Biochimica et Biophysica Acta, 688 (1982) 5–10
Elsevier Biomedical Press

BBA 71133

USE OF TRITIATED 3-*O*-METHYL-D-GLUCOSE FOR STUDIES OF MEMBRANE TRANSPORT CAVEAT

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(Received October 12th, 1981)

Key words: Methylglucose; Tritiated monosaccharide; Membrane transport; (Frog skeletal muscle)

Tritiated 3-*O*-methyl-D-glucose has many useful attributes as a model substance for studies of the transport of glucose across cell membranes. However, preparations of high specific radioactivity can decompose within a few months, producing radioactive impurities that can cause a several-fold increase in the apparent rate of sugar transport. In our investigation radioactive contaminants entered frog skeletal muscle cells by free diffusion rather than by facilitated transport. Much of the contaminating radioactive material could be removed by evaporating the solvent and redissolving the sugar. Tritiated sugar samples that had a specific activity below 0.1 Ci/mmol remained stable and suitable for transport measurements after several years of storage at −20°C. In order to evaluate the suitability of a given tritiated preparation of sugar for transport measurements, it is recommended that its behavior be compared with that of a stable reference standard of low specific activity.

Introduction

Tritiated 3-*O*-methyl-D-glucose is widely used to investigate the kinetics of sugar transport across cell membranes [1–4]. Among its advantages, this sugar is not metabolized by animal tissues [1,2,5], and it is handled by the same system of facilitated transport that carries glucose across cell membranes [1–3,6]. A disadvantage, not generally acknowledged in published descriptions (but see Whitesell and Gliemann [7]) is that under certain conditions decomposition of [^{3}H]methylglucose can form radioactive impurities which can seriously interfere with proper measurements of transport rates. In this report, the manner in which radioactive impurities can affect the apparent rate of transport of [^{3}H]methylglucose has been analyzed kinetically. Methods for the detection and removal of contaminants are described, and ways of improving radiochemical stability are evaluated.

Experimental procedures

Chemicals. 3-*O*-Methyl-D-glucose that contained only 0.06% glucose was a generous gift from Dr. John T. Snow of the Calbiochem-Behring Corp. The compound was nonselectively tritiated by the method of Wilzbach [8] at New England Nuclear Corp. In our laboratory readily exchangeable tritium was removed by repeated evaporation of aqueous solutions, and the labeled sugar was purified by preparative paper chromatography and recrystallized, as described previously [1]. Four preparations had specific activities ranging from 0.02 to 0.03 Ci/mmol and exhibited rates of penetration into frog muscle cells that remained essentially constant when used for periods of 2 to

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0005-2736/82/0000–0000/$02.75

5 years each. The single preparation to be used in the present experiments was further purified by chromatography on a column of Dowex 1-formate. Fractions from the center of the main peak that was eluted with H_2O were evaporated to dryness, redissolved in a small volume of H_2O at approx. 0.6 mCi/ml, and stored at −20°C.

3-*O*-[^{3}H]Methyl-D-glucose that had been synthesized with the use of [^{3}H]methyl iodide was obtained from New England Nuclear Corp. 3-*O*-[^{14}C]Methyl-D-glucose and a chromatographically repurified preparation of 3-*O*-[^{3}H]methyl-D-glucose were generously supplied by Nancy M. Wilbar of New England Nuclear Corp. 3-*O*-Methyl-D-[1-^{3}H]glucose that had been selectively labeled by a catalytic Wilzbach method was obtained from Amersham Corp. D-[1-^{14}C]Mannitol and D-[1-^{3}H]mannitol were obtained from New England Nuclear Corp.

Since some of the commercially supplied radioactive compounds were in solutions that contained alcohol, each preparation was evaporated to dryness at 35°C in a Buchler Rotary EvapoMix tube connected to a vacuum pump through two traps, which were chilled in baths of alcohol and solid CO_2. The dried radioactive sugars were immediately redissolved in cold H_2O to give a concentration of approx. 0.2 mCi/ml and were stored at −20°C.

