SYNTHESIS AND PROOF OF STRUCTURE OF TRITIATED GLUCOSE AND ITS USE IN THE STUDY OF THE MECHANISM OF UDPGAL-C-4-EPIMERASE[1]

R. D. Bevill, J. H. Nordin[2], F. Smith and S. Kirkwood

Department of Biochemistry, University of Minnesota, St.Paul 1, Minnesota

Received June 3, 1963

The conversion of UDPG to UDPGal, catalyzed by the enzyme UDPGal-C-4 epimerase, appears to involve an intermolecular oxidation and reduction between DPN^+ and the participating UDP hexoses. The liver enzyme contains loosely associated DPN^+ and the yeast enzyme contains tightly bound DPN^+ (Maxwell and de Robichon-Szulmajster, 1960). However, the yeast enzyme does not catalyze the incorporation of tritium from tritiated water into the sugar, nor is there any incorporation of tritium from DPN or DPNH labelled in both para positions with tritium (Maxwell, 1957). Kohn et al. (1963), working with an in vivo liver system, have demonstrated the retention of tritium in rat liver glycogen glucose after in vivo injection of D-galactose-4-T. We wish to report on an investigation of the mechanism of the yeast enzyme in vitro using chemically synthesized tritiated α-D-glucose 1-phosphate in which the position of the tritium was established unequivocally through the stepwise degradation of the tritiated glucose. This degradation scheme is generally applicable for the location of labelled positions and hence should prove of value in future studies of carbohydrate metabolism where hexoses labelled with both C^{14} and/or T are used. The procedure has the advantage that each fragment is isolated in good yield and as a crystalline derivative.

---

[1] Paper No. 5141 Scientific Journal Series, Minnesota Agricultural Experiment Station. This work was supported by a grant from the National Institutes of Health.

[2] Career Development Awardee, USPHS, 1962-63.

Experimental. All tritium counting was performed in a Packard Liquid Scintillation Spectrometer. Methyl 2,3.6-tri-O-methyl-D-glucopyranoside (I) was oxidized with CrO_3 in pyridine (Wolfrom and Hanessian, 1962) to give the 4-oxo derivative which was subsequently reduced with $NaBT_4$ (144 mc/mmole), and demethylated with BCl_3 in methylene chloride (Allen, et al. 1958). D-Glucose-4-T (II), isolated by paper chromatography, was acetylated with acetic anhydride and sodium acetate and the β-pentaacetate so formed was converted in anhydrous phosphoric acid (MacDonald, 1962), to tritiated α-D-glucose 1-phosphate (III), a product which was non-reducing. After mild acid hydrolysis the molar concentration of glucose formed, as evidenced by reducing power, was identical with the starting molar concentration of (III) as determined with phosphoglucomutase, G-6-P dehydogenase, and TPN^+.

In order to determine the position of the tritium in radioactive glucose, methyl α-D-glucopyranoside (IV) was prepared from (II), carrier IV added, and (IV) crystallized to a constant specific activity. The methyl glucoside (IV) was treated with periodic acid to yield formic acid (V^a) and D-methoxy-D-hydroxymethyldiglycolic aldehyde (VI). The formic acid (V^a) was converted into its ammonium salt and purified by sublimation. The dialdehyde (VI) was oxidized with Br_2 in the presence of $SrCO_3$ (Boothroyd, et al. 1955) to yield Strontium D-methoxy-D-hydroxymethyldiglycolate (VII). The dialdehyde (VI) was also reduced with $NaBH_4$ (Van Cleve and Smith, 1955) and hydrolyzed with N HCl to yield glycerol (VIII) and glycolic aldehyde (IX). The glycerol (VIII) was isolated as the tri-p-nitrobenzoate and the aldehyde (IX) as the crystalline methone derivative. Table I shows the results of the degradation. Since (VII) contained 40% of the activity, and positions 1 and 2 contained no activity, it was necessary to degrade (VII) and to establish the location of the label in positions 5 and 6. Accordingly (VII) was hydrolyzed with N HCl producing a mixture of D-glyceric acid (X) and glyoxylic acid (XI). The glyceric acid (X), isolated by paper chromatography, was treated with periodic acid to yield formic acid (V^b) and formaldehyde (XII). The formic acid (V^b)

was isolated in the same manner as (V^a), and (XII) was isolated as the crystalline methone derivative.

Table I

Compound	Position in Hexose of Carbon-bound H-atom	Counts/min m mole	% Total Activity
IV	all	71,000	100
IX	1, 2	nil	---
V^a	3	nil	---
VIII	4,5,6	71,300	100
VII	1,5,6	31,500	43.6
V^b	5	nil	nil
XII	6	28,400	40.2

It can be seen from Table I that the tritiated D-glucose has 60% of the total activity in position 4, the rest being located at position 6. The appearance of the tritium label in two positions does not prevent the use of this compound for testing the enzyme mechanisms since the position of all label has been established by the controlled chemical degradation referred to above.

