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The Estimation of Rates of Utilization of Glucose and Ketone Bodies in the Brain of the Suckling Rat Using Compartmental Analysis of Isotopic Data

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The brains of 18-day-old rats utilize glucose and ketone bodies. The rates of acetyl-CoA formation from these substrates and of glycolysis were determined *in vivo* from the labelling of intermediary metabolites after intraperitoneal injection of D-[2-^{14}C]glucose, L(+)-[3-^{14}C]- and L(+)-[U-^{14}C]-lactate and D(−)-3-hydroxy[^{14}C]butyrate. Compartmental analysis was used in calculating rates to allow for the rapid exchange of blood and brain lactate, the presence in brain of at least two pools each of glucose and lactate, and the incomplete equilibration of oxaloacetate with aspartate and of 2-oxoglutarate with glutamate. Results were as follows. 1. Glucose and ketone bodies labelled identical pools of tricarboxylate-cycle metabolites, and were in every way alternative substrates. 2. The combined rate of oxidation of acetyl-CoA derived from pyruvate (and hence glucose) and ketone bodies was 1.05 μmol/min per g. 3. Ketone bodies contributed 0.11–0.53 μmol/min per g in proportion to their concentration in blood, with a mean rate of 0.30 μmol/min per g at 1.24mM. 4. Pyruvate and ketone bodies were converted into lipid at 0.018 and 0.008 μmol/min per g respectively. 5. Glycolysis, at 0.48 μmol/min per g, was more rapid in most rats than pyruvate utilization by oxidation and lipid synthesis, resulting in a net output of lactate from brain to blood. 6. Rates of formation of brain glutamate, glutamine and aspartate were also measured. Further information on the derivation of the models has been deposited as Supplementary Publication SUP 50034 (18 pages) at the British Library, Lending Division (formerly the National Lending Library for Science and Technology), Boston Spa, Yorks. LS23 7QB, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1973) **131**, 5.

Moore *et al.* (1971) studied glucose transport and utilization in the brains of rats of different ages and concluded that the brain of the suckling rat used other substrates besides glucose. Hawkins *et al.* (1971) showed by arterio-venous difference measurements across the brain that the other substrates were predominantly the ketone bodies, acetoacetate and 3-hydroxybutyrate. They determined relative oxidation rates of glucose and the ketone bodies, but absolute rates could not be determined because the rate of blood flow in the brain was not known.

We decided to determine the rates of utilization of these substrates by using a method that does not require a knowledge of blood flow, but is based on the retention of label in brain after intraperitoneal injection of substrates labelled with ^{14}C. The method was applied to conscious rats at an age, 18 days, when all three substrates were known to be utilized by the brain.

Earlier studies had shown that ^{14}C entering the brain either as glucose (Cremer, 1964; Gaitonde, 1965) or as ketone bodies (Cremer, 1971) became incorporated via the tricarboxylate cycle into amino acids that sequestered much of the label. Initially therefore it seemed feasible to determine the rates from specific radioactivity–time curves for substrates in blood and the accumulation of label in brain without more than, at most, a one-pool model to correct for loss of label as $^{14}CO_2$. Two such methods gave accurate values for ketone bodies but not for glucose.

The main reason for the inapplicability of a simple model to the [^{14}C]glucose data was the rapid exchange of lactate between blood and brain combined with the inaccessibility to label of part of the brain lactate. In addition, it became apparent that only a portion of the brain glucose was rapidly labelled from blood glucose and that injected [^{14}C]glucose labelled blood lactate and vice versa during the course of the experiment (this is referred to below as 'cross-labelling' of blood glucose and lactate).

To allow for these several factors a compartmental analysis was carried out, the purpose of which was the precise determination of glycolysis and oxidation

rates. It gave the rates of oxidation correct to a few per cent, but the rate of glycolysis less precisely. It was not designed to simulate brain biochemistry, for which it was too simple, but it did show that ketone bodies and glucose were alternative substrates that fed identical pools of tricarboxylate-cycle metabolites, and it also revealed some other features of brain metabolism.

A preliminary account of part of this work has appeared elsewhere (Cremer, 1973).

Materials and Methods

Chemicals

Enzymes were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K., except glutaminase which was obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

The resins Dowex AG50 W (X4) and Dowex AG1 (X8) were purchased from Bio-Rad Laboratories Ltd., St. Albans, Herts., U.K., and phenylhydrazine hydrochloride was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Arcton 12 was obtained from I.C.I. Ltd., London W.C.1, U.K.

Radioactive chemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and New England Nuclear Chemicals, Frankfurt, Germany. D-[2-^{14}C]Glucose, L(+)-[3-^{14}C]lactate and L(+)-[U-^{14}C]lactate were used as supplied. D(−)-3-Hydroxy[3-^{14}C]butyrate was either prepared from [3-^{14}C]acetoacetate as described by Cremer (1971) or purchased.

Animals

Rats of the Porton strain were used. Groups of 18-day-old female rats were taken from their mothers and used within 1 h. To maintain their body temperatures they were kept in plastic beakers or small plastic-walled animal cages amongst loose tissue paper to simulate their nests. They did not shiver or feel cold to the touch.

Injections and tissue sampling

For the study of ketone-body utilization each rat received by intraperitoneal injection 1 μCi of D(−)-3-hydroxy[3-^{14}C]butyrate (sp. radioactivity either 13 or 26mCi/mmol) in either 0.1 or 0.2ml of 0.9% (w/v) NaCl. At selected time-intervals after injection rats were decapitated in such a way that the heads fell into liquid N_2 and the blood from the severed neck was collected into beakers containing heparin (approx. 50 units/ml of blood). The frozen brains were homogenized in 30ml of 1M-$HClO_4$, the protein was removed by centrifugation and the acid-soluble fraction brought to pH 8.0 with KOH. The $KClO_4$ precipitate was removed by filtration, the filtrate evaporated to dryness at 35°C and the dried brain extract redissolved in water.

For the study of glucose utilization, each rat received 3.3 μCi of D-[2-^{14}C]glucose (sp. radioactivity 3.1 to 3.7mCi/mmol) in 0.1ml of 0.9% (w/v) NaCl. Rats were either decapitated as described above and blood samples collected or they were dropped into Arcton 12 (Freon 12, dichlorofluoromethane) which was cooled to the temperature of liquid N_2. The frozen brains were removed and homogenized as described above. The latter killing procedure was used to decrease the extent of post-mortem glycolysis in the brain.

In experiments in which the entry of lactate into the brain and its further metabolism were studied rats were given by intraperitoneal injection either 0.8 μCi of L(+)-[3-^{14}C]lactate (sp. radioactivity 1.5mCi/mmol) or 1.56 μCi of L(+)-[U-^{14}C]lactate (sp. radioactivity 29mCi/mmol) in 0.1ml of 0.9% (w/v) NaCl. Rats from the same litters were divided into two groups such that some animals were decapitated for the collection of blood and others were dropped into Arcton 12 to obtain rapidly frozen brain tissue, as described above.

Assay and separation of metabolites

The concentrations of metabolites in the blood and brain samples were determined by standard assay procedures as described previously (Cremer, 1971) except alanine (see below), glutamate and glutamine which were determined as described by Lund (1971) and Lund (1970) respectively.

The separation of metabolites for determination of their specific radioactivities in experiments with 3-hydroxy[3-^{14}C]butyrate was carried out as described previously (Cremer, 1971). Essentially, the acid-soluble brain extracts were separated into a non-amino acid and an amino acid fraction on a Dowex AG50 W resin (H^+ form) and then the amino acids were further separated on a Dowex AG1 resin (acetate form).

In experiments with [^{14}C]glucose or [^{14}C]lactate the order of the procedure outlined above was modified depending on the particular parameters to be measured. For the determination of the specific radioactivity of glucose, lactate and amino acids in the brains of rats frozen in Arcton 12 the acid-soluble material was placed on a column (25cm × 0.9cm) of Dowex AG1 (X8; 200–400 mesh; acetate form) and the metabolites were eluted by gradient elution with acetic acid (Cremer, 1970). The first 40ml which contained glucose and glutamine were dried *in vacuo* and placed on a column (3.0cm × 1cm) of Dowex AG50 W (X4; 200–400 mesh; H^+ form). The washings (30ml of water) from this column contained glucose which

accounted for more than 90% of the radioactivity in this fraction. The amount of glucose was determined by enzymic assay and the radioactivity of glucose was taken as the total radioactivity in this fraction. Glutamine was removed from the column with 20ml of 4M-NH_3 and was converted into glutamate with glutaminase, as described by Lund (1970). The glutamate formed was then separated on a column of Dowex AG1 (X8; acetate form).

During the course of this study difficulties were encountered in obtaining reliable values for the specific radioactivity of brain lactate when assayed and counted for radioactivity after separation on a Dowex AG1 (X8; acetate form) column, owing to the elution from the column of excess of lactate from unknown sources. Since a reliable measure of the specific radioactivity of brain lactate was essential to the present study two alternative procedures were used. In both procedures the acid-soluble material of the brain extract was separated into a non-amino acid and an amino acid fraction on Dowex AG50 resin (H^+ form).

In procedure 1 glucose was separated from a portion of the non-amino acid fraction by using a Dowex AG1 (X8; acetate form) column. On a second portion of the fraction lactate was assayed enzymically (Hohorst, 1963) and the pyruvate formed was precipitated as its phenylhydrazone essentially as described by Freminet *et al.* (1972). The difference in radioactivity before and after the enzymic conversion and precipitation steps was taken as being due to lactate. Precipitation with phenylhydrazone and 'carrier' pyruvate without prior lactate dehydrogenase treatment indicated that radioactivity present as 2-oxo acids was negligible.

