The Incorporation of Isotopic Carbon (^{14}C) into the Cerebral Glycogen of Rabbits

By R. V. COXON, E. C. GORDON-SMITH AND J. R. HENDERSON

University Laboratory of Physiology, Oxford

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1. The incorporation of ^{14}C into the brain glycogen of conscious rabbits with labelled glucose, bicarbonate and glutamate as precursors has been studied. 2. Substantial incorporation from all these precursors was demonstrated after an interval of 5 hr. from their injection. 3. With [^{14}C]glucose maximal incorporation occurred at about 8 hr. from the time of injection. 4. Hydrocortisone led to increased incorporation of ^{14}C from labelled glucose. 5. Some comparisons between the turnover of brain glycogen and that of skeletal and cardiac muscle are reported.

The presence of glycogen in small amounts in cerebral tissue has been recognized for many years. The material was isolated and characterized chemically by Kerr (1938). His observations on the effect of various environmental factors on the quantity of the cerebral glycogen in rabbits led to the conclusion that this constituent of the brain was a relatively stable one, its concentration being unaffected by adrenaline or starvation. However, Kerr & Ghantus (1936) did confirm the earlier findings of Takahashi & Asher (1924) that hypoglycaemia of a sufficient degree to produce convulsions led to a fall in the cerebral glycogen content. Chance & Yaxley (1950) demonstrated that the glycogen content of brain undergoes a rapid fall after the death of the animal, the amount present being decreased by 75% between the end of the first and the fourth minutes after death. LeBaron (1955) demonstrated that the loss of cerebral glycogen which occurs when slices are cut from guinea-pig brain can be restored if the slices are incubated in glucose, but the rate of production of glycogen was considerably less than that found in diaphragm or in liver slices under similar conditions.

Some work has been published by Russian authors (summarized by Palladine, 1955) which indicated that ^{14}C administered to rats in the form of radioactive glucose is incorporated into the glycogen of their brains. However, our experiments were undertaken independently and initially without knowledge of the Russian results. In view of the apparent stability of the brain glycogen *in vivo* in spite of the presence in the brain of systems capable of destroying it *post mortem*, and in view of the special importance of carbohydrate metabolism in the central nervous system, it was decided to study the rate of incorporation of ^{14}C from labelled precursors into cerebral glycogen.

Preliminary accounts of some of the present work have been presented (Coxon & Henderson, 1958; Coxon & Gordon-Smith, 1961).

METHODS

Animals. The animals used were adult rabbits of both sexes. They were housed during the experiments in a gas-tight box out of which expired air was led away through a tower containing caustic alkali for absorption of expired radioactive CO_2 . The chamber was lined with polythene sheeting, which enabled the animals' excreta to be collected for disposal without contamination of the apparatus. At the beginning of the experiment the animals were anaesthetized with intravenous pentobarbitone or thiopentone, and while it was unconscious the labelled precursor was injected intravenously into the ear. Shortly after receiving the tracer the animal recovered consciousness and during the remainder of the experiment moved freely about in its box. At the conclusion of the experimental period the animal was deeply anaesthetized with pentobarbitone or thiopentone, the skin over the back of the head was incised and the vault of the cranium removed. The animal remained alive up to this point, and while the circulation was still vigorous the brain was quickly removed and dropped into 25 ml. of 60% (w/v) KOH in a water bath at 90° . Any samples of other tissues required for comparison with the brain were removed immediately after the brain, and were also placed in hot KOH solution. All tissue blocks once immersed in the hot alkali were rapidly dispersed by stirring.

Radioactive precursors. These were purchased from The Radiochemical Centre, Amersham, Bucks. [^{14}C]Glucose had a specific activity of 3 mc/m-mole; $\text{Na}_2^{14}\text{CO}_3$ had a specific activity of 1 mc/m-mole; DL-[^{14}C]glutamic acid had a specific activity of 1 mc/m-mole; L-[^{14}C]glutamic acid had a specific activity of 5 mc/m-mole. The original materials were diluted about fivefold with carrier before injection and were dissolved in approx. 4 ml. of 0.9% NaCl. The dose of each substance was thus between 5 and 25 μ -moles/kg. body wt.

