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Isolation and Purification of Mactins, Heparin-like Anticoagulants from Mollusca[1]

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RECEIVED MAY 14, 1956

Anticoagulants, designated mactin A and mactin B, were isolated from the mollusca *Spissula* (*Mactra*) *solidissima* and *Cyprina* (*Arctica*) *islandica*, respectively. *In vitro* anticoagulant activities of the purified neutral sodium salts were 130–150 U.S.P. units/mg. for mactin A and 150–180 U.S.P. units/mg. for mactin B, as compared with 120–145 U.S.P. units/mg. for the most active samples of heparin neutral sodium salts available. Both mactins were sulfated polysaccharides and contained glucosamine and glucuronic acid. Mactin A, mactin B and heparin neutral sodium salts appeared to be similar except for their molecular weights, which were 24,800, 28,700 and 14,200, respectively, and their optical rotations, which were +71, +61 and +47°, respectively.

Discussion

The presence of a heparin-like substance in the common surf clam, *Spissula* (*Mactra*) *solidissima*, has been demonstrated by Thomas.[2] The isolation and partial purification of such anticoagulants have been reported.[3,4] In the present study the isolation and purification procedures of Frommhagen, *et al.*,[3] were extended and improved to yield highly purified anticoagulants, designated mactin A and mactin B, from the two species of clams, *Spissula* (*Mactra*) *solidissima* and *Cyprina* (*Arctica*) *islandica*, respectively.

For the preparation of crude extracts of mactin A and mactin B procedures were employed which were similar to those already reported for the isolation of crude heparin from mammalian tissues.[5–9] Ground fresh clam meat was autolyzed at 45° for 24 to 72 hours, extracted with alkali and ammonium sulfate, and the solid isolated from this extraction by acidification to *p*H 1.5 to 2.0 was then digested with pancreatin or trypsin. Crude products isolated from these digestions by precipitation with such solvents as acetone or alcohol had *in vitro* anticoagulant activities which ranged between 15 and 20 U.S.P. units/mg.[10] Over-all yield of anticoagulant activity isolated in these crude products was 30,000 to 36,000 U.S.P. units/kg. of ground whole clam meat in the case of mactin A, and 20,000 to 26,000 U.S.P. units/kg. of ground whole clam meat in the case of mactin B. These values are appreciably greater than those reported for the isolation of crude heparin from mammalian tissues[9] and also for the isolation of similar products from *Spissula* (*Mactra*) *solidissima*.[4]

In spite of their strong negative charges, neither mactin A nor mactin B could be absorbed on anion-exchange resins. Presumably this is because of the large molecular size of these substances. Similar observations have been made by Ricketts and Walton[11] with dextran sulfate.

Accordingly, columns of IRA-400[12] and IR-120[12] were utilized for partial deionization of crude mactin solutions. Addition of lead acetate to the dilute aqueous effluents allowed the resultant insoluble mactin lead salts to be isolated by filtration. This treatment serves as a convenient means for isolation of the anticoagulants from the very dilute effluent and as an effective method for the separation of the anticoagulants from many impurities which are not normally removed in the conventional solvent precipitation procedures. However, complete removal of lead-precipitable ions other than the mactins was found to be undesirable, since the subsequent treatment of such thoroughly deionized effluents invariably led to colored products which could not be conveniently decolorized. Optimum results in the deionization step were obtained when the capacity of the resin was exceeded by approximately 30%, thereby obtaining a solution contaminated with a controlled quantity of extraneous ions. Preparation of the acid sodium salt of either mactin was accomplished by suspension of the corresponding lead salt in 20% sodium chloride solution at 60°, followed by the addition of sodium sulfide to *p*H 9 to 10, clarification of the warm mixture and addition of two volumes of glacial acetic acid to the filtrate at room temperature. The amorphous white acid sodium salt was isolated by filtration with filter aid, then redissolved in water and reprecipitated with glacial acetic acid in order to remove traces of salt. Conversion to the neutral sodium salt was effected by addition of sodium hydroxide to a suspension of the acid sodium salt in anhydrous 3A alcohol–ether (1:1) employing brom thymol blue as indicator. An alternate procedure for the preparation of the neutral sodium salt consisted of the passage of a solution of the corresponding acid sodium salt through a monobed column containing IRA-400[12] and IR-120,[12] followed by titration of the effluent to the equivalence point (*p*H 7.85) with sodium hydroxide and finally isolation of the neutral sodium salt by freeze drying.