Handling of tissues. Female *Rana pipiens* 4–5 cm long were obtained in winter from Rand McNally Bio-Centre (Somerset, WI) and kept in pans of water at 5°C. Isolated frog sartorius muscles were incubated at 19°C, with mechanical shaking, in 2 ml of frog Ringer's bicarbonate solution [9], and the rate of entry of 3-*O*-methylglucose was measured by procedures described previously [1].

Calculation of transport constants. All transport rates are here expressed as the rate of entry of 3-*O*-methylglucose, even though some of the radioactive material that entered the cells appeared to differ from the sugar in its transport kinetics and chemical properties. Kinetic analysis revealed that v, the observed rate of entry, has two components: v_C, a saturable or carrier-mediated system of transport, and v_D, which resembles free diffusion:

$$v = v_C + v_D$$

The carrier-mediated system approached saturation at 36 mM sugar. Higher concentrations were not employed because hyperosmolarity alters permeability [10].

The rate of free diffusion is proportional to the concentration of sugar, S

$$v_D = k_D S$$

The value of k_D was determined by measuring the increase in v between 20 and 36 mM sugar. Carrier-mediated transport was not completely saturated in this concentration interval, and v_C, as calculated from V_{max} and the apparent K_m, contributed approx. 10% to the value of k_D determined in this manner. However, this effect did not interfere with the pattern of observations reported below, and no correction was made in the value of k_D.

In the absence of radioactive contaminants the rate of entry of [^{3}H]methylglucose was only slightly higher at 36 mM than at 20 mM. The effect of variations in the permeability of muscles from different frogs was eliminated by calculating k_D for paired muscles from the same frog at the two concentrations of sugar.

For calculation of kinetic constants of the saturable component of transport, v was measured at 1, 4, 12, 20, and 36 mM 3-*O*-methylglucose, and v_C was determined by subtracting the calculated value of v_D from each value of v. V_{max} and the apparent K_m were calculated by the method of least squares from a plot of $1/v_C$ against $1/S$ [11].

Results

The kinetic behavior of a stable preparation of [^{3}H]methylglucose will be described as a basis of comparison for other, less stable ones. Preparation A′ had a k_D of 0.020 h^{-1} (Table I). When the solution was dried, redissolved in H_2O (preparation A″) and stored for 6 months longer, the k_D remained unchanged. The k_D for [^{14}C]methylglucose (preparation B′) was essentially the same as for preparation A′. Low k_D values like these have been obtained consistently in our studies with highly purified, apparently stable preparations of radioactively labeled 3-*O*-methylglucose.

Strikingly different results were obtained with C′, a preparation of 3-*O*-[^{3}H]methyl-D-glucose that entered frog muscle cells at an unusually fast rate,

TABLE I

APPARENT RATE OF ENTRY OF VARIOUS PREPARATIONS OF RADIOACTIVELY LABELED 3-*O*-METHYLGLUCOSE INTO MUSCLE CELLS

Preparation A was purified in our laboratory after tritiation at New England Nuclear Corp. Preparations B–E were obtained from New England Nuclear Corp., and preparations F and G were from Amersham Corp. Preparation D is an aliquot of preparation C that was chromatographically repurified at New England Nuclear Corp. The type of label on the sugar is indicated in parentheses. A prime indicates that the preparation was dried and dissolved in H_2O once, prior to storage and testing of transport behavior. A double prime indicates that the preparation was dried and dissolved a second time, before further storage and testing. In the third column the duration of storage refers to the interval between the last drying and assay. Preparation G had a specific activity of 2.3 Ci/mmol when it was received; after it had been dried and redissolved, carrier 3-*O*-methylglucose was added to bring the specific activity to 0.076 Ci/mmol before storage at −20°C. Values for v are stated as the mean ± S.E. for the number of muscles indicated in parentheses; for k_D, the number of calculations, based on muscle pairs, is indicated in parentheses.

