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SELECTIVE *N*-DESULFATION OF HEPARIN WITH DIMETHYL SULFOXIDE CONTAINING WATER OR METHANOL

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ABSTRACT

A solvolytic *N*-desulfation of heparin was developed by treatment of its pyridinium salt with dimethyl sulfoxide containing 5% of water or methanol for 1.5 h at 50°. Chemical and chromatographic studies showed that the solvolytic desulfation is a useful method for *N*-desulfation of heparin without depolymerization of the heparin molecule. The partially *N*-desulfated heparins were also obtained by treatment with dimethyl sulfoxide containing 5% of water at 20°, and their anticoagulant activity is related to the degree of *N*-desulfation.

INTRODUCTION

For the selective liberation of *N*-sulfate groups from heparin, a method that does not affect the glycosidic linkages or the *O*-sulfate groups would be important for the study of the chemical structure of heparin and for the elucidation of the relationship between chemical structure and biological activity. The methods of *N*-desulfation used until now are based on hydrolysis[1–3], and it has been suggested that the hydrolysis of *N*-sulfate groups is accompanied by some cleavage of the glycosidic linkages and *O*-sulfate groups[4–6]. In a previous paper[7], we reported that the pyridinium salt of 2-deoxy-2-sulfoamino-D-glucose is desulfated much more rapidly than that of D-glucose 6-sulfate in dimethyl sulfoxide containing a small amount of water or methanol. The present work shows that *N*-desulfation of heparin without depolymerization is successfully accomplished in dimethyl sulfoxide containing a small amount of water or methanol.

EXPERIMENTAL

Material. — Heparin prepared from porcine intestinal mucosa was purchased from Cohelfred Laboratories, Chicago, Ill., and had an anticoagulant activity of 171.6

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U.S.P. units. The properties of the heparin preparation are shown in Table I. All analytical data were corrected for moisture content after drying the analyzed sample under reduced pressure for 2 h at 100°.

Analytical methods. — The methods for the quantitative determination of amino groups and total sulfate groups have been reported previously[7]. The uronic acid content was determined by the method of Bitter and Muir[8] and by a modification of this procedure[9] that differs by the omission of borate ions from the reaction mixture, D-glucurono-6,3-lactone being the standard; in this paper, these two methods will be referred to as the carbazole–borate method and carbazole method, respectively. The hexosamine content was determined by a modification of the Elson–Morgan procedure[10], after hydrolysis in 4M hydrochloric acid for 14 h at 100°. Reducing power was measured by the 3,6-dinitrophthalic acid method[11]. The amount of inorganic sulfate liberated from heparin was determined by a modified method[12] of turbidimetry. For automatic amino acid analysis, samples were hydrolyzed in 6M hydrochloric acid, at a concentration of 2 mg/ml, in sealed tubes under nitrogen for 16 h at 100°. Electrophoresis was accomplished, after application of 1.5% solutions to Separax strips, in 0.05M lithium chloride–0.01M hydrochloric acid, pH 2.0 (I 0.06), as described by Breen *et al.*[13]. The strips were stained with 0.5% Alcian Blue solution.

The *N*-acetyl content was estimated by the ratio, in the n.m.r. spectra, of the intensity of *N*-acetyl proton signals to the total intensity of the signals due to the two anomeric protons of the heparin disaccharide unit. These spectra were recorded at 70° with a JNM-PS-100 n.m.r. spectrometer operated at 100 MHz on solutions in deuterium oxide containing sodium 4,4-dimethyl-4-silapentane-1-sulfonate as the internal standard. The preliminary deuterium-exchange was accomplished by repeated addition and evaporation of deuterium oxide.

The *N*-sulfate content was directly determined by turbidimetry of the inorganic sulfate liberated after treatment of the samples with nitrous acid[14]. To the sample solution (0.5 ml), 5% sodium nitrite (0.5 ml) and 33% acetic acid (0.5 ml) were added, and the solution was kept for 30 min. To the solution was added 3.8% trichloroacetic acid (4.5 ml), the mixture was shaken, and then the barium chloride–gelatin reagent (1.5 ml, prepared by dissolving 1.0 g of gelatin and 0.5 g of barium chloride in 100 ml of water) was added and the mixture shaken immediately. After the solution had been kept for 20 min, the turbidity was measured at 500 nm.

The anticoagulant activity was determined by the U.S.P. method.

