ISOLATION OF LIPID GLUCURONIC ACID AND N-ACETYLGLUCOSAMINE DERIVATIVES FROM A RAT FIBROSARCOMA[1,2]

John J. Hopwood[3] and Albert Dorfman

Departments of Pediatrics and Biochemistry,
Joseph P. Kennedy, Jr., Mental Retardation Research Center
Pritzker School of Medicine
University of Chicago
Chicago, Illinois 60637

Received February 18,1977

SUMMARY

Incubation of labeled UDP-GlcUA and UDP-GlcNAc with microsomes of a fibrosarcoma yielded labeled glycolipids resistant to hydrolysis with dilute alkali. These compounds have been tentatively identified as lipid-GlcNAc, lipid-GlcNAc-GlcUA and lipid-tetra and hexasaccharides containing both GlcUA and GlcNAc.

Lipid intermediates, first detected during studies of peptidoglycan synthesis in prokaryotes (1), participate in the synthesis of bacterial lipopolysaccharides and polysaccharides (2), and in the synthesis of glycoproteins by eukaryotes (3). While our work was in progress, Turco and Heath (4) reported the isolation of a lipid-P-GlcNAc-GlcUA component from SV40-transformed human lung fibroblasts. Using microsomal preparations from a rat fibrosarcoma we now report the isolation of lipid components containing GlcNAc and GlcUA.

MATERIALS AND METHODS

Unless otherwise stated, materials and methods were as previously described (5).

GlcNAc and GlcUA transfer from UDP-GlcNAc and UDP-GlcUA by microsomal preparations into lipid soluble and polysaccharide products was determined by incubation of a standard mixture, final volume of 3.24 ml, consisting of 150 μmoles of Na_2HPO_4-

1 This investigation was supported by National Institutes of Health Grants AM-05996, HD-04583 and HD-09402.

2 Preliminary results were presented at the Third International Symposium of Glycoconjugates, Brighton, England, July, 1975.

3 Kennedy Fellow In the Neurosciences. Present Address: Department of Chemical Pathology, Adelaide Children's Hospital, North Adelaide, S. A. 5006, Australia.

Abbreviation : diHA, 2-acetamido-2-deoxy-3-0-(β-D-glucopyranosyluronic acid)-D-glucose.

ISSN 0006-291X

KH_2PO_4 buffer, pH 7.15, 48 μmoles of $MgCl_2$, 6 μmoles of dithiothreitol, 60 μmmoles of UDP-GlcNAc and UDP-GlcUA, 1.92 μCi of UDP-[^{14}C]GlcUA, (0.20 Ci/mmole) and 3.96 μCi of UDP-[^{3}H]GlcNAc (6.6 Ci/mmole, New England Nuclear, Boston, Massachusetts), 30 μmoles of ATP and 6 mg of microsomal protein in 1.2 ml containing 150 μmoles of Tris-HCl buffer, pH 7.15 and 420 μmoles of sucrose. UDP-[^{14}C]GlcNAc used was 42 mCi/mmole (Schwarz, Bioresearch, Inc.).

Following incubation, mixtures were centrifuged at 15,000 x g for 20 min. Pellets were suspended in 5 ml of 0.1M phosphate buffer, pH 7.15, and extracted twice with 20 ml of water-saturated butanol by vigorously shaking for 15 to 30 s immediately and about 15 min after the addition of the butanol. The suspension was centrifuged (4000-5000 x g) to separate the two phases. The combined butanol extracts were washed twice with butanol-saturated H_2O and 4M KOH was added to adjust the alkali concentration to 0.1M. The mixtures were incubated at 37° for 20 min, cooled to 4° and neutralized with ice-cold 1.0M acetic acid. The butanol phase was washed twice with 20 ml of butanol-saturated water and stored at -20°.

Descending paper chromatography was performed on Whatman 3MM paper in Solvent A (isobutyric acid:2.0M NH_3, 5:3, v/v), Solvent B (1.0M NH_4acetate:ethanol, 3.5:6.5 v/v) and Solvent C (1-butanol: pyridine:0.1M HCl, 5:3:2 v/v/v).

