Deuterioglucose: Alteration of Biodistribution by an Isotope Effect

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Carbon-14 glucose, in which all carbon-hydrogen bonds were replaced by carbon-deuterium bonds (deuterioglucose), was extracted from algae growing in heavy water and exposed to [^{14}C]carbon dioxide. The identity of [^{14}C]deuterioglucose was confirmed by comparison with authentic material on two high performance liquid chromatography and two thin layer chromatography systems. Fermentation to lactate followed by oxidative decarboxylation demonstrated that 35% of the ^{14}C was on carbons 3 and 4 for deuterioglucose isolated from a 24-hr algal incubation, and 61% for a 20-min incubation. Mice were injected intravenously with either (24-hr) deuterioglucose or with ^{14}C-labeled (protio)glucose labeled uniformly. The deuterioglucose cleared more slowly from the blood, while heart and brain accumulated label more slowly. Tissue concentrations peaked at later times for deuterioglucose. Deuteration may be a useful feature of radiopharmaceutical design.

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Several radiotracers are available for the study of glucose metabolism and/or transport in the intact human by means of positron tomography. In addition to carbon-11 (^{11}C) glucose prepared by photosynthesis (*1,2*) or by chemical synthesis (*3*), a number of positron-labeled glucose analogs have been studied. The most widely used agent is fluorine-18 (^{18}F) 2-deoxy-2-fluoro-D-glucose (2FDG) (*4*) whose metabolism stops after formation of the 6-phosphate. Thus, as originally suggested by Sols and Crane (*5*) for 2-deoxy-D-glucose, 2FDG essentially isolates the hexokinase step in glycolysis. As an alternative to 2FDG, 2DG is available labeled with ^{11}C (*6,7*). Unlike these 2-substituted analogs, substitution of the —OH group at position 3, by a fluorine atom or a methoxy group results in well-transported but poorly phosphorylated analogs (*8,9*); local glucose transport rates in the human brain have been measured using both [^{18}F]3FDG (*10*) and [^{11}C] 3-O-methyl D-glucose (*11*). In principle, indices of both metabolism and transport can be extracted from measurement of tissue kinetics after administration of [^{11}C]glucose (*12,13*). However, the very rapid entry of carbon from labeled glucose into many intermediary metabolites must complicate the interpretation of kinetic data (*14*). In this respect the simplified metabolism of glucose analogs is advantageous. However, the use of analogs may require assumptions about the extent to which transport and phosphorylation of glucose and its analogs are constantly related in various metabolic and pathologic states (*15*).

A possible alternative to the principle of functional group replacement (—H, —F or —OCH_3 for —OH) in the design of substrate analogs is the substitution of carbon-deuterium for carbon-hydrogen bonds. Isotope effects for heavier atoms than hydrogen (e.g., replacement of oxygen-16 by oxygen-18) are very small because they are related to the difference in mass of the isotopes. However, when H is replaced by D the mass doubles and quite a difference in reaction rates can sometimes be observed (*16*). Glucose in which the hydrogen atom attached to C-1 is replaced with deuterium is oxidized three or four times more slowly than the unsubstituted molecule by the bacterial enzyme, glucose oxidase (*17*). In this reaction, the sugar aldehyde function (CHO) is oxidized to a carboxylic acid (CO_2H), and the C—H bond must be broken for this to occur. Large kinetic deuterium isotope effects are typically found when the splitting of a C—H bond is the slowest step in a sequence of reactions. Smaller secondary isotope effects occur in some reactions where C—H bonds are not broken.

Glucose in which all C—H bonds are replaced by

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C—D (deuterioglucose) is available commercially†. It is extracted from algae made to grow in 99.7% D_2O (*18*). Carbon-11-labeled deuterioglucose can thus be prepared from $[^{11}C]CO_2$ by algal photosynthesis in the same way as undeuterated glucose (protioglucose) (*2*). Although glucose oxidase is not involved in glycolysis, which is the dominant pathway of glucose metabolism in mammals, it seemed possible that isotope effects would be manifested at some points between entry of glucose into cells and production of CO_2. In fact, an overall isotope effect on whole-body glucose turnover was suggested by studies in which blood levels of deuterioglucose were measured mass spectroscopically during constant infusion experiments and compared with levels of [^{14}C]glucose (*19,20*). In contrast, glucose in which only the H atoms on C-6 were replaced by D did not appear to exhibit a significant isotope effect in vivo (*21,22*).