Gel-filtration of heparin and its derivatives on Sephadex G-75. — The polysaccharides (6.5 mg) dissolved in 0.1M sodium chloride (3 ml) were applied to a column (2.5 × 90 cm) of Sephadex G-75 and eluted with the same solvent at a flow rate of 20 ml/h. The eluate was collected in 5-ml fractions and each fraction was analyzed for uronic acid content.

Preparation of the pyridinium salt of heparin and N-*desulfation with dimethyl sulfoxide containing 5% of water or methanol.* — The sodium salt of heparin (3 g) was passed through a column of Dowex 50W (X-8, H^+, 20–50 mesh) at 4°, and the effluent was neutralized with pyridine and lyophilized to give a white powder (2.9 g).

N-Desulfation of the pyridinium salt with dimethyl sulfoxide containing 5% of water or methanol was performed by a method virtually identical with that described for monosaccharide sulfates[7]. The reducing power, the amount of liberated inorganic sulfate, and the content of amino groups of the reaction mixture were determined at regular time-intervals.

N-*Desulfation of heparin with 0.04*M *hydrochloric acid.* — The sodium salt of heparin (400 mg) was dissolved in 0.04M hydrochloric acid (10 ml). Aliquots (1 ml) of this solution were placed into Pyrex-glass tubes (1.3 x 10 cm) that were stoppered and heated at 100°. At regular time-intervals, one glass tube was removed and cooled in ice–water, and the content was neutralized with 0.04M sodium hydroxide and analyzed as just described.

Preparation of N-*desulfated heparin with dimethyl sulfoxide containing 5% of water.* — The pyridinium salt of heparin (900 mg) was dissolved in dimethyl sulfoxide containing 5% of water (45 ml), and the solution was kept for 1.5 h at 50°. The content was diluted with an equal volume of water and adjusted to pH 9.14 by the addition of 0.1M sodium hydroxide. The solution was dialyzed against running tap-water for 24 h, and then against distilled water for 20 h. Lyophilization of the dialyzate gave a white powder (660 mg).

Preparation of N-*desulfated heparin with dimethyl sulfoxide containing 5% of methanol.* — The pyridinium salt of heparin (400 mg) was suspended in dimethyl sulfoxide containing 5% of methanol (40 ml), and the mixture kept for 1.5 h at 50° to give a homogeneous solution. After the reaction, the content was diluted with an equal volume of water and treated by the same method as just described to give a white powder (270 mg).

Preparation of N-*desulfated heparin with 0.04*M *hydrochloric acid.* — The sodium salt of heparin (500 mg) was dissolved in 0.04M hydrochloric acid (30 ml), and the solution kept for 2 h at 100°. The content was neutralized with sodium hydroxide and treated by the same method as just described to give a white powder (330 mg).

N-*Acetylation of heparin.* — Heparin was acetylated with acetic anhydride in water at pH 6.5–7.0 at 0° by the method of Danishefsky *et al.*[15]. Acetylated heparin showed no free amino groups, as determined by 2,4,6-trinitrophenylation.

Sulfation of N-*desulfated heparins.* — The free amino residues of *N*-desulfated heparins were sulfated by a method virtually identical with that described by Lloyd *et al.*[3]. To a solution of *N*-desulfated heparin (100 mg) in water (4 ml), two portions of trimethylamine–sulfur trioxide (140 mg) were added at an interval of 1 h at 55°. The reaction mixture was kept at pH 9–10 under stirring for 6 h, and examined by electrophoresis on a Separax film. The resulfation procedure was repeated until the mobility of the product was equal to that of heparin. The reaction mixture was dialyzed against running tap-water for 24 h and then against distilled water for 20 h. Lyophilization of the dialyzate gave a white powder (98 mg).

RESULTS AND DISCUSSION

The pyridinium and sodium salts of heparin were treated with dimethyl sulfoxide containing 5% of water at 50° and with 0.04M hydrochloric acid at 100°, respectively, and the time course of their *N*-desulfation is shown in Fig. 1. In the solvolytic desulfation, liberation of inorganic sulfate and amino groups became approximately constant at 1.5 h after the start of the reaction. The reducing power (A_{450}) measured by the 3,6-dinitrophthalic acid method remained constant from the beginning of the reaction, suggesting that this treatment has no effect on the glycosidic linkage. Based on the reaction mechanism[16], *N*-sulfate groups are more reactive than *O*-sulfate groups. Although not shown here, it was observed that the desulfation of heparin showed approximately the same course in dimethyl sulfoxide containing 5% of methanol at 50°.

