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The preparation and purification of anomer-free methyl α-*D*- [gluco-^{14}C(U)] pyranoside

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SUMMARY

Methyl α-D-[gluco-^{14}C(U)] pyranoside was synthesized from D-glucose-^{14}C(U) and methanol using a cation exchange resin catalyst, but was found to contain 10 % of the β-anomer as a radiochemical impurity. This was conveniently removed by treatment with a β-glucosidase — almond emulsin — to yield the pure α-anomer whose analysis is fully described.

INTRODUCTION.

Methyl α-*D*-glucopyranoside (α-*D*-glucoside) has been used in cell transport studies. [1] It is believed to trace *D*-glucose, since it contains three structural features believed necessary for transport [2, 3], a 2-hydroxyl group, a six-membered ring and the α-configuration at C(1). It is not metabolisable. This paper describes the preparation of pure methyl α-*D*-[gluco-^{14}C(U)] pyranoside which contains no β-anomer.

Methyl glucosides can be prepared by several methods (see reference 4), but Fischer's method [5] requires the minimum of manipulations with radioactive material and so was the method of choice. However, the radiochemical purity determined by reverse isotope dilution analysis of material previously prepared by this method was suspected to be low (*ca.* 90 %) despite the fact that it was satisfactory by paper chromatography and by chemical tests of purity. Hence a new preparation was carried out and analyses performed to establish the nature of this impurity. This paper reports this new preparation, the analyses, and the final enzymatic purification of the material.

EXPERIMENTAL.

Materials and Methods.

D-Glucose-^{14}C(U) was prepared by the hydrolysis of sucrose-^{14}C(U) obtained from *Canna* leaves allowed to photosynthesise in an atmosphere of carbon-14 dioxide. It had a radiochemical purity of > 98 %.

Methyl α-*D*-glucopyranoside was obtained from Koch-Light Laboratories Ltd, Colnbrook, Bucks, England, and was recrystallised from aqueous ethanol. It had m.p. 165° C-167° C, $[\alpha]_D^{20} = +157°$ C (literature values m.p. 166° C, $[\alpha]_D^{20} = +158°$C). [4] The proton magnetic resonance spectrum determined at 60 MHz in dimethyl sulphoxide (14 % w/v solution) showed a doublet (to a first order approximation J = 3-4Hz) at 5.46τ, corresponding to one proton. Less than 2 % of one proton signal was obtained on integration of the range 5.90τ — 6.00τ.

Methyl β-*D*-glucopyranoside was obtained from Fluka, Buchs, Switzerland and was recrystallised from aqueous ethanol. It had m.p. 110° C — 113° C, $[\alpha]_D^{20} = -32°$ C (literature values m.p. 110° C, $[\alpha]_D^{20} = -32°$ C) [4]. The proton magnetic resonance spectrum (determined as for the α-anomer), showed a doublet (J = 7Hz) at 5.95τ, corresponding to one proton. Integration of the range 5.40τ — 5.50τ showed a negligible signal above background.

Amberlite IR 120 (H^+ form) cation exchange resin (10 g) was refluxed for 2 hrs with methanol (30 ml) and was stored under methanol.

Almond emulsin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, USA.

Purified diluted samples in the reverse isotope dilution analyses were obtained by recrystallisation to constant specific activity. The samples were dissolved in water and counted by liquid scintillation methods using a Nuclear-Chicago Mk. I counter and Triton X-100 scintillant [6]. Counting efficiency was determined by the Channels Ratio method and suitably predetermined background counts were subtracted from all counts.

Paper chromatography was carried out by the descending method on Whatman No. 1 paper in the following systems :

(A) *n*-butanol : ethanol : water (52 : 33 : 15),
(B) phenol saturated with water
(C) ethyl acetate : acetic acid : water (9 : 2 : 2),
containing 2 % w/v phenyl boronic acid,
(D) *tert*-pentyl alcohol : *n*-propanol : water (4 : 1 : 1.5) [7],
(E) ethyl acetate : pyridine : water : benzene (5 : 3 : 3 : 1 — upper layer) [8],
(F) ethyl acetate : *n*-propanol : water (5 : 3 : 2) [8].

