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CONVERSION OF CARBOXYL GROUPS OF MUCOPOLYSACCHARIDES INTO AMIDES OF AMINO ACID ESTERS

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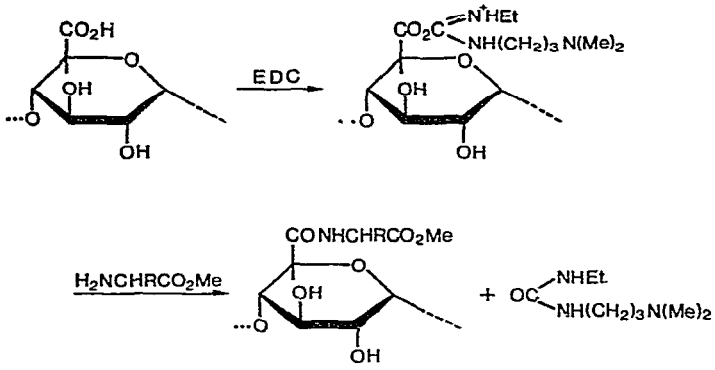
ABSTRACT

The carboxyl groups of mucopolysaccharides were converted into substituted amides of amino acids by the activation of the carboxyl group with a water-soluble carbodiimide followed by nucleophilic reaction of an amino acid methyl ester with the activated carboxyl group. The reaction is comparatively rapid and proceeds under mild conditions and no other modification of the polysaccharide occurs. Amides of the carboxyl groups of heparin were obtained with glycine methyl ester, phenylalanine methyl ester, and glycylphenylalanine amide. The procedure was also extended to other mucopolysaccharides and alginic acid by the use of radioactive glycine ester.

INTRODUCTION

The procedures which are currently available for modification of the uronic acid-carboxyl group of mucopolysaccharides (glycosaminoglycuronans) are conversion into the methyl ester and reduction to a primary alcohol goup[1–4]. However, the series of reactions required for the preparation of these derivatives also cause partial desulfation and degradation. Thus, although the esterified and reduced products are useful intermediates for specific structural studies, they are not derivatives in which the carboxyl group only is affected. This paper describes the reaction of mucopolysaccharides with amino acid esters in the presence of a water-soluble carbodiimide to yield an amide derivative of the uronic acid-carboxyl group. The mechanistic sequence involves the condensation of the uronic acid moiety with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to form the *O*-acylurea followed by the displacement of the substituted urea by the amino group. This yields an amide derivative of the mucopolysaccharide in which the uronic acid is linked to an amino acid or peptide as shown.

The usefulness of water-soluble carbodiimides for the synthesis of peptides in aqueous solution was reported by Sheehan and Hlavka[5]. It was demonstrated that these carbodiimides can effect modification of proteins[6,7] and form linkages between free carboxyl groups of proteins and various amino acids or peptides[8–10]. The general mechanism for the action of carbodiimides proposed by Khorana[11] was investigated in detail for the carboxyl groups of proteins (*e.g.*, ribonuclease, lysozyme,



R = H OR AMINO ACID SIDE CHAIN

insulin) and other model compounds by Hoare and Koshland[12]. It was also shown that under the conditions employed alcoholic hydroxyl groups, like those of serine or threonine, and simple amines did not undergo any reaction.

RESULTS AND DISCUSSION

The first experiments in the present study involved the interaction of glycine methyl ester and heparin in the presence of EDC at pH 4.75. The carbodiimide is comparatively unstable at lower pH; at higher pH, the reaction rate decreases considerably. The analytical data on the resulting heparin glycine derivative (Table I) are consistent with the reaction product shown in the above equation. It is composed of almost equimolar amounts of hexosamine, glycine, and methoxyl groups; the molar ratio of hexosamine to sulfur is similar to that of heparin itself, indicating that the sulfate groups were not hydrolyzed during the reaction. The presence of amide groups is shown by sharp infrared absorption bands at 1680 and 1550 cm^{-1}. The results of the carbazole and orcinol reactions for uronic acid were decreased by as much as 50% of those of heparin. Conversion of the carboxyl group into a substituted amide, which is not completely hydrolyzed during the assay procedure, apparently results in a low color-yield.