In procedure 2 a portion of the non-amino acid fraction was assayed for lactate and a second portion was placed on a column (6ml, 24cm) of Dowex AG1 (X8; acetate form) which was eluted with water (30ml) and a stepwise gradient of acetic acid (25ml of 0.3M, 25ml of 1M and 35ml of 2M). A fraction (3–16ml) of the water wash was taken for the determination of the specific radioactivity of glucose. A fraction (5–24ml) after the start of elution with 2M-acetic acid was taken for the measurement of radioactivity as lactate.

The specific radioactivities of glucose and lactate in blood were determined on neutralized $HClO_4$ extracts either as outlined in procedure 2 for brain samples or, for glucose only, after passage through a deionizing column (Heath & Rose, 1969).

After injection of 3-hydroxy[3-^{14}C]butyrate the total radioactivity in the acid-soluble material of blood was assumed to be due entirely to ketone bodies. A check on a few blood samples showed that 1% or less of the radioactivity was present as amino acids.

The specific radioactivities of acetoacetate and D-3-hydroxybutyrate in the blood after an intraperitoneal injection of D(−)-3-hydroxy[3-^{14}C]butyrate were determined by pooling blood samples from three individual rats killed at either 3 or 6min and separating each ketone body by column chromatography as described by Cremer (1971).

In some brain samples alanine was analysed by using a Technicon AutoAnalyzer (TSM1). For specific radioactivity measurements the column effluent was split equally for reaction with ninhydrin and for counting for radioactivity.

The C-1 label of glutamate isolated from the brain was determined as described by Heath & Phillips (1972).

Total brain lipids were isolated as described by Kanazawa *et al.* (1971) based on the original method of Folch *et al.* (1957).

Scintillation counting

Counting for radioactivity was done in either a model 4000 liquid-scintillation spectrometer (Packard Instrument Co. Inc.) and counting efficiencies were determined by using [^{14}C]toluene as an internal standard, or in a Philips model PW 4510/01 automatic liquid-scintillation analyser (Pye–Unicam, Cambridge, U.K.) where counting efficiencies were determined by an external standard channels ratio.

Calculation of rates

Several procedures were used in response to particular experimental findings and are described in the Results and Estimation of Rates section.

The compounds studied can be classified as primary substrates and products. Primary substrates are those compounds, blood glucose, ketone bodies and lactate, which donated label to intermediary metabolites in brain. Products are the intermediary metabolites which received label, e.g. amino acids. Brain lactate was shown to be a product with respect to glucose, but a primary substrate with respect to the tricarboxylate cycle.

Concentrations of compounds were, with one exception (Fig. 1), constant throughout each experiment so steady-state theory was used. A first-order differential equation for each product pool was therefore set up in the usual way. The equations are derived and listed in the Supplementary Publication (SUP 50034). Integration gave relations between rates of utilization of primary substrates, rates of transport of compounds between product pools and quantities of label (q) in product pools in terms of the values of specific radioactivity (S) for primary substrates. The basic data therefore consisted of S–t curves for primary substrates and q–t curves for products. Optimizing the fit of the integrated equations to the q–t data with rates as variables gave estimates of the rates and their S.E.M. values.

Integration was carried out analytically, not numerically, requiring S–t curves to be expressed as continuous functions, preferably as the sums of exponentials:

$$S = \sum A_i e^{-g_i t} \quad (1)$$

where A_i and g_i are constants. Experimental curves could always be represented very precisely in this way if the experiment was timed from a short interval (δ) after the injection to allow for the passage of label from the peritoneal cavity to blood and also, in the case of brain lactate labelled from [^{14}C]glucose, down the glycolytic chain. S–t data were therefore fitted to equations of the form:

$$S = \sum A_i e^{-g_i(t-\delta)} \quad (2)$$

(timing from the injection), or, in one case, to:

$$S = A(t-\delta)e^{-g(t-\delta)} \quad (3)$$

[Eqn. (3) is the limiting case of eqn. (2) when the latter has two terms and g_1 and g_2 converge]. Optimized fits were obtained, by using A values, g values and sometimes δ values as variables, with standard error bands. The last were required so that in estimating the errors on rates etc. allowance could be made for the errors on the mean values of S.

Integration using eqns. (2) and (3) is the equivalent of using eqn. (1) or the analogue to eqn. (3):

$$S = Ate^{-gt} \quad (4)$$

and treating each value of q in a product at time t as though it had been determined at time $t-\delta$. It follows that any value of q at times less than δ after injection would have been voided of meaning; but in fact no such value was determined. The use of a delay time as an approximation to the beginning of a highly sigmoid curve causes some error. However, an approximation to this part of each S–t curve is necessary in any treatment; the error grows rapidly less with time after δ; and the estimates of rates were not sensitive to early values of q. Overall it appears likely that the errors introduced into estimates of rates were well under 1%.

Optimized fits to data were obtained by the least-mean-squares method described by Deming (1964) using a desk calculator (Hewlett-Packard model 9810A) programmed to handle up to six variables. Since in most experiments the coefficient of deviation (c.d.) from variance within replicates was constant throughout for label in each particular pool, weights (w) were calculated by: n/(c.d. × smoothed mean)^2, where smoothed means were obtained from a preliminary fit. These smoothed means were always close enough to the final values to render further refinement unnecessary. In a few instances other methods of weighting were used. In Figures the S.E.M. data are values of $1/\sqrt{w}$, except where stated otherwise.

Results and Estimation of Rates

Concentrations and labelling of blood and brain glucose after injection of [2-^{14}C]*glucose*

The blood glucose concentration rose slightly but not significantly after injection. The overall mean ± S.D. was 5.88 ± 0.53 (104) mM.

Brain glucose concentrations (μmol/g) rose after injection to an ill-defined maximum (Fig. 1).

Blood glucose specific radioactivity (S_{bg}) rose rapidly through a maximum after a short delay period (Fig. 2) and the results fitted the equation:

$$S_{bg} = (57.14 \pm 8.21)\,[e^{-(0.0249\pm0.0096)(t-0.52\pm0.07)} - e^{-(0.275\pm0.041)(t-0.52\pm0.07)}] \quad (5)$$

Units are 10^{-3} × d.p.m./μmol. That of brain glucose rose rather more slowly, and the maximum was later and less well-defined (Fig. 2).

The data in Figs. 1 and 2 can be brought together by postulating that only part of the brain glucose is in rapid equilibrium with blood glucose and that the

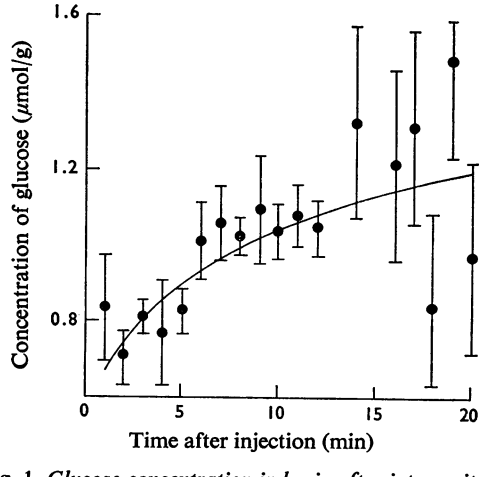


Fig. 1. *Glucose concentration in brain after intraperitoneal injection of* [^{14}C]*glucose*

From replicates the S.D. from 0 to 12 min was 0.14 μmol/g, and thereafter from deviations from a smoothed curve was 0.25 μmol/g (d.f. = 34 and 5, $P < 0.05$ by the F test). S.E.M. values shown were calculated as S.D./$\sqrt{n}$; total number of values = 54. The curve of best fit is shown (Supplementary Publication SUP 50034).

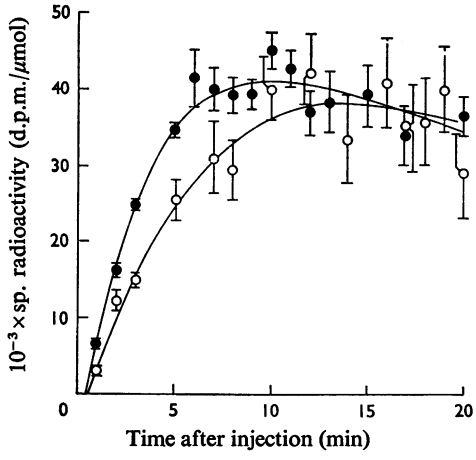


Fig. 2. *Specific radioactivities of glucose in blood* (●) *and brain* (○) *after an intraperitoneal injection of* 3.3 μCi *of* [2-^{14}C]*glucose*

Values were corrected to a body wt. of 45g. S.E.M. values shown were calculated by (mean × coefficient of deviation)/$\sqrt{n}$. Coefficient of deviations were calculated in each set from the variance of replicates. They were 0.10 for blood label and 0.15 for brain label. Total numbers of experimental values were: blood, 49; brain, 29. The best-fit curves are shown (Supplementary Publication SUP 50034).

remainder, which exchanges glucose only slowly with the blood, increases as a result of the injection (perhaps a stress effect) or, perhaps, decreases very rapidly and then returns slowly to normal. Model 1 shows a possible way of formulating these postulates and leads (SUP 50034) to the curves shown in Figs. 1 and 2 for brain glucose. The initial assumptions were as follows.

(*a*) Part of the brain glucose was in perfect equilibrium with blood glucose. The absence of a clear sigmoidal section in the early part of the brain specific radioactivity–time curve shows that equilibration must have been very rapid. Uptake in sheep and rabbit is in fact very fast (Pappenheimer & Setchell, 1973).

