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HYDROPHOBIC-INTERACTION CHROMATOGRAPHY OF GLYCOSAMINOGLYCURONANS: THE CONTRIBUTION OF *N*-ACETYL GROUPS IN HEPARIN AND HEPARAN SULFATE TO THE AFFINITY FOR HYDROPHOBIC GELS, AND VARIETY OF MOLECULAR SPECIES IN BEEF-KIDNEY HEPARAN SULFATE

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ABSTRACT

Contribution of *N*-acetyl groups in heparin and heparan sulfate to their affinity for hydrophobic gels was examined by use of a series of semi-synthetic, *N*-acetylated, hog-intestinal heparins, a whale-intestinal heparin, and a beef-kidney heparan sulfate. Chromatography on Phenyl–Sepharose CL-4B in 3.8–1.0M ammonium sulfate–10mM hydrochloric acid indicated that an increasing *N*-acetyl content, which is correlated to a decreasing *N*-sulfate content, results in a marked increase in the affinity for the gels.

The variety of molecular species in beef-kidney heparan sulfate, previously fractionated by conventional chromatographic procedures, was demonstrated by separating further, by hydrophobic-interaction chromatography, the polysaccharide into several fractions composed of molecular species distinctly different in *N*-acetyl and sulfate content, and in molecular size.

INTRODUCTION

Heparin, which is known to be the most heterogeneous glycosaminoglycuronan, has been fractionated into components that show different affinities for Phenyl–Sepharose CL-4B. It has been suggested that both *N*-acetyl content and molecular size of the polysaccharide are related to the affinity for the hydrophobic gels[1]. Hydrophobic-interaction chromatography, on Phenyl–Sepharose CL-4B, of a series of *N*-acetylchondrosine homopolymers, showed that the molecular size of the homopolymer is directly correlated with its affinity for the gel[2]. In connection with these previous results, the present report describes the contribution of the *N*-acetyl groups

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in heparin and heparan sulfate to the affinity for hydrophobic gels, and the variety of molecular species in beef-kidney heparan sulfate, as shown by fractionation by hydrophobic-interaction chromatography.

EXPERIMENTAL

Materials. — All reagents used were of analytical grade. Beef-kidney heparan sulfate, supplied by Seikagaku Kogyo Co. (Tokyo) was purified by fractionation on a column of Dowex 1 (Cl^-) anion-exchange resin[3]. The fraction eluted with 1.25M sodium chloride was used in this study.

Analytical methods. — The total sulfate, *N*-sulfate, *N*-acetyl, and uronic acid contents were determined by the methods previously reported[1].

Analytical gel-chromatography in Ultrogel AcA44 agarose–acrylamide gel (LKB-Produkter AB, Bromma) was performed according to the procedure described previously[1]. Analytical gel-chromatography on Sephadex G-100 or Sepharose 6B (Pharmacia Fine Chemicals, Uppsala) was carried out according to the following procedure: the sample (~2 mg), dissolved in 0.15M sodium chloride (1 mL), was applied to a Sephadex G-100 or Sepharose 6B column (1.5 × 82 cm) in 0.15M sodium chloride, and eluted with the same solvent at a flow-rate of 20 mL/h at 20–25°. The effluent was collected in 2-mL fractions, each of which was analyzed for uronic acid. V_0 and V_t were determined by the elution positions of Blue Dextran and 2.1M sodium chloride, respectively.

Anticoagulant activity was assayed by the whole-blood assay method of the United States Pharmacopoeia, and the activity is expressed as units/mg.

Preparation of semi-synthetic, N-acetylated heparins. — Semi-synthetic, *N*-acetylated heparins having various *N*-acetyl contents were prepared by *N*-desulfation of natural heparin, followed by *N*-acetylation as described previously[4]. Commercial hog-intestinal heparin (anticoagulant activity, 165 units/mg; Cohelfred Laboratories, Chicago, IL 60618) was *N*-desulfated separately in dimethyl sulfoxide containing

TABLE I

ANALYTICAL DATA FOR SEMI-SYNTHETIC, *N*-ACETYLATED, HOG-INTESTINAL HEPARINS

Material	*Total S*		*N-bound S*		N-*Acetyl*		K_{av} *on Sephadex G-100*
	%	*Mol*[a]	%	*Mol*[a]	%	*Mol*[a]	
Original heparin[b]	12.13	2.25	4.47	0.83	0.87	0.12	0.29
N-Acetylated heparin							
Preparation 1	11.33	2.05	3.22	0.58	1.79	0.24	0.29
Preparation 2	10.95	2.00	2.37	0.43	3.61	0.49	0.29
Preparation 3	8.07	1.34	0.29	0.05	7.18	0.89	0.29

[a]Mole ratio relative to disaccharide unit. [b]Product from Cohelfred.