The specificity of the reagents for the carboxyl groups and the absence of any other side reactions is also supported by results of electrometric titration experiments. The acidic groups of heparin itself, *i.e.* sulfate and carboxyl, can be titrated separately[1]. Thus, when heparin is isolated by precipitation with ethanol from an acidic solution, only the carboxyl groups can be titrated with alkali. On the other hand, titration of both sulfate and carboxyl groups can be performed after the heparin solution has been passed through a cation-exchange resin in the hydrogen form. In the present study, it was found that almost no carboxyl groups can be titrated after the heparin-glycine methyl ester derivative had been obtained by precipitation with ethanol from an acidic solution. After passing the product through Dowex-50 (H^+), the sulfate groups were titrated (at pH 1.8–2.5) and no free carboxyl groups could be

detected. Saponification of the heparin-glycine methyl ester with dilute alkali yielded the acid derivative in which both carboxyl and sulfate could be titrated (Fig. 1). Analysis of this material showed no loss in the original glycine. None of the titration curves showed the buffering effect from pH 7 to 10 that is observed when *N*-desulfation occurs[1], indicating that sulfoamino groups were not cleaved.

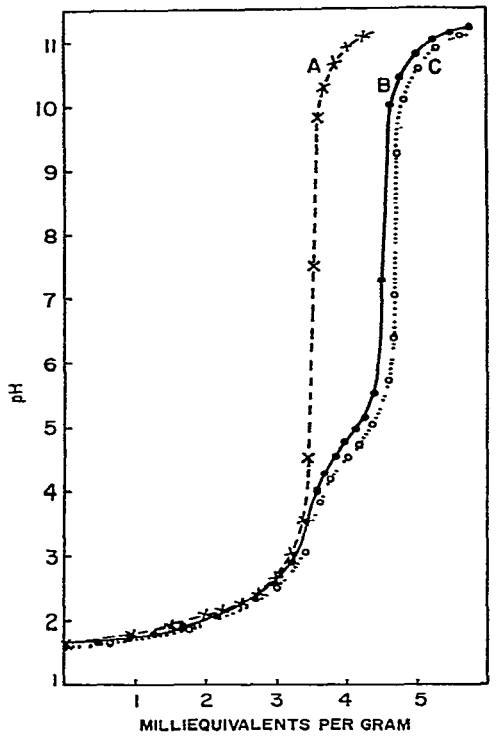


Fig. 1. Titration curves of heparin–glycine methyl ester (*A*), of its saponification product (B), and of heparin (C). Aqueous solutions of these materials were passed through a column of Dowex-50 (H^+), and the effluents were titrated with 0.0965M sodium hydroxide. For purposes of comparison, the results were converted to mequiv of alkali per g of mucopolysaccharide derivative.

In order to study a reactant which might present some steric hindrance, the reaction was also performed with phenylalanine methyl ester and glycylphenylalanyl amide. The results (Table I) show that the molar ratio of hexosamine to amino acids in these products was approximately 1 to 0.7. The degree of reaction is thus clearly decreased when bulky reagents are employed.

Amino acid incorporation into the products was also verified by paper chromatography of hydrolyzates wherein the respective amino acids were identified by comparison with standards. Heparin-phenylalanine methyl ester also showed the characteristic u.v. absorption spectrum (maxima at 246, 251, 257, 263 nm) exhibited by the free amino acid. The phenylalanine content of the product, calculated from the

known extinction coefficients, compared well with the values obtained by amino acid analysis.

TABLE I

COMPOSITION OF HEPARIN-AMINO ACID DERIVATIVES[a]

Sample	*2-Amino-2-deoxy-D-glucose*	*Sulfate*	*Glycine*	*Phenylalanine*
Untreated heparin	1.39	3.48		
Derivative with:				
glycine	1.22	3.16	1.17	
L-phenylalanine	1.21	3.13		0.90
glycyl-L-phenylalanine	1.13	2.89	0.92	0.85

[a]In mmol per g. The values in percent are given in the experimental section.

It is noteworthy that the product obtained with glycine methyl ester has practically no blood anticoagulant activity, suggesting that the free carboxyl groups are necessary for biological activity. The product of reaction with phenylalanine methyl ester, in which only about 70% of the carboxyl groups were substituted, retained about 20% of the anticoagulant activity.