MATERIALS AND METHODS

Fully Deuterated Algae

Scenedesmus lividus maintained for many generations in growth medium based on 99.6% deuterium oxide were used (*18*).

Preparation [^{14}C]Deuterioglucose

Procedure I. About 2 ml of packed algal cells were added to 100 ml of growth medium in a 1-l flask irradiated with a tungsten lamp. The flask was flushed with nitrogen gas for 10 min before generation of labeled carbon dioxide by addition of concentrated sulfuric acid to [^{14}C]barium carbonate in a center-well. After 24 hr, the flask was again flushed with nitrogen and the cell suspension centrifuged for 10 min at 1000 *g*. The algal pellet was refluxed (oil-bath) in 10 ml of 2N HCl for 60 min, and the acid removed by rotary evaporation. The residue was re-dissolved in 10 ml of water and passed sequentially through carbonate-form and hydrogen-form ion exchange resins (1 × 5 cm columns). Following rotary evaporation of the water, the residue was taken up in 10 ml of absolute ethanol and passed through a silica gel column (1 × 5 cm) and an activated charcoal column.

Procedure II. Algae (1 ml of packed cells) in 30 ml of deuterated growth medium were placed in a 250-ml flask which was then flushed with N_2, capped, and exposed to light for 10 min. Carbon-14 carbon dioxide was then introduced into the flask and irradiation continued for 20 min. The algae were added directly to 100 ml of boiling 95% ethanol and boiled for 5 min. After cooling to room temperature the mixture was filtered and added to an equal volume of dichloromethane. The dark green organic phase was discarded and the almost colorless aqueous phase (which contained 600 mCi) was rotary evaporated to dryness. Invertase (1 mg) in 5 ml of water was added and portions of the solution taken directly for purification by high performance liquid chromatography (HPLC).

Chromatography

Two HPLC systems were used for carbohydrate analysis: (a) an amino column eluted at room temperature with 70% aqueous acetonitrile at 1.5 ml/min, and (b) an HPX-87P‡ column maintained at 80° and eluted with water at 0.6 ml/min. In both systems, glucose and deuterioglucose had identical retention times (10 min for system I and 14 min for system II). Additionally, two thin layer chromatography (TLC) systems were used: two-dimensional chromatography on cellulose, with butanol/acetic acid/water (4:1:5, v/v) followed by 80% phenol; and standard TLC on silica gel with ethyl acetate/ethanol (1:1, v/v).

Metabolite Analyses

Animals were killed and brains, hearts, and samples of liver and blood transferred rapidly to beakers containing 3 ml of 0.3*M* $HClO_4$ at 0°. The tissues were minced with scissors and then homogenized. Precipitated protein was removed by centrifugation and the supernatants carefully neutralized (pH 5–9) with KOH. After removal of the $KClO_4$ precipitate, 1-ml samples were passed through two 1 × 5 cm ion exchange columns in tandem. The first contained H^+-form Dowex 50 (×8) resin and the second HCO_2^--form Dowex 1 (×8) resin. The columns were washed with 10 ml of water which was collected in a scintillation vial. The H^+-form column was then washed with 2*M* NH_4OH and the HCO_2^--form column was washed successively with 0.2M HCO_2H and 4*M* HCO_2H which were collected in separate vials. Each vial was dried at 60° under a stream of N_2. Neutral compounds such as glucose are collected in the water, aminoacids in the NH_4OH, carboxylates in the 0.2*M* HCO_2H, and phosphate esters in the 4*M* acid (*23*).