5% of water under three different conditions (20 min, 20°; 120 min, 20°; and 90 min, 50°). The *N*-desulfated heparins obtained were *N*-acetylated by the method of Danishefsky *et al.*[5] to afford three *N*-acetylated heparins having different *N*-acetyl content (Preparations 1–3 in Table I).

Purification of whale-intestine heparin. — Whale-intestine heparin (anticoagulant activity, 91 units/mg, Lot AEO 1) was obtained from Tokyo Kasei Kogyo, Tokyo. The whale heparin (1.4 g) was dissolved in water (20 mL), and the solution was applied to a column (2.6 × 94 cm) of Dowex 1-X2 (Cl^-) anion-exchange resin. The column was eluted stepwise with 0.5M sodium chloride (960 mL), 1.25M sodium chloride (1400 mL), 1.5M sodium chloride (1400 mL), and 2.0M sodium chloride (500 mL) at a flow-rate of 30 mL/h. The effluent was collected in 15-mL fractions, each of which was analyzed for uronic acid. The fractions eluted with 1.5M sodium chloride were pooled, dialyzed, and freeze-dried (290 mg). The material obtained was treated with chondroitinase ABC (Seikagaku Kogyo Co.) according to the procedure described by Laurent *et al.*[6], to afford a preparation free from contaminant dermatan sulfate (200 mg; anticoagulant activity, 172 units/mg).

Fractionation of beef-kidney heparan sulfate in Sepharose 6B gels. — The heparan sulfate (450 mg), obtained from the fractions eluted with 1.25M sodium chloride on Dowex 1-X2 (Cl^-) anion-exchange resin, was dissolved in 0.5M sodium hydroxide. The mixture was kept for 20 h at 4°, and then made neutral with M acetic acid. The solution was diluted with ethanol (3 vol.) and the precipitate formed washed successively with 75% (v/v) ethanol, ethanol, and ether, and then dried *in vacuo* (420 mg).

Anal.: GalN to total HexN <0.5%; serine content <0.6 μmol/g; anticoagulant activity, 3 units/mg.

The purified polysaccharide thus obtained (411 mg) was dissolved in 0.5M sodium acetate (adjusted to pH 7.0 with acetic acid, 5 mL), and applied to a column (5 × 80 cm) of Sepharose 6B prepared in 0.5M sodium acetate (pH 7.0). The column was eluted with the same solvent at a flow-rate of 40 mL/h at 20–25°. The effluent was collected in 10-mL fractions, each of which was analyzed for uronic acid. Three main fractions containing equal amounts of total uronic acid were collected. Each of these was diluted with ethanol (3 vol.) and the precipitate formed was washed sequentially with 75% (v/v) ethanol, ethanol, and ether and then dried *in vacuo* (yield of Preparation 1, 103.8 mg; Preparation 2, 107.7 mg; and Preparation 3, 108.2 mg).

Purification of hog-intestinal heparin. — The commercial hog-intestinal heparin (anticoagulant activity, 166 units/mg; Sigma Chemical Co., St. Louis, MO 63178) contained dermatan sulfate in the amount of ~2.5% (calc. by ratio of GalN to total HexN). A heparin preparation free from dermatan sulfate was prepared from the commercial heparin by the method of Cifonelli and associates[7,8], and was purified further by gel-filtration according to the procedure of Laurent *et al.*[6] to obtain a heparin sample having a molecular-weight distribution similar to those of whale-intestinal heparin and Preparation 3 of heparan sulfate (see Table II).