The reaction with glycine methyl ester was also investigated with other polysaccharides containing carboxyl groups. In these studies, glycine ester labeled with ^{14}C on the carboxyl carbon was employed, and the extent of reaction was determined by the specific activity of the isolated product. The conditions for the reaction were similar to those employed with the nonradioactive amino acid esters, except that the concentrations of the reactants were somewhat lower (Table II). The considerable

TABLE II

REACTION OF GLYCINE METHYL ESTERS WITH VARIOUS POLYSACCHARIDES

Polysaccharide	*C.p.m. per mg* $\times 10^{-4}$	*Glycine*[a], *mmol/g*	*Hexosamine*[b], *mmol/g*	*Percent of reaction*[c]
Heparin	5.54	1.06	1.22	86.9
Heparan sulfate	5.87	1.12	1.30	86.2
Chondroitin 4-sulfate	2.62	0.50	1.52	32.9
Chondroitin 6-sulfate	2.76	0.53	1.47	36.0
Dermatan sulfate	2.22	0.43	1.52	28.3
Hyaluronic acid	4.50	0.86	2.23	38.6
Alginic acid	8.06	1.55		38.4

[a]Calculated from the specific activity of glycine ester employed for the reaction ($52{,}145 \times 10^3$ c.p.m. per mmol of glycine). [b]Determined by the Elson–Morgan reaction on a hydrolyzate of the isolated reaction product. [c]Based on the total number of carboxyl groups, assuming equimolar amounts of hexosamine and uronic acid. With alginic acid, the value is based on the theoretical equiv. wt of a uronic acid unit.

difference in amount of glycine methyl ester incorporated by heparin or heparan sulfate, as compared to the other polysaccharides investigated, is probably due to the

greater accessibility of the carboxyl groups in the former materials. One structural characteristic of heparin and heparan sulfate which differentiates them from hyaluronic acid and the chondroitin sulfates is that the former substances possess an α-D-(1→4) glycosyluronic linkage, whereas the chondroitin sulfates and hyaluronic acid possess a β-D (α-L for dermatan sulfate) linkage. Considering models of such compounds, it is quite conceivable that the carboxyl groups in heparin and heparan sulfate are more exposed than those of the other polysaccharides investigated. Should this explanation be true, it would be expected that disaccharides or oligosaccharides of the other substances would react to a greater degree. This concept is presently under investigation.

The preparation of amide derivatives of the uronic acid unit with amino acids and peptides has a number of applications, both in structure determinations and in studies of biological activity. By blocking the carboxyl function in a mucopolysaccharide, hydrolysis of the acid resistant glycosyluronic linkage may be increased considerably, so that different oligosaccharides and better yields may be obtained on graded hydrolysis. In the case of biologically active polysaccharides, such as heparin, it is also of interest to study the effect of the structural modification on anticoagulant or lipemia-clearing effects. Studies of the three-dimensional structure or conformational changes in polysaccharides may also be possible by employing amino acids having suitable steric properties or other nucleophiles having specific optical characteristics. Finally, the procedure may be employed for the synthesis of polysaccharide–peptides for immunochemical investigation. It may thus be possible to elicit antibodies directed at polysaccharides which would otherwise not be antigenic. This is conceivable in view of the findings of Rüde *et al.*[14] with various galactose–peptide conjugates.

EXPERIMENTAL

Materials and methods. — Heparin and heparan sulfate were generously supplied by the Upjohn Company. The heparan sulfate was purified by fractionation of the cetylpyridinum complex[15]. Hyaluronic acid, dermatan sulfate, and chondroitin 6-sulfate were prepared from umbilical cord[16]. Chondroitin 4-sulfate and the amino acid and peptide derivatives were products of Miles Laboratories, Inc. [1-^{14}C]-Glycine ester (3.78 mCi per mmol) was purchased from New England Nuclear Corp. and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) from Ott Chemical Company.

The hexosamine content was determined, after hydrolysis with 4M hydrochloric acid for 16 h, by the Elson–Morgan reaction as modified by Boas[17]. The uronic acid content was measured by the carbazole[18] and orcinol[19] reagents. Nitrogen, sulfur, and methoxyl determinations were performed by the Schwarzkopf Microanalytical Laboratory. For the determination of amino acids, samples were hydrolyzed with 6M hydrochloric acid for 22 h at 110°, and the hydrolyzates were analyzed with the amino acid analyzer[20].