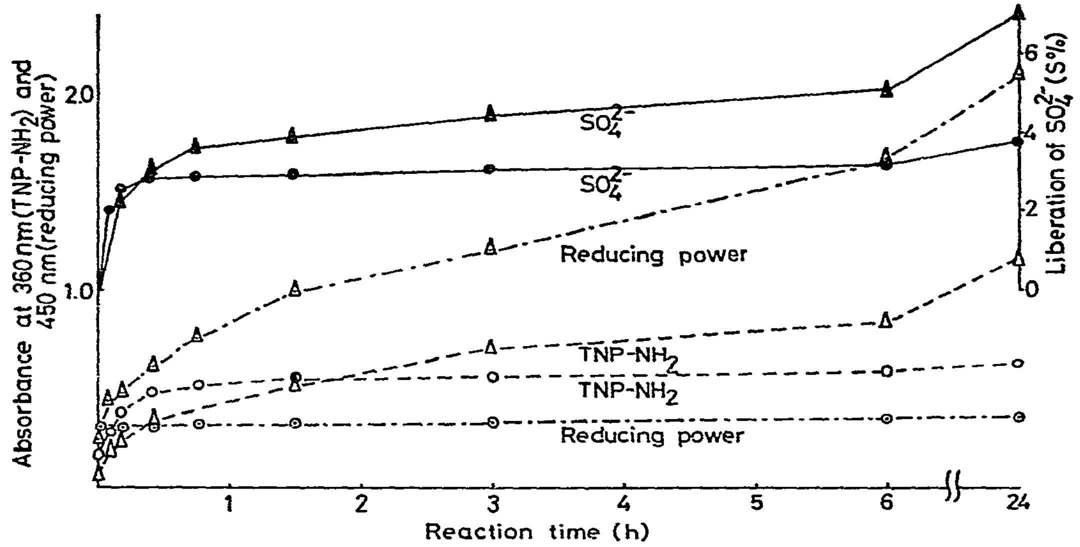

Fig. 1. *N*-Desulfation of the pyridinium salt of heparin with dimethyl sulfoxide containing 5% of water at 50° (○, ⊙, ●) and 0.04M hydrochloric acid at 100° (△, ◬, ▲).

In the hydrolytic desulfation, the liberation of inorganic sulfate and the formation of free amino groups, as determined by the increase in optical density after 2,4,6-trinitrophenylation, were slightly slower than in the solvolytic desulfation, at the beginning of the reaction, but the liberation of inorganic sulfate gradually increased with time, and the reducing power increased markedly. These results indicate that treatment of heparin with 0.04M hydrochloric acid at 100° results in the hydrolysis of a part of the glycosidic bonds and *O*-sulfate groups, in addition to *N*-desulfation.

Therefore, heparin was treated with (a) dimethyl sulfoxide containing 5% of water for 1.5 h at 50°, (b) dimethyl sulfoxide containing 5% of methanol for 1.5 h at

50°, and (c) 0.04M hydrochloric acid for 2 h at 100°. The properties of the desulfated heparins are shown in Table I. The contents of uronic acid, as determined by the carbazole[9] and the carbazole–borate methods[8], and the ratio of these two values indicate that no degradation of the uronic acid occurred under the three sets of conditions. The small proportion of serine in the starting material was not released and the extent of desulfation was the same. The proportion of *N*-sulfate groups in the starting heparin corresponded to 0.79 mol/mol of hexosamine, assuming that the polysaccharide chain consists of repeated disaccharide units of hexuronic acid and hexosamine. The content of *N*-acetyl groups in heparin and *N*-acetylated heparin was 0.11 and 0.18 mol/mol of hexosamine, respectively, indicating a proportion of 0.18 mol of 2-acetamido-2-deoxy-D-glucose/mol of hexosamine, a value in agreement with the proportion of *N*-sulfate groups. From the value of *N*-sulfate groups in *N*-desulfated heparins obtained by solvolysis and hydrolysis and from the ratio of *N*-sulfate to *O*-sulfate groups per repeating disaccharide, it can be deduced that the majority of the *N*-sulfate groups had been liberated, accompanied by some liberation of *O*-sulfate groups under both sets of conditions; solvolysis seems to show a slightly better selectivity for *N*-desulfation than does hydrolysis. The extent of amino group liberation was measured by the absorbance at 360 nm after 2,4,6-trinitrophenylation. The sample obtained by hydrolysis showed the highest value (103.8), which is probably due to the higher absorbance of 2,4,6-trinitrophenylamino groups in partially depolymerized heparin.