Preparation	Specific activity	Duration of storage at −20°C	v at 20 mM (μmol·ml^{-1}·h^{-1})	v at 36 mM (μmol·ml^{-1}·h^{-1})	k_D (h^{-1})
A′(^{3}H)	0.02	5 years	0.73 ± 0.11(12)	1.05 ± 0.10(12)	0.020 ± 0.002(12)
A″		6 months	0.92 ± 0.16 (8)	1.26 ± 0.17 (8)	0.021 ± 0.003 (8)
B′(^{14}C)	0.36	1 week	1.13 ± 0.16 (6)	1.43 ± 0.14 (6)	0.019 ± 0.003 (6)
C′(^{3}H)	80.0	1 year	8.2 ± 0.3 (6)	16.0 ± 0.5 (6)	0.49 ± 0.02 (6)
C″		4 days	2.5 ± 0.1 (4)	4.4 ± 0.2 (4)	0.12 ± 0.01 (4)
C″		3 months	6.1 ± 0.2 (4)	9.6 ± 0.3 (4)	0.22 ± 0.02 (4)
D′(^{3}H)	80.0	1 month	1.01 ± 0.13(10)	1.46 ± 0.13(10)	0.028 ± 0.003(10)
D′		3 months	2.8 ± 0.1 (6)	4.5 ± 0.1 (6)	0.11 ± 0.01 (6)
E′(^{3}H)	3.6	3.5 years	1.4 ± 0.1 (8)	2.0 ± 0.1 (8)	0.038 ± 0.004 (8)
F′(^{3}H)	7.7	3 days	1.4 ± 0.2 (4)	2.6 ± 0.2 (4)	0.075 ± 0.004 (4)
G′(^{3}H)	0.076	2 months	0.98 ± 0.10 (8)	1.33 ± 0.09 (8)	0.022 ± 0.002 (8)

especially at high concentrations in the medium (Table I). The faster rate was accounted for mainly by an increase in v_D, rather than in v_C. The k_D increased progressively during storage, reaching 0.49 h^{-1} after 1 year (Table I). When preparation C′ was evaporated to dryness and redissolved (preparation C″), the rate constant fell to 0.12 h^{-1}, but after three more months of storage it rose to 0.22 h^{-1}.

The decrease in k_D of preparation C soon after the second evaporation may have been related to removal of volatile tritiated impurities. When an aliquot was evaporated after 1.5 years of storage at −20°C, 7.6% of the total radioactivity was volatile (Table II, preparation C′). Evaporation of the redissolved material after it had been stored for 6 months (Table II, preparation C″) revealed that 3.2% of the total radioactivity was volatile, which demonstrated that further decomposition had occurred following the first evaporation. Preparation A′ did not exhibit this kind of instability. An aliquot that had been stored for 5 years at −20°C released only 0.3% of its radioactivity into the trap during evaporation; when a separate aliquot was evaporated after 5.5 years of storage, there was no further formation of volatile radioactive material (Table II, two separate A′ preparations). In a control study, evaporation of a freshly obtained [^{14}C]methylglucose solution produced a negligible amount of volatile radioactivity (Table II, preparation B). Thus, radioactive sugar itself does not appear to be carried into the vacuum traps. Under the conditions of these experiments the conversion of more than 0.5% of the radioactivity of [^{3}H]methylglucose to a volatile form appears to reflect a degree of instability that can interfere significantly with measurements of transport across cell membranes.

When preparation C was chromatographically purified, evaporated, and redissolved (preparation D′), the k_D fell to a value almost as low as that of preparation A′. However, the material was quite unstable, and k_D increased to 0.11 h^{-1} after two more months of storage (Table I).

TABLE II

DEMONSTRATION OF VOLATILE RADIOACTIVE MATERIAL IN PREPARATIONS OF [^{3}H]METHYLGLUCOSE

Preparations of labeled sugar are designated by the same letters as in Table I. For B, D, and G, volatile radioactivity was measured when the preparations were evaporated to dryness immediately after receipt from the company. A prime indicates that the sample was evaporated once, redissolved, and stored before it was evaporated again for determination of volatile radioactivity. A double prime indicates that after the second evaporation the redissolved material was stored again, for the period of time indicated, before volatile radioactivity was measured. The purity of preparation A was determined by paper chromatography as described earlier [1]; for the other preparations purity is that stated by the commercial source. The addition of carrier to preparation G is described in Table I.