Hormonal treatment. Adrenaline, when given, was injected intramuscularly in a dose of 0.15 mg. repeated at hourly intervals during the experimental period. The dose of hydrocortisone was 15 mg./day given intramuscularly on each of the 2 days preceding the experiment and again immediately before the [^{14}C]glucose.

Blood samples. These were obtained from an ear artery for glucose estimations and by cardiac puncture for the preparation of glucosazone and for $^{14}\text{CO}_2$ analyses.

Isolation of glycogen. The mixture of tissue and alkali was kept at 100° for 20 min. and after being cooled was treated with 2.5 vol. of ethanol. After standing at room temperature for 5 hr. the precipitate was collected by centrifugation at 1200g for 10 min. and washed with methanol and chloroform as described by Kerr (1936) to remove cerebrosides. The glycogen, which remained, was reprecipitated from 77% (v/v) ethanol and heated with 30% (w/v) KOH at 100° for 2 hr. to remove nitrogenous impurities. It was then reprecipitated from aq. 66% (v/v) ethanol after treatment with dilute HCl to remove any traces of carbonate. The final precipitate was dried over CaCl_2 *in vacuo*.

Identification of the precipitate as glycogen. The precipitate, when dissolved in water and treated with iodine, gave the typical colour of glycogen. The precipitate was hydrolysed with H_2SO_4 and the hydrolysate subjected to chromatography on paper with a phenol-water system (Putman, 1957). The unhydrolysed precipitate behaved like an authentic sample of animal glycogen by failing to move from the starting line. The hydrolysate gave a single spot having the same R_F as a standard sample of glucose. The sugar was identified by spraying with a mixture containing 1.66 g. of phthalic acid and 9 g. of aniline in 100 ml. of butan-1-ol saturated with water. When hydrolysates of radioactive glycogen were chromatographed, approx. 75% of the counts recoverable from the chromatograms were found to be associated with the glucose spot. Of the remaining counts recoverable from the paper about half moved ahead of the stainable glucose spot into an area that overlapped into the galactose spot on the paper in this system. Since an equal number of counts were found on the paper immediately behind the glucose spot, whereas none were found in the middle and forward areas of the galactose spot, it seemed reasonable to conclude that glucose was the principal product of hydrolysis of the radioactive material. Finally, some radioactive glycogen was subjected to dialysis against water in the presence of carrier glucose. In this experiment 80% of the added material was recovered from inside the dialysis sac and the whole of the radioactivity was contained within this compartment, which confirmed that the radioactivity was in a macromolecular substance. Elementary analysis of the product of our isolation procedure was undertaken on a mixed sample of glycogen obtained from the brains of 15 rabbits. This was not radioactive but was prepared in exactly the same manner as the radioactive samples. The results were compatible with a formula $\text{C}_6\text{H}_{10}\text{O}_5 \cdot \text{H}_2\text{O}$. This monohydrate has frequently been isolated from organs other than brain and is said by Slater (1924) to be a very stable form of glycogen.

Allowance for the possible contaminating effect of glycogen contained in leucocytes within the blood vessels of the brain. Since Wagner (1946) reported that mammalian leucocytes contain approx. 5 mg. of glycogen/100 ml. of blood, it was clearly desirable to investigate possible interference from this source with measurements on samples of glycogen

obtained from brains, since the brains used contained a certain amount of blood.

By collecting the leucocytes from a sample of blood from a rabbit injected 6 hr. previously with [^{14}C]glucose and determining the specific activity of the glycogen contained in its leucocytes, it was calculated that contamination from this source was unlikely to contribute more than 1% to the measured counts in glycogen prepared from the brain samples.

Determination of radioactivity. With glycogen the dry material was subjected to combustion as described by Van Slyke & Folch (1940) and the total carbon and its radioactivity were measured as described by Van Slyke, Plazin & Steele (1951), the results being expressed as counts/min./mg. of C.

With glucose an osazone was prepared by the method of Hassid & McCready (1942) after the addition of carrier glucose. The osazone was then subjected to combustion in the same way as the glycogen and, since the amount of carrier added was known, the radioactivity of the original glucose was calculated in counts/min./mg. of C.

Peripheral blood glucose. This was determined by the method of Hagedorn & Jensen (1923).