Isotopic Labeling Pattern

Deuterioglucose (10 nCi) was incubated with 100 mg (wet wt) of freshly harvested cells of *Lactobacillus casei* (*24*). After 1 hr HPX-87P HPLC showed the absence of deuterioglucose, while on anion exchange HPLC (SAX column; mobile phase 5 m*M* potassium phosphate, pH 4.7) 90% of the radioactivity eluted with lactate (RI detector) at 10 min with a flow rate of 1.4 ml/min. Samples of this [^{14}C]lactate were placed in scintillation vials equipped with center wells containing 0.2 ml of 2*M* KOH. Decarboxylation was initiated by addition of 0.1 ml of 1*M* $KMnO_4$ and the vials were quickly capped and left at 60°. After 8 hr the alkaline trapping solution was transferred to a fresh vial for liquid scintillation counting.

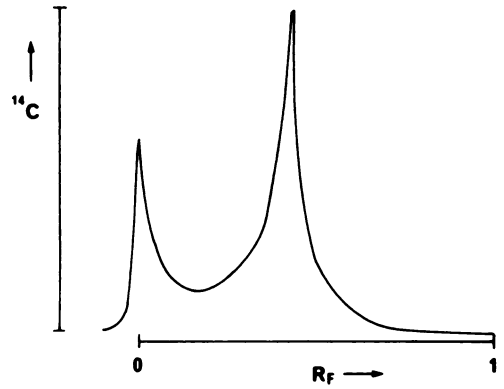


FIGURE 1
Silica gel TLC of algal extract after passage through Dowex and charcoal columns

Tissue Distributions

Mice from local inbred strains were injected through the tail vein with 0.05 ml of 0.9% saline containing 0.1 μCi of [^{14}C]protioglucose or deuterioglucose. The animals were killed at 1, 15, 30, 60, or 120 min postinjection and samples of blood, brain, heart, and liver were taken. The weighed tissue samples, and the entire tail, were digested for 12 hr at 50° in 2 ml of Scintigest plus 0.5–1.0 ml of water. After digestion, the blood samples were decolorized with a drop of 30% hydrogen peroxide. Scintillation cocktail (15 ml) was added to each vial and, after waiting for 3–7 days for chemiluminescence to decay, samples were counted. Quench correction was performed by re-counting samples after addition of 25 nCi of ^{14}C to each vial.

Distribution data were expressed as percent injected ^{14}C per g of tissue. Animals whose tails contained more than 10% of the injected dose were discarded from consideration.

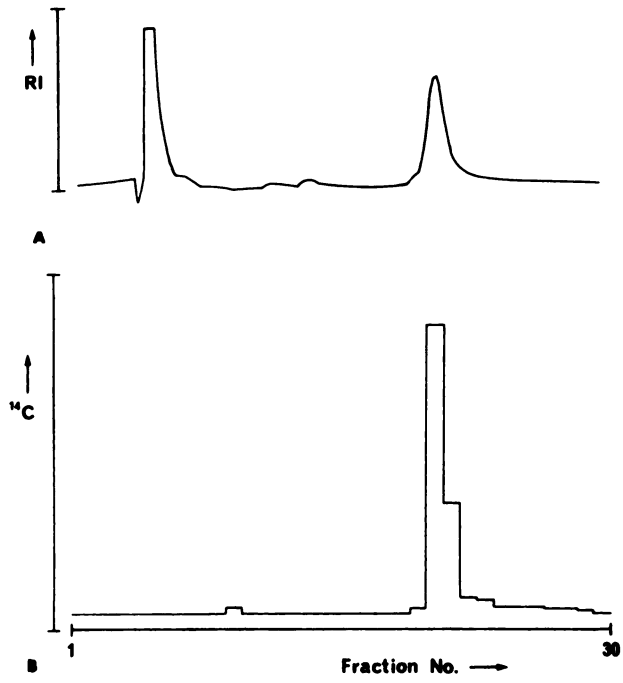


FIGURE 2
A: Aminopropyl normal phase HPLC of final product. B: Retention time of authentic deuterioglucose was established with refractive index detector