In vitro anticoagulant activities of various preparations of the acid and neutral sodium salts of mactin A ranged between 130 and 150 U.S.P. units/mg., whereas those of the corresponding sodium salts of mactin B were between 150 and 180 U.S.P. units/

(1) Presented at the 129th Meeting of the American Chemical Society, Dallas, Texas, April 11, 1956.

(2) L. J. Thomas, Jr., *Biol. Bull.*, **101**, 230 (1951).

(3) L. H. Frommhagen, M. J. Fahrenbach, J. A. Brockman, Jr., and E. L. R. Stokstad, *Proc. Soc. Exptl. Biol. Med.*, **82**, 280 (1953).

(4) L. J. Thomas, Jr., *Biol. Bull.*, **106**, 129 (1954).

(5) D. A. Scott and A. F. Charles, *J. Biol. Chem.*, **102**, 425 (1933).

(6) A. F. Charles and D. A. Scott, *Biochem. J.*, **30**, 1927 (1936).

(7) A. F. Charles and A. R. Todd, *ibid.*, **34**, 112 (1940).

(8) L. B. Jaques, E. T. Waters and A. F. Charles, *J. Biol. Chem.*, **144**, 229 (1942).

(9) M. H. Kuizenga and L. B. Spaulding, *ibid.*, **148**, 641 (1943).

(10) Heparin sodium, U. S. Pharmacopeia XIV, 271 (1950).

(11) C. R. Ricketts and K. W. Walton, *Chem. & Ind.*, 869 (1952).

(12) Rohm & Haas, Inc., Philadelphia, Pennsylvania.

TABLE I

ANALYTICAL DATA[a] (NEUTRAL SODIUM SALTS)

	Mactin A (134 units/mg.)	Mactin B (176 units/mg.)	Heparin (146 units/mg.)	Heparin (140 units/mg.)	Calcd. for mucoitin disulfuric acid Neutral salt	Acid salt
C (%)	25.8	26.7	26.0	25.2	25.6	26.6
H (%)	3.2	2.9	3.4	2.8	2.8	3.2
N (%)	2.4	2.5	2.6	2.4	2.5	2.6
S (%)	12.0	11.6	11.6	12.2	11.3	11.8
Na (%)	12.2[b]	12.3[b]	12.0[c]	12.2[c]	12.2	8.5
H_2O (by Karl Fischer analysis, %)	15.8	15.3	12.3	9.6		
Glucosamine (%)[15]	25.2	30.8	28.8	29.8	31.7	33.0
Glucuronic acid (%)[16]	26.5	34.7	24.8	25.8	34.4	35.8
N-Acetyl (%)	2.0	1.7	1.6	2.0		
O-Acetyl (%)	2.3	1.5	1.5	2.4		
P, Amino-N, N-CH_3 and C-CH_3	Nil	Nil	Nil	Nil		
$[\alpha]^{25}_D$ in water	+71° (*c* 1.2)	+61° (*c* 1.5)	+47° (*c* 1.5)			

[a] Calculated on anhydrous basis. [b] By flame photometer. [c] From sulfated ash.

mg. Recovery of *in vitro* anticoagulant activity from various mactin crudes was 50 to 60%. By means of the various chemical and physical analyses discussed below these products were found to be reasonably pure and homogeneous. Repeated application of the purification procedures did not yield anticoagulants of greater purity or activity.

Crystallization of mactins was attempted by the procedures of Wolfrom[13] and Smith.[14] Although both methods yielded the typical crystalline barium acid heparinate from sodium heparinate, the mactin sodium salts could not be converted to crystalline acid barium salts.