The synthesis of UDPG was accomplished by the use of a coupled enzyme system which promotes the completion of the reaction. The various reaction mixtures at pH 7.5 contained the following in micromoles in a final volume of 0.5 ml: tritiated G-1-P 0.78, UTP 3.0, Tris buffer 34, $MgCl_2$ 3.4 and ethyl-mercaptan 3.0. In addition, 384 units of UDPG pyrophosphorylase (60 μg protein, Munch-Petersen, et al. 1955) and 180 units of crystalline inorganic pyrophosphatase (7.4 μg protein, Hepple and Hilmoe, 1951) were added. After 60 minutes at room temperature, the pH of the reaction mixture was adjusted to 8.7 with 2 M glycine buffer, pH 9.0, and 990 units of Saccharomyces

fragilis UDPGal-C-4 epimerase were added (1.62 mg protein, Maxwell and de Robichon-Szulmajster, 1960). Incubations with epimerase lasted 60-240 minutes. The reactions were terminated by the addition of HCl and the nucleotide bound sugars hydrolyzed at 100° for 5 min. The hexose mixture, obtained after deionizing the reaction mixture, was separated on Whatman No. 1 paper using pyridine:ethyl acetate:water-2:5:7 (McFarren, et al. 1951) as the irrigating solvent. By reference to marginal glucose and galactose markers, the areas corresponding to glucose and galactose were cut out, eluted with water, and the extracts diluted to an appropriate concentration for radioactivity analyses and reducing sugar determinations (Park and Johnson, 1949).

The results of four experiments are summarized in Table 2.

Table 2

Experiment	Incubation time (min) (epimerase)	Glucose		Galactose		% Recovery [a]
		µm	Cpm µm	µm	Cpm µm	
1	90	0.610	9300	0.132	10,700	94
2	60	0.540	9380	0.114	9,650	84
3	120	0.555	9200	0.147	8,180	90
4	240	b	10,200	b	9,230	b
Average	-	0.568	9,780	0.131	9,440	90

(a) Micromoles glucose + micromoles galactose — based on 0.78 micromoles G-1-P added; specific activity 10,150 cpm/µm.

(b) Sugars were not quantitatively recovered and therefore not included in the average.

The values obtained for the specific activities of each sugar indicate clearly the tritium is retained by the hexose during epimerization at position 4.

The complete conversion of added tritiated G-1-P, with excess UTP, to UDPG was confirmed by the use of UDPG dehydrogenase and DPN^+ (Strominger,

et al. 1957). UDPGal was detected in the second incubation by use of the epimerase after heating at 100° and measuring UDPGal with fresh epimerase.

No galactose was detected on paper chromatograms when 4-epimerase, G-1-P, or UTP was withheld from the reaction mixture. Moreover, when G-1-P was withheld no glucose or galactose was observed, thus indicating that the system was free of endogenous glucose and galactose.

One may conclude, therefore, that in the reaction catalyzed by S. fragilis UDPGal-C-4 epimerase, the carbon bound hydrogen in position 4 is either stereospecifically removed and reintroduced, or it never departs from the hexose molecule. The possibility of the stereospecific removal of an hydroxyl group and its reintroduction has not yet been ruled out. Pyridine nucleotide dependent hydroxylation of steroids is well known (Grant, 1956) and interestingly the oxygen of hydroxyl group comes from molecular O_2 and not from water.

References

1. Allen, S., Bonner, T. C., Bourne, E. J. and Saville, N. M., Chem. and Ind. (London) 630 (1958).
2. Boothroyd, B., Brown, S. A., Thorn, J. A. and Neish, A. C., Can. J. Biochem. and Physiol. 33, 62 (1955).
3. Grant, J. K., Biochem. J. 64, 559 (1956).
4. Heppel, L. A. and Hilmoe, R. J., J. Biol. Chem. 192, 87 (1951).
5. Kohn, B. D., Hoffman, R. C. and Kohn, P., J. Biol. Chem. 238, 1193 (1963).
6. MacDonald, D. L., J. Org. Chem. 27, 1107 (1962).
7. Maxwell, E. S., J. Biol. Chem. 229, 139 (1957).
8. Maxwell, E. S. and de Robichon-Szulmajster, H., J. Biol. Chem. 235, 308 (1960).
9. McFarren, E. F., Rand, K. and Rutkowski, H., Anal. Chem. 23, 1146 (1951).
10. Munch-Petersen, A., Kalckar, H. M. and Smith, E. E. B., Kgl. Danske Videnskab. Selskab. Biol. Med. 22, 3 (1955).
11. Park, J. T. and Johnson, M. J., J. Biol. Chem. 181, 149 (1949).
12. Smith, F. and Van Cleve, J. W., J. Am. Chem. Soc. 77, 3091 (1955).
13. Strominger, J. L., Maxwell, E. S., Axelrod, J. and Kalckar, H. M., J. Biol. Chem. 224, 79 (1957).
14. Wolfrom, M. L. and Hanessian, S., J. Org. Chem. 27, 2107 (1962).