REFERENCES

1. MIRONOV, V. A., SOBOLEV, E. V. and ELIZAROVA, A. N. — *Dokl. Akad. Nauk. SSSR*, **143** : 112 (1962).
2. MCBEE, E. T., MEYERS, R. K. and BARANAUCKAS, C. F. — *J. Am. Chem. Soc.*, **77** : 86 (1955).
3. LEITCH, L. C. — Patent No. 564, 064 (Sept. 30, 1958).
4. LEITCH, L. C. — *Can. J. Chem.*, **32** : 813 (1954).
5. SCHISSLER, D. O., THOMPSON, S. O. and TURKEVICH, J. — *Disc. Farad. Soc.*, **10** : 46 (1951).
6. KURSANOV, D. N. and PARNES, Z. N. — *Proc. Acad. Sci. USSR*, **106-111** : 385 (1956).
7. FRASER, R. R. and RENAUD, R. N. — *J. Am. Chem. Soc.*, **88** : 4 365 (1966).

The synthesis and chromatographic behavior of D-mannitol-1-^{14}C-1-PO_4, D-mannitol-1-$^{32}PO_4$ and ^{32}PPA*

Received on 10 July 1967

In the course of an investigation dealing with the utilization of D-mannitol and inorganic phosphate by *Absidia glauca* it was found necessary to confirm the existence and establish the identity of the 1-Phosphate ester of this polyol. This paper describes the synthesis of radioactive mannitol-1-phosphate labeled with carbon-14 and phosphorus-32 by a modification of the procedure of Seegmiller and Horecker (1951) for the synthesis of glucose-6-phosphate. In addition we document the paper and gel chromatographic behavior on Sephadex G-15 of the products and ^{32}PPA prepared by the method of Bell (1948) for the synthesis of polyphosphoric acid.

Polyphosphoric Acid. Orthophosphoric acid (100 ml, 85 %) was cautiously added with stirring in the cold to 200 g of reagent grade P_2O_5. After the bulk of the P_2O_5 had dissolved, the stirred mixture was heated for 3 hours on a steam bath, allowed to cool and the heavy syrup of polyphosphoric acid (PPA) transferred to a dry bottle for storage. Titration equivalent of the material was 112 % ortho. ^{32}PPA was prepared as described by adding 8 mC of carrier-free $H_3{}^{32}PO_4$ (Cambridge Nuclear Corp., Cambridge, Mass.) to the 85 % ortho phosphoric acid.

D-mannitol-1-$^{32}PO_4$. D-mannitol (5.0 g) was added to 150 g of ^{32}PPA followed by 10 ml of deionized water. The reaction was stirred at 25° C for 10 hours and then terminated by the addition of 300 ml of water. Sodium

* Phosphorus-32 labeled Polyphosphoric Acid.

carbonate (150 g) was added to the solution with stirring at 60° C. The reaction vessel was evacuated for 20 minutes. The solution was stirred overnight at 4° C and filtered. To 250 ml of this filtrate was added 55 ml of concentrated HBr. This reaction mixture was refluxed for 16 hours and cooled (Davidson, 1967). Barium carbonate (120 g) and a few drops of Dow Corning antifoam H-10 were added to the hydrolysis mixture which was stirred 4 hours at room temperature and filtered. The residue was washed with 2-100 ml portions of water. The combined filtrate and washings were treated with 2 liters of 95 % ethanol. The resulting precipitate was washed with 200 ml of 95 % ethanol, the resulting slurry was centrifuged and then extracted with 3-150 ml portions of water. Supernatant and extracts were combined and decolorized with 2.0 g of activated charcoal. The filtrate was treated with 2.5 liters of 95 % ethanol and the precipitate which formed was washed with 100 ml of absolute ethanol and dried in air. Yield, 1.5 g, specific activity, 0.5 μC/mM.

D-Mannitol-l-^{14}C-l-PO$_4$. This synthesis was carried out as described above with PPA replacing ^{32}PPA and 0.05 mC (or higher activity) of D-mannitol-l-^{14}C (International Chemical and Nuclear Corp., City of Industry, Calif.) added to 5.0 g of D-mannitol. Yield, 0.5, g specific activity, 0.03 μC/mM.

Gel Chromatography. Individual samples of the final products and the filtrates taken prior to HBr hydrolysis were loaded in sucrose dense layers on a column of Sephadex G 15F (2.5 × 41 cm) and eluted by gravity-feed with 0.1M ammonium acetate 0.02 % (w/v) in sodium azide. The column effluent was monitored continuously in an anthracene-packed (Eastmen NO. X-480) flow cell mounted in a Nuclear-Chicago (N-C) liquid scintillation counter (LSC, Model 703P). The ^{14}C label was monitored in the N-C 2 ml flow cell while the ^{32}P label was monitored using a Packard 2 ml or a Picker 1 ml or 2 ml flow cell as required, mounted in the N-C counter. The radioactivity accumulated in 12-minute intervals was counted in integral mode. Void (V_0) and elution (V_e) volumes were recorded to the detector.