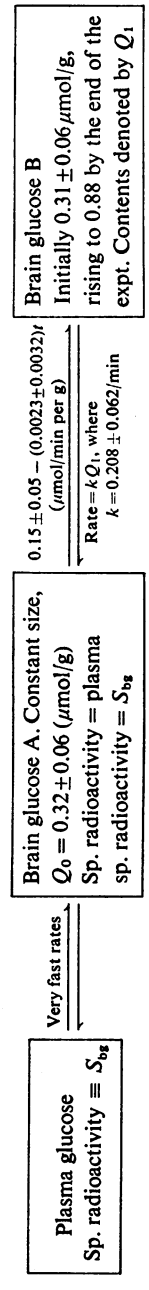
(*b*) There was only one pool which equilibrated slowly, and this had a turnover rate, kQ (Model 1), proportional to its size.

(*c*) This pool received glucose at a rate which varied linearly with time. (In fact, the data did not require a variation in rate.)

Labelling of blood lactate by injected [^{14}C]*lactate*

The results, shown in Fig. 3 for the specific radioactivity of blood lactate (S_{bL}), were best fitted by the eqn:

$$S_{bL} = (27.5 \pm 5.0)(t-0.5)e^{-(0.494\pm0.087)(t-0.5)} \quad (6)$$



Model 1. *Brain glucose and brain glucose specific radioactivity*

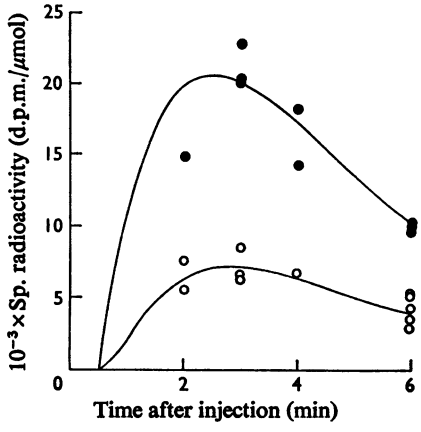


Fig. 3. *Specific radioactivity of lactate in blood* (●) *and brain* (○) *after injection of* 1.56 μCi/*rat of* L(+)-[^{14}C]*lactate*

Results were corrected to a body wt. of 45g. The best-fit curves are shown (see the text and Supplementary Publication SUP 50034).

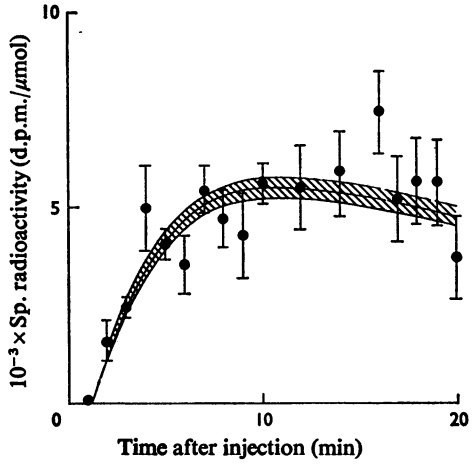


Fig. 4. *Specific radioactivity of brain lactate after injection of* [2-^{14}C]*glucose*

For details see the legend to Fig. 2. The curve of best fit is shown with standard error range (see the Materials and Methods section). Total number of experimental values was 38.

Units are 10^{-3}×d.p.m./μmol. The delay time of 0.5min was assumed by analogy with glucose [see eqn. (5)].

Labelling of brain lactate and the rate of glycolysis

The specific radioactivity of brain lactate after injection of [^{14}C]glucose is shown in Fig. 4. Three experimental values are omitted as they were much higher than those shown. Comparison with the data of Fig. 2 showed that brain glucose as a whole could not have been the precursor of brain lactate because the specific radioactivity of the latter reached half its maximal specific radioactivity in less time than the former. Thus the effective precursor of lactate label after [2-^{14}C]glucose injection was blood glucose, i.e. if the actual precursor was a pool of brain glucose then its specific radioactivity was always very close to that of blood glucose.

By about 7min after injection the specific radioactivity ratio of brain lactate/blood glucose had reached a constant value of about 0.13. The ratio must become constant if brain lactate is a product of blood glucose and turns over very rapidly; but if blood glucose were the only source of lactate, as 1mol of glucose gives 2mol of lactate, the ratio would be close to 0.5. The low value of 0.13 showed that blood glucose was not the major source.

Experiments with [^{14}C]lactate showed that the major source was blood lactate which exchanged rapidly with brain lactate. Fig. 3 shows the specific radioactivities of blood and brain lactate, estimated on different rats (see Materials and Methods section), after injection of [3-^{14}C]lactate. The specific radioactivity ratio of brain lactate/blood lactate was nearly constant at about 0.36.

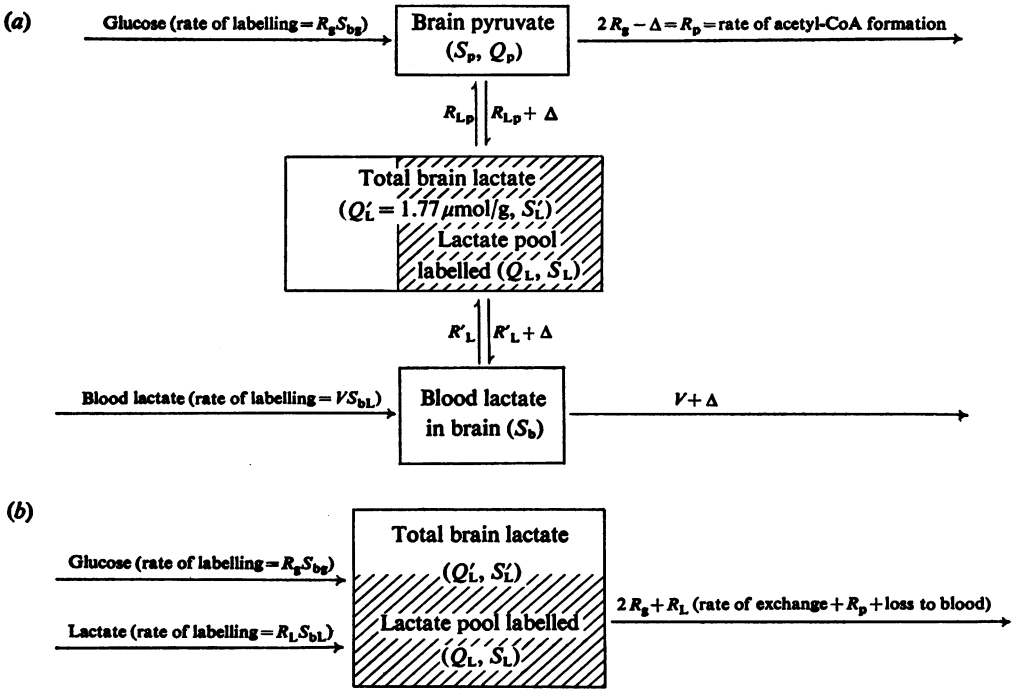
The combined results showed that not all of the brain lactate originated as either blood lactate or blood glucose, the former accounting for 0.36 of the whole, and the latter for 2×0.13 of the whole, i.e. only 0.62 in all.

To obtain more accurate values of these ratios and to estimate the rate of glycolysis a kinetic treatment was developed based on Model 2(*a*). Only a part of the total brain lactate became labelled. This part exchanged label with the lactate which was in the blood in the brain and with the pyruvate pool from which acetyl-CoA was formed. (There may be other pyruvate in the brain, but if so it is not relevant here.) Model 2(*a*) could be simplified to Model 2(*b*) because three conditions were satisfied.

(1) The turnover time of the blood lactate pool in brain was very short, about 3s, in terms of the timescale of the experiment. (2) Pyruvate was in equilibrium with the lactate pool labelled, i.e. $R_{Lp} \gg R_p$. (3) [^{14}C]Glucose and [^{14}C]lactate labelled the same part of the brain lactate pool. Points 2 and 3 are demonstrated later under 'Labelling of amino acids by [^{14}C]-lactate'.

In the symbolism of Model 2, the rate of exchange of lactate between blood and brain, R'_L, is related to the rate of input of lactate into the lactate pool labelled in Model 2(*b*), i.e. R_L, by:

$$R_L = VR'_L/(R'_L + V + \Delta) \quad (7)$$



Model 2. *Models for brain pyruvate and lactate*

Definitions are as follows. Specific radioactivities: blood glucose, S_{bg}; blood lactate, S_{bL}; blood lactate in brain, S_b; total extravascular brain lactate, S'_L; lactate in pool labelled, S_L; brain pyruvate labelled, S_p. Pool sizes: total extravascular brain lactate, Q'_L; pool labelled, Q_L. Rates: glycolysis, R_g; rate of conversion of brain lactate into brain pyruvate, R_{Lp}; rate of pyruvate dehydrogenase reaction, R_p; net rate of production of lactate by brain, Δ; lactate input into brain, V (blood flow × concn.); other rates are exchange rates between pools.

Defining the specific radioactivities of brain lactate (S_L) after the injection of [^{14}C]glucose and [^{14}C]-lactate by (glucose) and (lactate) respectively, and the equilibrium value of S_L(glucose)/S_{bg} by α and of S_L(lactate)/S_{bL} by β, it can be shown (see the Supplementary Publication SUP 50034) that:

$$2\alpha + \beta = 1 \qquad (8)$$

The equilibrium values are those that would be reached eventually during constant infusion of labelled substrate if the system was in a steady state. The analogous experimental ratios: S'_L(glucose)/S_{bg} and S'_L(lactate)/S_{bL} are denoted by α' and β'. Then, as:

$$\alpha/\alpha' = \beta/\beta' = Q'_L/Q_L,$$
$$Q_L/Q'_L = S'_L/S_L = 2\alpha' + \beta' \qquad (9)$$

Thus estimation of α' and β' gives an estimate of Q_L, the labelled portion of the brain lactate pool,

It is now possible to write two equations which include the rate of glycolysis (R_g) (see the Supplementary Publication SUP 50034):

$$dS_L(\text{glucose})/dt = R_g S_{bg}/Q_L - k_L S_L(\text{glucose}) \qquad (10)$$

$$dS_L(\text{lactate})/dt = \beta' R_g S_{bL}/\alpha' Q_L - k_L S_L(\text{lactate}) \qquad (11)$$

where

$$k_L = R_g/\alpha' Q'_L \qquad (12)$$

Integration of eqns. (10) and (11) and substitution of eqn. (9) gave S'_L(glucose) and S'_L(lactate), i.e. the quantities actually determined, in terms of Q'_L, t, R_g, α' and β'. The best-fit curves to the data are shown in Figs. 3 and 4. The fit to S'_L(glucose) required an additional delay of 0.5 min to allow glucose label to reach lactate via the glycolytic chain, i.e. a total delay time of 1.0 min. The parameter values were: $\alpha' = 0.135 \pm 0.006$, $\beta' = 0.353 \pm 0.013$, $R_g = 0.72 \pm 0.45$ μmol/min per g. By eqn. (9) only 0.622 ± 0.018 of the total brain lactate received label.

