Incorporation of Isotopic Carbon into Cerebral Glycogen from Non-Glucose Substrates

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1. Measurable incorporation of radioactive carbon from [U-¹⁴C]pyruvate, [U-¹⁴C]-glutamate and [¹⁴C]bicarbonate into the glycogen synthesized by brain slices *in vitro* was demonstrated. 2. The fructose diphosphatase activity of guinea-pig brain was determined and found to be about 0.03 μ mol of substrate degraded/min per g of fresh tissue. 3. The specific radioactivity of the glucose carbon from glycogen relative to that of the precursor added to the incubation medium gave approximate values of 0.195 for glucose, 0.006 for pyruvate, 0.039 for glutamate and 0.001 for bicarbonate.

There is considerable evidence (Elliot & Wolfe, 1962) for the oxidation of substances other than glucose by brain tissue, but the question as to whether an alternative can also be used in the synthesis of cerebral glycogen has attracted much less attention.

Coxon *et al.* (1965) reported that the intravenous injection of [¹⁴C]glutamate and [¹⁴C]bicarbonate led to the appearance of label in the cerebral glycogen of rabbits, and Khaikina & Goncharova (1964) have also described the incorporation of ¹⁴C, administered subcutaneously as acetate, into the cerebral glycogen of rats. Labelling of the glycogen of the brain in both instances may have been brought about indirectly by gluconeogenesis outside the brain, with [¹⁴C]glucose reaching the brain via the bloodstream. An alternative possibility is that gluconeogenesis occurred within the brain itself, and this is compatible with the occurrence of CO₂ fixation (Waelsh *et al.*, 1964) and the presence of gluconeogenic enzymes (Scrutton & Utter, 1968) in this organ. However, the unequivocal demonstration of gluconeogenesis in the brain requires the use of preparations other than the whole animal in order to exclude the complicating effects of other organs. For this reason we have studied the problem in brain slices, and since attempts in this laboratory to promote net synthesis of glycogen by incubating such slices with several possible precursors other than glucose were no more successful than those of earlier workers (e.g. Benoy & Elliot, 1937), we resolved to study the incorporation of ¹⁴C by using pyruvate, glutamate and bicarbonate as potential donors. In agreement with LeBaron (1955) we have previously observed that incubation of slices in the presence of 15 mM-glucose induces the re-synthesis of glycogen, and accordingly in the present study we have used this concentration of unlabelled glucose to ensure that detectable synthesis was taking

place. While our experiments were in progress there came to our notice a paper by Ide *et al.* (1969) in which incorporation of ¹⁴C from lactate into glycogen was described in slices of rat brain. We, however, used guinea pigs as the source of our material because of their larger brains. To minimize possible errors due to artifacts we hydrolysed the glycogen and confirmed the presence of ¹⁴C in the resulting glucose by paper chromatography. As a further precaution against such errors the slices were always incubated in parallel with control slices from the same brain which had been inactivated. For comparison, some slices were also incubated with [¹⁴C]glucose.

In addition, since the evidence on the activity of fructose diphosphatase in brain is conflicting (Krebs & Woodward, 1965; Scrutton & Utter, 1968), we assayed this enzyme in the guinea-pig brain.

Materials and Methods

Animals

Albino Duncan Hartley guinea pigs of both sexes, weighing between 200 and 350 g, were used. They were fed *ad lib.* on Oxoid diet SG1, supplemented daily with cabbage.

Preparation of tissue

The method used for removing and slicing the brains was based on that described by McIlwain & Rodnight (1962). The animals were stunned by a light blow on the back of the neck, caudal to the occiput, and the brain was removed. Slices (0.3 mm thick) were cut, one from each hemisphere, without wetting the tissue, and were immediately placed in the incubation medium.

It had previously been shown that paired slices from the two hemispheres behaved quite similarly on incubation and so in the present work a slice from one

side was used as the 'test' slice and a corresponding one from the other side as the 'control' slice. The control slice was inactivated at the commencement of incubation (see below).

Incubation

A Krebs-Ringer type of medium was used for the experiments with pyruvate, glucose and glutamate that had the following composition: NaCl, 118.5mM; KCl, 4.7mM; CaCl₂, 2.54mM; KH₂PO₄, 1.19mM; MgSO₄, 1.19mM; NaHCO₃, 24.9mM; pH 7.4. It was gassed with O₂+CO₂ (95:5) and 15mM-unlabelled glucose was added to ensure a good yield of glycogen (see above). The weight of tissue per flask was about 100mg and the volume of medium was 3ml.

A modified medium was used for the experiments with [¹⁴C]bicarbonate to permit an increase in the specific radioactivity of the substrate. It contained only 4.92mM-NaHCO₃ and, in addition to the salts and glucose mentioned above, it contained 9.6mM-Na₂HPO₄. It was gassed with O₂+CO₂ (99:1) and the final pH was 7.3.