RESULTS

Preparation and Purification of [^{14}C]Deuterioglucose

Chromatographic analysis showed the ^{14}C in the final ethanol extract of Procedure I to be 90% glucose, in a yield based on the carbon dioxide of 4%. Material of higher purity but low specific activity was obtained by recrystallizing authentic glucose from a portion of the ethanolic extract. Silica gel TLC was used to monitor fractions during isolation of [^{14}C]deuterioglucose from the algae. An isotope effect was manifested between protio (Rf = 0.5) and deuterio (Rf = 0.45) glucoses when plates were eluted with ethanol/ethylacetate (1:1, v/v). The major radioactive peak (Fig. 1) in the final ethanol extract of Procedure I coincided with the deuterioglucose spot produced by charring the chromatogram. In chromatograms of the initial neutralized acid extract of the algae, most of the ^{14}C was contained in two broad peaks with $R_f = 0$ and $R_f = 0.1$. Passage through carbonate-form ion exchange resin removed the material with $R_f = 0.1$, so that this peak presumably represents carboxylic acids and/or acid-stable phosphates. Material which stayed at the origin on silica gel TLC was removed by passage through silica gel.

The radioactivity trace (Fig. 2A) was obtained with the recrystallized fraction on the amino column. The corresponding refractive index trace (Fig. 2B) shows co-elution of a mixture of protio and deuterio glucose. Peak widths for protio and deuterio compounds and for the mixture were identical, showing that there is no isotope effect for chromatography in this system. Similar results, indicating 90% purity and co-elution of protio- and deuterioglucoses were found with the HPX-87P column. Figure 3 is a radiochromatogram of the supernatant fluid remaining after most of the ^{14}C was removed by crystallization. Impurities are thus concentrated in this fraction. Although glucose was still the major component, several other peaks were apparent, and expected retention times for several other sugars are indicated.

Deuterioglucose purified by HPX-87P HPLC was subjected to two-dimensional cellulose TLC (Fig. 4). A single spot indistinguishable from that given by [^{14}C]glucose was found on autoradiography.

Procedure II was faster than Procedure I (about 2 hr, but this could be considerably reduced) and gave a yield of chromatographically purified ^{14}C deuterioglucose of about 6%.

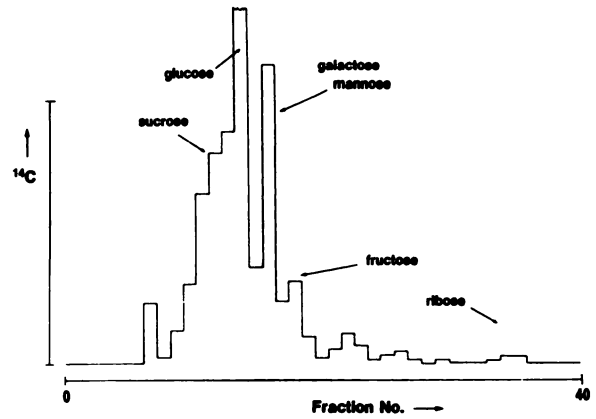


FIGURE 3
Trace impurities in deuterioglucose preparation. HPX-87P column was used, eluted with water at 80°. Glucose peak is approximately ten times larger than galactose/mannose peak

Isotopic Labeling Pattern

Deuterioglucose isolated from algae exposed to [^{14}C]carbon dioxide for 24 hr contained 35% of the label on carbons 3 and 4 (Table 1). This is consistent with universal labeling. In contrast, deuterioglucose made with a 20-min exposure had 61% of the ^{14}C on the central carbons.

Tissue Distributions

Distributions of [^{14}C]deuterioglucose and universally labeled protioglucose were rather similar (Table 2). Blood radioactivity fell more slowly for deuterioglucose, while radioactivity in the brain rose more slowly and reached a maximum value at 60 min, compared with 30 min for protioglucose. Radioactivity in the heart also peaked later for deuterioglucose. The distribution of labeled protioglucose was broadly consistent with that reported in rats for photosynthetic [^{11}C]glucose by Jones et al. (*25*). The time course of label from