It can be shown that:

$$R_L/2R_g = \beta'/2\alpha' \qquad (13)$$

where R_L is the rate at which molecules of lactate

entered the brain from blood and $2R_g$ is the rate at which they were formed by glycolysis in brain. From the values of α' and β' above it follows that more brain lactate molecules came from blood than from glycolysis.

The very big estimated error in R_g reflects the error in the difference between the times taken to reach half-maximal specific radioactivity by brain lactate and its precursor, blood glucose. This difference was small (see Figs. 2 and 3), could not be estimated accurately, and errors on it caused highly asymmetrical errors on R_g so that even the mean value is in error. The most probable value and lower limit of R_g could, however, be calculated another way. Calculation showed that the estimate of R_g was almost entirely dependent on the 10 experimental values of S'_L(glucose) at 2 and 3 min after injection. Unless R_g exceeded 0.41 μmol/min per g more than 8 of the 10 were higher than those calculated, i.e. by the binomial theorem $P>0.98$ that $R_g>0.41$ μmol/min per g, which rate is taken as the lower limit. The most probable value, that in which 5 of the 10 were higher than those calculated, was 0.48 μmol/min per g. The upper limit given by this method was greater than 10 μmol/min per g, which is essentially meaningless.

A glycolysis rate of 0.48 μmol/min per g and a delay of 0.5 min for passage of label through glycolytic intermediates is consistent with the latter being composed of a multiplicity of pools totalling about 0.25 μmol/g, which is in fair accordance with the total concentration in adults of about 0.4 μmol/g (Nahorski, 1972; Hawkins *et al.*, 1973).

The preceding treatment of data needed correcting for cross-labelling of blood lactate and glucose (see above). As its effects on the estimates of rates proved to be small no attempt was made to accumulate enough data for an exact treatment. The very small correction for labelling of blood glucose by injected [^{14}C]lactate was based on the finding that at 6 min after injection the specific radioactivity of blood glucose was 5% of that of blood lactate. The larger correction for labelling of blood lactate by injected [^{14}C]-glucose was made from the mean curve shown in Fig. 5. (Equations used are in Supplementary Publication SUP 50034.) For various reasons (probably mainly due to animal husbandry) we think this curve overcorrected, and that the best estimates of rates in this section and later lie between those obtained by using Fig. 5 and those without correction.

When corrected for cross-labelling the following values were obtained, $\alpha' = 0.103 \pm 0.005$, $\beta' = 0.353 \pm 0.016$, the lower limit of $R_g = 0.44$ μmol/min per g and the most probable value of $R_g = 0.56$ μmol/min per g. Thus correction affected the estimates of rates very little.

Ketone bodies in blood after injection of D(−)-3-*hydroxy*[3-^{14}*C*]*butyrate*

The concentrations of acetoacetate+3-hydroxybutyrate in blood were very variable at 1.24± 0.42 μmol/ml (arithmetical mean±S.D., $n = 22$), with an overall range of 0.44–2.15 μmol/ml. Animals of the same litter had comparable concentrations, so that variation was mainly between litters. There was no systematic drift with time after injection. Where determined the ratio [3-hydroxybutyrate]/[aceto-

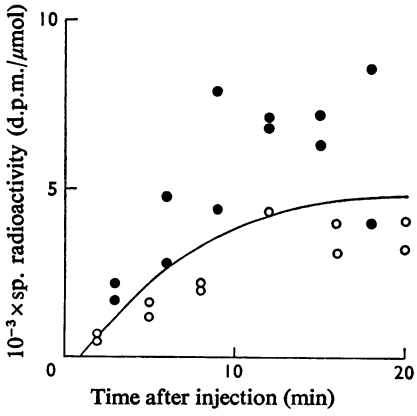


Fig. 5. *Specific radioactivity of lactate in blood after injection of* 3.3 μ*Ci of* [2-^{14}*C*]*glucose/rat*

Results are corrected to a body wt. of 45 g. Two experiments, separated by 2 weeks are denoted separately by ○ and ●. The best-fit curve to all the data is shown.

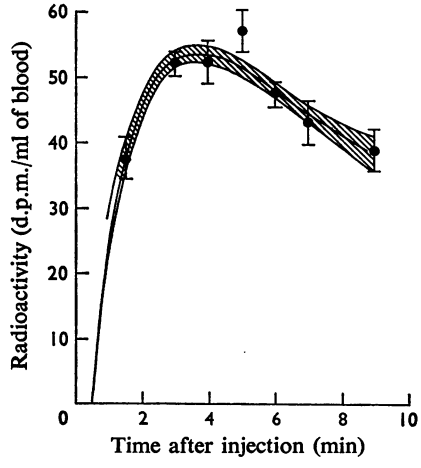


Fig. 6. *Quantity of ketone-body label in blood after injection of* 1 μ*Ci of* D(−)-3-*hydroxy*[3-^{14}*C*]*butyrate/rat*

Results are corrected to a body wt. of 45 g. S.E.M. values were calculated as in the legend to Fig. 2. Total number of values, 18. The best-fit curve is shown with standard error range (see Materials and Methods section).

acetate] was 3.10±0.18 (S.E.M., $n = 7$), and a value of 3.0 has been used throughout.

The quantity of label in ketone bodies in blood (q_k) was very much less variable than the specific radioactivity (F test on variance of replicates, $P<0.01$) as was to be expected if the rate of uptake of label from the peritoneal cavity was independent of the concentration of ketone bodies in the blood and if the rate of disposal of blood ketone bodies in the whole animal was roughly proportional to concentration (see Heath & Barton, 1973, for a discussion of this point), as it is in adults (Barton, 1973). Therefore only the q_k–t curve is shown (Fig. 6). The best-fit equation was:

$$q_k/\text{ml} = (78 \pm 12)\,[e^{-(0.084\pm0.026)(t-0.5)} - e^{-(0.80\pm0.18)(t-0.5)}] \quad (14)$$

and is shown with standard error range in Fig. 6. Units are $10^{-3}\times$d.p.m./ml. The 'delay time' of 0.5min was required to explain the time-course of citrate-cycle labelling.

To determine the mean rate of utilization of ketone bodies a mean S–t curve was obtained by dividing eqn. (14) by the mean concentration of ketone bodies, 1.24μmol/ml. This will give a correct mean rate on three assumptions. (1) The rate of utilization is proportional to concentration, as it was (next section). (2) The specific radioactivity of acetoacetate is a constant fraction of that of 3-hydroxybutyrate throughout the experimental period. This cannot be true immediately after injection, when only 3-hydroxybutyrate is labelled, but in adults a constant ratio is reached very rapidly (Barton, 1973) and in our rats the values at 3 and 6min after injection, 0.63 and 0.58, were very close. Under these conditions errors from the assumption will have been negligible. It is shown in the Appendix that the ratio need not be unity, only constant. (3) The rate constant for utilization of acetoacetate, k_a, must be the same as that of 3-hydroxybutyrate, k_b. In fact Hawkins *et al.* (1971) found $k_a/k_b = 1.85$. It can be shown, however (see the Appendix), that errors from this source can be corrected for by decreasing the mean specific radioactivities of ketone bodies by 6% for calculation of the rates of labelling of products.

Estimation of rates in the tricarboxylate cycle

The reactions considered are shown in Model 3(*a*), which was converted into Model 3(*b*) for the purposes of calculation.

The rates of oxidation of the acetyl-CoA coming from ketone bodies and pyruvate are denoted by R_k and R_p respectively. Their sum, R, is the rate of rotation of the tricarboxylate cycle. The rate of labelling of acetyl-CoA by ketone bodies is $0.5\,R_k S_{bk}$, where S_{bk} was the specific radioactivity of blood ketone bodies, and the factor, 0.5, allows for the formation of 2mol of acetyl-CoA from each mol of ketone body; and of acetyl-CoA by pyruvate is $R_p S_L$, where S_L was the specific radioactivity of the lactate pool labelled and with which pyruvate was in isotopic equilibrium (see under 'Labelling of brain lactate and the rate of glycolysis').