The inactivating agents used in the control flasks were 10% (w/v) trichloroacetic acid when pyruvate, glutamate and glucose were the labelled substrates and 15mM-NaF, used as an enzyme poison, when [¹⁴C]bicarbonate was present.

The amounts of radioactivity added were 0.1, 0.5, and 0.5μCi of [U-¹⁴C]glucose, [U-¹⁴C]pyruvate and [U-¹⁴C]glutamate respectively. The total concentrations of substrate were 15mM for glucose, 4mM for pyruvate and 15mM for glutamate. In the experiments with [¹⁴C]bicarbonate 1μCi was added to the modified medium already described. All the ¹⁴C-labelled materials were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and the specific radioactivities were: glucose 3mCi/mmol, pyruvate 10mCi/mmol, glutamate 15mCi/mmol, bicarbonate 42mCi/mmol.

Control and experimental slices were incubated for 2h at 37.5°C and after incubation were washed in substrate-free medium and placed in ethanolic KOH [60% (w/v) KOH in aq. 60% (v/v) ethanol] at 85°C for digestion.

Isolation of glycogen and determination of radioactivity

Isolation and purification of glycogen was as described by LeBaron (1955). The glycogen recovered from each slice was dissolved in 0.5ml of water and 10ml of N.E. 240 or 250 scintillation fluid [Nuclear Enterprises (G.B.) Ltd., Edinburgh 11, U.K.] was added to each sample. Radioactive counting was done on a Nuclear-Chicago Unilux TM II, the channels-ratio method being used for the determination of efficiency.

Chromatography

Because of the paucity of glycogen in individual slices, the chromatographic experiments were performed on material obtained by pooling 20–24 slices, which had been subjected to identical treatment; similar control samples were obtained by pooling 20–24 slices incubated in a medium containing an inactivator. After purification the glycogen samples were hydrolysed with 0.5M-H₂SO₄ at 100°C for 3h. The hydrolysates were neutralized with 0.1M-Ba(OH)₂ to precipitate the SO₄²⁻ ions, and the supernatant was freeze-dried.

The powder obtained from freeze-drying was dissolved in a minimum volume of water and spotted on to Whatman no. 1 chromatography paper. Spots of unlabelled glucose were also placed on the same paper on each side of the unknown. The solvent system used for development was butan-1-ol-ethanol-water (26:15:9, by vol.) and the running time was 18h at room temperature (about 20°C). After being dried, the paper was cut, in the direction of the run, into three strips between the original spots. The peripheral strips were sprayed with a reagent composed of 0.91g of aniline plus 1.55g of phthalic acid, which stained the unlabelled glucose. The central strip, carrying the hydrolysate, was then cut into numbered sections, 16mm in length, of which the first straddled the starting line, and these sections were placed in separate vials, together with 10ml of scintillator and counted for radioactivity. The location of maximal radioactivity was thus established relative to the glucose markers.

To demonstrate the spread of radioactivity to be expected on the paper as a result of solvent movement, a run was also performed in which 1μCi of pure [U-¹⁴C]glucose (sp. radioactivity 3μCi/μmol) was spotted on the starting line and the chromatogram developed under the same conditions as those used for the samples.

Experiments were also performed to establish that any contaminating glutamate and pyruvate, which might have been carried through the precipitation procedure, would be separated from glucose on the chromatograms. (This was not considered necessary for bicarbonate, since ¹⁴CO₂ should have been driven off during acid hydrolysis.) Labelled glutamate and pyruvate were run on a paper together with non-labelled glucose, and the positions of maximum count were shown to be remote from the glucose spot.

Determination of fructose diphosphatase activity

Fructose diphosphatase in cortical matter from guinea-pig brain was assayed by the method of Underwood & Newsholme (1965). The activity of the enzyme was measured on a Gilford recording spectrophotometer at room temperature (about 22°C).

Table 1. *Incorporation of ^{14}C from several substrates into the glycogen of brain slices*

The numbers in the second, third and fourth columns are d.p.m./2 h per g of fresh brain. n is the number of paired comparisons from which the differences were calculated. The specific radioactivities of the substrates were as follows: glucose, 2 nCi/ μmol , glutamate, 11.1 nCi/ μmol , pyruvate, 41.6 nCi/ μmol and bicarbonate, 66.7 nCi/ μmol .

