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The Preparation of Labeled Carbohydrates For Biomedical Uses[1,2]

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This study was undertaken to develop general methods for labeling carbohydrates and other polyhydroxy compounds for biomedical uses.

Obviously ^{14}C and ^{3}H-labeled inulin would be most similar to natural inulin in chemical composition and biological behavior, but such labeled materials are costly, not readily obtainable and not as conveniently evaluated as a radioiodinated inulin would be. An ^{131}I-labeled inulin or other compound would permit external scanning and localization.

The labeled compounds should be readily prepared, of adequate specific activity and stable over a practical shelf-life period.

A stable radioiodinated inulin is a much needed, desirable compound. The need for a readily evaluated labeled inulin for measuring glomerular filtration and extracellular fluid was recognized by Brooks *et al* (1). Such a labeled compound would eliminate the difficulties of the colorimetric determination of inulin in the assessment of glomerular filtration. Brooks and co-workers (1) prepared a radioiodinated inulin by first forming an allyl ether of the carbohydrate which contained one allyl group per 14 fructose units and then radioiodinating the unsaturated bonds with iodine-131. These workers found the same glomerular filtration rate in rabbits, whether calculated from the colorimetric determinations or radiometry of plasma and urine. Flear and Graber (2) of the same institution, used the radioiodinated allyl inulin for the measurement of extracellular fluid volume, *in vitro*. They concluded that such a labeled inulin could be used, but that there was a need to use standardized preparations with known molecular weight dispersions and freedom from contaminating radioiodide, for accurate measurement.

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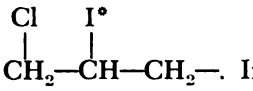
More recently, several studies (3-6) have been reported on the use of radioiodinated and chlor-radioiodinated allyl inulin for the measurement of glomerular filtration in dogs and man. The chlor-radioiodinated allyl inulin is sometimes referred to as chloriodopropylinulin-^{131}I (6-8) on the basis that saturation of the allyl radical $CH_2{=}CH{-}CH_2{-}$ produces a substituted propyl radical

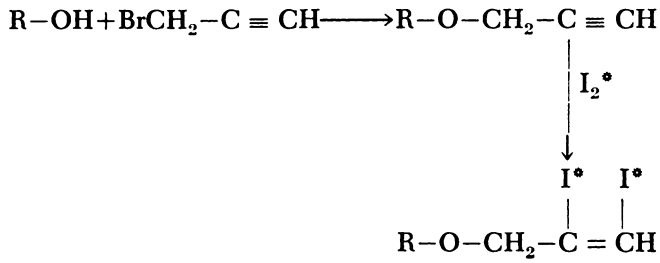


$CH_2{-}CH{-}CH_2{-}$. In this preparation, the allyl inulin is treated with ^{131}ICl (4,9). In the former study (4), it was reported that the material contained no free radioiodide shortly after preparation, but that after a few days, the preparation contained nine per cent free radioiodide. In the blood of some animals the free radioiodide reached 20% and in the urine, 15%.

Recently an ^{125}I-labeled allyl inulin was used to measure the glomerular filtration rate (GFR) in dogs, but was found usable only if freshly purified just prior to use (3). Excellent correlation between the chemical and radioactive assays for the ^{125}I allyl inulin in blood and urine showed the distribution and excretion to be similar to that of inulin (3).

In view of the separation of radioiodide from the radioiodinated allyl inulin observed by us and others, a more stable derivative of inulin and other carbohydrates was sought. Therefore, we postulated that a carbohydrate ether containing an unsaturated triple bond might provide a site for radioiodination resulting in a more stable, partially saturated bond between the carbon atoms. It was presumed that in such compounds the C→I bonds would be more stable than those in the corresponding allyl derivatives.

To provide such a structure, the propargyl ethers were proposed. The formation of the mono-propargyl ether and its radioiodination is indicated in the following equations:



Small quantities of dipropargyl ethers might possibly be formed and radioiodinated. Brooks et al (1) found that in the case of the allyl inulin there was one allyl group per 14 fructose units and one iodine molecule per 35 fructose units.

The radioiodinated propargyl ethers of inulin and of dextrans having molecular weights of 4100 and 9400 have been prepared. The use of these compounds is planned in a new approach to brain tumor localization based on the concept of controlled diffusion of these intermediate molecular weight substances into the extracellular fluid of the tumor tissue. These compounds will be used with the 'high speed section scanner' of Cassen (10, 11). They may prove superior for

studies of glomerular filtration, inulin space and dextran distribution. Propargyl mannitol was prepared by a modification of a similar method (12). These methods can be used for the preparation and labeling of other polyhydroxy compounds for other biomedical purposes.