RESULTS

Time-course of appearance in cerebral glycogen of ^{14}C administered as [$\text{U}-^{14}\text{C}$]glucose. This is shown in Fig. 1, which demonstrates that the radioactivity of the cerebral glycogen reaches a peak at about 8 hr., after which a slow decline sets in. The effects of hydrocortisone and of adrenaline on this process are shown in Fig. 2. It appears that hydrocortisone increases the extent of labelling at any given time, whereas adrenaline does not have any appreciable effect.

Comparison of the degree of labelling of cerebral glycogen with that of heart and skeletal muscle. Not much information is available about the rate of incorporation of ^{14}C into the glycogen either of skeletal or of cardiac muscle in the rabbit. Some measurements of incorporation of ^{14}C into the

Fig. 1. Specific activity of brain glycogen after the injection of $8\mu\text{C}$ of [$\text{U}-^{14}\text{C}$]glucose/kg. body wt. Each point shown represents the mean of duplicate samples from one rabbit's brain. Experimental details are given in the text.

Fig. 2. Effect of hydrocortisone and adrenaline on the incorporation of ^{14}C into brain glycogen from $[\text{U-}^{14}\text{C}]$ glucose ($8\mu\text{C/kg. body wt.}$). Each point represents the mean of duplicate samples from one rabbit. The broken curve and the control values are reproduced from Fig. 1. Experimental details are given in the text. ●, Normal animals; ○, animals treated with hydrocortisone; ■, animals treated with adrenaline.

Fig. 3. Incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]$ glucose into glycogen. The dose was as given in Fig. 1. Other experimental details are given in the text. ●, Brain; ▲, heart; □, skeletal muscle.

glycogen of these organs after the injection of ^{14}C glucose were therefore made for purposes of comparison with the values obtained for cerebral glycogen. The results of these measurements are given in Fig. 3. There is some scatter of points, but the general picture that emerges suggests that cerebral glycogen is labelled no less speedily than the glycogen of cardiac muscle and is labelled more speedily than the glycogen of skeletal muscle. Cardiac muscle was chosen for inclusion in this comparison because it shows continuous activity even in an animal not undertaking any particularly vigorous exertion.

Fig. 4. Effect of adrenaline and hydrocortisone on the incorporation of ^{14}C into heart glycogen after the injection of $8\mu\text{C}$ of $[\text{U-}^{14}\text{C}]$ glucose/kg. body wt. Experimental details are given in the text. ▲, Normal animals; ■, animals treated with adrenaline; △, animals treated with hydrocortisone. The broken line has been fitted to the values for the normal animals by eye.

To provide a comparison with their effects on the incorporation of ^{14}C from labelled glucose into cerebral glycogen the influence of adrenaline and hydrocortisone on the labelling of cardiac glycogen was also studied. The results shown in Fig. 4 contrast with those for cerebral glycogen in that adrenaline appears to enhance the incorporation of label into cardiac-muscle glycogen whereas hydrocortisone is without consistent effect.

Labelling of cerebral and skeletal-muscle glycogen after the injection of ^{14}C carbonate and ^{14}C glutamate. The administration of ^{14}C in the form of labelled carbonate led to the appearance of radioactivity in brain glycogen. However, the specific activity of the glycogen carbon was about one-quarter of that observed when the ^{14}C had been administered in the form of ^{14}C glucose, but the dose of radioactive material administered as labelled carbonate was also one-quarter of that given as labelled glucose.

When ^{14}C was injected either as $[\text{U-}^{14}\text{C}]$ glutamate or as $[1\text{-}^{14}\text{C}]$ glutamate the cerebral glycogen became labelled as shown in Table 1. The labelling, however, at 5 hr. after the injection was about six times as great when $[\text{U-}^{14}\text{C}]$ glutamate rather than $[1\text{-}^{14}\text{C}]$ glutamate had been administered. The total amount of radioactivity injected was the same in both instances, but the singly labelled material was racemic whereas the generally labelled precursor was in the L-form.

In the experiments with ^{14}C carbonate and both types of ^{14}C glutamate no labelling of muscle glycogen was detected.