Hydrophobic-interaction chromatography on Phenyl-Sepharose CL-4B. —

TABLE II

ANALYTICAL DATA FOR HOG- AND WHALE-INTESTINAL HEPARINS, AND FOR BEEF-KIDNEY HEPARAN SULFATE

Material	*Total S*		*N-bound S*		N-*Acetyl*		K_{av} *on*		*Anticoagulant activity (USP units/mg)*
	%	*Mol*[a]	%	*Mol*[a]	%	*Mol*[a]	*Ultrogel AcA44*	*Sepharose 6B*	
Hog-intestinal heparin[b]	12.20	2.27	4.40	0.82	0.85	0.12	0.32		167
Whale-intestinal heparin	9.75	1.61	4.18	0.70	2.06	0.25	0.31		172
Beef-kidney heparan sulfate									
Starting heparan sulfate	5.98	0.90	2.98	0.44	4.55	0.50			3
Fractions separated on Sepharose 6B									
Preparation 1	6.12	0.90	3.00	0.44	4.55	0.50		0.44 (1:0.8)[c]	
Preparation 2	6.01	0.88	3.08	0.45	4.46	0.49		0.53 (1:0.9)	
Preparation 3	6.11	0.90	2.95	0.44	4.64	0.51	0.30	0.62 (1:0.9)	

[a]See footnote to Table I. [b]Product from Sigma. [c]In parentheses, ratio of width to height for each peak as an indication of the elution profile.

Separation of semi-synthetic, N-acetylated, hog-intestinal heparins. A solution of the sample (5 mg) in 3.8M ammonium sulfate in 10mM hydrochloric acid (pH 3.4, 1 mL) was loaded onto a column (0.6 × 6 cm) of Phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals) prepared in the same solution. The column was eluted, stepwise, at a flow-rate of 15 mL/h, with 30-mL portions of 3.4, 3.0, 2.5, 2.0, and M ammonium sulfate in 10mM hydrochloric acid at 20–24°. The effluent was collected in 5-mL fractions, each of which was analyzed for uronic acid.

Separation of whale-intestinal heparin. — The sample (90 mg) dissolved in 3.8M ammonium sulfate in 10mM hydrochloric acid (10 mL) was loaded onto a column (2 × 9.5 cm) of Phenyl-Sepharose CL-4B, prepared in the same solvent. The column was eluted stepwise, at a flow-rate of 30 mL/h, with each 300-mL portion of 3.4, 3.0, 2.0, and M ammonium sulfate in 10mM hydrochloric acid at 20–22°. The effluent was collected in 7-mL fractions, and each fraction was analyzed for uronic acid. Each of the fractions pooled (3.8–2.0M) was recovered by cetylpyridinium chloride complex-formation, solubilization with 2.1M sodium chloride, and precipitation with ethanol, according to the method reported previously[1]. The isolated 3.8–2.0M fractions were assayed for anticoagulant activity.

Fractionation of hog-intestinal heparin. — The purified, hog-intestinal heparin (167 units/mg, 450 mg) was fractionated on a column (2.5 × 31 cm) of Phenyl-Sepharose CL-4B, prepared in 3.8M ammonium sulfate in 10mM hydrochloric acid. The column was eluted stepwise with the same solvent (1000 mL), 3.4M (600 mL), 3.0M (600 mL), 2.0M (300 mL) and M ammonium sulfate in 10mM hydrochloric acid (300 mL) at 20–22°. Each of the fractions pooled (3.8–M) was isolated according to the same procedure just described, and assayed for anticoagulant activity.

Fractionation of beef-kidney heparan sulfates. — Each sample (Preparation 1, 85.5 mg; Preparation 2, 89.8 mg; and Preparation 3, 97.0 mg) was dissolved in 3.8M ammonium sulfate in 10mM hydrochloric acid (10 mL), and the solution was applied to a column (1.6 × 16 cm) of Phenyl-Sepharose CL-4B prepared in the same solvent. The column was eluted successively, at a flow-rate of 25 mL/h, with 3.8M, 3.4M, 3.0M, 2.5M, 2.0M, and M ammonium sulfate in 10mM hydrochloric acid (300 mL each) at 20–25°. The effluent was collected in 10-mL fractions, each of which was analyzed for uronic acid. The two main fractions (3.0 and 2.5M) were pooled and the material was recovered by the procedure described earlier. The 3.0 and 2.5M fractions isolated were subjected to chemical and gel-filtration analyses.