Reaction of heparin with glycine methyl ester ("*heparinylglycine*"). — Sodium heparinate (1.0 g) and glycine methyl ester (1.08 g) were dissolved in water (5.5 ml)

and the pH was adjusted to 4.75. To this solution was added EDC (982 mg) dissolved in water (2 ml) at pH 4.75. The mixture was stirred at room temperature, and the pH was maintained at 4.75 by intermittent addition of 0.1M hydrochloric acid for 4 h. The mixture was diluted with water to a final volume of 70 ml, and the heparin derivative was precipitated as the cetyltrimethylammonium salt by addition of 5% cetyltrimethylammonium bromide. The precipitate was centrifuged off and washed with water (10 ml) to remove the excess precipitant and adhering water-soluble reactants. The quaternary ammonium salt was then converted into the sodium salt by exhaustive extraction of the precipitate with a 5% solution of sodium iodide in ethanol[21]. The remaining sodium salt of the heparin derivative was washed with ethanol, dissolved in water (8 ml), and precipitated with ethanol (3 vol.); yield, 902 mg; ν_{max}^{KBr} 1680 (CO), 1550 (NH), 1760 (COO) cm^{-1}.

Anal. Found: N, 3.63; S, 10.10; OMe, 3.84; glucosamine, 21.8; glycine, 8.79; see molar ratios in Table I.

In another experiment with the same components, the reaction mixture was dialyzed exhaustively, concentrated to a small volume, and precipitated with ethanol to give 850 mg of product showing similar analytical results.

Anal. Found: N, 3.56; S, 10.41; OMe, 4.01; glucosamine, 22.2; glycine, 8.84.

The amino acids in this preparation and those described below were also characterized by paper chromatography of the hydrolyzates. The solvents employed were 1:1:1 2,6-lutidine–collidine–water and 10:3:7 butyl alcohol–acetic acid–water. These mixtures clearly separate glycine, phenylalanine, and the hexosamines from each other.

Saponification of heparin-glycine methyl ester. — The methyl ester (100 mg) was dissolved in 0.1M sodium hydroxide (2 ml), and the solution was kept in a nitrogen atmosphere for 2 days at 0–5°. The pH was brought to 5 with acetic acid, and the product was isolated by precipitation with ethanol (72 mg). The saponification procedure did not affect significantly the molar ratios of nitrogen, sulfur, and glycine.

Anal. Found: N, 3.51; S, 11.02; glycine, 8.90.

Reaction of heparin with L-phenylalanine methyl ester. — The conditions for this reaction were similar to those used for the preparation of the glycine methyl ester. The components consisted of heparin (1 g), L-phenylalanine methyl ester hydrochloride (1.8 g), and EDC (900 mg) in water (7 ml). Isolation by the quaternary ammonium salt method yielded 993 mg of product.

Anal. Found: N, 3.18; S, 10.03; OMe, 2.78; glucosamine, 21.62; phenylalanine, 14.66; see molar ratios in Table I.

Reaction of heparin with glycyl-L-phenylalaninamide. — This reaction was performed with heparin (250 mg), EDC (250 mg), and glycyl-L-phenylalaninamide hydrochloride (250 mg) in water (3 ml). The conditions and isolation procedure were similar to those employed in the reactions with the amino acids and yielded 270 mg of derivative.

Anal. Found: N, 4.56; S, 9.26; glucosamine, 20.2; glycine, 6.92; phenylalanine, 13.95; see molar ratios in Table I.

Reaction of other polysaccharides with radioactive glycine methyl ester. — The radioactive glycine ester was diluted with nonradioactive glycine methyl ester to a mixture having a specific activity of $52{,}145 \times 10^3$ c.p.m. per millimole. This material (15 ml) was added to a mixture of polysaccharide (listed in Table II) (25 mg) and EDC (25 mg) in a total volume of 1 ml. The procedure and isolation were similar to those described in the other experiments. As a final step, each product was reprecipitated from aqueous solution with ethanol until constant specific activity was obtained.

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