Table 1. *Radioactivity of glycogen after the injection of 12 μ C of [14 C]glutamate or Na $_2^{14}$ CO $_3$*

Experimental details are given in the text. 'None detected' implies less than 20 counts/min./mg. of C of glycogen.

Animal no.	Time after injection (hr.)	Precursor (and dose in μ C/kg. body wt.)	Radioactivity (above background) (counts/min./mg. of C of glycogen)		
			Brain		Muscle
			Duplicate samples	Mean	
1	5	DL-[1- 14 C]Glutamate (5)	114 137	125	None detected
2	5	DL-[1- 14 C]Glutamate (5)	97 84	91	None detected
3	5	L-[U- 14 C]Glutamate (5)	612 674	643	None detected
4	5	Na $_2^{14}$ CO $_3$ (2)	254 276	265	None detected
5	5	Na $_2^{14}$ CO $_3$ (2)	224 206	215	None detected

Fig. 5. Effect of hydrocortisone on the rate of decay of the specific activity of blood glucose after the injection of 8 μ C of [U- 14 C]glucose/kg. body wt. Experimental details are given in the text. ●, Normal animals; ○, animals receiving hydrocortisone.

Time-course of changes in the specific activity of blood glucose after the intravenous injection of [U- 14 C]glucose. To interpret the experiments in which hydrocortisone was given it was necessary to know what effect, if any, the hormone produced on the rate of decay of the specific activity of the blood glucose. Fig. 5 demonstrates that there was no substantial effect under our experimental conditions.

DISCUSSION

From the foregoing results certain simple inferences can be drawn with some confidence. First, it is evident that the cerebral glycogen in a conscious rabbit undergoes turnover to a substantial extent during a period of 24 hr. (Fig. 1). Secondly, treatment of the animal with hydrocortisone promotes an increased incorporation of 14 C from labelled glucose into the cerebral glycogen, whereas adrenaline is without effect on this process. Thirdly, 14 C introduced into the animal in the form of labelled bicarbonate or glutamate as well as glucose appears in the glycogen of the brain. Fourthly, the fraction of administered 14 C that appears in cerebral glycogen is rather small; when glucose is the precursor it amounts to about 0.01%. Fifthly, it seems probable that carbon atoms derived from glutamate are incorporated into cerebral glycogen by way of some intermediate other than carbon dioxide because of the difference in behaviour of [U- 14 C]- and [1- 14 C]-glutamate. Precise quantitative evaluation of this difference is difficult, owing to the fact that [1- 14 C]glutamate was available only in the racemic form. However, if it be assumed that the D-isomer made no contribution to the labelling of glycogen, then one-half the dose of radioactivity given as [1- 14 C]glutamate produced only one-fifth of the intensity of labelling to be expected from the results with [U- 14 C]glutamate. The D-isomer was present only in trace amount, so that it should not interfere with the metabolism of the L-form, whereas if it were itself utilized this would magnify the difference between [1- 14 C]- and [U- 14 C]-glutamate.

Any attempt at a more detailed interpretation

of the data must take account of the inhomogeneity of glycogen, which has been extensively studied by Stetten and his co-workers. Their main findings have been reviewed by Stetten & Stetten (1960), and from these it is clear that, not only is glycogen a heterogeneous material with regard to molecular size, but it also exhibits metabolic inhomogeneity of a somewhat complex kind. For when glycogen is isolated from the livers and muscles of animals that have received labelled glucose it is found on the one hand that the molecules of different sizes become labelled at different rates whereas on the other hand different portions of individual molecules also become labelled serially. Hence the extent of labelling measurable at any particular time will be determined by the method of preparation of the glycogen samples used. This may select out of any initially heterogeneous collection of molecules those having sizes within a certain range or may cause some degradation of the molecules isolated, with either preferential removal or preferential preservation of labelled atoms depending on the location of these within the original macromolecular aggregate. Though the metabolic inhomogeneity of glycogen has been demonstrated with material derived from either liver or muscle, it seems reasonable to expect that the same considerations will apply to cerebral glycogen, although, owing to the small amounts present, the direct demonstration of this may prove technically difficult. Hence it is meaningless to attempt to calculate a single half-life of cerebral glycogen. However, the presence of labelled atoms in the glycogen of brain may safely be taken as evidence that some synthesis of glycogen has occurred since the injection of labelled precursor and this does give a definite indication of lability. Moreover, although Stetten, Katzen & Stetten (1956) found that muscle and liver glycogen differed with respect to the relative extent of labelling in small and large molecules, evidently well-marked dissimilarities between organs with respect to the time-course of labelling of their contained glycogen must imply differences in some aspects of glycogen turnover in the two situations. Similarly, if an agent such as a hormone alters the time-course of labelling of glycogen in a particular organ this again must imply an effect, direct or indirect, on one or more steps in the process of glycogen synthesis or glycogen breakdown in that organ. At the same time, it must be recognized that a straightforward isolation of the glycogen by the so-called 'classical' method of Pflüger (1909), involving hot potassium hydroxide precipitation from aqueous ethanol followed by counting the radioactivity of the product, can give only an incomplete picture of what is going on in the glycogen of the tissue.

