A SIMPLE PROCEDURE FOR THE PREPARATION OF UDP-N-ACETYLGLUCOSAMINE-^{14}C

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Summary - A simple, efficient method is described for the preparation of UDP-N-acetylglucosamine (UDP-GlcNAc)-^{14}C. D-Glucosamine-^{14}C is first incubated with growing mycelia of the fungus Helminthosporium sativum in the presence of polyoxin D, an inhibitor of the chitin synthetase of fungi. The sugar nucleotide is then isolated from the incubation mixture by paper electrophoresis and paper chromatography. The overall yield of the radioactivity in UDP-GlcNAc-^{14}C is about 65% of the dose of glucosamine-^{14}C added.

The nucleotide sugar UDP-N-acetylglucosamine (UDP-GlcNAc) is presumed to be the precursor of the N-acetylglucosamine residue of glycoproteins, mucopolysaccharides and chitin. For studies of enzymatic synthesis of these substances, the availability of this compound labeled in glucosamine is essential. Several procedures for this purpose have been described (1 - 4). In most cases, D-glucosamine-^{14}C is used as the starting material, which is first phosphorylated chemically (5) or enzymatically (6) to form glucosamine 6-phosphate. Glucosamine 6-phosphate is acetylated chemically (7) or enzymatically (8) to give N-acetylglucosamine 6-phosphate. Subsequently N-acetylglucosamine 6-phosphate is converted to UDP-GlcNAc by the sequential actions of phosphoacetylglucosamine mutase

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and UDP-GlcNAc pyrophosphorylase of baker's yeast (1). The overall yield of those processes is around 10% (4). Radioactive UDP-N-acetylhexosamine (a mixture of UDP-GlcNAc and UDP-N-acetylgalactosamine (UDP-GalNAc)) has been prepared by injecting rats with glucosamine-^{14}C, followed by isolating the nucleotide sugars from the livers (3). UDP-GlcNAc has also been chemically synthesized by reacting N-acetyl-glucosamine 1-phosphate with uridine-5'-phosphomorpholidate (2).

A previous paper from our laboratories (9) has shown that the antibiotic polyoxin D is a specific inhibitor of chitin synthetase of fungi. Furthermore, some experiments on the metabolism of glucosamine-^{14}C by Neurospora crassa have shown that this amino sugar is efficiently incorporated into the UDP-GlcNAc pool but not into the cell wall chitin when the nucleotide sugar utilization for chitin synthesis is blocked by the antibiotic (10). These findings suggested the use of this system for the preparation of radioactive UDP-GlcNAc.

EXPERIMENTAL

Fungal strain - Under the conditions employed, glucosamine-^{14}C was incorporated consistently into the UDP-GlcNAc pool of all the tested strains sensitive to polyoxin D when they were grown in the presence of the antibiotic. An exhaustive survey has not been made, but Helminthosporium sativum was selected for the purpose of this study and used throughout the experiments, since the acid-soluble fraction of this fungal mycelia was found to contain no detectable amounts of UDP-GalNAc. The separation of UDP-GlcNAc from UDP-GalNAc is quite laborious. Other two strains, Piricularia oryzae and Cochliobolus miyabeanus, could also be effectively utilized for this purpose.

Incubation - H. sativum was grown from conidia in Potato Dextrose Broth-Bacto (Difco) by shaking at 28°C for 48 hours. The mycelia were

harvested by centrifugation, washed twice with 0.05 M Tris-HCl, pH 6.0 containing 10% L-sorbose (Merck, Germany), and then suspended in the same buffer (3.4 mg dry weight/ml). To a 20-ml flask containing 5 ml of the suspension, were added 250 μg of polyoxin D and 10 μC of D-glucosamine-1-^{14}C hydrochloride (3.4 μC/μmole, Radiochemical Centre, England). The flask was shaken at 28°C. At various time intervals, an aliquot of 0.1 ml of the incubation mixture was taken out and added to 2 ml of cold 5% trichloroacetic acid. Nucleotides in the acid-soluble fraction obtained was adsorbed on Norit A charcoal as described below and counted. As shown in Fig. 1, the level in the nucleotide fraction was maximal after 3.5 hours of incubation.