RESULTS AND DISCUSSION

Contribution of N-acetyl groups in heparin and heparan sulfate to their affinity for hydrophobic gels. — Fractionation of semi-synthetic N-acetylated hog-intestinal heparins on Phenyl-Sepharose CL-4B. The gel-filtration diagrams of hog-intestinal heparin and its *N*-acetylated derivatives were similar with respect to their elution profiles (diagrams not shown), and the K_{av} values were identical (see Table I). The total sulfate content of these materials decreases with increasing degree of *N*-acetyl

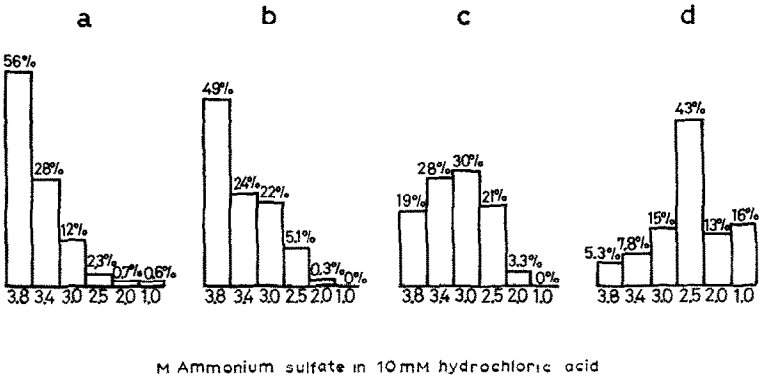

Fig. 1. Elution diagrams of semi-synthetic, *N*-acetylated, hog-intestinal heparins on Phenyl-Sepharose CL-4B: (a) Original heparin, and (b–d) semi-synthetic, *N*-acetylated heparins (b, Preparation 1; c, Preparation 2; and d, Preparation 3). The percentage indicated above each bar indicates the distribution of the fractions (3.8–M ammonium sulfate).

content, mainly because of the reduction of *N*-sulfate content. The diagrams of elution of these materials from Phenyl-Sepharose CL-4B with 3.8–M ammonium sulfate in 10mM hydrochloric acid (Fig. 1) shows an increase of the *N*-acetyl content of the *N*-acetylated heparins that is related to a decrease of the sulfate content, and clearly results in a decrease of the flow-through fraction (3.8M ammonium sulfate in 10mM hydrochloric acid). In the case of the original heparin having 0.12 *N*-acetyl group per repeating unit, only a portion (3.6%) of the heparin applied was retained on Phenyl-Sepharose CL-4B gel in 3.0M ammonium sulfate in 10mM hydrochloric acid, whereas in the case of the *N*-acetylated heparin having 0.89 *N*-acetyl groups (Preparation 3, Table I), 72% of the *N*-acetylated heparin applied was retained by the gel under the same conditions. These data strongly suggest that the *N*-acetyl groups in heparin and its *N*-acetylated derivatives contribute considerably to the affinity for hydrophobic gels.

Fractionation of hog- and whale-intestinal heparins, and of a preparation of beef-kidney heparan sulfate on Phenyl-Sepharose CL-4B. — Two natural heparins and one heparan sulfate that are different in both *N*-acetyl and sulfate content but similar in molecular-weight distribution, were examined for their hydrophobic interaction with Phenyl-Sepharose CL-4B (see Table II). Whale heparin has been known to have a higher *N*-acetyl and lower sulfate content than have heparins of terrestrial-animal origin[9] (see also Table II). The heparan sulfate (Preparation 3) used in this experiment had a much higher *N*-acetyl and lower sulfate content than had whale heparin. The gel-filtration diagrams of the two heparins and one heparan sulfate on Ultrogel AcA44 gels were analogous in all cases (diagrams not shown), and their K_{av} values were similar (Table II). As can be seen in the elution diagrams (Figs. 2a, b, and e), the difference in the distribution of the fractions (3.8–2.0M) in percentage of the total amount of applied sample plainly reflects the difference in the content of *N*-acetyl and sulfate groups. This is especially clear for the former groups, because

