# INCORPORATION OF $C^{14}$ FROM LABELED GLUCURONIC ACID AND N-ACETYL GLUCOSAMINE INTO POLYSACCHARIDE BY A CELL FREE PREPARATION FROM MOUSE MAST-CELL TUMOR*

Jeremiah E. Silbert**

Department of Medicine, Harvard Medical School
Massachusetts General Hospital, Boston, Massachusetts

Received September 10, 1962

Heparin is a sulfated polysaccharide consisting of equal parts of glucuronic acid and glucosamine. In addition, galactosamine has been found in the heparin fraction from mouse mast-cell tumors (Ringertz, 1960, Silbert and Brown, 1961); the presence of iduronic acid in heparin has also been reported (Cifonelli and Dorfman, 1962). Sulfate is present in both ester and sulfamide linkages; no acetylated hexosamines have been found. Heparitin sulfate, however, has partly acetylated and partly sulfated amino groups. Since the possibility exists that heparitin sulfate is a precursor of heparin, it was of interest to determine if heparin is formed from an acetylated intermediate. The presence of UDPGNAc*** (Silbert and Brown, 1961) in large quantities in the heparin synthesizing DBA mouse mast-cell tumor of Dunn and Potter (1957) is consistent with the probability that heparin synthesis proceeds through nucleotide linked sugars. The synthesis of UDPGA (Hambraeus, Rodén and Boström, 1959), as well as UDP-glucosamine (Silber and Brown, 1961) by cell free preparations from this tumor has also been describe

In the present communication the incorporation of radioactivity into a polysaccharide moiety by a cell-free fraction of mast-cell tumor homogenate is described. UDPGA and UDPGNAc were substrates; UDP-glucosamine was not.

UDPGA-$C^{14}$, uniformly labeled in the glucuronic acid moiety, was synthesized as follows: Uniformly labeled glucose-$C^{14}$ (Putnam, Hassid, Krotkov and Barker,

---

*Supported by research grant No. A-3564 (C2) of the National Institute of Arthritis and Metabolic Disease, National Institutes of Health. This is publication No. 319 of the Robert W. Lovett Memorial for the Study of Diseases Causing Deformities, Harvard Medical School, Massachusetts General Hospital.

**Postdoctoral Research Fellow of the National Foundation.

***Abbreviations: UDPGNAc, uridine diphosphate-N-acetyl glucosamine; UDPGA, uridine diphosphate glucuronic acid.

1948) was phosphorylated with ATP and hexokinase, then reacted with rabbit muscle phosphoglucomutase in the presence of α-D-glucose-1,6-diphosphate, UTP, and yeast UDPG pyrophosphoylase. The UDPG was then oxidized to UDPGA in the presence of UDPG dehydrogenase and purified by chromatography on a Dowex-1-formate column, charcoal adsorption, and paper chromatography using the ethanol-ammonium acetate system of Paladini and Leloir (1952). A radioactive spot corresponding to the $R_{UMP}$ position of authentic UDPGA was obtained. This had an ultraviolet spectrum typical of uridine and a specific activity of $2 \times 10^8$ cpm/μmole using a G-M counter with a thin (micromil) end window.

UDPGNAc-$C^{14}$ was prepared from glucosamine-1-$C^{14}$ (New England Nuclear) using hexokinase and ATP followed by acetylation with acetic anhydride and then reaction with phosphoglucomutase, UTP, and yeast UDPGNAc pyrophosphorylase. The UDPGNAc which was isolated and identified as above had a specific activity of $10^6$ cpm/μmole

UDP-Glucosamine was synthesized from glucosamine-1-phosphate (Maley, Maley and Lardy, 1956) and UTP by enzymes from the DBA mast-cell tumor (Silbert and Brown, 1961) or similar preparations from yeast.

A microsomal fraction was prepared by grinding 4 g of fresh DBA tumor in a Potter-Elvehjem Teflon homogenizer in 40 ml of 0.25 M sucrose. The homogenate was then centrifuged at 20,000 x g for 20 minutes and the supernatant centrifuged at 105,000 x g for 20 minutes. The 105,000 x g pellet was resuspended in 0.25 M sucrose and centrifuged two more times at 105,000 x g. The pellet was suspended in 0.25 ml of 0.25 M sucrose.

Mast-cell granules were obtained from the DBA tumor by homogenization as above, followed by centrifugation for 20 minutes at 600 x g. The supernatant was then centrifuged at 10,000 x g for 20 minutes, and the pellet resuspended in 1 ml of 0.25 M sucrose. This was layered onto a sucrose gradient (1.0-2.0 M) and centrifuged at 173,000 x g for 1 hour in a swinging bucket rotor. The granules are found tightly packed at the bottom of the tube in 2 M sucrose. Granules were then resuspended in 0.25 ml of 0.25 M sucrose.

The results using UDPGA-$C^{14}$ are shown in Table I. After reaction the mixtures were boiled, centrifuged, and the sediment washed two times with water by centrifugation. The sediment was resuspended in water and digested with pancreatin overnight at pH 8-9 and 37° (Korn, 1959). Following boiling and centrifugation, the supernatant was dialyzed against water overnight and the radioactivity of an aliquot of this supernatant (polysaccharide fraction) determined. Radioactivity of the washed reaction sediment was found to be made completely soluble by pancreatin digestion and 98% of this was retained with heparin in the dialysis bag.

