In vivo Incorporation of D-Glucosamine-1-C^{14} into Acid Mucopolysaccharides of Rabbit Liver. (28602)

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During the last few years, knowledge has developed concerning the biosynthesis of the heteropolysaccharides. A biosynthetic pathway for hyaluronic acid has been established (1); from glucose this involves the formation of uridinediphospho-D-glucuronic acid and uridinediphospho-N-acetyl-D-glucosamine, which are precursors of the heteropolysaccharide(2).

It has been shown that D-glucosamine-1-C^{14} injected into rats and rabbits(3,4) is incorporated into the glycoproteins of the body tissues and fluids. In this investigation, we have studied the incorporation of D-glucosamine-1-C^{14} into the acid mucopolysaccharides of rabbit liver.

Experimental. Fasting young white male rabbits weighing 1504 (1342-1666) g were injected intraperitoneally with 60 microcuries of D-glucosamine-1-C^{14}. At intervals of 0.5 to 72 hours following the injection, the animals were sacrificed by exsanguination from the abdominal aorta. The liver was removed, perfused with ice cold saline, lyophilized and extracted with n-butanol and acetone. The acid mucopolysaccharides were extracted from 2 g of this preparation by a modification of the method described by Schiller *et al.*(5). This involved extraction with 0.5 N NaOH, neutralization, ethanolic precipitation, digestion with proteolytic enzymes, trichloroacetic acid precipitation of protein, and dialysis followed by passage of the acid-free preparation through Dowex 50 resin at 0°. Mucopolysaccharides were precipitated with cetylpyridinium chloride and fractionated by selective solubilization of the cetylpyridinium salts of the mucopolysaccharides (Scott, 6). By this technique the cetylpyridinium salts were resolved into three fractions: those soluble in 0.5, 1.5 and 2.0 molar sodium chloride. Cetylpyridinium chloride was removed by precipitation with KCNS (7); soluble salts were then removed by dialysis.

The resulting polysaccharide preparations were analyzed for uronic acid by a modification of the carbazole method(8), and for hexosamine(9).

For further studies of the hexosamine constituents, samples of the polysaccharide preparations were hydrolyzed in 4N HCl in closed tubes at 100°C for 6 hours. After removal of HCl *in vacuo* at room temperature, the hydrolysates were dissolved in water, again taken to dryness, and finally dissolved in 10% isopropanol. Aliquots were subjected to descending paper chromatography on Whatman No. 1 paper using an ethyl acetate-pyridine-water (12:5:4) solvent system which separates glucosamine and galactosamine. Amino sugars were detected by dipping the chromatogram in a 0.2% ninhydrin solution (in acetone with 4% acetic acid), followed by development at room temperature. Spots with the mobility of glucosamine were found in all hydrolysates; however, galactosamine was never detected. Radioisotope studies of the glucosamine areas of the paper chromatograms were made by cutting out the appropriate areas and placing them directly in vials for counting in a liquid scintillation system. Comparable areas, where galactosamine would be present as indicated by the standards, were similarly counted. Definite radioactivity was always associated with the glucosamine areas, but only negli-

TABLE I. Analytical Data for Mucopolysaccharides Isolated from Rabbit Liver (Average Values for 10 Samples Expressed in μM/g of Dry Defatted Liver).

	Fraction			
	.5M	1.5M	2.0M	Total
Uronic acid (μM)	.444	.263	.200	.907
Hexosamine (μM)	.343	.158	.105	.606

Supported in part by a predoctoral fellowship from Nat. Inst. Health. Heparitin sulfate was kindly supplied by Dr. J. A. Cifonelli and keratosulfate by Mr. M. W. Hurst.

gible counts were found in galactosamine areas.

Studies of the uronic acid constituents were made by hydrolyzing samples of the preparation with 5 g AG 50 × 12 resin, 100-200 mesh (Bio Rad Laboratories) at 90°C for 72 hours. This hydrolysate was concentrated *in vacuo* and aliquots subjected to paper chromatography on Whatman No. 1 paper, using an ethyl acetate-pyridine-water (12:5:4) solvent system. Sugars were detected by dipping in aminobiphenyl reagent (3 g aminobiphenyl, 100 ml acetic acid, 1.3 ml 85% H_3PO_4) followed by heating at 105°C for 5-10 minutes. Spots with the mobility and staining characteristics of standard glucuronic acid were found in all preparations; however, no radioactivity was found associated with these areas when counted in the liquid scintillation system. No other sugars were detected.

The acid mucopolysaccharide preparations were subjected to paper electrophoresis on S & S 2043A paper with a potential gradient of 2 volts per cm in lithium sulfate buffer, pH 5.4 ($\mu = 0.66$), for 16 hours(10). A Durrum type cell was used. After electrophoresis of the 0.5 M fraction, 2 bands were noted: one at the origin and the other with a mobility similar to a standard preparation of hyaluronic acid. Both of these bands stained with Alcian blue, thus indicating the presence of an acid mucopolysaccharide, but they did not stain metachromatically with toluidine blue, indicating the absence of sulfation. The presence of a small amount of protein at the origin was indicated by a faint band after staining with Nigrosin.