Preparation	Initial chromatographic radiochemical purity (%)	Duration of storage at −20°C	Volatile radioactivity (% of total)
A′(^{3}H)	>98	5 years	0.34
A′		5.5 years	0.29
B(^{14}C)	>99	None	0.0040
C′(^{3}H)	98.5	1.5 years	7.6
C″		6 months	3.2
D(^{3}H)	>98	None	4.8
D′		7 months	1.3
F′(^{3}H)	98	7 months	0.64
G(^{3}H)	>97	None	2.4
G′		6 months	0.33

Variations in the rate of entry of [^{3}H]methylglucose into frog muscle cells were attributable to changes in the k_D. The apparent K_m for the saturable component of transport ranged from 3.6 to 6.9 mM for all preparations, and showed no correlation with changes in transport rates. Similar values have been found in the past for stable preparations of [^{3}H]methylglucose [1]. The V_{max} for all of the preparations in the experiments reported here were in the range of 0.56 to 0.84 μmol of 3-*O*-methylglucose entering per ml of intracellular water per hour. Higher values of V_{max} in some earlier experiments on basal muscles are attributable in part to differences in the handling of animals; more precautions are now taken to minimize contractions during dissection.

Stable tritiated preparations differed markedly from the less stable ones in their specific radioactivities, 0.02 Ci/mmol for preparation A and 80.8 for preparations C and D. Additional evidence indicated that stability was influenced more by differences in specific activity than by differences in the mode of chemical synthesis of the compounds. E′, an older preparation of [^{3}H]methylglucose that had been synthesized by the same method as preparation C but at a lower specific activity (3.6 Ci/mmol) was tested 3.5 years after evaporation. It had a relatively low K_D of 0.038 h^{-1}.

An Amersham preparation of 3-*O*-methyl-D-[1-^{3}H]glucose with a specific activity of 7.7 Ci/mmol exhibited a k_D of 0.075 h^{-1} when tested 3 days after evaporation (Table I, preparation F′). After seven additional months of storage at −20°C, 0.64% of volatile radioactive contaminant was found in this solution (Table II).

A second preparation of [^{3}H]methylglucose from Amersham Corp. was received as an aqueous solution at a specific activity of 2.3 Ci/mmol. It had been kept at the company for approx. 6 months at 2°C. It was evaporated upon arrival and contained 2.4% of volatile radioactive contaminant, which suggests that some decomposition had occurred at this specific activity and under these conditions of storage. We added nonradioactive 3-*O*-methylglucose carrier to decrease the specific activity to 0.076 Ci/mmol. After 2 months of storage at −20°C this modified preparation exhibited a low k_D of 0.022 h^{-1} (Table I, preparation G′). After 6 months of storage there was only 0.33% of volatile radioactive contamination (Table II).

Addition of alcohol can improve the stability of certain radioactively labeled compounds [12] and can exert a bacteriostatic effect. Such solutions are

customarily stored above the freezing point. However, if the specific activity is very high, the presence of alcohol may not be adequate to prevent significant decomposition from taking place. Preparation D (specific activity 80.8 Ci/mmol) was purified chromatographically at New England Nuclear Corp., dissolved in ethanol/H_2O (9:1, v/v) and kept at 5°C for approx. 10 months before shipment. When it was evaporated immediately after receipt it contained 4.8% of volatile impurity (Table II).

Discussion

The specific activity of commercially available organic compounds has undergone a progressive upward shift as methods of chemical synthesis have improved. However, this trend has been accompanied by a decrease in stability for some preparations. Increasing the number of tritium atoms per molecule, in particular, increases the possibility of forming radioactive decomposition products [12]. One tritium atom per molecule corresponds to a specific activity of approx. 30 Ci/mmol [13].