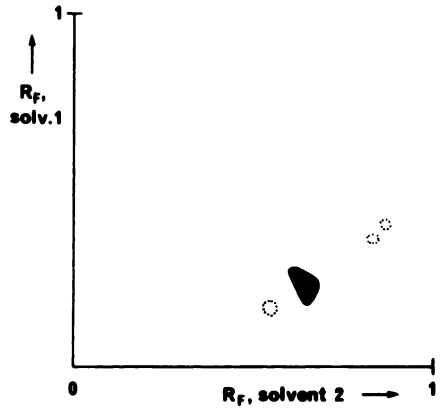


FIGURE 4
Two-dimensional cellulose TLC of deuterioglucose after purification on HPX-87P column. Three minor spots were located by overexposure of autoradiograph

TABLE 1
Percent of ^{14}C in Carbon Atoms 3 and 4 of Samples of Deuterioglucose and Commercial Protioglucose*

Sample	Exposure time	Percent in carbons 3 and 4
Deuterioglucose	24 hr	35 ± 2
Deuterioglucose	20 min	61 ± 5
U-[^{14}C]glucose	—	37 ± 4
3,4-[^{14}C]glucose	—	96 ± 3

* Values are the mean ± s.d. for three measurements of ^{14}C evolved.

protioglucose in brain (Table 2) was consistent with the variously labeled ^{14}C glucoses examined by Sacks et al. (*14*).

Tissue Metabolites

Analysis of ^{14}C in tissues, 10 min after administration of glucose or deuterioglucose, according to broad classes of compounds is presented in Table 3. Several points may be made.

1. Most of the label in the blood is neutral, presumably unchanged glucose.
2. A large fraction of the label in brain behaves like aminoacids, as expected (*26*).

TABLE 2
Distribution of [^{14}C]Deuterioglucose and Universally Labeled [^{14}C]Protioglucose in Mice*

Tissue	Minutes	Protioglucose	Deuterioglucose
Blood	1	3.84 ± 1.02	3.68 ± 1.06
	15	2.62 ± 0.61	2.69 ± 0.51
	30	1.89 ± 0.58	2.53 ± 0.35
	60	0.70 ± 0.22	2.18 ± 0.24
	120	0.41 (0.29–0.54)	0.77 (0.61–0.99)
Brain	1	1.18 ± 0.38	0.61 ± 0.26
	15	2.85 ± 0.67	2.05 ± 0.54
	30	5.20 ± 1.15	4.19 ± 0.93
	60	2.02 ± 0.29	4.32 ± 1.06
	120	1.09 (0.67–1.38)	2.21 (1.34–2.69)
Heart	1	1.89 ± 0.58	1.57 ± 0.61
	15	3.23 ± 0.86	1.95 ± 0.58
	30	2.75 ± 0.48	2.53 ± 0.42
	60	1.92 ± 0.54	3.14 ± 0.48
	120	1.76 (1.12–2.50)	1.54 (1.18–2.37)
Liver	1	2.50 ± 0.56	1.31 ± 0.51
	15	4.38 ± 0.99	2.02 ± 0.98
	30	3.97 ± 1.38	5.76 ± 1.63
	60	2.46 ± 0.67	3.17 ± 1.34
	120	1.47 (0.93–2.27)	0.77 (0.54–1.15)

* Values are mean ± s.d. for five animals, or mean and range for four animals, in % injected dose per g of tissue.

TABLE 3
Analysis of Metabolites of [^{14}C]Glucose and Deuterioglucose in Tissues

Tissue	Fraction			
	H_2O	0.2*M* HCO_2H	4*M* HCO_2H	NH_3
Deuterioglucose				
Blood	71 ± 18	13 ± 5	9 ± 2	11 ± 4
Brain	9 ± 2	32 ± 6	15 ± 5	44 ± 10
Heart	21 ± 9	26 ± 11	23 ± 12	31 ± 13
Liver	71 ± 12	14 ± 4	15 ± 1	19 ± 7
Protioglucose				
Blood	79 ± 7	8 ± 3	4 ± 2	9 ± 3
Brain	4 ± 3	27 ± 6	10 ± 3	60 ± 11
Heart	27 ± 17	21 ± 2	15 ± 4	38 ± 17
Liver	78 ± 4	9 ± 2	6 ± 1	8 ± 2

* Values are the mean ± s.d. for tissues from four animals, for percent of total ^{14}C in each tissue.