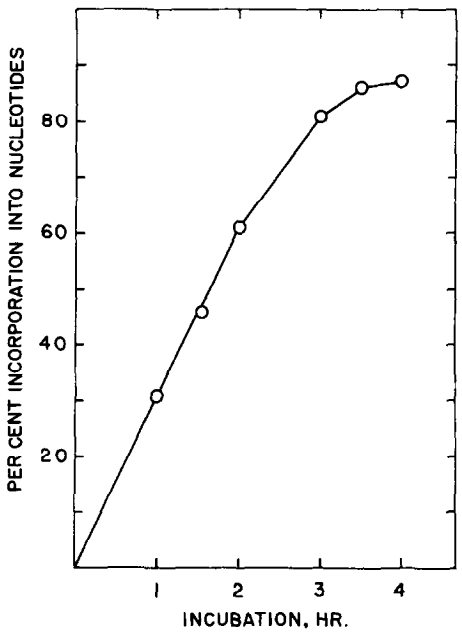

Fig. 1. Incorporation of D-glucosamine-^{14}C into UDP-N-acetylglucosamine of H. sativum. Each point represents the percentage of the dose added at zero time, as described in the text. Polyoxin D was a generous gift from Dr. S. Suzuki of Institute of Physical and Chemical Research, Saitama, Japan.

Purification - After 4 hours of incubation, 5 ml of cold 10% trichloroacetic acid solution was added to the flask. The acid-soluble

fraction was obtained by centrifugation and then washed 3 times with 10-ml portions of diethylether. The ether layer was back-washed with water. The volume of the aqueous layer was adjusted to 18 ml with water. The nucleotides were adsorbed on Norit A charcoal, using 2 mg charcoal for every unit of absorbancy at 262 mμ (110 mg). The charcoal was collected by centrifugation at 20,000 xg for 30 min. and washed once with 10 ml of 0.01 N acetic acid. The nucleotides were twice eluted with 15-ml portions of 0.1% concentrated NH_4OH in 50% ethanol. The combined eluates were concentrated to a small volume under reduced pressure and submitted to paper electrophoresis in pyridine-acetic acid-water (1:10:69), pH 3.5 at 3,000 volts for 40 min. A 0.5-cm guide strip was cut out and scanned for localization of the area corresponding to authentic UDP-GlcNAc. Material in this area was eluted with water and further purified by paper chromatography in ethanol-1M ammonium acetate (75:30), pH 7.4 for 16 hours. The filter paper had been previously washed by descending chromatography in the same buffer and then in water. Labeled UDP-GlcNAc was located and eluted from the paper as described above after removing salt from the paper by treating with absolute ethanol. The overall yield of the radioactivity in the purified UDP-GlcNAc-^{14}C was approximately 65% of the dose of glucosamine-^{14}C added. Its specific activity was 3.3 μC/μmole, which was calculated from ultraviolet absorption at 262 mμ, assuming a value of 10,020 for ε_{max} at pH 7 (11).

Proof and purity - The purified product migrated as a single ultraviolet-absorbing spot when submitted to paper electrophoresis in pyridine-acetic acid-water (1:10:69), pH 3.5 and paper chromatography in the following solvents: ethanol-1M ammonium acetate (75:30), pH 3.8; isobutyric acid-ammonium hydroxide-water (57:4:9). The ultraviolet absorption spectrum was characteristic of uridine at pH 2 and 7 (12), having a maximum at 262 mμ.

The ratio of uridine (ultraviolet absorption) to total phosphate (13) was 1:2.05 and that of uridine to N-acetylglucosamine (14), 1:0.96. Sequential treatment with venom phosphodiesterase and *E. coli* alkaline phosphatase (both from Worthington Biochemical Corp.) gave a single radioactive component which migrated with authentic N-acetylglucosamine on borate-impregnated paper (15) in ethyl acetate-pyridine-water (10:4:3). This technique readily separates N-acetylglucosamine from both N-acetylgalactosamine and N-acetylmannosamine. Mild acid hydrolysis (0.1N HCl, 100°C, 10 min.) yielded a single compound migrating as N-acetylglucosamine, as judged from the above technique. After strong acid hydrolysis (4N HCl, 100°C, 2 hours), all of the radioactivity was in glucosamine, as identified by paper chromatography in *n*-butanol-pyridine-water (6:4:3).

When the purified product was employed as a substrate for chitin synthetase system of *N. crassa* (9), it proved to be an active sugar donor.

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