Elementary microchemical analyses (Table I) of the neutral sodium salts of mactin A (134 U.S.P. units/mg.), mactin B (176 U.S.P. units/mg.) and two samples of commercially available heparin (146 and 140 U.S.P. units/mg.) for C, H, N, S and Na agreed reasonably well with the calculated values for a mucoitin disulfuric acid neutral sodium salt. However, the C analysis for mactin B and the S analyses for mactin A and one of the commercial heparin samples (140 units/mg.) were slightly higher than theory.

Glucosamine was identified and determined quantitatively by the method of Gardell.[15] Hydrolysis of the various polysaccharides was effected by 6 *N* hydrochloric acid at 100–110° for 24 hours. The resultant values for glucosamine (Table I) were all lower than theory for a mucoitin disulfuric acid neutral sodium salt, although the content of glucosamine in mactin B was in rather close agreement. The reason for the low glucosamine results is not apparent. Some slight destruction of glucosamine could occur under the conditions of the hydrolysis. Furthermore, in our hands the accuracy of the Gardell procedure was at best ±5%. Nevertheless, it is felt that the variations in glucosamine content reflect to some extent the purity of the various mactin and heparin samples.

The method of Tracey[16] was employed for the quantitative determination of uronic acid; in order to obtain the maximum liberation of carbon dioxide, treatment with 6 *N* hydrochloric acid at 115° for 24 hours was necessary. Again the values for the two heparin samples and mactin A were lower than theory for a mucoitin disulfuric acid neutral sodium salt, whereas mactin B agreed with the calculated value (Table I). Thus, except for the slightly high carbon content, the analytical results from mactin B correspond most closely to a mucoitin disulfuric acid neutral sodium salt.

By the paper chromatographic method of Fischer and Dörfel[17] the presence of glucuronic acid was established in both mactins (Table II). Although glucuronic acid and glucosamine were the only substances detected in mactins under the acid hydrolysis conditions employed (3 *N* hydrochloric acid for 6 hours, or 6 *N* hydrochloric acid for 1 hour at 100–110°), it is possible that other compounds were present which were either unstable to acid or undetected by the spray reagents used. All of the samples after acid hydrolysis gave a barium precipitate, indicating the presence of ester sulfate. Heparin exhibited the same behavior as the mactins in all of the qualitative tests investigated.

The explanation for the presence of approximately 2% acetyl in the mactin and heparin samples (Table I) is not clear. Although the exact process for the preparation of the heparin neutral sodium salts is not known, the mactin neutral sodium salts were prepared by first passing a solution of the mactin neutral sodium salt through a monobed of IRA-400[12] (OH form) and IR-120[12] (H form) in order to remove all cations and all anions except the mactin free acid. Then the effluent was exactly neutralized to the equivalence point with sodium hydroxide, and this solution was freeze dried to yield the mactin neutral sodium salt. By this procedure the presence of free acetate in the final product would appear highly unlikely. Furthermore, the fact that the N-acetyl and O-acetyl determinations gave essentially the same results indicates that the nitrogen atoms are not acetylated. No phosphorus, free amino-nitrogen, N-methyl or C-methyl were found in mactin A, mactin B or heparin.

The specific rotations of the neutral sodium salts of mactin A (+71°) and mactin B (+61°) were sig-

(13) M. L. Wolfrom, D. I. Weisblat, J. V. Karabinos, W. H. McNeeley and Jay McLean, THIS JOURNAL, **65**, 2077 (1943).

(14) H. Smith, R. C. Gallop, P. W. Harris-Smith and J. L. Stanley, *Biochem. J.*, **52**, 23 (1952).

(15) S. Gardell, *Acta Chem. Scand.*, **7**, 207 (1953).

(16) M. V. Tracey, *Biochem. J.*, **43**, 185 (1948).

(17) F. G. Fischer and H. Dörfel, *Hoppe-Seyler's Z. Physiol. Chem.*, **301**, 224 (1955).

