserine was present in the incubation media, indicating that the tissue acetylcholine was mainly in the form of bound acetylcholine.

Control experiments carried out with brain slices incubated for 1h at 0°C instead of 37°C showed the formation of apparently small quantities of acetylcholine, containing approx. 7ng-atoms of ^{14}C incorporated into the acetylcholine/g wet wt., with both the labelled glucose and the labelled acetoacetate. It is, however, likely that much of this radioactivity is due to some residual contamination still present on the washed acetylcholine chloroaurate precipitate.

Addition of glucose accelerates the rate of synthesis of labelled acetylcholine from labelled acetoacetate in adult rat brain-cortex slices (Table 8), but has relatively little effect on that taking place in infant rat brain. The action of glucose is probably greater than that shown by the results in Table 8, because the presence of unlabelled glucose gives rise to unlabelled acetyl-CoA and therefore to isotopic dilution of the labelled acetylcholine.

Table 8. *Rates of formation of* [^{14}C]*acetylcholine in adult and infant rat brain-cortex slices incubated in the presence of eserine*

Rat brain-cortex slices were incubated in the Krebs–Ringer phosphate medium, containing labelled glucose (0.33μCi/ml), or labelled acetoacetate (0.33μCi/ml), or a mixture of labelled acetoacetate and unlabelled glucose, in the presence of eserine (0.5mM) in O_2 at 37°C for 1h. Labelled acetylcholine was assayed as described in the text. Other experimental details are given in Table 1. Values of labelled acetylcholine formed are expressed as ng-atoms of ^{14}C incorporated into acetylcholine/h per g wet wt.

Substrate (5mM)	Adult	Infant
[2-^{14}C]Glucose	43.8+2.5	28.5±1.5
[3-^{14}C]Acetoacetate	16.6±3.9	29.0±2.9
[3-^{14}C]Acetoacetate+glucose	23.5±1.7	31.4±3.7

The results demonstrate that acetoacetate cannot effectively replace glucose as a precursor of acetylcholine in adult rat brain slices. Nevertheless its activity in this respect approaches that of glucose in infant rat brain slices. Moreover the formation of labelled acetylcholine from [3-^{14}C]acetoacetate proceeds, in the absence of glucose, at a greater rate in infant brain slices than in adult rat brain slices.

DISCUSSION

The addition of glucose accelerates the oxidative utilization of acetoacetate to a much larger extent in adult rat brain slices than in infant rat brain

slices, a result to be expected if acetoacetate oxidation depends on the operation of the citric acid cycle, which proceeds more vigorously in adult brain than in infant brain.

That acetoacetate oxidation in both infant and adult brain tissue depends on the operation of the citric acid cycle is shown by the inhibitory effects of malonate. This substance, which was shown to block oxidative breakdown of acetoacetate in kidney cortex (Quastel & Wheatley, 1935), inhibits oxidative utilization of acetoacetate in both adult and infant brain. It also abolishes the accelerative effect of glucose on the rate of $^{14}CO_2$ evolution from [3-^{14}C]acetoacetate with adult brain, lowering this rate to that found in the absence of glucose. These results indicate that the rate of cerebral oxidation of acetoacetate depends on its conversion into acetyl-CoA, which is then utilized by the citric acid cycle operating in both the adult and infant brain. This conclusion is supported by the fact that the rate of evolution of $^{14}CO_2$ from either [U-^{14}C]-glucose or [2-^{14}C]glucose, in both infant and adult brain, is suppressed by the addition of acetoacetate, as would be expected from the pooling of labelled acetyl-CoA derived from labelled glucose with unlabelled acetyl-CoA derived from the acetoacetate.

The decreased rate of utilization of glucose in infant brain compared with that in adult brain (Tables 1 and 5) is doubtless partly responsible for the lowered rate of operation of the citric acid cycle in infant brain and for the fact that the addition of glucose has so little effect on the utilization of acetoacetate in infant brain. The diminished succinic dehydrogenase activity of infant brain, compared with that of adult brain (Potter *et al.* 1945), is presumably also a factor contributing to the lowered rate of operation of the citric acid cycle in infant brain.

Experiments carried out with [1-^{14}C]pyruvate show that inhibition of its oxidation to $^{14}CO_2$ by acetoacetate does take place. The inhibition is small in adult brain in the absence of glucose but marked (33%) in the presence of glucose. This result would be expected if the actual inhibitory agent is acetoacetyl-CoA or acetyl-CoA derived from acetoacetate. Since thiolase exists in brain and the equilibrium position of the thiolase reaction is highly in favour of the formation of acetyl-CoA (Goldman, 1954), acetyl-CoA is more likely to be the inhibitory agent, a conclusion supported by the fact that acetyl-CoA is known to inhibit pyruvate oxidation in the heart (Garland & Randle, 1964; Davis & Quastel, 1964). It is unlikely that acetoacetate itself inhibits pyruvate oxidation by competition of both molecules for a limited supply of CoA, for it would then be expected that increase of acetoacetate concentration would bring about a corresponding increase of inhibition. The results show, however, that the inhibition of oxidation of 5mM-pyruvate is at a maximum with 2mM-acetoacetate. Moreover such an explanation would not account for the fact that the presence of glucose increases the inhibitory effect of acetoacetate.