When tritiated organic compounds are stored in solution, volatile impurities can arise by slow exchange of tritium in the compound or its degradation products with hydrogen atoms in the solvent [12]. Volatile radioactive contaminants were demonstrated when solutions of [^{3}H]methylglucose were evaporated under vacuum (Table II). Decomposition during storage was found to be greater for compounds of higher specific activity (Tables I and II).

Although the investigator bears the primary responsibility for ascertaining that the materials he uses are suitable for his research, limitations of time and facilities often lead to a heavy dependence on commercial sources for evaluations of chemical purity. Those assays are, for the most part, reliable, but there are occasional pitfalls. For example, paper chromatography, the most common method of determining radiochemical purity, may fail to detect volatile impurities. Furthermore, decomposition can occur after an assay has been performed. Therefore, statements of initial purity may not always alert the user to the presence of significant impurities.

An abnormal increase in the apparent rate of transport of [^{3}H]methylglucose into cells can be a highly sensitive indicator of the presence of radioactive impurities. In such cases there is an increase in k_D, the rate constant for free diffusion, with no appreciable alteration in the V_{max} and apparent K_m of facilitated transport. A small-percentage contamination of [^{3}H]methylglucose can cause a several-fold increase in its apparent rate of free diffusion into cells (Tables I and II).

Removal of volatile radioactive contaminants by evaporation is a simple procedure that can improve the transport characteristics of some preparations (Table I, preparation C″). Dried tritiated sugars should be redissolved promptly, since tritiated compounds are in general more stable when in solution. Chromatographic purification can achieve a greater improvement in transport rates than evaporation alone (Table I, preparation D′), which suggests that decomposition of [^{3}H]methylglucose can produce nonvolatile as well as volatile impurities. Whatever the method of purification, decomposition products will reaccumulate during further storage when the specific radioactivity is high (Tables I and II).

For [^{3}H]methylglucose preparations with specific activities of 3.6 to 80.8 Ci/mmol elevation of the transport rate is more pronounced at higher specific activities and after longer periods of storage. In contrast, preparations with specific activities less than 0.1 Ci/mmol show no significant alteration of the rate of transport after comparable periods of storage. A specific activity of 0.02 Ci/mmol in the stock solution provides ample radioactivity for transport measurements in muscle cells.

Biological variation among tissue samples from individual animals makes it difficult to assess the significance of small changes in transport rates unless comparisons can be made with labeled compounds of high purity and dependable stability. Unfortunately the instability of most currently offered commercial preparations of [^{3}H]methylglucose significantly impairs their suitability for transport studies. [^{14}C]Methylglucose is more stable, but our experimental design requires [^{3}H]mannitol for measuring the extracellular fluid volume in each muscle when a ^{14}C-labeled sugar is used. [^{3}H]Mannitol of moderate specific activity

can decompose during storage; diffusion of tritiated impurities into cells appears to account for occasional calculations of negative rates of sugar entry when old preparations of [^{3}H]mannitol are used. Valuable reference material would be provided if some commercial samples of [^{3}H]methylglucose that contained no more than one tritium atom per molecule were diluted with carrier 3-*O*-methylglucose, immediately after chromatographic purification, to reduce the specific activity to approximately 0.1 Ci/mmol prior to storage.

Although lowering the specific activity of [^{3}H]methylglucose appears to be the most dependable way of enhancing chemical stability, the mode of storage is also an important consideration. The observation that unfrozen solutions of tritiated thymidine are more stable than frozen ones has led to the general recommendation that all tritiated compounds be stored either above the freezing point or at an extremely low temperature of -196°C [14]. However, we have found that storage of aqueous solutions of [^{3}H]methylglucose in narrow test tubes at -20°C is convenient and satisfactory when the specific activity is low. The tubes should be inspected periodically to guard against separation of solvent and solute by sublimation.

Acknowledgment

This work was supported in part by Research Grant AM-14917 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, PHS/DHHS.

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