RESULTS

It was previously shown that microsomal preparations from a rat fibrosarcoma synthesize hyaluronic acid (5). When incubation mixtures were further examined, butanol-soluble components containing label derived from UDP-[^{3}H]GlcNAc and UDP-[^{14}C]GlcUA were found.

Extraction of the reaction mixture with butanol yielded 13,000 cpm of ^{3}H and 1220 cpm of ^{14}C in the butanol phase. The labeled butanol-soluble products chromatographed on paper in Solvent A near the solvent front and well ahead of the nucleotide sugar intermediates or products of their degradation. Two peaks of ^{14}C-labeled material (R_f 0.79 and 0.87, Fig. 1a) and ^{3}H-labeled material (R_f 0.79 and 0.94) were observed. Following dilute acid hydrolysis, three major peaks of radioactive material separated on paper chromatography. Peak A material (Fig. 1b) co-migrated with diHA and contained 32% and 65% of the total ^{3}H and ^{14}C respectively. Peak B material co-migrated with authentic GlcNAc, contained 46% of the total ^{3}H radioactivity and was free of ^{14}C. Peak C material contained 26% and 32% of the total ^{3}H and ^{14}C respectively. Doubling the period of alkali treatment of the butanol-soluble material described in the butanol extraction procedure lowered the yield of Peak C material to 5% and 8% of

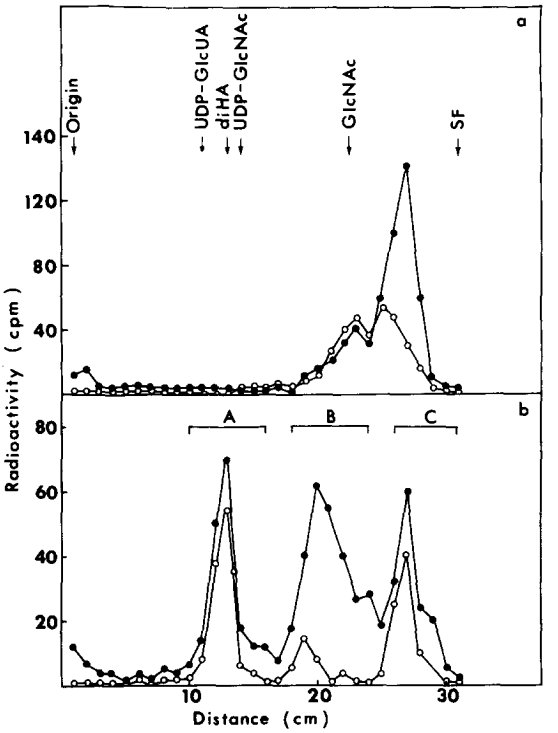

Fig. 1. Paper chromatography in Solvent A of a) butanol-soluble components, and b) water-soluble material produced from acid hydrolysis of butanol-soluble components labeled with [^{3}H]GlcNAc (●——●) and [^{14}C]GlcUA (o---o). The incubation mixture was maintained at 37° for 30 mins and extracted with butanol. One quarter of the butanol-soluble components were concentrated at 10° under a stream of N_2 to a volume of 0.25 ml, applied to Whatman 3MM paper and chromatographed in Solvent A. One quarter of the butanol-soluble components were mixed with 8 ml of isopropanol and 1.6 ml of 1.0N HCl. The mixture was heated at 50° for 1 hr, cooled to 4°, vigorously shaken with a mixture of 9 ml of H_2O, 8 ml of chloroform and 8 ml of methanol and centrifuged at 2000 x g for 20 min. The aqueous layer was freeze-dried, dissolved in 0.15 ml of water and chromatographed as described above. Paper chromatograms were dried, cut into 1 cm segments and radioactivity assayed.

the total [^{3}H]GlcNAc and [^{14}C]GlcUA, respectively. In another preparation of butanol-extractable labeled material, the Peak C material contained less than 2% of the total of both labels.