MATERIALS, METHODS, AND RESULTS

The inulin used was the commercial material treated with N NaOH according to Walser *et al* (13) to remove lower molecular weight materials and alkali-labile substances, inasmuch as the later preparation of the allyl and propargyl ethers involved heating with alkali. It was felt that alkali-stable inulin might represent a more molecularly homogeneous material. Our radioiodinated inulin preparations did not show the presence of any smaller polymers when chromatographed.

The dextrans[1] used had average molecular weights of 4100 and 9400 obtained by light-scattering measurements and were used without further treatment. They are referred to as Dextrans 4 and 9.

The propargyl ethers of inulin and the dextrans were prepared by modifications of the method proposed by Brooks and co-workers (1) for the preparation of allyl inulin.

The method was as follows: 5 g of the alkali-stable inulin or dextran was dissolved in 50 ml H_2O at 70° C in a three-neck flask carrying a reflux condenser, thermometer and a dropping funnel for volatile liquids. The temperature was then reduced to 60° C and 0.5 g allyl or propargyl bromide dissolved in 3.5 ml methyl ethyl ketone added dropwise with stirring to produce a fine dispersion.

[1]Samples were generously supplied by Pharmacia Fine Chemicals, Inc., 800 Centennial Ave., Piscataway, N.J. 08854.

TABLE I

SPECIFIC ACTIVITIES AND FREE RADIOIODIDE CONTENT OF ^{125}I AND ^{131}I ALLYL AND PROPARGYL INULINS

Compound	*RI*	*Sp. Act. in μC/mg.*	*% Free Radioiodide On day of preparation*	*% Free Radioiodide On day of last test*
Allyl	I 125	0.7	0.4	0.5 (19)[1]
Allyl	I 125	0.9	0	0.1 (35)
Allyl	I 131	2.1	1.8	1.8 (15)
Allyl	I 131	0.5	0.1	3.2 (27)
Propargyl	I 125	1.1	0	0.7 (35)
Propargyl	I 131	1.7	1.3	3.3 (15)
Propargyl	I 131	1.1	0.6	0.5 (14)

[1]Days after preparation.

The temperature was raised to 65°-75° C and 10.4 ml N NaOH added gradually over a 30-minute period. The mixture was maintained at this temperature for six hours, after which it was cooled and neutralized with acetic acid. Electrolytes were removed by passage through an ion exchange column containing 75 ml of AG 501-X8 (Biorad) resin and eluting with six bed volumes of H_2O. The solution was flash evaporated to a small volume and lyophilized. The dried materials were extracted in a Soxhlet apparatus with boiling ethanol to remove impurities. The yield of the propargyl inulin was 68% of the weight taken. The recovery of allyl inulin was 44% of the original weight. Both materials were subjected to ascending paper chromatography on Whatman No. 1 paper in n propanol:H_2O, 70:30, and sprayed with 1,3-Dihydroxynaphthalene (naphthoresorcinol) (14) and showed no fructose or other breakdown products. The allyl and propargyl ethers are quite stable for months.

The propargyl and allyl ethers of mannitol were prepared by the same method (1) and the monopropargyl and monoallyl ethers isolated by the method of Wrigley and Yanovsky (12).

Mannitol 5.6 g (30.7 mM) and 11.2 g (93mM) propargyl or allyl bromide and 18.6 g of 20% NaOH (93mM) were used in the etherification. The mono ethers were isolated by precipitation with benzene from the methanolic solution (12).

The ethers were radioiodinated by the method of Brooks *et al* (1) with modifications of the quantities of reagents used to release the radioiodine. Generally, the radioiodine, preferably as preservative- and stabilizer-free sodium radioiodide, 100 to 500 μC, was released by mixing with 0.48 ml 0.1M KI solution (8 mg), 0.47 ml 0.01 M KIO_3 (1.0 mg) and 1.0 ml 0.05 N H_2SO_4. These quantities of reagents were added to each 100 mg of the propargyl or allyl ether of the carbohydrate dissolved in 1 ml or so of H_2O contained in a serum vial, stoppered and allowed to stand at room temperature for 24 hours. To each vial

TABLE II

SPECIFIC ACTIVITIES AND FREE RADIOIODIDE CONTENT OF ^{125}I AND ^{131}I PROPARGYL DEXTRANS

Compound	*RI*	*Sp. Act. in μC/mg.*	*% Free Radioiodide On day of preparation*	*% Free Radioiodide On day of last test*
Dextran 4[2]	I 125	1.3	0	0.7 (35)[1]
Dextran 4	I 125	0.5	4.6	8.9 (19)
Dextran 4	I 131	0.3	3.8	3.2 (27)
Dextran 9[3]	I 125	0.7	0	0.6 (35)
Dextran 9	I 131	0.6	3.8	7.5 (15)

[1]Days after preparation.
[2]4 = 4100 M.W.
[3]9 = 9400 M.W.

was added 0.25 ml of 0.1N $Na_2S_2O_3$ solution to react with the free iodine and each solution passed through a column of anion exchange resin AG 1-X8 in the Cl^- form, 28 cm long and 5.5 mm in diameter, to remove unbound radioiodide.