In order to establish whether solvolytic *N*-desulfation would degrade the polysaccharide chain of heparin, gel chromatography on Sephadex G-75 of the *N*-desulfated heparins was performed together with the starting material and *N*-desulfated heparin obtained by hydrolysis (Fig. 2). The reducing power (Table I) and

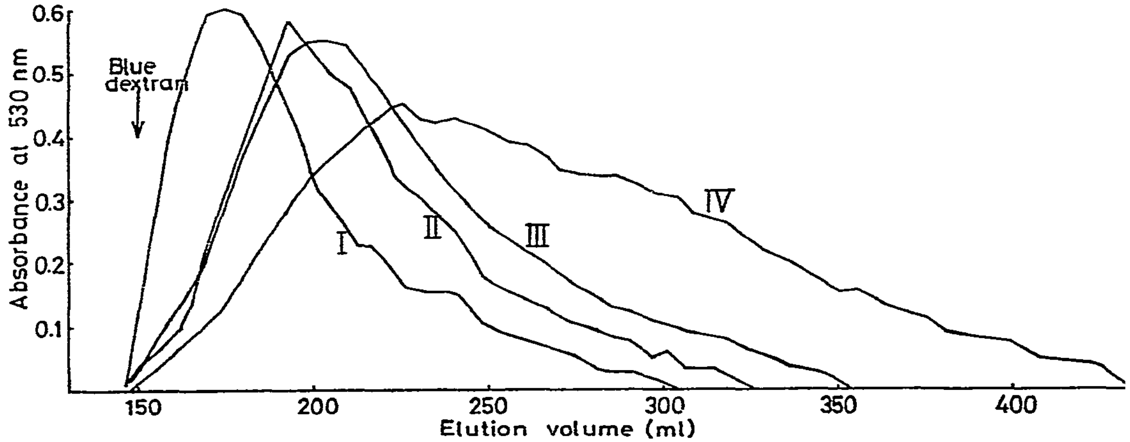

Fig. 2. Gel-filtration of heparin and *N*-desulfated heparins on Sephadex G-75. I, Heparin; II, *N*-desulfated heparin (5% water–dimethyl sulfoxide); III, *N*-desulfated heparin (5% methanol–dimethyl sulfoxide); IV, *N*-desulfated heparin (0.04M hydrochloric acid).

TABLE I

CONDITIONS OF PREPARATION, CHEMICAL COMPOSITION AND PROPERTIES, AND ANTICOAGULANT ACTIVITY OF HEPARIN, *N*-DESULFATED HEPARINS, AND RESULFATED PRODUCTS OF *N*-DESULFATED HEPARINS

Compound	*Condition of* N-*desulfation*			*N* (%)	*Uronic acid* (%)[a]		*Hexosamine* (%)
	Solvent	*Temp.* (°)	*Time* (*h*)		(A)[b]	(B)[c]	
Heparin				2.13	48.0 (0.77)	36.9	25.6
N-Desulfated heparin	5% H_2O–Me_2SO	50	1.5	2.38	56.9 (0.77)	44.0	29.3
N-Desulfated heparin	5% MeOH–Me_2SO	50	1.5	2.30	56.3 (0.77)	43.5	29.8
N-Desulfated heparin	0.04M HCl	100	2	2.26	56.6 (0.77)	43.8	29.4
Resulfated	5% H_2O–Me_2SO	50	1.5				
Resulfated	0.04M HCl	100	2				

[a]In parentheses, ratio (*A*):(*B*) [b]Determined by the carbazole method[9]. [c]Determined by the carbazole–borate method[8]. [d]*N*-Sulfate content as sulfur. In parentheses, the amount of *N*-sulfate remaining in each *N*-desulfated heparin, taking the amount of *N*-sulfate in heparin as 100. [e]In parentheses, the relative value of each sample when the optical density value of the sample, in which *N*-desulfation has progressed the most by treatment with dimethyl sulfoxide containing 5% of methanol, is taken as 100. [f]Given as molar ratio to hexosamine. [g]Calculation based on the assumption that the polysaccharide chain in heparin is made up of repeating disaccharide units of hexuronic acid and hexosamine only. [h]*O*-Sulfate content as sulfur content was estimated from total sulfate less *N*-sulfate content.