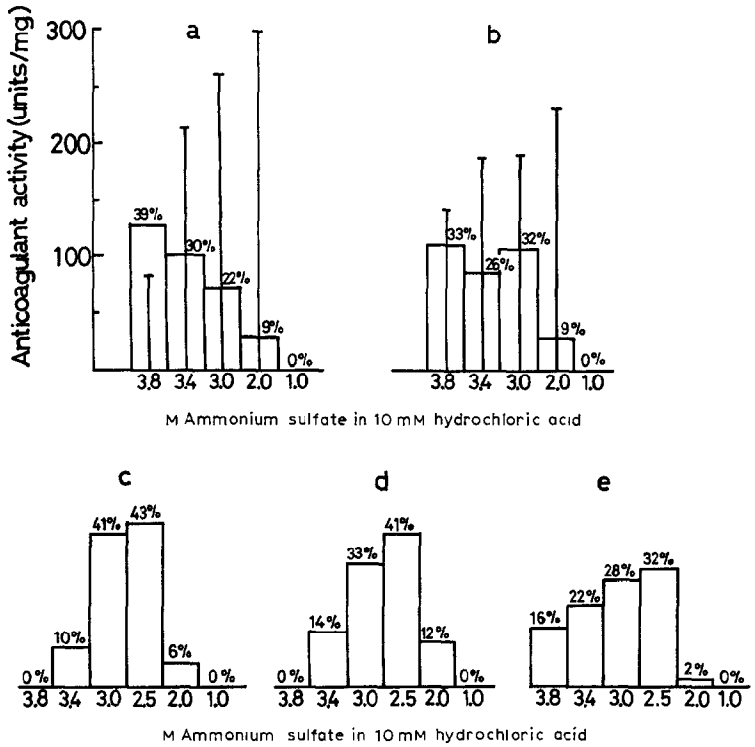

Fig. 2. Elution diagrams of hog- and whale-intestinal heparins, and of beef-kidney heparan sulfates on Phenyl-Sepharose CL-4B: (a) Hog-intestinal heparin; (b) whale-intestine heparin; and (c–e) beef-kidney heparan sulfates (c, Preparation 1; d, Preparation 2; and e, Preparation 3). The height of each upright solid line (in a and b) corresponds to the anticoagulant activity (USP units/mg). The percentage indicated above each bar (a–e) indicates the distribution of the fractions (3.8–M ammonium sulfate).

the molecular-size distributions were nearly identical. The anticoagulant activity of the whale heparin increases with the increasing degree of hydrophobic interaction with Phenyl-Sepharose gels. This behavior of whale heparin is quite similar to that of hog-intestinal heparin, as shown in Fig. 2a (see also ref. 1). These data, and those[2] of the fractionation of *N*-acetylchondrosine homopolymers on Phenyl-Sepharose CL-4B, suggest a marked influence of the *N*-acetyl group of the hexosamine unit of glycosaminoglycuronans, including heparin and heparan sulfate, on their affinity for hydrophobic gels.

Variety of molecular species of beef-kidney heparan sulfate. — No appreciable difference in the analytical data, except the K_{av} value, was observed for Preparations 1–3 obtained by filtration of beef-kidney heparan sulfate on Sepharose 6B gels (Table II). As can be seen in the elution diagrams (Figs. 2c–e), the heparan sulfates of Preparations 1 and 2 were all retained on the hydrophobic gels in 3.8M ammonium sulfate in 10mM hydrochloric acid, and the similar elution profiles show the 3.0 and 2.5M fractions as being the main products. In the case of Preparation 3

TABLE III

ANALYTICAL DATA FOR BEEF-KIDNEY HEPARAN SULFATE FRACTIONS OBTAINED ON PHENYL-SEPHAROSE CL-4B

Heparan sulfate fraction		*Total S*		*N-bound S*		N-*Acetyl*		K_{av} *on Sepharose 6B*
		%	*Mol*[a]	%	*Mol*[a]	%	*Mol*[a]	
Preparation 1	3.0M	6.48	0.97	3.23	0.48	4.21	0.47	0.43 (1:1.6)[b]
	2.5M	5.95	0.87	2.91	0.43	4.62	0.50	0.40 (1:1.5)
Preparation 2	3.0M	6.35	0.95	3.05	0.45	4.46	0.49	0.54 (1:1.9)
	2.5M	6.07	0.89	2.90	0.43	4.69	0.52	0.50 (1:1.6)
Preparation 3	3.0M	6.51	0.98	3.12	0.47	4.60	0.51	0.58 (1:1.4)
	2.5M	5.64	0.82	2.50	0.36	5.21	0.57	0.55 (1:1.6)

[a]See footnotes to Table I. [b]See footnotes to Table II.