The dried chromatograms were autoradiographed on « Kodirex » X-ray film and the active areas marked, cut out and placed into counting vials containing toluene-PPO liquid scintillant. These were counted by liquid scintil-

lation methods and the activity in the major spot expressed as a percentage of the total activity along the solvent track.

Paper electrophoresis was carried out using saturated sodium borate (0.2M) solution [12], at a potential gradient of 30V/cm and at a temperature of 4° C. Methyl α-*D*-glucopyranoside was found to have a mobility of 7mm/hr, and the β-anomer 11 mm/hr under these conditions.

Inactive methyl glucosides were detected on paper chromatograms using the periodato-cuprate reagent of Bonner. [9]

pH 5.3 acetate buffer was made up according to formulae in reference [10].

The preparation of crude methyl α-D-[gluco-^{14}C(U)] pyranoside.

D-Glucose-^{14}C(U) (1 mmole, 30 mCi) was dried to a syrup by evacuation over phosphorus pentoxide. The dry syrup was refluxed for 6 hr with Amberlite IR 120 (H^+ form) cation exchange resin (1g) and methanol (10 ml). The resin was removed by filtration and washed with methanol until no more activity was removed. The combined filtrate and washings were rotary evaporated to yield a syrup which was applied in water to a column of Amberlite XE78 (OH^- form) anion exchange resin (25 ml).

The eluate (100 ml, 28 mCi) was concentrated by rotary evaporation and purified by preparative paper chromatography using Whatman 3MM paper and solvent system (A). Autoradiography showed the major band of activity to be opposite a marker spot of methyl α-*D*-glucoside.

The solution of crude product (26 mCi) from the chromatography papers was deionised using a column of mixed anion-cation exchange resin. Inactive methyl α-*D*-glucopyranoside (262mg) was added and the crude product crystallised from aqueous ethanol. Yield = 297 mg (13 mCi).

On addition of carrier to the mother liquors, a second crop of crystals (256 mg, 4mCi) was obtained. The combined product (553 mg, 17mCi) was found to have a mean radiochemical purity of 90 % by duplicate reverse isotope dilution analysis with carrier methyl α-*D*-glucopyranoside. Duplicate reverse isotope dilution analysis with carrier methyl β-*D*-glucopyranoside gave a β-content of 10 %. Radiochemical purity was found to be > 99 % by paper chromatography in systems A, B, C, D, E and F. Paper electrophoresis in 0.2M sodium borate showed 10 % of the activity opposite an inactive marker spot of β-anomer.

Purification of crude methyl [α-D-[gluco-^{14}C(U)] pyranoside.

A portion (80 μCi, 3 mg) of product was incubated at 37° C in pH 5.3 buffer with almond emulsin (1 mg). The solution was sampled at time intervals, and the samples were paper chromatographed in system (A), to give the proportion of activity present as *D*-glucose-^{14}C(U) (see Table I).

The resolution of crude methyl α-*D*-[gluco-^{14}C(U)] pyranoside with almond emulsin — the proportion of *D*-glucose-^{14}C(U) produced at different times.

TABLE 1

Time (hr)	Percentage of total activity as *D*-Glucose-^{14}C (U)
0	0
0.1	1.2
0.2	2.1
1.0	3.8
4.0	9.5
24.0	10.2

A solution of crude methyl-α-*D*-[gluco-^{14}C(U)] pyranoside (14 mCi) in water (5 ml) was incubated at 37° C with almond emulsin (50 mg) in pH 5.3 acetate buffer (5 ml). After 4.5 hr the reaction was terminated by pouring the mixture through a column of Amberlite XE78 (OH^- form) anion exchange resin (25 ml) which also removed the *D*-glucose-^{14}C(U) formed.