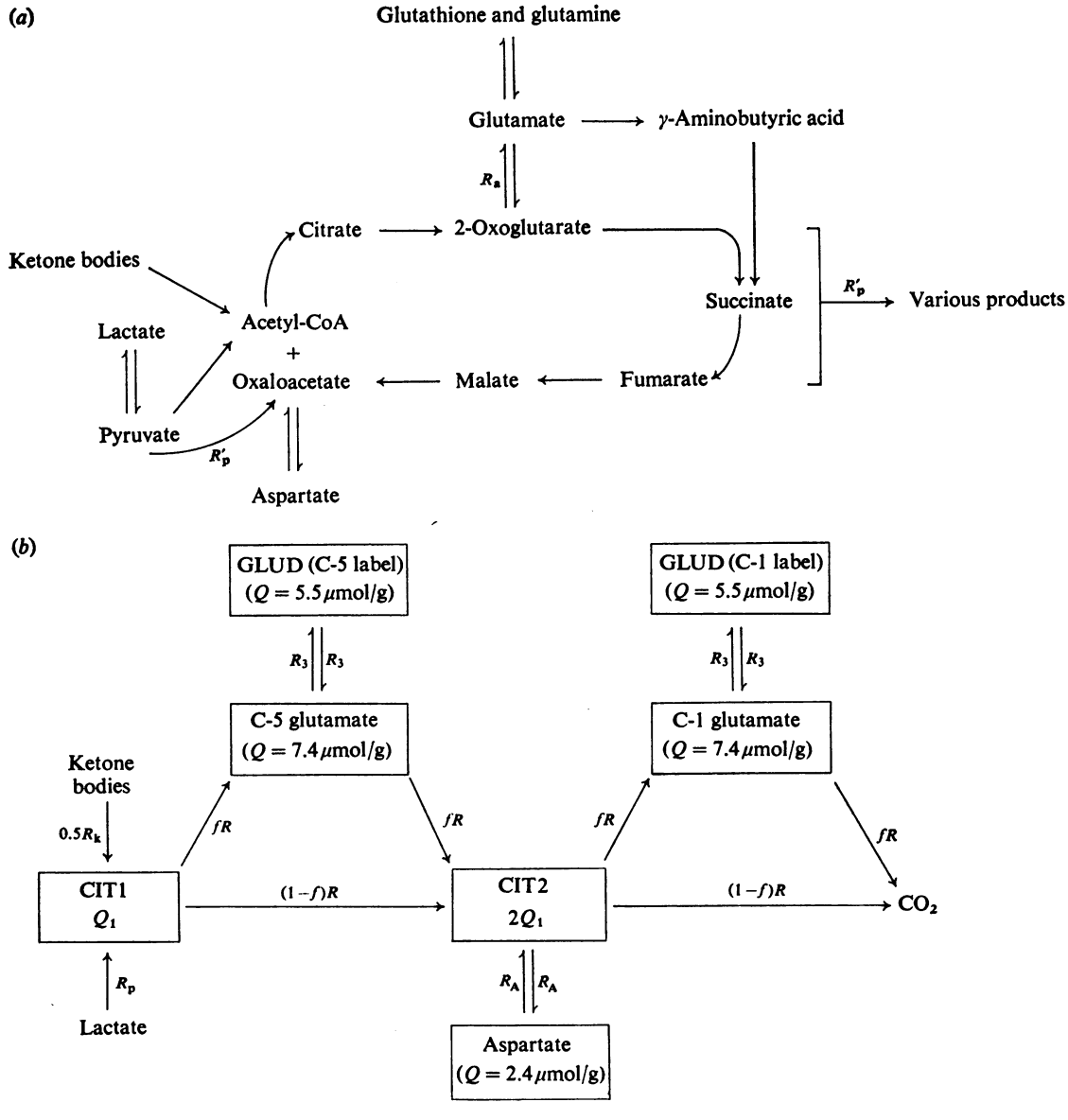
The acetyl-CoA was combined with citrate and the other compounds round the cycle up to and including part of the 2-oxoglutarate to form the pool, CIT1, that labelled glutamate in the C-5 position. Only the fraction, f, of the label was supposed to enter glutamate, the rest bypassing it. If R_a is the rate of amination of 2-oxoglutarate it can be shown that:

$$f = R_a/(R_a + R) \quad (15)$$

Both the label bypassing glutamate and that returning from it entered the second citrate cycle pool, CIT2, composed of the complete carboxylate components of the cycle. This pool labelled glutamate in the C-1 position. Half of the label is lost as $^{14}CO_2$ when citrate is converted into 2-oxoglutarate for the second time, before C-1-glutamate labelling. The pool also labelled aspartate, formed at the rate R_A. The bulking of cycle carboxylates into only two pools was justified by their small size and rapid turnover. In adults CIT2 would be about twice as big as CIT1 (from data given by Nahorski, 1972; Hawkins *et al.*, 1973) and CIT2 was assumed to be exactly twice as big, i.e. of size $2Q_1$.

The derivatives of glutamate, i.e. glutamine, 4-aminobutyrate and glutathione were bulked as a single, GLUD pool exchanging label with glutamate. Although the rate of formation of these compounds, R_3, can be regarded as a single entity, the rate of return is more complex than is shown. Thus 4-aminobutyrate returns label directly to succinate in the cycle and not via glutamate; and the labelling of the constituents of the pool is not necessarily proportional to their individual concentrations. The GLUD pool therefore represented a big approximation.

Carboxylation of pyruvate to oxaloacetate, known to take place in adults (Koeppe & Hahn, 1962), was ignored for reasons given later, i.e. $R'_p = 0$. Model 3 excludes lipid synthesis, which also involves acetyl-CoA formation. Labelling of total brain lipids was determined after [2-^{14}C]glucose and D(−)-3-hydroxy-[3-^{14}C]butyrate injection and gave the mean rates 0.018 and 0.008μmol/min per g for conversion of pyruvate and ketone bodies respectively into lipids. (Data and calculation are in the Supplementary



Model 3. *Chemical reactions (3a) and pool model (3b) for brain metabolism*

Many reverse reactions are omitted from 3(*a*) as they are not relevant to the discussion. The rate R_k is that of acetyl-CoA formation from ketone bodies, which are therefore utilized at a rate 0.5 R_k.

Publication SUP 50034.) These rates, which are relatively very small, should be added to R_p and 0.5 R_k to obtain the total rates of utilization of pyruvate and ketone bodies.

The experimental data were as follows. (1) Labelling from D(−)-3-hydroxy[3-^{14}C]butyrate. Label was determined in glutamate, aspartate, glutamine, total amino acids and total non-amino acids. In each amino acid fraction the quantities of label were more constant at each time and fell on smoother curves than the specific radioactivities, indicating, as shown by Heath & Barton (1973), that the rate of utilization in the citrate cycle was more nearly proportional to ketone-body concentration in blood than it was

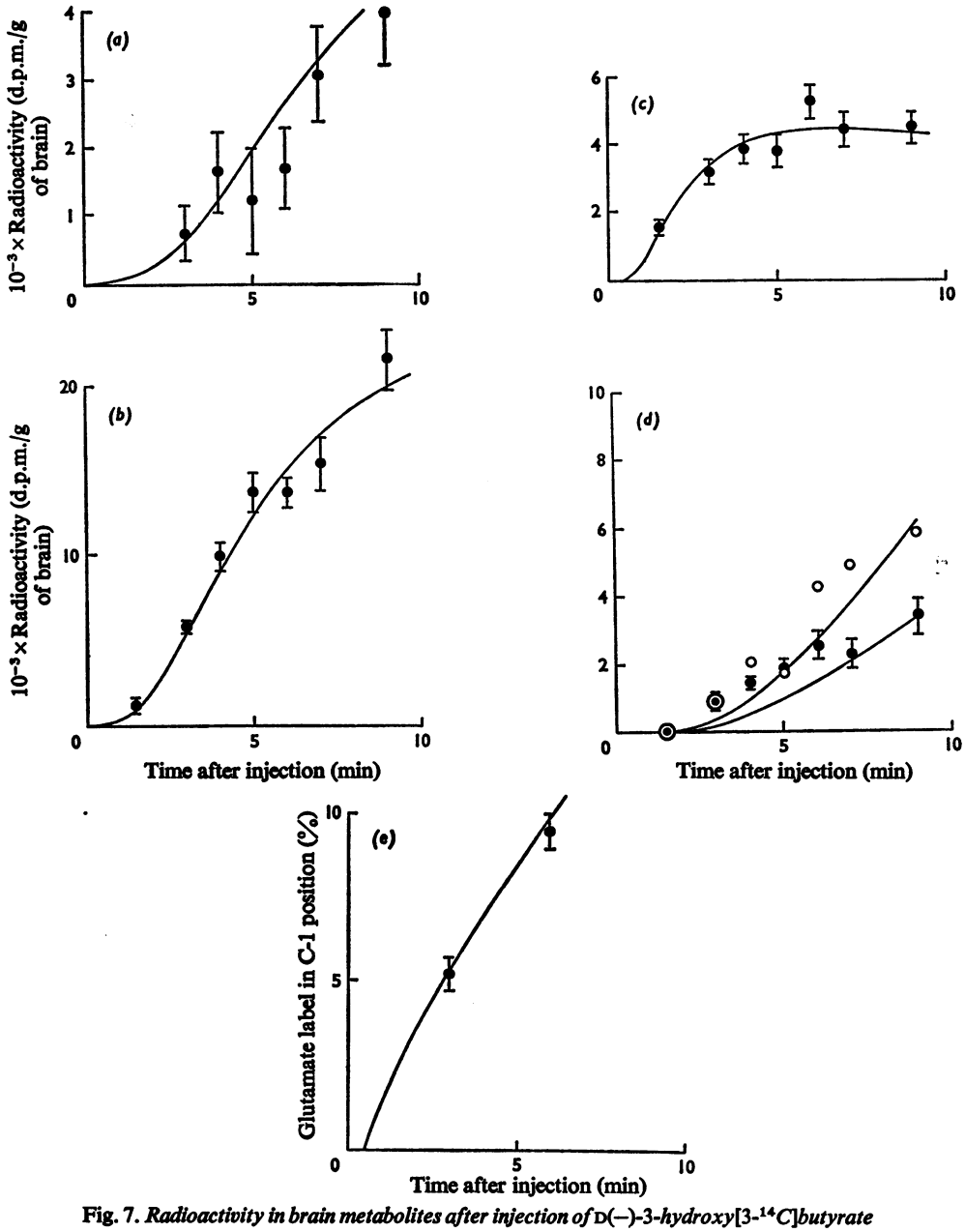


Fig. 7. *Radioactivity in brain metabolites after injection of* D(−)-3-*hydroxy*[3-^{14}*C*]*butyrate*

(*a*) Aspartate; (*b*) glutamate; (*c*) citrate cycle carboxylates; (*d*) 'GLUD' amino acids, ○; glutamine, ●; (*e*) [1-^{14}C]glutamate/(total glutamate label) as a percentage. For S.E.M. values see the Materials and Methods section. Best-fit curves are shown calculated from the parameters in Table 1, column 2.

independent of it. Consistent with this conclusion the simplifying assumption of strict proportionality was made for all calculations.