containing the heparan sulfate species having the smaller molecular-size, 16% of the polysaccharide applied was eluted in the flow-through fraction, and the rest was scattered between the 3.4, 3.0, and 2.5M fractions. The polysaccharides from the 2.5 and 3.0M fractions of Preparations 1–3 were recovered as sodium salt, and their analytical data are reported in Table III. In all Preparations 1–3, the 2.5M fraction having a higher affinity for the hydrophobic gels had a larger molecular-size and a higher *N*-acetyl content than the corresponding 3.0M fraction with a lower affinity for the gels. These data again show that both molecular size and *N*-acetyl content are the principal factors for the fractionation, on hydrophobic gels, of heparin and its analogs, as suggested previously[1]. Furthermore, comparison between the 3.0M fractions of these preparations, as well as between the 2.5M fractions, indicates that the heparan sulfate species having the smaller molecular-size and some affinity for the hydrophobic gels have a higher *N*-acetyl content than those species with a larger molecular-size. These data suggest that the contributions of the molecular-size and of the *N*-acetyl content of heparan sulfate (heparin also seems to be the case) to its affinity for hydrophobic gels are complementary.

The heparan sulfate preparations, separated on Sepharose 6B gels or Phenyl-Sepharose CL-4B gels, were analyzed by gel-chromatography in a Sepharose 6B column to determine the K_{av} values (Tables II and III). The ratio of width to height for each peak obtained was also determined as an indicator of the elution profiles. A comparison between the ratios obtained for the peaks of Preparations 1–3 (Table II) and those obtained for their 3.0 and 2.5M fractions separated on hydrophobic gels (Table III) clearly indicates that the molecular-size distribution of the former materials is much more heterogeneous than that of the latter. This suggests that a separation based on the interaction between heparan sulfate molecules and hydrophobic gels results in a superior molecular-sieving of the polysaccharide molecules (see ref. 2).

Although many heparan sulfates have been obtained from various animal

organs, and their various chemical and physical properties have been reported[10,11], the variety of molecular species in a polysaccharide originating from a single source has been little studied, except for that reported by Dietrich and Nader[12]. The present work shows, in Table III, the variety of molecular species existing in a beef-kidney heparan sulfate that had been previously fractionated by conventional chromatographic procedures, the polysaccharide being separated further, by hydrophobic-interaction chromatography, into several fractions composed of molecular species distinctly different in *N*-acetyl and sulfate content, and in molecular-size. Like the previous reports[1,2], the present work shows that the hydrophobic-interaction chromatography is useful for the fractionation of glycosaminoglycuronans.

REFERENCES

1 A. OGAMO, H. UCHIYAMA, AND K. NAGASAWA, *Biochim. Biophys. Acta*, 626 (1980) 477–485.
2 H. UCHIYAMA, A. OGAMO, AND K. NAGASAWA, *Carbohydr. Res.*, 99 (1982) 87–92.
3 J. A. CIFONELLI AND A. DORFMAN, *J. Biol. Chem.*, 235 (1960) 3283–3286.
4 K. NAGASAWA, T. TOKUYASU, AND Y. INOUE, *J. Biochem. (Tokyo)*, 81 (1977) 989–993.
5 I. DANISHEFSKY, H. B. EIBER, AND J. J. CARR, *Arch. Biochem. Biophys.*, 90 (1960) 114–121.
6 T. C. LAURENT, A. TENGBLAD, L. THUNBERG, M. HÖÖK, AND U. LINDAHL, *Biochem. J.*, 175 (1978) 691–701.
7 J. A. CIFONELLI AND J. KING, *Biochim. Biophys. Acta*, 320 (1973) 331–340.
8 L. RODÉN, J. BAKER, J. A. CIFONELLI, AND M. B. MATHEWS, *Methods Enzymol.*, 28 (1972) 73–140.
9 Z. YOSHIZAWA, *Biochem. Biophys. Res. Commun.*, 16 (1964) 336–341.
10 A. LINKER AND P. HOVINGH, *Carbohydr. Res.*, 29 (1973) 41–62.
11 R. L. TAYLOR, J. E. SHIVELY, H. E. CONRAD, AND J. A. CIFONELLI, *Biochemistry*, 12 (1973) 3633–3637.
12 C. P. DIETRICH AND H. B. NADER, *Biochim. Biophys. Acta*, 343 (1974) 34–44.