Thus it seems to be well established from the

work of Stetten *et al.* (1956) that the heating of glycogen in the presence of caustic alkali or even water leads to a considerable decrease in the molecular weight and may result also in the actual conversion of macromolecular material into diffusible derivatives that might well escape precipitation by ethanol in the concentrations that we have employed. The molecular weight of a sample of cerebral glycogen prepared according to the procedure described in the present paper was kindly determined by Dr A. Roger in the Department of Biochemistry at Oxford by the method of Bell, Gutfreund, Cecil & Ogston (1948). It was found to be between 1.5×10^6 and 2.0×10^6 , which is of the same order as that found for rabbit-liver glycogen after treatment with potassium hydroxide as reported by Stetten *et al.* (1956).

In view of all these potential complicating factors it must be recognized that, had we been able to prepare cerebral glycogen for analysis by some procedure not involving exposure to hot alkali, our curves for the time-course of labelling might well have presented a different appearance. Nevertheless, it seems fair to claim that, judged by this admittedly imprecise criterion, the glycogen of brain appears to turn over no less rapidly than that of cardiac and non-exercising skeletal muscle, and by comparison with the results of Stetten & Boxer (1944) not very much more slowly than that of liver.

The absence of any detectable incorporation of ^{14}C into the muscle glycogen after the injection of $\text{Na}_2^{14}\text{CO}_3$, whereas considerable incorporation into cerebral glycogen was observed, is of doubtful significance, since when the ^{14}C was administered as labelled glucose the labelling of glycogen in the muscles was only one-quarter as heavy as that in the brain. If the same ratio held after the injection of labelled carbonate the labelling in muscle would be too slight to be measured at all accurately. However, Waelsch, Berl, Rossi, Clarke & Purpura (1964) have produced evidence of carbon dioxide fixation by brain, and this may be relevant to the present findings.

The enhancement of ^{14}C incorporation from glucose by treatment with hydrocortisone raises the question whether this is to be attributed to increased uptake of glucose, as precursor from the blood stream, or involves gluconeogenesis from non-carbohydrate sources. Timiras, Woodbury & Baker (1956) found an increased total glycogen in brain after treatment with a glucocorticoid, and our finding might therefore at first sight appear to indicate an increased rate of condensation of glucose molecules derived from the blood. However, Gaitonde, Marchi & Richter (1963) have shown that when radioactive glucose is injected into a cat some 55–70% of the radioactivity taken up by the brain appears in the amino acids of the organ within

22 min. Hence, if the net increase in glycogen found by Timiras *et al.* were due to gluconeogenesis, the rapid labelling of the glycogen after giving [^{14}C]-glucose could be brought about via the amino acid pool. Evidence for gluconeogenesis in brain is lacking, and in fact in a recent study of the distribution of one of the essential enzymes involved, fructose 1,6-diphosphatase, Krebs & Woodford (1965) failed to find it in rat brain, although traces of activity were found in sheep and ox retina. This enzyme does, however, show considerable variations in activity between species, and failure to demonstrate it in rat brain does not necessarily exclude the possibility of its occurrence in this organ in the rabbit. In any event, it seems clear that the finding of Timiras *et al.* (1956) cannot be regarded as a consequence of diminished degradation of cerebral glycogen, for if this were so the rate of increase in specific activity should have been decreased rather than augmented.

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