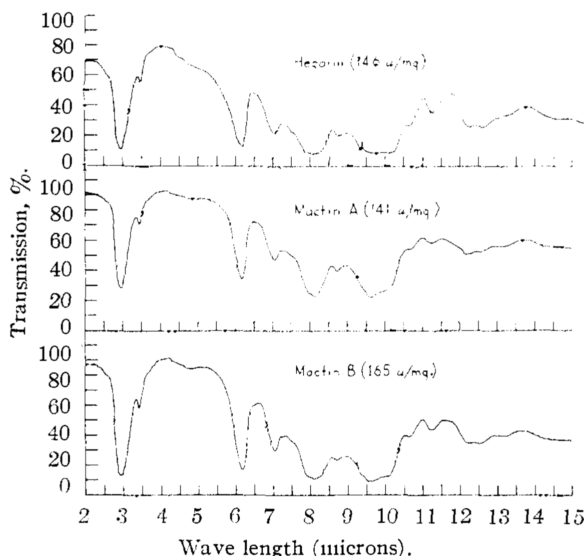


Fig. 1.—Infrared absorption.

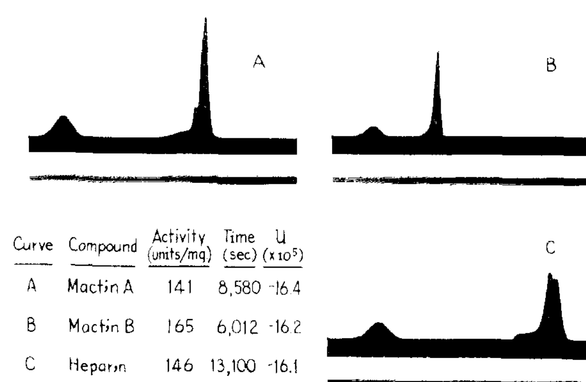
nificantly higher than that of heparin (+47°).

The curves shown in Fig. 1 demonstrate that mactin A, mactin B and heparin have essentially identical infrared absorption characteristics. The flat maximum between 9.50 and 10.25 μ in the heparin curve may indicate a difference in either purity or molecular size of the heparin as compared with the two mactins. It is evident, however, that there are no gross differences between any of the three compounds observable in their respective infrared absorption spectra.

Examination of 0.01% aqueous solutions of heparin and the mactins showed no ultraviolet absorption between 210 and 400 mμ. This is in accord with the findings of Lathrop and Allen[18] for heparin.

The titration curves of mactin A and mactin B were identical with that of heparin[19] and demonstrated the presence of carboxyl and the absence of free amino groups. The molecular weight of mactin A neutral sodium salt (558), obtained by titration of the acid sodium salt, denotes the weight of the smallest possible unit, the sulfated glucosamine–glucuronic acid disaccharide. Since the N:S ratio of 1:2 indicates two sulfate groups per disaccharide in addition to the carboxyl group, the molecular weight obtained by titration of the free acid would be triple the neutral equivalent (185) or 555. These values are in agreement with the calculated weight of mucoitin disulfuric acid neutral sodium salt (563). The neutral equivalent obtained by titration of mactin B free acid (193) on the basis of one carboxyl and two sulfuric acid groups gives a molecular weight of 579 for the disaccharide. The higher value in this case may possibly be attributed to a small loss of mactin B on the ion exchange resins used for preparation of the free acid. In the titration of mactin A free acid the weight of neutral salt actually isolated by freeze drying the neutralized effluent was used for calculation of the equivalent weight, whereas in the case of mactin B the weight of the starting sample of neutral sodium salt which was run through the monobed was employed.

The electrophoretic patterns for the three preparations are shown in Fig. 2. Although mactin A and heparin showed evidence of two peaks, mactin B traveled as a single peak.



Curve	Compound	Activity (units/mq)	Time (sec)	U (x10⁵)
A	Mactin A	141	8,580	-16.4
B	Mactin B	165	6,012	-16.2
C	Heparin	146	13,100	-16.1

Fig. 2.—Electrophoretic patterns (ascending).