After 5 mins of mild acid hydrolysis (0.01N HCl, 100°) of butanol-soluble material, more than 50% of ^{3}H and ^{14}C-labeled material was converted to water-soluble products (Fig. 2). Alkaline hydrolysis (0.1N NaOH, 100° for 30 mins) yielded 83%

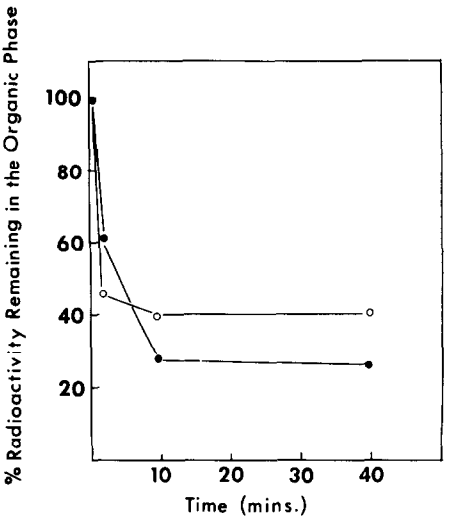

Fig. 2. Butanol-soluble components, labeled with [^{3}H]GlcNAc and [^{14}C]GlcUA were isolated as described under Materials and Methods. Mixtures, adjusted to 0.01N HCl with 1.0N HCl, containing 2.5 ml of butanol extract, 1.5 ml of water and 1.5 ml of isopropanol were heated to 100°. After the intervals indicated the samples were cooled to 4° and neutralized with 2N KOH, thoroughly mixed with 1.3 ml of chloroform, 1.3 ml of methanol and 1.4 ml of water, and centrifuged at 2000 x g for 20 min. The organic phase was washed with 2.0 ml of methanol:water (1:1 v/v), placed in glass counting vials, dried under a stream of nitrogen and radioactivity measured, ●——●, ^{3}H; o---o, ^{14}C.

and 85% water-soluble ^{14}C and ^{3}H-labeled products, respectively. Bacterial alkaline phosphatase (Worthington, Biochemical Corp., code BAPF) treatment (6) of this water-soluble material was required to produce ^{3}H and ^{3}H/^{14}C-labeled components that co-chromatographed in Solvent A with GlcNAc and diHA, respectively.

Two separate incubation mixtures (total volume reduced from 3.24 ml to 0.27 ml) one containing 0.13 μCi of UDP-[^{14}C]GlcNAc but without UDP-[^{3}H]GlcNAc and UDP-GlcNAc, the other without UDP-GlcUA and UDP-[^{3}H]GlcNAc were incubated at 37° for 30 min. Butanol-soluble labeled materials, separated from these incubation mixtures, were retarded on DEAE-cellulose under conditions which do not lead to the absorption of polyisoprenol monophosphate derivatives (7). More than 78% of the [^{14}C]GlcUA-labeled products were eluted in a single peak with 0.1M ammonium acetate, whereas two peaks of radioactive material were obtained for [^{14}C]GlcNAc-labeled products.