3. The large neutral fraction in the liver may include glycogen, as well as glucose.

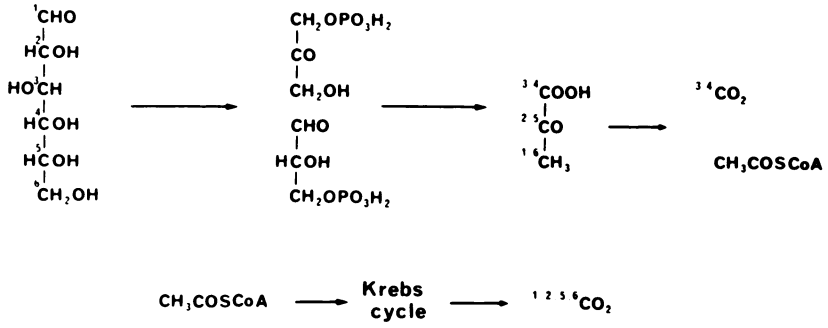
4. There was more label in the 4M-HCO_2H fraction of the brain, heart, and liver of animals given deuterioglucose, which presumably represents sugar phosphates.

DISCUSSION

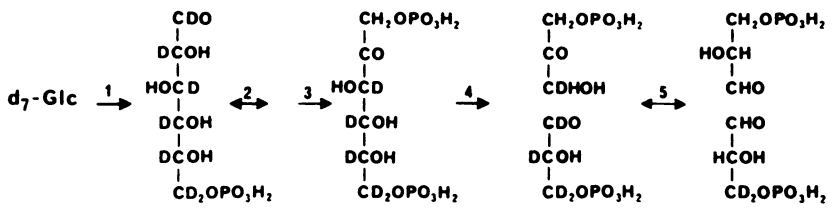
It is of the utmost importance, when isolating one component of a complex mixture of labeled compounds from a biological system, to be sure of the identity of that component. That our major product is [^{14}C]deuterioglucose is strongly supported by identical chromatographic behavior with commercially available deuterioglucose on both cellulose and silica gel TLC, and on ion moderated partition HPLC and aminopropyl bonded phase HPLC. All these systems are individually capable of separating glucose from many other sugars and other metabolites. The two HPLC systems in addition to gas chromatography were used by Ehrin et al. (*2*) to demonstrate the identity of [^{14}C]protioglucose produced by algae. The assignment as deuterioglucose is also supported by the ability of *L. casei* to ferment the material to lactate. Silica gel TLC was able to partially separate protio and deuterioglucoses. Deuterioglucose also travels slightly more slowly then protioglucose on some paper chromatographic systems (*27*). An interesting aspect of purity arises in this work; the possible synthesis of glucose containing both protium and deuterium atoms by algae, which grow better in light water. To avoid this possibility every precaution was taken to exclude traces of light water from glassware and algal suspensions. In principle, nonbiological routes to deuterioglucose could be employed. Carbon-14-labeled material could be made by catalytic deuteration of [^{14}C]protioglucose (*27*) while either [^{11}C] or [^{14}C]deuterioglucose could be prepared by chemical synthesis using deuterated precursors and reagents.

A major problem with photosynthetically produced [^{11}C]protio or deuterioglucose is the uncertainty in the relative amount of label on each of the six carbon atoms. This is important because the fate of each carbon atom is different (Scheme 1). The major biochemical pathway of glycolysis involves production of two molecules of pyruvate which are decarboxylated to acetyl CoA; carbons 3 and 4 are lost as carbon dioxide at this point. Carbons 1, 2, 5, and 6 are lost in the subsequent activity of the Krebs cycle; the appearance of labeled carbon dioxide from these carbons is slower because of the dilution of isocitrate and alpha-oxoglutarate (the decarboxylase substrates of the cycle) by the pool of metabolites in equilibrium with the cycle intermediates. Additionally, in tissues where the pentose phosphate shunt is important a fraction of label on carbon 1 is separately oxidized to carbon dioxide. These considerations, which have been discussed by Sacks et al. (*14*), make interpretation of the clearance phase of ^{11}C kinetics impossible unless the labeling pattern is defined.