Paper Chromatography. D-mannitol-1-^{14}C, hydrolyzed (by heating 5 mg samples of the sugar phosphates with 6.0 ml of 6 N HCl for 3 hours at 80° C in sealed tubes), unhydrolyzed samples of D-mannitol-1-^{14}C-1-PO$_4$ and D-mannitol-1-^{32}PO$_4$ and a mixture of the latter two phosphates and D-mannitol-1-PO$_4$ prepared by the reduction of D-mannose-6-PO$_4$ as described by Wolff and Kaplan (1956) were spotted in 5 μl aliquots on Whatman No. 1 paper and chromatographed in an ascending system with the solvent n-propanol : ammonia : water (6 : 3 : 1).

Duplicate chromatograms were run, the first was developed for carbohydrate by the periodate-benzidine method of Cifonelli and Smith (1954), and the second was developed for phosphate by the method of Hanes and Isherwood (1949). R_f values were recorded and the spots cut out and examined for radioactivity using static scintillation counting techniques.

The multiplicity of peaks evident in the elution profile for the ^{14}C and the ^{32}P containing filtrates presented in Table 1 reflects the heterogeneity of the ^{32}PPA. The peak(s) emerging at $V_e = V_o = 71$ ml indicates the presence of high molecular weight polyphosphates and possibly cyclic metaphosphates. The latter because of their rigid structure could reasonably be expected to be excluded from this gel, hence mimic the behavior of a larger molecule (Jolly, 1966). Hydrolysis using HBr completely degrades these polymeric forms to

TABLE I. Gel Elution Profile of Reaction Mixture.

Run	Sample	Elution Volumes (V_e, ml)						No. Peaks
50	^{32}P Filtrate	71		98	112	119		4-5
49	^{14}C Filtrate	71			111	118		3-4
53	D-mannitol-1-^{14}C-1-PO_4				111			1
15	D-mannitol-1-^{14}C						133	1
47	D-mannitol-1-$^{32}PO_4$				112			1
54	^{32}PPA [a]	71		95		118		4-5
55	^{32}PPA [b]	71		95		118		4-5
56	^{32}PPA [c]		85	95		118		2-3
60	^{32}PPA [d]			95		118		2
59	^{32}PPA [e]					117		1
32	$H_3{}^{32}PO_4$					118		1

[a-d] Hydrolyzed with water : [a] 2-hr 60° C; [b] 3-hr 60° C; [c] 6-days 25° C; [d] 15-days; [e] 16-hr HBr hydrolysis.

orthophosphate (V_e118 ml) which is easily separated from the ^{14}C and ^{32}P labeled polyophosphates (V_e 111-112 ml). Prolonged exposure (up to 15 days at 25° C) to water (H_2O : ^{32}PPA — 200 : 1) was not sufficient to completely hydrolyze the acid. Slow hydrolysis does occur since the 71 ml peak disappears and an 85 ml peak appears then disappears within the 3-hour-15-day exposure. In addition the increase of radioactivity in the o-phosphate peak correlates well with the disappearance of the ^{32}P activity of the other peaks.

The unhydrolyzed mannitol phosphate preparations (^{14}C and ^{32}P labeled, ^{14}C and ^{32}P labeled mixture, and unlabeled mannitol-1-phosphate (from the mannose-6-phosphate reduction)) all gave a single spot on paper with an R_f value of 0.13. The spot was color positive for carbohydrate and phosphorus and showed ^{14}C and ^{32}P activity separately and in the mixture when assayed by differential LSC. (Partial) acid hydrolysis of the polyol phosphates yielded 3 spots, a polyol-positive spot (R_f 0.59) identical with a sample of Mannitol-1-^{14}C in color and radioactivity, a spot corresponding to the unhydrolyzed polyol ester and an immobile phosphate positive spot. The (two) color positive phosphate spots of the mannitol-1-$^{32}PO_4$ hydrolysate and the polyol color positive spots of the mannitol-1-^{14}C-1-PO_4 hydrolysate contained the

totality of the respective nuclide radioactivities while all three spots of the ^{32}P-^{14}C labeled mixture contained the totality of both nuclide radioactivities. ^{32}PPA was immobile in this solvent system.

LITERATURE CITED

BELL, R. N. — *Ind. Eng. Chem.*, **40** : 1464 (1948).
CIFONELLI, J. A. and SMITH, F. — *Anal Chem.*, **26** : 1132 (1954).
JOLLY, W. L. — *The Chemistry of the Non-Metals*, Prentice-Hall Inc., Englewood Cliffs, N. J., 1966, page 96.
HANES, C. S. and ISHERWOOD, F. A. — *Nature*, **164** : 1107 (1949).
DAVIDSON, E. A. — *Carbohydrate Chemistry*, Holt, Rinehart and Winston, Inc., New York, 1967, page 327.
SEEGMILLER, J. E. and HORECKER, B. L. — *J. Biol. Chem.*, **192** : 175 (1951).
WOLFF, J. B. and KAPLAN, N. O. — *J. Biol. Chem.*, **218** : 849 (1956).

E. T. MCGUINNESS * and J. L. BEEBE **

* Department of Chemistry, Seton Hall University, South Orange, New Jersey 07079
** Department of Biology, Seton Hall University, South Orange, New Jersey 07079