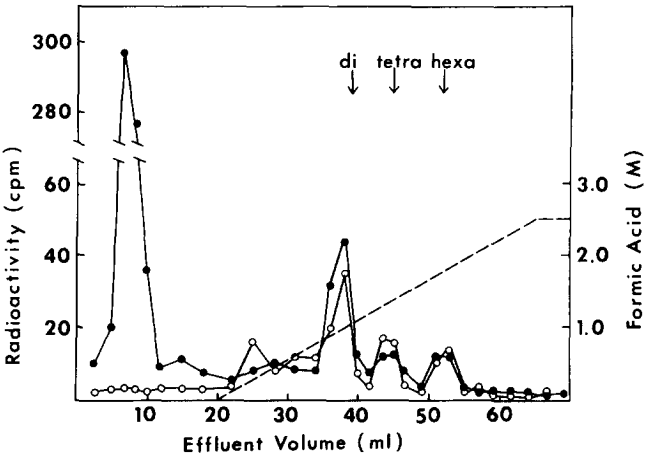

Fig. 3. Butanol-soluble components were labeled with [^{3}H]GlcNAc and [^{14}C]GlcUA, isolated and acid hydrolysed as described in the legend to Fig. 1. Water-soluble material, isolated after acid hydrolysis of the butanol-soluble components, was freeze-dried, dissolved in 4.0 ml of water and applied to a column (1 x 14 cm) of Dowex 1 (formate) equilibrated with 0.05M HCOOH. After washing with 16 ml of 0.05M HCOOH, the column was eluted with a HCOOH gradient from 0.05M to 2.5M concentration (-----). An aliquot (0.5 ml) of each effluent fraction was analyzed for ^{3}H and ^{14}C radioactivity, ●——● and o---o, respectively. Arrows indicate the peak elution positions of di, tetra and hexasaccharide fragments isolated from a testicular hyaluronidase digest of hyaluronic acid.

In another experiment, butanol-soluble products, [^{14}C]GlcUA (1400 cpm) and [^{3}H]GlcNAc (12,000 cpm), were hydrolyzed with 0.01N HCl at 100° for 20 min. Water-soluble materials (more than 75% of both) were applied to a column of Dowex 1 Cl^{-} (Fig. 3). Sixty-eight percent of the applied ^{3}H-labeled material passed through the column with water and more than 80% of this fraction co-chromatographed on paper with authentic GlcNAc in Solvents A and C. The column was then eluted with a linear gradient (0.05M to 2.5M) of formic acid. A major peak of radioactivity containing 74% of the total applied ^{14}C and 12% of the ^{3}H was eluted at a concentration of 1.0M formic acid which also eluted authentic diHA (Fig. 3). This ^{3}H and ^{14}C-labeled material co-chromatographed with diHA in Solvent A as a single radioactive spot. Although the activity was low, the remaining ^{3}H and ^{14}C-labeled material appeared to be eluted from the Dowex 1 column in two peaks at

formic acid concentrations required to elute tetra- and hexasaccharides of hyaluronic acid (Fig. 3). Total recovery of ^{14}C- and ^{3}H from the Dowex 1 column was 95% and 86%, respectively.

In order to determine which monosaccharide was at the reducing end of the lipid-linked oligosaccharide, material labeled only with [^{14}C]GlcUA (1150 cpm) (prepared by omission of UDP-[^{3}H]GlcNAc from the reaction mixture) was acid hydrolyzed and the resultant water-soluble material was fractionated on a column of Dowex 1 as described in the legend to Fig. 3. Approximately 90% of the applied ^{14}C was eluted in a single peak at a formic acid concentration corresponding to that which elutes diHA. This material was treated with Na[^{3}H]BH_4 at pH 8.5 and 25° in 1M Tris. After 45 min , the pH was adjusted to 4.0 with acetic acid, passed through a column of Dowex 50-H^+, dried by repeated addition and evaporation of methanol, dissolved in 3 ml of H_2O and applied to a column of Dowex 1 (formate). On elution with a linear gradient (from 0.05M to 2.5M) of formic acid, more than 78% of the applied ^{14}C appeared as a single peak between 0.85N and 1.08N formic acid and contained ^{3}H which co-chromatographed in Solvent C as a single peak with material produced by reduction of diHA with Na[^{3}H]BH_4 by the method described above.

The products of strong acid hydrolysis (4M HCl, 8 hrs at 100°) of the [^{3}H][^{14}C]labeled material were applied to a column of Dowex 50-H^+ and the column eluted with a linear gradient (0.1 to 1.0N) of HCl. Approximately 75% of the total ^{3}H applied eluted as a single peak in the same area (0.4 to 0.5N HCl) as does authentic glucosaminitol. This material was pooled and an aliquot chromatographed in Solvent C and subjected to electrophoresis under conditions which separate glucosaminitol from galactosaminitol (8). More than 85% of the ^{3}H-labeled material migrated with authentic glucosaminitol. These data indicate that the major sugar at the reducing end of the [^{14}C]GlcUA-labeled disaccharide is glucosamine.