Studies of the kinetics of the inhibition indicate a non-competitive inhibition of oxidation of [1-^{14}C]-pyruvate to $^{14}CO_2$ by acetoacetate, and this would be expected if the inhibition of pyruvate oxidation takes place by competition between acetyl-CoA and CoA for the pyruvate-oxidation system, as seems to occur with muscle (Garland & Randle, 1964).

Inhibition of $^{14}CO_2$ evolution from [1-^{14}C]lactate by acetoacetate takes place in a manner similar to that for [1-^{14}C]pyruvate.

The suppression of oxidative pyruvate utilization by acetoacetate probably accounts for the fact that the rate of aerobic lactate formation from glucose is accelerated by acetoacetate in both infant and adult rat brain slices (Table 5), without apparent change in glucose utilization. Rolleston & Newsholme (1967) have noted that 5mM-β-hydroxybutyrate increases the rate of lactate formation from glucose by brain-cortex slices derived from guinea pigs starved for 48h, without affecting glucose utilization. A report by Mourek (1968) indicates that in brain slices of 5-day-old rats the presence of 3.2mM-sodium acetoacetate increases the rate of lactate formation but diminishes it in the brains of adult rats. We have not in our work observed any depression of cerebral aerobic glycolysis in adult rats by acetoacetate. As observed above, glucose utilization (i.e. glucose broken down by a combination of oxidative breakdown and lactate formation) is not affected, within the experimental error, by the addition of acetoacetate. It is therefore evident that any suppression of glucose oxidation is balanced by increased lactate formation. Nor is the rate of oxygen consumption of the brain-cortex slices (adult or infant) significantly affected by the presence of acetoacetate. The diminished rate of oxygen consumption due to diminished oxidative glucose breakdown is obviously compensated by oxidative acetoacetate utilization.

We have found that the ATP concentration of the brain cell obtained in the presence of glucose is not significantly affected by the presence of acetoacetate. For example, values of 162 and 165nmol of ATP/100mg wet wt. of adult brain are found in the presence of 5mM-glucose and a mixture of 5mM-glucose and 5mM-acetoacetate respectively. Any loss of ATP due to diminished oxidation of glucose is compensated for by a rise due to acetoacetate oxidation. The fact that acetoacetate exerts a notable inhibition (45%) of oxidation of [1-^{14}C]-pyruvate in infant brain in the absence of glucose,

this inhibition being increased only to 53% in the presence of glucose, indicates that a considerable conversion of acetoacetate into acetyl-CoA takes place in infant brain in the absence of glucose. This helps to explain the fact that oxidative utilization of acetoacetate in infant rat brain is as great as that in the adult rat brain in the absence of glucose.

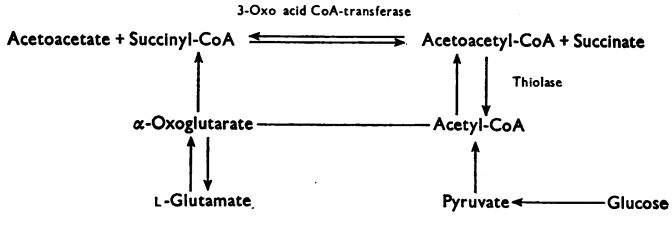
Some of the results recorded above are in qualitative agreement with those of Openshaw & Bortz (1968), who incubated rat brain minces for 6h in oxygen at 37°C in the presence of labelled and unlabelled glucose or acetoacetate, or of mixtures of these substances. They also found that the presence of acetoacetate diminishes the rate of glucose oxidation, as judged from the rate of $^{14}CO_2$ evolution from [U-^{14}C]glucose, and that it increases the rate of lactate formation from glucose. However, as is well known, metabolic rates (e.g. respiratory rate, rate of glucose utilization) differ so greatly in a minced brain, depending on the experimental conditions, from those in brain slices (Quastel, 1957, 1961; Elliott & Wolfe, 1962; McIlwain, 1959) that it is difficult to assess the quantitative significance of glucose–acetoacetate interrelations found in minced brain tissue incubated over a period of 6h.

The fact that acetoacetate may increase the rate of lactate formation from glucose has been demonstrated in experiments with perfused heart, where there is considerable inhibition of glucose oxidation (Hall, 1961; Williamson & Krebs, 1961).