Glucose produced during photosynthesis in the first few seconds after introduction of labeled carbon dioxide is labeled predominantly on carbons 3 and 4, but as the Calvin cycle intermediates become radioactive the situation tends towards universal labeling after several hours (*28,29,30*). The [^{14}C]deuterioglucose used in animal experiments, made from a 24-hr incubation, had one third of the label on C-3 and C-4, and all six carbon atoms probably had the same specific activity. Carbon-



SCHEME 1
Oxidation of glucose carbon atoms to carbon dioxide. Carbon atom 3 and 4 are lost as carbon dioxide at pyruvate dehydrogenase step. Other carbons proceed, as acetyl coenzyme A, into Krebs cycle and are thus diluted by cycle intermediates and other metabolites which interact with them



SCHEME 2
Exchange of deuterium from deuterioglucose during metabolism. Deuterium is lost from deuterioglucose, not necessarily completely, as consequence of enzymatic reactions. 1: Hexokinase; 2: Hexose phosphate isomerase; 3: Phosphofructokinase; 4: Aldolase; 5: Triose phosphate isomerase

14 deuterioglucose made by a 20-min exposure had 60% of the label on carbons 3 and 4 (Table 1), and therefore was quite unsymmetrically labeled. A shorter exposure time should yield a more specifically 3,4-labeled product.

Loss of deuterium from deuterioglucose occurs during metabolism; deuterium is removed (not necessarily completely) from carbons 1 and 2 by hexose phosphate isomerase and from carbons 3, 4, and 5 by triose phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase (Scheme 2). Loss of tritium from carbons 2, 3, and 5 of glucose in rat liver cells was discussed by Katz et al. (*23*). Deuterium remaining on pyruvate and acetyl CoA is lost at the first two steps of the Krebs cycle, the citrate synthetase and aconitase reactions (*31*). Possible isotope effects in the kinetics of deuterioglucose are therefore limited to the enzymes before aconitase.

It was originally hoped that a pronounced overall deuterium isotope effect in the metabolism of deuterioglucose might slow the tissue kinetics to the point where effective "metabolic trapping" would occur (*32*). Work with isolated enzymes has revealed an isotope effect of 1.4 for tritium substitution at either C-3 or C-4 of aldolase (*33*). The effect should be more pronounced with substitution at both carbons. Similarly, an isotope effect of 1.9 for deuterium substitution at C-2 was found for hexose phosphate isomerase (*34*). Although the content of ^{14}C in heart and brain was greater at 30 and 60 min for deuterioglucose than for [^{14}C]glucose (Table 2), the metabolite assays (Table 3) argue against substantial metabolic trapping, since there was only a small increase in the 4N formic acid fraction (which would include phosphate esters) in brain, heart, and liver. An explanation for these small differences may be that an isotope effect for an individual enzyme will only be fully expressed in the flux through the whole glycolytic pathway to the extent that the enzyme is rate limiting. Together with the slower blood clearance of deuterioglucose, the metabolite patterns and organ distributions do demonstrate that per-deuteration alters the transport and/or metabolism of glucose. Two other possible explanations for the different behavior of the two sugars are radiochemical impurity of the deuterioglucose and nonuniform labeling. These appear unlikely because of the chromatographic data (Figs. 1-3) and the degradation experiment (Table 1).

It may be possible to exploit deuterium isotope effects in the design of other positron radiotracers. Large isotope effects which lead to qualitative changes in metabolism have been demonstrated on partial deuteration of caffeine (*35*). A 13-fold change in anti-tumor potency has been reported after deuteration of cyclophosphamide (*36*). Ways in which similar effects could be important for radiopharmaceuticals thus include reducing radiation doses to critical organs by altering patterns of metabolism and excretion as well as changing target tissue uptake and clearance kinetics.

FOOTNOTES

† Merck Sharp & Dohme, Rahway, NJ.
‡ Bio-Rad Laboratories, Richmond, CA.

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