the gel-filtration pattern indicate that the solvolytic conditions did not produce fragmentation of the heparin chain to any great extent, but that the hydrolytic procedure caused some cleavage. These conclusions were confirmed by resulfation[3] of two samples of *N*-desulfated heparin that had been obtained by solvolysis and hydrolysis. The Sephadex gel-filtration pattern (Fig. 3) of the resulfated product of the sample obtained by solvolysis was nearly identical with that of intact heparin, whereas the pattern of the resulfated product of the sample obtained by hydrolysis had more-retarded fractions, indicating the presence of lower-molecular-weight substances. As shown in Table I, 73.0% of the biological activity of the intact heparin was recovered on resulfation of the sample obtained by solvolysis, whereas only 34.7% of this activity was recovered in the sample obtained by hydrolysis.

In a similar study, Lloyd *et al.*[3] have reported that resulfation with trimethylamine–sulfur trioxide, in alkaline medium, of heparin that had been desulfated by auto-acid hydrolysis for 24 h at 55° gave a product having a gel-filtration pattern and

Total S (%)	N-*S* (%)[d]	*TNP-NH$_2$* (*Abs$_{360}$*)[e]	*Serine*[f]	*Reducing power* (*Abs$_{450}$*)	*Mole*[g]			*Anti-coagulant activity*
					Total S	N-*S*	O-*S*[h]	
12.44	4.24 (100)	0.060 (7.6)	0.012	0.051	2.31	0.79	1.52	171.6
8.20	0.30 (7.1)	0.789 (99.5)	0.013	0.128	1.25	0.05	1.20	1.46
8.16	0.16 (3.8)	0.793 (100)	0.013	0.149	1.24	0.03	1.21	1.17
7.93	0.67 (15.8)	0.823 (103.8)	0.009	0.693	1.19	0.10	1.09	2.24
11.83	3.89 (91.7)	0.046 (5.8)		0.150	2.13	0.70	1.43	125.2
10.93	3.80 (89.6)	0.021 (2.6)		0.370	1.88	0.65	1.23	59.6

anticoagulant activity similar to those of the starting heparin. The differences between the results of Lloyd *et al.*[3] and the present ones seem to be mainly due to the different hydrolysis conditions used.

The relation between the anticoagulant activity of heparin and the content of *N*-sulfate groups has been previously studied mainly by determination of the deactivation[2,17–19]. The partially *N*-desulfated heparins that were studied had been obtained by partial acid hydrolysis with dilute hydrochloric acid or acetic acid, with the possibility, as mentioned earlier, for other chemical changes to occur. Therefore, in order to re-examine the relationship between the degree of *N*-desulfation of heparin and its biological activity, several samples of partially *N*-desulfated heparin were prepared by treatment in dimethyl sulfoxide containing 5% of water at 20° (see Table II). It has been observed[20] that, under these conditions, 2-deoxy-2-sulfoamino-D-glucose is desulfated to 64% after 24 h, and D-glucose 6-sulfate to only 0.4%. Therefore, selective liberation of the *N*-sulfate group in heparin was anticipated. It is

TABLE II

CONDITIONS OF PREPARATION, CHEMICAL COMPOSITION AND PROPERTIES, AND ANTICOAGULANT ACTIVITY OF PARTIALLY *N*-DESULFATED HEPARINS[a]

Compound	*Conditions of* N-*desulfation*			*Total S* (%)	N-*S* (%)	*TNP-NH$_2$* (*Abs$_{360}$*)	*Mole*			*Anticoagulant activity*
	Solvent	*Temp.* (°)	*Time* (*h*)				*Total S*	N-*S*	O-*S*	
Heparin				12.44	4.24 (100)	0.060 (7.6)	2.31	0.79	1.52	171.6
N-Desulfated heparin	5% H_2O–Me_2SO	20	0.25	11.70	3.24 (76.4)	0.168 (21.2)	2.09	0.59	1.50	147.9
N-Desulfated heparin	5% H_2O–Me_2SO	20	1	11.23	2.79 (65.8)	0.231 (29.1)	1.96	0.49	1.47	115.6
N-Desulfated heparin	5% H_2O–Me_2SO	20	2	10.36	2.22 (52.4)	0.275 (34.7)	1.73	0.38	1.35	52.2
N-Desulfated heparin	5% H_2O–Me_2SO	20	7	9.69	1.86 (43.9)	0.470 (59.3)	1.57	0.31	1.26	38.0

[a] See footnotes to Table I.

of interest that the preparation having almost no *O*-sulfate group and 24% of the *N*-sulfate groups removed still retained 86% of the activity of the starting heparin, and to compare this result with past studies[18,19].