Solutions were deionized by passage through a column of swollen Sephadex G-25, coarse, 2.2 cm in diameter and 26.3 cm in height with a total volume of 100 cc.

This column was not as efficient in the removal of radioiodide as an anion exchange resin such as AG 1-X8. If used subsequent to resin column, it serves to deionize the solution, which may be desirable.

The pH of the column effluents was between five and six, which is desirable (15).

The solutions were sterilized by Millipore filtration and stored in sterile dark containers in the refrigerator. Samples were taken for the determination of specific activity and chromatography and electrophoresis for the determination of free radioiodide. All were sterile and several representative samples were pyrogen-free. Many preparations were lyophilized and were examined for the release of radioiodide on storage in the dry state.

Free radioiodide content was determined by descending chromatography on Whatman No. 1 paper using a solvent of n butanol:acetic acid:water, 4:1:1, followed by autoradiography and radiometry of the separated radioiodide.

Electrophoretic separations of the radioiodide were made on S & S paper 2043A in Veronal buffer 0.05 M at pH 8.6 at a constant voltage of 200 v. for one hour. The labeled compounds remained at the origin and the radioiodide migrated. Electrophoresis usually showed a greater concentration of free iodide than the corresponding chromatographic separation of the same sample.

It was found that on standing in solution in the refrigerator or in the lyophilized state that there was a progressive separation of radioiodide with time in both states and that the separation was proportional to the initial value.

Typical results are shown in Tables I, II, and III for the radioiodinated propargyl-inulins, dextrans, and mannitols, respectively. The corresponding allyl compounds are shown for comparison.

TABLE III

SPECIFIC ACTIVITIES AND FREE RADIOIODIDE CONTENT OF ^{125}I AND ^{131}I ALLYL AND PROPARGYL MANNITOLS

Compound	*RI*	*Sp. Act. in μC/mg.*	*% Free Radioiodide*	
			On day of preparation	*On day of last test*
Allyl	I 125	0.9	2.9	4.3 (19)[1]
Allyl	I 131	0.5	2.1	1.1 (27)
Propargyl	I 125	0.2	5.3	5.4 (19)
Propargyl	I 131	0.1	2.5	6.2 (27)

[1]Days after preparation.

Occasionally, a preparation showed inexplicable high initial free iodide content, which was not reducible by anion exchange treatment (or Sephadex G-25) and this became progressively greater with age.

The radioiodide content of most preparations was within a usable range for the first few days after preparation without further treatment. If analysis shows that the radioiodide content had increased to an undesirable degree, then passage through a small ion exchange column and resterilization by cellulose membrane filtration is recommended.

Longer periods of radioiodination of 48 and 96 hours did not increase the specific activity significantly and in some instances produced more free radioiodide. Increasing the ratio of mC to mgs of the ethers did not increase the specific activity and only necessitated the removal of larger amounts of unbound radioiodide. Radioiodination at 37° did not increase the specific activity over that at room temperature. Radioiodination at 50° for two hours in presence of 65 mcg ICl produced large and variable quantities of radioiodinated breakdown products. It has been reported that a method utilizing ^{131}ICl yielded radioiodinated allyl inulin of specific activities up to 20 μC/mg (6).

The addition of radioiodine portion-wise to the ethers did not increase the incorporation.

The propargyl derivatives were generally comparable in specific activity and free radioiodide content to the corresponding allyl derivatives and in several instances somewhat better. The formation of the propargyl analogs provides an alternate means of labeling other polyhydroxy compounds that may prove more advantageous in some cases.

It has been reported (15) that chloriodopropyl inulin is stable as a solid. This was not shown to be the case with a group of our lyophilized allyl and propargyl inulins, allyl and propargyl mannitols and propargyl dextrans which showed a considerable increase in free radioiodide, 15 days after preparation.

In isotonic saline at pH 5, at room temperature and in the refrigerator, 5% free iodine activity is split out in four weeks but if the pH is raised to 9, in 70 hours, 80% of the activity is released. At pH 7.6 in 70 hours some 20% is released (15). This indicates that a slightly acid, buffered solution might be a preferable dosage form.

Specific activities of the radioiodinated propargyl and allyl derivatives were adequate and the free radioiodide content was low and acceptable for the uses indicated.

Lyophilized preparations showed a progressive detachment of radioiodide similar to a solution of the same material of the same age retained in the refrigerator.

SUMMARY

Methods are presented for the preparation of propargyl ethers of inulin, dextrans, and mannitol. These can be prepared and radioiodinated on demand. These methods can be advantageously used to label other carbohydrates and polyhydroxy compounds.

The radioiodinated materials were prepared of adequate specific activity, sterile and pyrogen-free and of acceptable free radioiodide content.

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