The 1.5 M fraction was also resolved into 2 bands by electrophoresis, one of which remained at the origin and had staining characteristics identical with that of the nonmobile component in the 0.5 M fraction. The second band did not have a mobility which matched any of the standard preparations tried (hyaluronic acid, chondroitin sulfate, heparitin sulfate, keratosulfate and heparin). The mobility of this band was slightly less than the mobility of heparitin sulfate. It stained with Alcian blue and metachromatically with toluidine blue.

TABLE II. Radioactivity of Isolated Liver Mucopolysaccharides.

Hr after inj of D-glucosamine-1-C^{14}	1000 c/m per μM hexosamine		
	0.5M fraction	1.5M fraction	2.0M fraction
	Specific activity		
.5	3	2	6
1	28	62	54
2	29	57	56
4	22	42	5
6	6	42	11
8	5	25	12
12	9	12	3
48	8	3	2
72	7	4	1

The 2.0 M fraction appeared to contain only one mucopolysaccharide. After paper electrophoresis, only one band was detected which stained with both Alcian blue and metachromatically with toluidine blue, and did not contain detectable protein. It had a mobility equal to heparitin sulfate. In contrast, Schiller *et al.*(5) found that heparitin sulfate dissociated from its cetylpyridium salt at a 1.2 M concentration of sodium chloride.

No appreciable activity could be found associated with any of the amino acids of the protein bound fractions or with the uronic acid. All of the preparations contained radioactive glucosamine; however, the specific activity (c/m per μM hexosamine) in the different fractions was found to vary with the time after injection of labeled glucosamine (Table II).

The maximum incorporation of the label occurs in the 1 to 6 hour interval. In the 2.0 M fraction, this initial uptake is followed by a rather rapid decrease in activity indicating a more rapid turnover of this fraction than in the case of those in the 0.5 and 1.5 M fractions.

It is of interest to compare the specific activity of the liver mucopolysaccharides with that of the liver glycoproteins. Comparative data are presented in Table III.

During the interval of maximum incorporation of the label into the polysaccharide moiety (1 to 6 hours), the specific activity of the polysaccharides is usually higher than that of the glycoproteins. This seems to indicate that D-glucosamine is utilized more

TABLE III. Liver Mucopolysaccharide and Glycoprotein Radioactivity.

Hr after inj of D-glucosamine	% of total bound radioactivity in mucopoly-saccharides	Ratio of specific activity (c/m per μM hexosamine) mucopolysaccharide to glycoprotein		
		.5M fraction	1.5M fraction	2.0M fraction
.5	4.7	1.0	.6	1.9
1	9.3	1.4	3.0	2.7
2	10.5	1.7	3.3	3.3
4	5.2	1.3	2.6	.3
6	3.4	3.2	3.2	.8
8	4.5	.3	1.6	.8
12	2.5	1.2	1.7	.4
48	8.8	3.3	1.3	.6
72	4.2	1.8	1.2	.4

readily by the system synthesizing mucopolysaccharides. The mechanism of incorporation of amino sugar moieties into protein molecules is unknown. The difference in specific activity of the liver glycoproteins and mucopolysaccharides may indicate different mechanisms of incorporation of glucosamine. It is of interest that no chondroitin sulfate or heparin were detected in these rabbit liver preparations. However, Marx *et al.*, have reported a very low heparin concentration (1.11 units/g) in rabbit liver(11).

Summary and conclusion. Studies have been made of the *in vivo* incorporation of radioactive glucosamine into the mucopolysaccharides of rabbit liver. After intraperitoneal injection of radioactive glucosamine, acid mucopolysaccharides with high specific activity were isolated from the liver. Radioactively labeled substances with electrophoretic mobility (on paper) and staining characteristics of hyaluronic acid and heparitin sulfate were found. Glucosamine was the only radioactive substance found in the mucopolysaccharide preparations. This radioactivity amounted to only 2.5 to 10.5% of the total bound radioactivity. A system is apparently present which utilizes preformed glucosamine for the synthesis of acid mucopolysaccharides.

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Received July 3, 1963. P.S.E.B.M., 1963, v114.

Metabolism of Injected $Na_2S^{35}O_4$ in the Rat. I. Effect of Liver Injury on Serum Sulfate.* (28603)

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When inorganic $S^{35}O_4$ is injected intraperitoneally in rats, it rapidly becomes associated with plasma proteins and resists prolonged dialysis even at high pH(1,2,3). All present

* This study was supported in part by USPHS grant.