^{14}C -labelled substrate	Control (mean and range)	Experimental (mean and range)	Mean of differences $\pm$ S.E.M. (experimental—control)
15 mM-Glucose ($n=57$)	600 (200–1350)	5350 (1250–10550)	4750 $\pm$ 320
15 mM-Glutamate ($n=36$)	750 (300–1300)	6450 (1600–11650)	5700 $\pm$ 450
4 mM-Pyruvate ($n=24$)	1500 (600–2400)	7300 (3600–10300)	5800 $\pm$ 350
5 mM-Bicarbonate ($n=21$)	300 (50–650)	3650 (1900–7600)	3350 $\pm$ 230

Results

Table 1 shows the amounts of radioactivity recovered in the glycogen with labelled glucose, glutamate, pyruvate and bicarbonate as donors. Although there was considerable variability between the individual experiments the radioactivity of the glycogen in the slice from the experimental flask on every occasion exceeded that of the control by a substantial amount and statistical analysis by the 'method of paired comparisons' (Bailey, 1959) showed the differences to be highly significant. Since the differences were invariably positive the non-parametric sign test (Siegel, 1956) also indicated that such a result would only be expected to occur by chance once in more than 1000 trials.

The outcome of a representative set of chromatographic runs is summarized diagrammatically in Fig. 1. It shows that, for each of the three precursors tested, a peak of radioactivity was found with an R_F corresponding to that of glucose. It is possible that the apparent glucose spot may have contained some galactose (whose behaviour in the solvent system used is very similar), but this would not invalidate the conclusion that ^{14}C had been incorporated into a hexose sugar. The counting rates shown in Fig. 1 do not, of course, represent the total radioactivity recoverable from glycogen but only the distribution of it in the sample of hydrolysate actually applied to the paper. The peak of activity in the pyruvate scan can be seen to spread over several squares. This is possibly due to a higher concentration of glycogen in that sample. It is known that the degree of spread on a chromatogram increases with increasing concentration of the sample. In the run performed with a uniformly labelled standard glucose sample, the resulting peak of radioactivity spread over three squares indicating that the spread did not necessarily depend on the purity of the sample.

Both the pyruvate and glutamate scans show other

peaks. Ideally it would have been desirable to remove all contaminants by such procedures as ion-exchange, but the scarcity of material made further purification very difficult. Carbon could have become incorporated into many intermediates, which the incomplete purification has not removed. This, however, does not affect the conclusion that there are peaks of radioactivity appearing on the chromatograms in the glucose position.

The fructose diphosphatase activities in three specimens of guinea-pig brain were 0.042, 0.023 and 0.030 μmol of substrate degraded/min per g of fresh tissue at 22°C.

The mean value of 0.030 is low compared with that found in muscle and liver by Krebs & Woodward (1965), who reported the enzyme to be absent from brain, and is also less than that quoted for rat brain by Scrutton & Utter (1968). However, AMP is known to inhibit fructose diphosphatase and previous experiments (Phillips, 1970) have revealed that the concentration of AMP in the mixture on which our measurements were made could be as high as 20 μM . Such a concentration, if the brain enzyme resembles its counterpart in muscle, could exert a powerful inhibitory effect.

Discussion

These experiments have demonstrated the incorporation of ^{14}C derived from a number of substances into the glycogen of brain slices in the presence of unlabelled glucose as well as the incorporation from labelled glucose. It is of interest to compare the specific radioactivity of the glucose carbon of glycogen relative to the specific radioactivities of the individual precursors added to the incubation medium. Previous work in this laboratory has shown that no net synthesis of glycogen could be obtained on incubation with these substances (unlabelled) alone.

Fig. 1. Location of radioactivity on a paper chromatogram of glycogen hydrolysate

The numbered boxes in each panel refer to 16mm strips of paper and the radioactivity recovered from each strip is indicated by the symbols above and below the boxes. ●, ○, correspond to hydrolysates from the experimental and control flasks respectively. The arrows represent the positions of the starting lines (S) and the glucose marker (G). The panels relate to the several ^{14}C -labelled precursors as follows: (a) pyruvate; (b) bicarbonate; (c) glutamate; (d) glucose.

However, slices incubated in 15mm-glucose for 2h regularly synthesized glycogen to a maximum of about $5\mu\text{mol/g}$ of brain, expressed as glucose equivalents. There was a marked fall in glycogen concentration during the first 30min from 2 to 0.7

μmol of glycogen/g of brain, and then a steady re-synthesis up to $5.7\mu\text{mol/g}$ in 2h. Further increases in incubation time caused fragmentation of the slice (Phillips, 1970; M. E. Phillips, unpublished work).

Although the actual concentrations of glycogen in the present study have not been measured, it is reasonable to assume, since the conditions were similar to those in the experiments just described, that the extent of synthesis would be about $5\mu\text{mol}$ of glycogen/g of brain and calculations of the specific radioactivity of the glucose carbon from glycogen given in Table 2 were based on this value.