The aqueous eluate (12.5 mCi, 100 ml) was decationized and rotary evaporated to remove all solvent. Crystallisation from aqueous ethanol gave pure methyl α-*D*-[gluco-^{14}C(U)] pyranoside (331 mg, 8.1 mCi). The mother liquors contained a further 4 mCi of product.

Duplicate reverse isotope dilution analysis with carrier methyl α-*D*-gluco pyranoside gave a mean radiochemical purity of 98 %. Duplicate reverse isotope dilution analysis with methyl β-*D*-glucopyranoside gave a β-content of less than 1 %. Radiochemical purity was found to be greater than 99 % in paper chromatography systems (A) and (C). Paper electrophoresis of the product in 0.2M sodium borate gave 99 % of the activity of the product opposite the methyl α-*D*-glucopyranoside marker. Incubation of a portion (20 μCi) of the product with almond emulsin under the above conditions and subsequent paper chromatography of the solution in system (A) showed that no *D*-glucose-^{14}C(U) was formed in 4.5 hr and that more than 99 % of the activity was present in the methyl glucoside spot.

DISCUSSION.

Crude methyl α-*D*-[gluco-^{14}C(U)] pyranoside was prepared by a method adapted to the millimole scale from that of Cadotte *et al.* [11], using a cation exchange resin as a catalyst.

By carrying out the reaction at higher temperatures (60° C), and over prolonged periods, negligible amounts of methyl furanosides are formed. [4] Thus the crude methyl α-*D*-[gluco-^{14}C(U)] pyranoside contained β-pyranoside as the only major impurity. It was hoped that by a six-fold dilution of the

radioactive product with a pure α-glucoside carrier (which had been checked to ensure that the β-content was minimal) the pure α-glucoside-^{14}C(U) could be crystallized out.

Paper chromatography of the recrystallised product in six systems showed one active spot with a radiochemical purity of greater than 99 %. Three of these systems (D), (E), (F) (see experimental section) are used to separate α and β-glucosides, but development of α- and β-glucoside carriers in these systems showed that they could not be significantly separated in them.

Reverse isotope dilution analysis suggested the presence of 10 % impurity as the β-anomer, the presence of which wasconfirmed by paper electrophoresis in borate buffer. [12]

A sample of the crude product (containing 10 % of β-glucoside) was further diluted tenfold with more carrier methyl α-*D*-glucoside and recrystallised. Reverse isotope dilution analysis still showed the presence of 8 % β-impurity. It was evident that the last traces of β-glucoside could not be removed by fractional crystallisation. Since at this stage, the β-glucoside made up only 0.2 % of the chemical weight, although comprising 8 % of the radioactivity present, it was not possible to detect the presence of an impurity by conventional physical methods, for example rotation, melting-point, infra-red spectrum.

A trial enzymic resolution of the crude material was attempted using almond emulsin, which is a β-glucosidase but as extracted is contaminated with other activities [13]. The enzymic hydrolysis of the β-component of the crude methyl glucoside was followed by estimation of the *D*-glucose-^{14}C(U) content of the reaction mixture by paper chromatography which showed that the hydrolysis of 10 % of the initial activity to *D*-glucose-^{14}C(U) was almost complete after 4 hr and that no more radioactive *D*-glucose was produced after 24 hr (indicating the absence of any α-glucosidase activity).

Hence the crude methyl [*D*-gluco-^{14}C(U)] pyranoside was treated in this way and the resulting purified methyl α-*D*-[gluco-^{14}C(U)] pyranoside was crystallized. The pure material gave satisfactory reverse isotope dilution analyses for α-content (> 98 %) and β-content (< 1 %). It showed no β-glucoside on paper electrophoresis. When a sample of it was incubated with almond emulsin for 4.5 hr no radioactive *D*-glucose could be detected on paper chromatograms of the mixture. Hence it was free of β-anomer.

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