DISCUSSION

The results presented indicate the presence in a rat sarcoma of lipid pyrophospho-GlcNAc-GlcUA compound. The designation of a pyrophosphate bond in all butanol-soluble products containing GlcUA is based on the behavior of the GlcUA-GlcNAc-lipid component on DEAE-cellulose and its pattern of alkaline decomposition. The unknown contribution of the charged GlcUA residue to the

total charge of the GlcUA-GlcNAc-lipid product, complicates interpretation of the result from DEAE-cellulose chromatography. Obviously further study of these products is required before a final and accurate definition of their composition and structure is possible. The presence of a disaccharide containing GlcUA and GlcNAc raises the possibility that this compound may be an intermediate in the synthesis of hyaluronic acid, heparan sulfate or heparin, all of which contain GlcUA-GlcNAc disaccharide sequences. In hyaluronic acid, GlcUA is linked $\beta 1 \rightarrow 3$ to GlcNAc, whereas the bond in heparan sulfate and heparin is $\beta 1 \rightarrow 4$. The linkage in the disaccharide present in the lipid compound has not yet been determined.

In addition to the lipid-containing disaccharide, evidence has been obtained for the presence of trace amounts of tetra and hexasaccharides linked to lipid. A lipid-linked GlcNAc was also demonstrated. This compound could serve as acceptor for transfer of GlcUA or may be present simply as intermediate for glycoprotein synthesis (3).

Preliminary experiments with tunicamycin, an inhibitor of lipid-pyrophosphate-GlcNAc synthesis (10,11) showed little inhibition of hyaluronic acid synthesis by a cell-free preparation of the rat sarcoma. No inhibition was found by tunicamycin of hyaluronic acid synthesis by cultured rat glial cells (Horwitz, A. and Dorfman, A., unpublished results) or in cell-free preparations of Group A streptococci (Sugahara, K., Schwartz, N. B. and Dorfman, A., unpublished results). Previous studies of the streptococcus system gave no evidence of involvement of a lipid intermediate in hyaluronic acid synthesis (12,13).

These findings raise doubt whether hyaluronic acid is synthesized *via* a lipid-GlcNAc-GlcUA intermediate, but a final decision regarding the role of the lipid disaccharide in either heparin (or heparan sulfate) or hyaluronic acid synthesis must await further study.

REFERENCES

1. Anderson, J. C., Matsuhashi, M., Haskin, M. and Strominger, J. L. (1965) Proc. Natl. Acad. Sci. USA 53, 881-889.
2. Hemming, F. W. (1974). In: "Biochemistry of Lipids". Biochem. Ser. One. Ed: Goodwin, T. W. Chapter 4, pp. 39-98. University Park Press, Baltimore.
3. Waechter, C. J. and Lennarz, W. J. (1976). Ann. Rev. Biochem. 45, 95-112.

4. Turco, S. J. and Heath, E. C. (1976). Fed. Proc. 35, Abst. 104.

5. Hopwood, J. J., Fitch, F. W. and Dorfman, A. (1974). Biochem. Biophys. Res. Commun. 61, 583-590.

6. Lucas, J. J., Waechter, C. J. and Lennarz, W. J. (1975). J. Biol. Chem. 250, 1992-2002.

7. Leloir, L. F., Staneloni, R. J., Carminatti, H. and Behrens, N. H. (1973). Biochem. Biophys. Res. Commun. 52, 1285-1291.

8. Takasaki, S. and Kobata, A. (1974). J. Biochem. (Tokyo) 76, 783-789.

9. Roseman, S. and Ludowieg, J. (1954). J. Amer. Chem. Soc. 76, 301-302.

10. Takatsuki, A., Kohno, K. and Tamura, G. (1975). Arg. Biol. Chem. 39, 2089-2091.

11. Tkacz, J. S. and Lampen, J. O. (1975). Biochim. Biophys. Res. Commun. 65, 248-257.

12. Ishimoto, N. and Strominger, J. L. (1967). Biochim. Biophys. Acta 148, 296-297.

13. Stoolmiller, A. C. and Dorfman, A. (1969). J. Biol. Chem. 244, 236-246.