Fig. 7 shows the *q*–*t* curves. GLUD label was calculated by total amino acid label less that in glutamate and aspartate, and 'citrate cycle' label by total non-amino acid label less the ketone-body label in that portion of the blood in the brain. The fraction of glutamate label in the C-1 position is also shown (Fig. 7*e*). (2) Labelling from [2-^{14}C]glucose (Fig. 8).

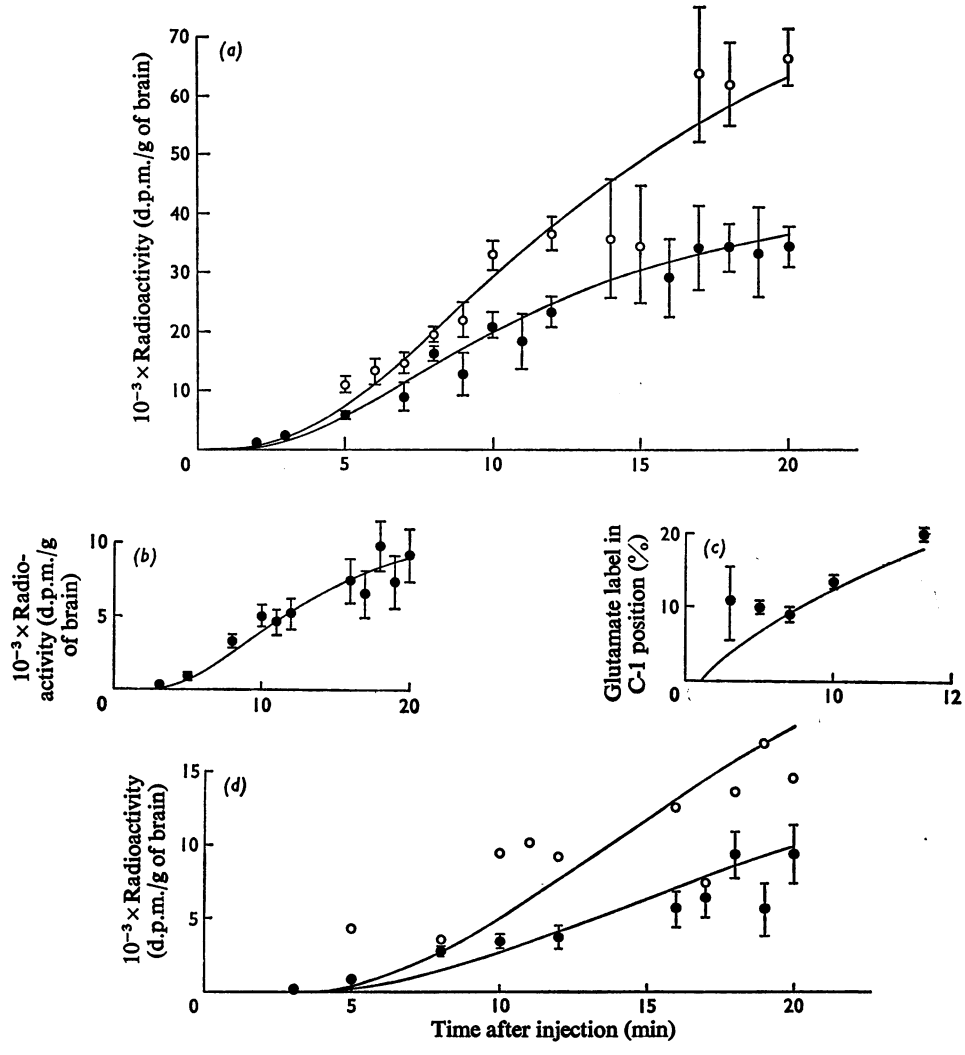


Fig. 8. *Radioactivity in brain metabolites after injection of* [2-^{14}C]*glucose*

(*a*) Glutamate, ●; total amino acids, ○; (*b*) aspartate; (*c*) [1-^{14}C]glutamate/(total glutamate label) as a percentage; (*d*) 'GLUD pool' amino acids, ○; glutamine, ●. For S.E.M. values see the Materials and Methods section. Best-fit curves are shown calculated from the parameters in Table 1, column 2.

Of the total amino acid label 6±1% (S.E.M., $n = 5$) was in alanine from 5 to 16min after injection. For mathematical analysis data had to be comparable with those obtained after injection of 3-hydroxy[^{14}C]butyrate, which does not label alanine. Therefore 6% was subtracted from all estimates of total amino acid label after [^{14}C]glucose injection to correct them for alanine label, and these corrected estimates were used to calculate GLUD label. In some experiments only total amino acid label, and not that in individual amino acids, was determined. These values, corrected for alanine label, are shown in Fig. 8(*a*). No data are presented for the non-amino acid fraction as it was not separated into glycolytic and tricarboxylate-cycle intermediates. (3) Pool sizes (μmol/g). The mean value for glutamate was 7.40±0.05 (S.E.M., $n = 120$) and for aspartate was 2.40±0.06 (S.E.M., $n = 29$). Of the label in the GLUD pool 55% was in glutamine. The glutamine concentration was 3.0±0.1 μmol/g (S.E.M., $n = 31$), so the GLUD pool was taken to be 3/0.55,

Table 1. *Rates and pool sizes calculated from Model 3(b)*

R was calculated as R_p+R_k; R_a by eqn. (15); R_G by finding the mean ratio of (glutamine label)/(calculated GLUD pool label), and multiplying R_3 by it. Both R_p and R_k are mean rates at the mean blood concentration of ketone bodies of 1.24 mM. Standard errors are shown. The curves shown in Figs. 7 and 8 are those calculated from the second column.

	All data	Excluding early data (best fit)	
Rates (μmol/min per g)	No cross-labelling	No cross-labelling	Cross-labelling
R_p, acetyl-CoA from pyruvate	0.762±0.035	0.743±0.032	0.661±0.034
R_k, acetyl-CoA from ketone bodies	0.314±0.016	0.302±0.015	0.304±0.015
R, citrate cycle rotation	1.08 ±0.04	1.05 ±0.03	0.97 ±0.04
R_a, 2-oxoglutarate to glutamate	3.39 ±0.24	3.36 ±0.22	2.81 ±0.17
R_3, glutamate to other amino acids	0.76 ±0.14	0.70 ±0.10	0.70 ±0.11
R_G, glutamate to glutamine	~0.42	~0.39	~0.39
R_A, aspartate formation	0.92 ±0.21	1.01 ±0.20	0.84 ±0.16
Pool size (μmol/g); 2×CIT1, citrate cycle carboxylates	0.92 ±0.06	0.99 ±0.06	0.91 ±0.05
Fraction of label bypassing glutamate, $(1-f)$	0.241±0.021	0.237±0.019	0.256±0.019
Goodness of fit: degrees of freedom	73	58	58
Goodness of fit: χ^2	148	59.5	68
Goodness of fit: P	≪0.01	0.5	0.2

i.e. 5.5 μmol/g. This assumes that the rates of formation and breakdown were proportional to the size of the constituent pools.

The variables were R_p, R_k, R_3, R_A, f and Q_1 (pool size of CIT1). An early trial with the pool size of glutamate as a variable showed that it was within the experimental range of total brain glutamate.

In fitting data the lowest S.E.M. was taken to be 0.2×10^3 d.p.m./g, as the experimental methods were not more precise than this. Experimental means and not calculated means were used to weight all GLUD data, which the model plainly only represented in a general way. Trials showed that calculated values of amino acid label at times less than 4 min after 3-hydroxy [3-^{14}C]butyrate injection and less than 6 min after [2-^{14}C]glucose injection were consistently lower than the experimental values. These were therefore also weighted by using their experimental means, as the bias towards low values introduced in this way would still be much smaller than that introduced by using calculated means.

It was found, however, that a good fit was only obtained when these data were excluded (Table 1). The fit was then good whether cross-labelling of blood glucose and lactate was allowed for or not.

The fit excluding cross-labelling and early values is shown in Figs. 7 and 8. None of the estimates of the parameters in the 3 sets (Table 1) differed significantly, although inclusion of cross-labelling decreased R_p. The small differences between the sets do not affect any conclusions so that the choice of which to discuss is essentially arbitrary. We think those in Table 1 (column 2) and Figs. 7 and 8 are the most realistic and have confined our discussion to these values.

Not only are data from ketone body and glucose label fitted equally well; even the systematic deviations (discussed below) are in the same direction for both. There is little doubt that ketone bodies and glucose were in all ways alternative substrates, feeding the same pools. This was confirmed by a statistical analysis of the data. (*a*) If after labelling with glucose the label in one amino acid was higher than average there was a significant tendency for label in the others also to be higher ($P<0.01$, non-parametric test comparing neighbouring pairs). (*b*) The variance within replicates of amino acid labelling after [^{14}C]glucose was higher than after 3-hydroxy[3-^{14}C]butyrate by an amount very close to that expected if increased acetyl-CoA formation from ketone bodies caused a corresponding decrease in that from glucose (see the Supplementary Publication SUP 50034).