The weight average sedimentation constants, diffusion constants and the calculated average molecular weights are presented in Table III. The sedimentation constants were extrapolated to zero concentration. The diffusion constants were not; however, they were measured at a mean concentration of 0.2%. Since there was very little skewing of the peaks, the concentration dependence of the diffusion constants is small, and the difference between the quoted and extrapolated values should be quite small. It is believed that one can measure the diffusion constant of a heterogeneous preparation by the method of second moments to 0.1–0.2% if sufficient fringes are used. However, only 40 fringes were used in these experiments and the values are probably accurate only to 0.5%.

The molecular weights were calculated using an average value of 0.41 for the partial specific volumes of the three preparations. The measured values were the same within the experimental error. Since the sodium salts were used for the measurements of the partial specific volumes, the quoted molecular weights correspond to the salts. The mactins and heparin are very highly charged substances and charge effects will tend to make calculated molecular weights too low.[20,21] The error may be as large as 10%.

At 0.6% the apparent diffusion constants for the three preparations calculated from the centrifuge patterns were less than those measured in the diffusion apparatus and corrected to the same temperature. The sharpening of the peaks caused by the concentration dependence of the sedimentation constant prevented the detection of any heterogeneity. In the diffusion apparatus it was obvious that mactin A and heparin were heterogeneous. Mactin B did not appear heterogeneous. The diffusion constants calculated by symmetrical fringes[22] were constant across the peak.

(18) K. Lathrop and J. G. Allen, *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 412 (1954).

(19) O. Wilander, *Skand. Arch. Physiol.*, **81**, Suppl. XV, 25 (1938).

(20) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, London, 1940, p. 23.

(21) A. F. Johnson, K. O. Kraus and S. Scatchard, *J. Phys. Chem.*, **58**, 1034 (1954).

(22) J. M. Creeth, THIS JOURNAL, **77**, 6428 (1955).

The first-order term for the concentration dependence of the diffusion constants is an odd function[22] and does not contribute to the even moments of the gradient curves. To a good approximation the weight average diffusion constant and the mean square weight average diffusion constant can be obtained from the second and fourth moments, respectively. Since the fourth moments can be accurately determined,[23] it is possible to calculate the standard deviation (1) of the diffusion constant as a

$$\sigma^2_D = \frac{1}{12t^2}(\overline{x^4} - \overline{x^2}^2) \qquad (1)$$

measure of the heterogeneity of the preparations. These values are tabulated in Table III.

The mactins have molecular weights about twice that of heparin (Table III). Our values for the molecular weight, diffusion constant and sedimentation constant of the neutral sodium salt of heparin are comparable to those obtained by Creeth and Record[24] (mol. wt., 16,500) for the neutral sodium salt of polysaccharide A,[14] a mucoitin disulfuric acid isolated from hog gastric mucin; the molecular weight, however, is somewhat lower than the 20,000 reported by Wolfrom, *et al.*,[25] for heparin sodium salt. As one would expect for a stiff random coil polymer, the higher molecular weight mactins are more asymmetric than heparin. Mactin B appeared to be a very good preparation; no heterogeneity was detected in these experiments.