It is noteworthy that, in the case of glucose, only about one-fifth of the glycogen synthesized appears to arise from the glucose of the medium. Presumably therefore the remainder must have been produced via some pool of glucose which was not freely exchangeable with that in the medium. There is some evidence (Stetten & Stetten, 1960) that glycogen granules may be associated in the cell with enzyme protein, and it may be that when degradation takes place, as appears to happen during the early stages of incubation *in vitro*, some of the glucose liberated could remain loosely bound to the granule and be preferentially utilized when synthesis is resumed.

The fact that ^{14}C presented as bicarbonate can appear in the glycogen is entirely consistent with the finding of CO_2 fixation in brain reported by Welsh *et al.* (1964). It also suggests very strongly that gluconeogenesis is by the same metabolic pathway as in other tissues (see Krebs, 1964). Our finding (Table 2) of measurable fructose diphosphatase activity in guinea-pig brain is also compatible with this suggestion, as is the report by Scrutton & Utter (1968) of the presence of pyruvate carboxylase and phosphoenolpyruvate carboxykinase in rat brain. Our values for fructose diphosphatase activity and those given by Scrutton & Utter (1968) for the other two enzymes (0.5 and $0.3\mu\text{mol/min per g}$ of fresh brain for pyruvate carboxylase and phosphoenolpyruvate carboxykinase respectively) are adequate to account for the amount of incorporation of pyruvate and glutamate carbon into glycogen that we have observed.

Another possibility is that incorporation may take place via a transaldolase reaction of the type described by Landau & Bartsch (1966), but, although this would by-pass fructose diphosphate, it would still require the production of labelled glyceraldehyde phosphate.

The present findings, and the calculations based on them, thus point to the conclusion that under normal conditions gluconeogenesis can occur but is not a major metabolic pathway in the guinea-pig brain. It could, nevertheless, become important under abnormal conditions. For example, Prasannan & Subrahmanyam (1968) have reported a net synthesis of glycogen in slices of cerebral cortex from alloxan-diabetic rats incubated in a medium containing only

Table 2. *Estimated extent of incorporation of ^{14}C into cerebral glycogen*

The specific radioactivities of the U- ^{14}C -labelled precursors were calculated from the data given in the legend to Table 1. In the case of bicarbonate allowance was made for the presence of CO_2 in the gas-phase, which was assumed to be in isotopic equilibrium with the CO_2 dissolved in the medium. The specific radioactivity of the glycogen carbon was calculated by using the data in the last column of Table 1 and making the assumption (see the Discussion section) that 5 μmol of glycogen (expressed as glucose equivalents) were synthesised/g of brain during the incubation period. All values are rounded off to two significant digits.

Precursor added to the medium	Specific radioactivity of precursor (d.p.m./ μg -atom C)	Specific radioactivity of glucose from glycogen (d.p.m./ μg -atom C)	Relative specific radioactivity of glucose from glycogen (d.p.m./ μg -atom C)
15mM-Glucose	810	160	0.20
15mM-Glutamate	4900	190	0.039
4mM-Pyruvate	31000	190	0.0061
5mM-Bicarbonate	100000	110	0.0011

pyruvate and acetate as substrates, but they found no synthesis in such a medium with slices from normal rats.

Although the definitive demonstration of glycogen in brain was reported by Kerr (1938) its functional importance to the organ is still something of an enigma, despite the very considerable number of subsequent publications on its metabolism. At the present time, the glycogen found in brain is widely regarded to be an 'energy reserve', but this notion appears to be largely based on the observation that it is rapidly converted into lactate during the first few minutes of hypoxia after circulatory arrest (Lowry *et al.*, 1964). Unlike skeletal muscle the brain is rarely, if ever, called on to function under hypoxic conditions in ordinary life, so that the capacity to mobilize glycogen very rapidly when such conditions do arise might be regarded as an emergency 'fail-safe' device. Similarly the occurrence of increased glycogen deposition during recovery from hypoxia (Ibrahim, *et al.*, 1970) and other stress situations such as irradiation, might be regarded as a response to tissue injury. On the other hand, the fact that the rate of turnover inferred from the incorporation of label from [^{14}C]glucose *in vivo* is comparable with that in cardiac muscle (Coxon *et al.*, 1965) and the retardation of this process under barbiturate anaesthesia (Watanabe & Passonneau, 1973) indicate a more continuous role in normal cerebral metabolism. The incorporation of ^{14}C from non-glucose precursors likewise points to an active involvement in the biochemical organization of the undamaged central nervous system. However, it should be noted in this connexion (see Ibrahim *et al.*, 1970) that such participation may to a great extent be related to the activities of neuroglial elements rather than the neurons themselves.

M. E. P. was a M.R.C. scholar.

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