This finding implies that pyruvate from glucose was not converted into oxaloacetate by carboxylation. Label from 3-hydroxybutyrate cannot reach this pathway. Consequently it would not be possible unless $R_p'=0$ to fit equally well the aspartate q–t curves after both [^{14}C]glucose and 3-hydroxy[^{14}C]butyrate injection.

As only the mean values of R_k and R_p were estimated, and R_k was proportional to blood concentration, the rates of acetyl-CoA formation can be related by:

$$R=R_p+K_k C_k \qquad (16)$$

where K_k is a proportionality constant and C_k is the concentration of ketone bodies in the blood. As R_k was 0.30 μmol/min per g when C_k was 1.24 μmol/ml, eqn. (16) can be rewritten:

$$R=1.05=R_p+0.244C_k \qquad (17)$$

Providing proportionality of R_k and C_k was maintained over the whole concentration range, this range, 0.44–2.15 μmol/ml, corresponded to a range of R_k of 0.11–0.53 μmol/min per g and of R_p from 0.93–

Table 2. *Amino acid labelling after injection of [U-^{14}C]- or [3-^{14}C]-lactate*

Values were corrected to a body weight of 45g and dose of 1.564μCi/animal. Results are given as $10^{-3}\times$d.p.m./g of brain. Results were calculated from Model 3(*b*) by using the second parameter set in Table 1. Values are means±S.E.M. with the numbers of experiments in parentheses.

Time after injection (min)	[U-^{14}C]Lactate		[3-^{14}C]Lactate			
	Total amino acid label		Glutamate label		Aspartate label	
	Experimental	Calculated	Experimental	Calculated	Experimental	Calculated
2	—	—	5.1±0.4 (2)	2.7	1.3±0.4 (2)	0.2
3	11.7±0.8 (3)	6.0	—	—	—	—
4	—	—	12.4±1.5 (2)	13.4	4.1±1.0 (2)	2.1
6	21.1±1.6 (3)	18.3	23.6±1.8 (2)	21.2	8.3±2.5 (2)	4.5

0.51μmol/min per g. Thus, although glucose was nearly always the major substrate, ketone bodies could be equally important.

The values of the parameters agree with other work in two respects. First the estimated contents of the tricarboxylate cycle, $2Q_1$, roughly equivalent to [citrate]+[oxoglutarate]+[succinyl-CoA]+[succinate]+[fumarate]+[malate], are at 0.99±0.06μmol/g, in fair agreement with the values in adults (Nahorski, 1972; Hawkins *et al.*, 1973). Secondly the rate of pyruvate utilization, R_p, was below the lower limit of the estimate of the rate of glycolysis, i.e. although there was exchange of brain lactate with blood the brain did not depend upon blood lactate as a substrate, but was probably a net producer of lactate, in agreement with the findings of Hawkins *et al.* (1971).

Labelling of amino acids by [^{14}C] lactate

In the preceding section it was assumed that lactate in the pool labelled was in equilibrium with brain mitochondrial pyruvate [Models 2(*a*) and 2(*b*)]. Unless this is so the specific radioactivity of the lactate pool after [^{14}C]glucose injection will have been lower than that of the pyruvate whereas after [^{14}C]lactate injection it will have been higher. Consequently parameter values which fitted the [^{14}C]glucose data (Table 1) would have given over-estimates of labelling after [^{14}C]lactate injection. This was not the case. Injection of [^{14}C]lactate gave labelling in glutamate and total amino acids which was initially higher than calculated but soon became very similar (Table 2). This is an unlikely finding unless mitochondrial and cytosolic lactate and pyruvate were all close to equilibrium with each other.

Aspartate labelling was, however, consistently higher than was calculated. This may indicate that lactate fed a pool of pyruvate that was readily carboxylated, unlike glucose, and that the pools of brain lactate from blood lactate and from glucose did not exactly coincide. If this were the case then calculation showed that R_p would have been over-estimated, but probably by less than 7%.

Although Model 3(*b*) gives a consistent account of the utilization of glucose, lactate and ketone bodies there were two discrepancies.

(1) The ratio of C-1 label/total label in glutamate at the two shortest times after injection of [^{14}C]glucose seemed impossibly high on any theory, probably owing to an impurity in the glutamate isolated for analysis.

(2) The labelling of aspartate, glutamate and the GLUD pool were all systematically under-estimated at short times after injection of any of the three substrates (Figs. 7 and 8 and Table 2). An effect of this sort is to be expected after [^{14}C]glucose injection unless equilibration between pyruvate and lactate was instantaneous; but it was also present after injection of 3-hydroxy[3-^{14}C]butyrate, when a close fit to the label in CIT1+CIT2 (Fig. 7*c*) was associated with an under-estimate of the label in its products, aspartate and glutamate, during the first 3min. A likely explanation is compartmentation of the tricarboxylate cycle into two parallel systems as in adults (Dzubow & Garfinkel, 1970), one containing only small pools of carboxylates so that label gets transferred to glutamate and aspartate more rapidly. If, moreover, as in adults, the associated pool of glutamate is small and confers label more rapidly on glutamine than the glutamate associated with the bulk of the carboxylates the discrepancies in amino acid labelling could be explained. There is good evidence that such compartmentation is present in the brain of 18-day-old rats (see Balázs *et al.*, 1973). A system of parallel tricarboxylate cycles when treated as one cycle will give estimates of R_p and R_k which are either correct or too low. Although this seems intuitively obvious, and we have checked it in several cases, not all simple, we have not found a general proof.

Discussion

In this study rats of only one age, 18 days, have been used. The procedures described for determining the rates of glucose and ketone-body utilization should

be applicable to rats of other ages. High concentrations of acetoacetate and 3-hydroxybuytrate are present in the blood throughout the suckling period from the first day onwards (Drahota *et al.*, 1964; Page *et al.*, 1971), probably as a consequence of the relatively high fat content of rat milk (Dymsza *et al.*, 1964). Since the concentrations of blood ketone bodies and blood glucose are high until weaning both forms of substrates are available to the brain of young rats. Previous work has indicated that in well-nourished, suckling rats up to 20 days of age ketone bodies are important as sources of oxidizable substrates (Hawkins *et al.*, 1971), which we have confirmed on 18-day-old rats.

The present work based on the use of ^{14}C-labelled precursors has provided more conclusive evidence than was previously available to support the idea that ketone bodies can replace glucose as oxidizable fuels for the brain. The specific-radioactivity–time curves for those brain metabolites measured were virtually identical after an injection of either [2-^{14}C]glucose or D(−)-3-hydroxy[3-^{14}C]butyrate. Not all substances that enter the brain and are metabolized via the formation of acetyl-CoA give rise to a similar pattern of labelling; many give a high ratio of glutamine to glutamate labelling (see Cremer, 1971). The differences in labelling patterns are thought to reflect metabolism in defined morphological structures, such as specific cell types or parts of neurones (Balázs *et al.*, 1973) and also to reflect the permeability properties of these structures to various precursors. From our kinetic data the morphological structures metabolizing the pyruvate plus lactate formed from the glucose entering the brain appear to be the same as those metabolizing the acetoacetate and 3-hydroxybutyrate entering from the blood. It is notable that when ^{14}C-labelled acetoacetyl-CoA is formed within the brain during the oxidation of leucine (Patel & Balázs, 1970) or butyrate (Tarkowski & Cremer, 1972) the specific-radioactivity–time curves for glutamate and glutamine are completely different from those observed when acetoacetyl-CoA is formed from blood-borne ketone bodies.

Measurement of rates

Arterio-venous difference measurement across the brain of young suckling rats had shown there was a net output of lactate (Hawkins *et al.*, 1971). The rates of glycolysis and conversion of carbohydrate into acetyl-CoA as estimated by us are in accordance with this finding. Although our data did not allow a precise estimate of the rate of glycolysis it did permit an estimate of the lower limit, 0.41 μmol/min per g. This minimal rate of formation of pyruvate plus lactate (0.82 μmol/min per g) was greater than the calculated rate at which they were converted into acetyl-CoA, 0.51–0.93 μmol/min per g, except for animals with the lowest concentrations of blood ketone bodies. The probable mean rate of glycolysis was 0.43 μmol/min per g. The concentration of brain lactate showed a fairly small variation (pyruvate was not measured but evidence is given elsewhere that it was in equilibrium with lactate) so that the excess of lactate plus pyruvate produced must have left the brain.

One of the most important features taken into consideration in the estimation of rates of glycolysis and the tricarboxylate cycle by labelling experiments was the exchange of lactate between the blood and brain. No estimate of this rate appears to have been given before for the young rat brain, but the results in Figs. 3 and 4 show that the minimum rate was 2 μmol/min per g [by substituting $R_g = 0.41$ in eqn. (13), and the value of R_L obtained into eqn. (7) with $V = 1$ (blood flow rate through brain in adults; Goldman & Sapperstein, 1973) and $\Delta = 0$; see legend to Model 2]. Consequently, a fraction of the glucose label passing down the glycolytic chain was washed out by blood lactate. This could be expressed as $\beta'/(2\alpha' + \beta')$, which in our experiments was 0.57 (mean values for α' and β' are given under 'Labelling of amino acids by [^{14}C]-lactate'). When, as was usually the case, the glycolytic rate exceeded the rate of pyruvate dehydrogenation this fraction was more. The exchange between blood and brain lactate greatly affected tricarboxylate-cycle labelling after injection of [^{14}C]glucose by diluting the label in the precursor, pyruvate. Neglecting it would cause rates of utilization of glucose by the tricarboxylate cycle to be grossly underestimated.

Simple methods of estimating rates of utilization in brain can be used when there is little washout of label, as appears to be the case after injection of ketone bodies.