Experimental

Preparation of Crude Mactin A.—The procedure employed for the preparation of crude mactin A followed quite closely the essential steps already reported by Charles, Scott, Jaques, Kuizenga and others[5-9] for the preparation of crude heparin from mammalian tissues. Thus, to 1022 kg. of ground eviscerated clam meat from the common surf clam, *Spissula* (*Mactra*) *solidissima*,[26] was added 450 l. of water and then 20 l. of toluene in order to inhibit bacterial action. This mixture was stirred vigorously at 45° for two hours then allowed to stand without stirring at 45° for 72 hours in order to achieve autolysis. At the end of this time 2247 l. of 1 *N* sodium hydroxide in 225 l. of saturated ammonium sulfate solution was added. The well-stirred mixture was heated to 55° for 3 hours and to 80° for 1 hour, then cooled to 25°. Hyflo (34 kg.) was added and the mixture was clarified through a filter press; the press was washed with sufficient water to bring the total volume of the filtrate to 4300 l. Addition of 131 kg. of concentrated sulfuric acid to *p*H 1.5 resulted in the formation of an amorphous cream colored precipitate. This suspension was allowed to settle overnight, then the supernatant solution was removed by decantation. To the residual 400 l. was added 22 kg. of Hyflo and the solid was isolated by filtration through a filter press. The wet filter cake was slurried with 100 l. of water at 40°. After the addition of 2.3 l. of 10 *N* sodium hydroxide to raise the *p*H to 8.5, 2250 g. of trypsin (1–110) was added, and the mixture was stirred for 16 hours at 37 to 40°; during the first few hours of the digestion it was necessary to add several small quantities of sodium hydroxide in order to maintain the *p*H between 8.0 and 8.5. At the end of this time the mixture was clarified through a filter press, and to the filtrate 1.5 volumes (180 l.) of 2B alcohol was added. The resultant crude mactin A was removed by filtration and dried at 50°; wt. 2120 g.; *in vitro* anticoagulant activity, 15 U.S.P. units/mg. This, therefore, represents a recovery of approximately 31,000 U.S.P. units/kg. of ground clam meat.

(23) R. A. Brown, M. C. Davies and M. Englert, in manuscript.

(24) J. M. Creeth and B. R. Record, *Biochem. J.*, **52**, 30 (1952).

(25) M. L. Wolfrom, R. Montgomery, J. V. Karabinos and P. Rathgeb, THIS JOURNAL, **72**, 5796 (1950).

(26) Bluepoints Company, West Sayville, New York.

The filtrate from the isolation of the above crude mactin A was adjusted to *p*H 5.0 with hydrochloric acid, then another volume (120 l.) of 2B alcohol was added. The resultant precipitate was isolated by filtration and dried at 50°; wt. 1200 g.; *in vitro* anticoagulant activity was less than 2.6 U.S.P. units/mg. Because of the low activity of this second precipitate, it was discarded.

Preparation of Crude Lead Salt of Mactin A.—Two columns were arranged in series. The first, containing 7400 ml. of Amberlite IRA-400[12] was backwashed, regenerated with 110 l. of 4% sodium hydroxide solution and washed with distilled water to a neutral effluent. The second column, containing 3700 ml. of Amberlite IR-120[12] was treated as above using 62 l. of 10% hydrochloric acid solution for regenerant. Both columns were backwashed with deionized water immediately before use. A suspension of 1350 g. of crude mactin A (*in vitro* anticoagulant activity, 9.3 U.S.P. units/mg.) in 270 l. of distilled water was stirred for 30 minutes then clarified by passing through a Sharples centrifuge. The resulting solution was allowed to flow through the column system at a rate of 2300 ml. per minute. The columns were washed with 30 l. of water which was added to the effluent; the entire 300 l. of solution was then stirred vigorously during the addition of 5.5 kg. of lead acetate. After one hour 550 g. of filter aid was added and the precipitate removed in a filter press; weight of wet cake, 5570 g.

The quantity of crude mactin to be used was determined in each instance by running an aliquot through a small quantity of resin. Lead acetate testing of the effluent immediately gave a fluffy precipitate of mactin lead salt. Eventually the capacity of the column for removal of ionic contaminants was exceeded, as evidenced by the formation of a heavy white precipitate on addition of lead acetate to the effluent. The proportion of crude to resin was increased 30% over this amount.

Regeneration of Mactin A from its Lead Salt.—A suspension of 557 g. of the wet mactin A crude lead salt cake in 2000 ml. of 20% sodium chloride solution was heated to 60° during the addition of finely ground sodium sulfide to *p*H 9.0. The resulting black suspension was filtered and washed with 950 ml. of 20% sodium chloride solution at 60°. After the filtrate and wash (2670 ml.) had been cooled, 7240 ml. of glacial acetic acid and 100 g. of Hyflo Supercel were added. The mixture was filtered; the wet cake was resuspended in 600 ml. of water and again filtered. The slightly turbid filtrate was clarified through a filter paper mulch to yield 900 ml. of clear pale yellow solution. The amorphous cream colored solid which was precipitated by the addition of 7200 ml. of glacial acetic acid was removed by centrifugation, suspended in acetone and filtered. This precipitate was washed on the funnel with 1000 ml. of acetone and 1000 ml. of petroleum ether (30–60°).