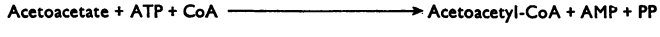
The conclusion that the citric acid cycle operates at a lower rate in infant rat brain slices than in adult rat brain slices in the presence of glucose, due partly to lowered utilization of the glucose, is supported by observations on the rates of formation of amino acids from glucose. For example, the yield of labelled glutamate from 5mM-[2-^{14}C]glucose is smaller with infant rat brain slices than with adult rat brain slices (Table 7). Moreover the results of two experiments carried out with 5mM-[2-^{14}C]pyruvate show that the yield of labelled glutamate from this substance corresponds to the incorporation of 1.11 and 0.237 μg-atoms of ^{14}C/h per 100mg wet wt. with adult and infant rat brain slices respectively under the experimental conditions described in Table 7.

The possibility that the lower yield of labelled glutamate from labelled glucose in infant rat brain is due to diminished activity of glutamate transferase is unlikely, because the yields of labelled glutamate from labelled acetoacetate in the presence of glucose are about the same with both infant and adult rat brain (Table 7).

In view of the diminished metabolism of glucose and pyruvate taking place in infant brain slices compared with that in adult brain slices, and the diminished operation of the citric acid cycle in the infant brain, the problem arises of how a marked conversion of acetoacetate into acetyl-CoA takes place in infant brain. At present little is known of the enzymes concerned with cerebral oxidation of acetoacetate, but the following possibilities (see Schemes 1 and 2) may be considered: (*a*) conversion of acetoacetate into acetoacetyl-CoA may take place by the initial operation of an acetoacetate kinase, a reaction that will depend on the cell concentration of ATP, followed by the activity of thiolase; (*b*) conversion of acetoacetate into acetoacetyl-CoA may occur by the activity of 3-oxo acid CoA-transferase with succinyl-CoA (Stern, Coon & Campillo, 1953) derived from the oxidation in the brain cell of L-glutamate. This amino acid is present in as large a concentration in infant brain as in adult brain (Waelsche, 1955), and glutamate dehydrogenase exists in infant brain at about one-third the activity of that in adult rat brain (Kuhlman & Lowry, 1956). It is also known that the permeability of infant brain cells to L-glutamate is greater than that of adult brain cells (Himwich & Himwich, 1955).



Scheme 1.



Scheme 2.

It does not seem likely that activity of acetoacetate-CoA kinase is the only reaction, preliminary to that of thiolase, operating in the oxidative breakdown of acetoacetate in infant brain. In the absence of glucose the ATP concentration is low (51nmol/100mg wet wt.) and yet oxidation of acetoacetate proceeds almost as vigorously as that in the presence of glucose, where the ATP concentration has risen to 174nmol/100mg wet wt. (Itoh & Quastel, 1969). Moreover the presence of 0.1mM-2,4-dinitrophenol does not exhibit (Table 6) as large an inhibitory effect on the rate of $^{14}CO_2$ formation from [3-^{14}C]acetoacetate in brain slices as that (90% inhibition) taking place in the formation of $^{14}CO_2$ from [1-^{14}C]acetate (Gonda & Quastel, 1966), a reaction involving the activity of acetate–CoA kinase (Stern & Ochao, 1951).

It seems that acetoacetate conversion into acetoacetyl-CoA by reaction with succinyl-CoA, which by analogy with other mammalian tissues is likely to occur in brain, plays a role in infant brain at least as important as that of acetoacetate-CoA kinase. Further investigations are needed to throw light on the activities of the cerebral enzymes involved in acetoacetate and activation oxidation and on their relative quantitative significance in brain metabolism.

Labelled acetoacetate gives rise in both infant and adult rat brain slices to labelled amino acids (chiefly glutamate and aspartate), the total yield being at least as great in infant rat brain slices as that in adult rat brain slices. It is not, however, as effective as glucose for the formation of amino acids in adult rat brain slices but it is at least as effective as glucose in infant rat brain slices. A similar conclusion applies to the results obtained on the rates of formation of acetylcholine.

Whether acetoacetate can act as an efficient source of acetyl-CoA in the living animal brain will depend on the speed with which it will cross the blood–brain barrier. It is known that transfer may take place in the rabbit and in man, though the speed of transfer is not known. For example, slow intravenous injection of acetoacetate into rabbits produces coma even under conditions where there is no acidosis (Schneider & Droller, 1938) and studies on three obese patients indicate that β-hydroxybutyrate and acetoacetate are taken up by the brain and can apparently replace glucose as a primary fuel in the brain during starvation (Owen *et al.* 1967). Preliminary experiments that we have carried out show that acetoacetate can pass from blood to brain in the rat but that the speed of transfer is considerably smaller than that of an equivalent concentration of glucose.

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