Table I

Incorporation of $C^{14}$ from UDPGA-$C^{14}$ into a Polysaccharide Fraction

| Additions | Total Incorporation into Polysaccharide Fraction | |
|---|---|---|
| | cpm | µmoles |
| UDPGNAc | 21,000 | 0.000105 |
| UDP-glucosamine | 1600 | 0.000008 |
| None | 1800 | 0.000009 |
| Controls | 100, 150 | |

All reaction mixtures were incubated at 37° for 2.5 hours in a total volume of 0.05 ml containing 0.05 M Tris pH 7.3, 0.01 M $MgSO_4$, 0.001 M EDTA, 0.0005 M mercaptoethanol, 0.01 ml of granule preparation, 0.01 ml of microsomal preparation and 0.00005 M UDPGA-$C^{14}$ containing 5 x $10^5$ cpm. Additions, as indicated above, were at final concentrations of 0.001 M. The controls contained 0.001 M UDPGNAc and had either boiled enzyme or had the UDPGA-$C^{14}$ added after the 2.5 hour incubation, just before boiling the mixture. The reaction mixtures were then trea ed and analyzed as described in the text.

The radioactive material from the dialyzed polysaccharide fraction was eluted from a DEAE cellulose column with a gradient of 2 M LiCl, and a broad series of peaks overlapping the corresponding heparin area was obtained, plus one sharp peak which came off the column before heparin. Three-fourths of this same dialyzed polysaccharide fraction could be precipitated with cetyltrimethylammonium bromide (Korn, 1959) and then eluted with various concentrations of NaCl (1-4 M). The lack of a single discrete peak on elution of the column, and the range of NaCl concentrations necessary to elute from ceyltrimethylammonium bromide is interpreted as indication of a mixture rather than a single polysaccharide.

When aliquots of the boiled reaction mixtures were chromatographed directly on Whatman No. 1 paper in the ethanol-ammonium acetate solvent, radioactivity appeared at the origin in proportions similar to those in Table I. Mast-cell heparin, identified by methylene blue staining (Foster and Pearce, 1961), also stayed at the origin. There was some minor, slow-moving activity in addition to the non-moving larger peak, but unreacted UDPGA-$C^{14}$ moved well down the paper and was distinctly separate.

When the granules were boiled prior to the reaction, the amount of radioactivity isolated with the polysaccharide fraction remained the same as with fresh granules. When no granules were added to the system, or when commercial heparin was added instead of granules, the radioactivity incorporated into the polysaccharide fraction was only 60% as high as that in the system containing granules. The enzyme preparation was found to contain small amounts of metachromatic material (Azure A) which was not separated from it during its preparation. Therefore, no conclusions are drawn as to primer requirements except that granules appear to enhance synthesis.

In order to verify further the carbohydrate composition of the labeled substance, UDPGNAc-$C^{14}$ was incubated with the same enzyme preparations in the presence and absence of non-labeled UDPGA (Table II). After reaction, the mixtures were boiled, spotted in entirety on Whatman No. 1 paper, and chromatographed in the ethanol-ammonium acetate solvent. The origins were cut out and eluted by incubation at 37° for 48 hours in a pancreatin solution pH 8-9. The solutions were then dialyzed against water, and activity in the dialysis bags (polysaccharide fraction) determined.

These studies indicate that the microsomal fraction of the heparin-producing DBA mast-cell tumor contains enzymatic activity which catalyzes the incorporation of $C^{14}$ from UDPGA-$C^{14}$ and UDPGNAc-$C^{14}$ into products which behave like polysaccharides. These products are thought to be heparin-like and possibly represent precursors of heparin. Further investigation is being undertaken to identify the products more completely as well as to determine their acetyl and sulfate content.

Table II

Incorporation of $C^{14}$ from UDPGNAc-$C^{14}$ into a Polysaccharide Fraction

| Additions | Total Incorporation into Polysaccharide Fraction | |
|---|---|---|
| | cpm | μmoles |
| UDPGA | 75 | 0.000075 |
| None | 10 | 0.000010 |
| Control | 5 | |

All reaction mixtures were incubated at 37° for 2.5 hours in a total volume of 0.1 ml containing 0.05 M Tris pH 7.3, 0.01 M $MgSO_4$, 0.001 M EDTA, 0.0005 M mercaptoethanol, 0.01 ml of granule preparation, 0.01 ml of microsomal preparation and 0.00020 M UDPGNAc-$C^{14}$ containing 2 x $10^4$ cpm. Additions as indicated above were at final concentrations of 0.001 M. The control had the UDPGNAc-$C^{14}$ added to the system containing 0.001 M UDPGA after the 2.5 hour incubation, just before boiling the mixture. The reaction mixtures were then treated and analyzed as described in the text.

Acknowledgment

Advice and encouragement of Dr. Stephen M. Krane in all phases of this work is gratefully acknowledged.

References

Cifonelli, J. A., and Dorfman, A., Biochem. Biophys. Res. Communs., 7, 41 (1962).
Dunn, T. B., and Potter, M., J. Natl Cancer Inst., 18, 587 (1957).
Foster, T. S., and Pearce, R. H., Can. J. Biochem. Physiol., 39, 1771 (1961).
Hambraeus, L., Rodén, L., and Boström, H., Acta Soc. Med. Upsal., 64, 247 (1959).
Korn, E. D., J. Biol. Chem., 234, 1325 (1959).
Maley, F., Maley, G. F., and Lardy, H. A., J. Am. Chem. Soc., 78, 5303 (1956).
Paladini, A. C., and Leloir, L. F., Biochem. J., 51, 426 (1952).
Putnam, E. W., Hassid, W. Z., Krotkov, G., and Barker, H. A., J. Biol. Chem., 173, 785 (1948).
Ringertz, N., Acid Polysaccharides of Mast Cell Tumors, Uppsala 1960.
Silbert, J. E., and Brown, D. H., Biochem. Biophys. Acta, 54, 590 (1961).