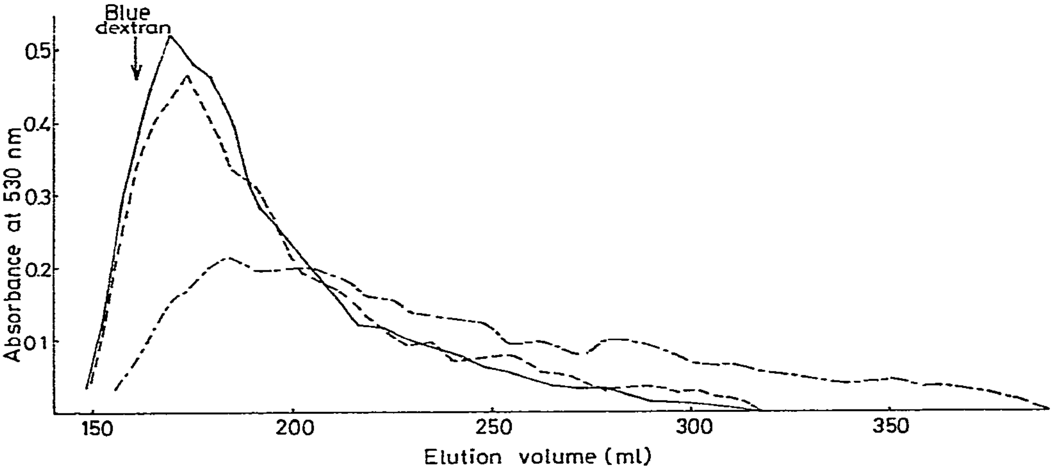

Fig. 3. Gel-filtration of heparin and resulfated products of *N*-desulfated heparins on Sephadex G-75. ————, Heparin; — — — —, resulfated product of *N*-desulfated heparin (5% water–dimethyl sulfoxide); — · — · —, resulfated product of *N*-desulfated heparin (0.04M hydrochloric acid).

REFERENCES

1 M. L. Wolfrom and W. H. McNeely, *J. Amer. Chem. Soc.*, 67 (1945) 748–753.
2 A. B. Foster, E. F. Martlew, and M. Stacey, *Chem. Ind.* (London), (1953) 899–900.
3 A. G. Lloyd, G. Embery, and L. J. Fowler, *Biochem. Pharmacol.*, 20 (1971) 637–648.
4 J. R. Helbert and M. A. Marini, *Biochemistry*, 2 (1963) 1101–1106.
5 J. A. Cifonelli, *Carbohydr. Res.*, 2(1966) 150–161.
6 A. H. Johnson and J. R. Baker, *Biochim. Biophys. Acta*, 320 (1973) 341–351.
7 K. Nagasawa and Y. Inoue, *Carbohydr. Res.*, 36 (1974) 265–271.
8 T. Bitter and H. Muir, *Anal. Biochem.*, 4 (1962) 330–334.
9 Z. Dische, *J. Biol. Chem.*, 167 (1947) 189–198.
10 L. A. Elson and W. T. J. Morgan, *Biochem. J.*, 27 (1933) 1824–1828.
11 T. Momose, A. Inaba, Y. Mukai, and M. Watanabe, *Talanta*, 4 (1960) 33–37.
12 Y. Kawai, N. Seno, and K. Anno, *Anal. Biochem.*, 32 (1969) 314–321.
13 M. Breen, H. G. Weinstein, M. Anderson, and A. Veis, *Anal. Biochem.*, 35 (1970) 146–159.
14 K. Nagasawa and Y. Inoue, *Anal. Biochem.*, submitted.
15 I. Danishefsky, H. B. Eiber, and J. J. Carr, *Arch. Biochem. Biophys.*, 90 (1960) 114–121.
16 K. Nagasawa and H. Yoshidome, *Chem. Pharm. Bull.* (Tokyo), 17 (1969) 1316–1323.
17 S. E. Lasker and S. S. Stivala, *Arch. Biochem. Biophys.*, 115 (1966) 360–372.
18 Z. Yoshizawa, T. Kotoku, F. Yamauchi, and M. Matsuno, *Biochim. Biophys. Acta*, 141 (1967) 358–365.
19 J. A. Cifonelli, *Carbohydr. Res.*, 37 (1974) 145–154.
20 K. Nagasawa and Y. Inoue, unpublished data.