(1) If retention of label is complete the rate of acetyl-CoA formation from ketone bodies is given by:

$$0.5R_k = [q_B(t_2) - q_B(t_1)]/\int_{t_1}^{t_2} S'_k \, dt \qquad (18)$$

where $q_B(t)$ is the total label in metabolites in brain at time t after injection, '0.5' allows for the formation of 1 mol of acetyl-CoA from 0.5 mol of ketone body and S'_k is the mean specific radioactivity of ketone bodies in blood corrected for inequality in the rate coefficients for 3-hydroxybutyrate and acetoacetate utilization (see under 'Ketone bodies in blood after injection of D(−)-3-hydroxy[3-^{14}C]butyrate'). The first time taken, t, is best not zero, the time of injection, but a time shortly afterwards, as the kinetics immediately after injection are uncertain. From the data in Figs. 6 and 7 average values of R_k over the ranges 1.5–3 to 1.5–9 min were obtained with an overall mean value of 0.263 μmol/min per g. The values, however, tended to fall as the range of time increased because retention of label by brain was incomplete. Extrapolation back to the effective zero time, 0.5 min after injection (Fig. 6), gave $R_k = 0.312$

$\pm 0.031\,\mu$mol/min per g in good agreement with the best estimate (Table 1).

(2) Some incompleteness of retention of label can be allowed for by treating the brain as a single homogeneous pool:

$$\text{Precursor} \rightarrow (\text{brain}, q_B) \xrightarrow{k} \text{products}$$

so that:

$$dq_B/dt = 0.5R_k S_k' - kq_B \qquad (19)$$

The turnover coefficient, k, is without clear physical meaning, as the brain is treated as a black box with an output which corrects for loss of label when this loss is small. In this instance integration of eqn. (19) and fitting to the data gave $R_k = 0.316 \pm 0.028\,\mu$mol/min per g. Thus both methods are satisfactory for ketone bodies. The second is likely in general to prove the better.

Initially in this study an attempt was made to apply these methods to the glucose data. It became obvious that neither was suitable.

Compartmentation of glucose and lactate

Reasons have been given above why the total brain glucose could not be the precursor of labelled brain metabolites, including lactate. A division of brain glucose into two metabolically distinct pools was proposed (Model 1). Only one of them is in rapid equilibrium with plasma glucose, and it is from this pool that further metabolism occurs. A very similar proposal has been made by Betz *et al.* (1974) to account for data obtained for glucose flux in dog brain.

The existence of two metabolically distinct pools of lactate, one of which did not become labelled in the course of an experiment (Model 2), was required to explain the labelling of brain lactate. The specific radioactivity and size of the labelled pool, which was the effective precursor of acetyl-CoA, could only be estimated by determining the specific radioactivity of brain lactate relative to that of blood glucose and of blood lactate after injection of [^{14}C]glucose and [^{14}C]lactate respectively. The source of the unlabelled pool of brain lactate and its morphological location are not known.

Disequilibrium between oxo and amino acids

From Model 3(*b*) and Table 1 it can be seen that not all label in the oxo acids becomes incorporated into the corresponding amino acids. We calculate that there is a 24% bypass of glutamate and a 50% bypass of aspartate. This means that for each molecule of carboxyl-labelled acetyl-CoA entering the tricarboxylate cycle from [2-^{14}C]glucose or D(−)-3-hydroxy[3-^{14}C]butyrate, 76% of the label passes into glutamate and of the remaining 24% that enters carboxylates of the cycle, half the label enters aspartate. The rate of exchange of label between oxaloacetate and aspartate was about 3 times less than exchange between 2-oxoglutarate and glutamate (Table 1). A similar difference between these two exchange rates was proposed by Van den Berg & Garfinkel (1971) in a simulation study of [^{14}C]-glucose metabolism in mouse brain. Both exchange rates are low compared with the total activity of the enzyme L-aspartate–2-oxoglutarate aminotransferase (EC 2.6.1.1) when assayed in whole brain homogenates; 21 μmol/min per g in 16–25-day-old rats (Bayer & McMurray, 1967).

The specific radioactivity of alanine increased for at least 16 min after the injection of [^{14}C]glucose unlike that of brain lactate, showing that the interconversion of pyruvate and alanine was much slower than that of pyruvate and lactate.

Regulation of enzymes

From measured blood concentrations of 3-hydroxybutyrate and acetoacetate and the total activities (Krebs *et al.*, 1971) and K_m values for the brain enzymes D(−)-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30; K_m given by Meinzel & Hammes, 1973) and acetoacetyl-CoA transferase (EC 2.8.3.5; K_m given by Tildon & Sevdalian, 1972) it would seem that the capacity of these enzymes is several times greater than the actual rate through these enzymic steps *in vivo*. A possible explanation is restricted entry of ketone bodies into brain.

Flux through the step acetoacetyl-CoA to acetyl-CoA was estimated to be between 0.055 and 0.265 μmol/min per g. These rates are only 1 to 5% of the total activity of the enzyme acetoacetyl-CoA thiolase (EC 2.3.1.9) in mitochondria of brain as determined by Middleton (1973). Flux through the step pyruvate to acetyl-CoA was estimated to be between 0.51 and 0.93 μmol/min per g and these values were remarkably close to those obtained for the total activity of pyruvate dehydrogenase in the 18-day-old rat brain (Cremer & Teal, 1974). If assays of pyruvate dehydrogenase *in vitro* do indeed reflect the activity of the enzyme *in situ* then the results would imply that the capacity of the young rat brain to oxidize carbohydrate is strictly limited.

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APPENDIX

The Effects on Estimates of Rates of Utilization of Ketone Bodies of Inequalities in their Specific Radioactivities and Fractional Rates of Utilization

Properties of acetoacetate are denoted by subscript 'a' and of 3-hydroxybutyrate by subscript 'b'. Other symbols are: concn. in blood, C; specific radioactivity, S; rate of acetyl-CoA formation from precursor, R; fractional rate of utilization, k; quantity of label in metabolites in brain, q_B. The total rate of acetyl-CoA formation from ketone bodies, R_k, is the sum of the individual rates, each proportional to the concn. in blood, i.e.

$$R_k = R_a + R_b = k_a C_a + k_b C_b \qquad (A.1)$$

At all times after injection R, k and C are assumed to be constant, as is S_a/S_b. [The constancy of S_a/S_b is discussed in the main text under 'Ketone bodies in blood after injection of D(−)-3-hydroxy[3-^{14}C]-butyrate' and below.] Under these conditions the following constant ratios can be defined:

$$r_c = C_a/C_b; \quad r_k = k_a/k_b; \quad r_s = S_a/S_b \qquad (A.2)$$

At any time the rate of input of label into the brain is given by:

$$R_a S_a + R_b S_b = R_b S_b (1 + r_c r_k r_s) \qquad (A.3)$$

obtained by substituting eqns. (A.1) and (A.2) into the left hand side of eqn. (A.3.) At any particular time after injection the quantity of label in brain can be expressed in the form:

$$q_B = M_1 R_b S_b (1 + r_c r_k r_s) \qquad (A.4)$$

where M_1 is a multiplier (obtained by integration of

the relevant differential equations). The value of M_1 is time-dependent.

In practice the specific radioactivities used were the mean values, S_k, of both ketone bodies, i.e.:

$$S_k = (C_a S_a + C_b S_b)/(C_a + C_b) = S_b(1 + r_c r_s)/(1 + r_c) \quad (A.5)$$

by substituting eqn. (A.2) into the central form of eqn. (A.5). If the rate of input of label into brain is taken to be $R_k S_k$ instead of $R_a S_a + R_b S_b$ (eqn. A.3), then, substituting for R_k by $R_b(1 + r_c r_k)$ by eqn. (A.2) in eqn. (A.1) and for S_k by eqn. (A.5):

$$\text{Input} = R_k S_k = R_b S_b(1 + r_c r_s)(1 + r_c r_k)/(1 + r_c) \quad (A.6)$$

The only variable in this equation is the same as that in eqn. (A.3), S_b. Consequently integrating as before to the same time as before will give the same multiplier, M_1, as in eqn. (A.4), but a different value of brain label, namely:

$$q'_B = M_1 R_k S_k = M_1 R_b S_b(1 + r_c r_s)(1 + r_c r_k)/(1 + r_c) \quad (A.7)$$

Hence, dividing eqn. (A.4) by eqn. (A.7):

$$\frac{q_B}{q'_B} = \frac{(1 + r_c r_k r_s)(1 + r_c)}{(1 + r_c r_s)(1 + r_c r_k)} = m \quad (A.8)$$

Thus the ratio q_B/q'_B is time-invariant. Consequently to calculate R_k from S_k by eqn. (A.7) S_k should be multiplied by this ratio, m, as q_B can be identified with the correct value of brain label, and q'_B with that which would be calculated by using $R_k S_k$ for $R_a S_a + R_b S_b$.

The numerical values found experimentally were: $r_c = 0.33$, $r_k = 1.85$ and $r_s = 0.065$, giving $m = 0.94$ by eqn. (A.8). Values of S_k were multiplied by this before computation of rates.

Eqn. (A.8) has some interesting properties.

(*a*) If $r_k = 1$, $m = 1$; i.e. if $k_a = k_b$ the results do not depend on the values of r_c or r_s.

(*b*) At $t = 0$, $r_s = 0$ (see the text). Then for $r_c = 0.33$ and $r_k = 1.85$, $m = 0.83$, which is not very different from 0.94. The label in brain, q_B, depends on the areas under the S_a–t and S_b–t curves. After intraperitoneal injection S_a/S_b reaches an equilibrium value in a time t such that the area defined by the S_b–t curve, integral of $(S_b\,dt)$ from $t = 0$ to t, is only a small fraction of the area to infinite time. Consequently except in very short experiments the value of m given by eqn. (A.8) will be close to its mean value. This would not be the case after intravenous injection, when, at least in post-absorptive adult rats, a high proportion of the area under the S_b–t curve is defined before S_a/S_b nears equilibrium (Barton, 1973).

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