The above solid was suspended in 100 ml. of anhydrous 3A alcohol–anhydrous ether (1:1) and stirred during neutralization to brom thymol blue with 1% sodium hydroxide solution in anhydrous 3A alcohol–ether (1:1). The neutral mixture was filtered and the precipitate was washed with 150 ml. of anhydrous 3A alcohol–ether (1:1), 100 ml. of acetone, 100 ml. of petroleum ether (30–60°) and dried *in vacuo*. The yield of mactin A neutral sodium salt was 5.43 g.; *in vitro* anticoagulant activity, 135 U.S.P. units/mg. This represented a 58% recovery of anticoagulant activity from the crude mactin A.

Mactin B.—By means of essentially the same procedures as those employed for mactin A, 1784 kg. of ground eviscerated clam meat from the species *Cyprina* (*Arctica*) *islandica* (ocean quahog clam)[27] yielded 2440 g. of crude mactin B; *in vitro* anticoagulant activity, 17 U.S.P. units/mg. The yield of *in vitro* anticoagulant activity was, therefore, approximately 24,000 U.S.P. units/kg. of ground clam meat.

The above crude mactin B, purified through isolation of the lead salt as described for mactin A, yielded 118 g. of white amorphous neutral sodium salt; *in vitro* anticoagulant activity, 175 U.S.P. units/mg. Recovery of anticoagulant activity from the crude mactin B was, therefore, approximately 52%.

Paper Chromatography.—Descending chromatography on Whatman #1 strips six inches wide was accomplished using pyridine–ethyl acetate–acetic acid–water (5:5:1:3) as developer and pyridine–ethyl acetate–water (11:40:6)[17] in

(27) Morim Brothers Shellfish Co., Oakland Beach, Rhode Island.

the cylinder bottom to saturate the atmosphere. The cylinder was lined with filter paper saturated with the pyridine-ester-water solution and was covered with aluminum foil. A 100-μg. sample of glucose standard was put on the paper. The quantity of heparin and mactin hydrolysates applied was such that 240 μg. of glucuronic acid (calculated on the basis of complete hydrolysis and no destruction) was on the starting line. All experiments were performed in a room at 20° and were run for 46 hours, after which the paper strips were dried at 40° for 2 hours then sprayed with naphthoresorcinol-phosphoric acid.[28] The results appear in Table II.

TABLE II

PAPER CHROMATOGRAPHY OF POLYSACCHARIDE HYDROLYSATES[a]

Sample	Hydrolysis conditions[b] HCl, N	Time, hr.	Rg Found	Lit.[17]
Glucuronic acid			0.27	0.27
Galacturonic acid			.20	.18
Mannuronic acid				.35
Heparin hydrolysate	3	6	.27	
Mactin A hydrolysate	3	6	.27	
Mactin A hydrolysate	6	1	.27	
Mactin B hydrolysate	3	6	.27	
Mactin B hydrolysate	6	1	.27	

[a] *In vitro* anticoagulant activities of the neutral sodium salts investigated were: heparin, 146 U.S.P. units/mg.; mactin A, 141 U.S.P. units/mg.; and mactin B, 165 U.S.P. units/mg. [b] Samples were hydrolyzed at 100–110° and then neutralized with silver carbonate after hydrolysis roughly according to the procedure of Fischer.[17]

Potentiometric Titration.—The neutral equivalent values were determined on either the free acid or the acid sodium salts of various mactin preparations by means of titration using a glass electrode.

Since earlier experiments had demonstrated that both mactins were essentially unabsorbed when passed through a mixed resin ion-exchange column containing IRA-400[12] (OH form) and IR-120[12] (H form), it was possible to employ such a column to remove the sodium and then titrate the mactin free acid in the effluent with standard alkali.

Thus, 102.8 mg. of mactin B neutral sodium salt (180 U.S.P. units/mg.; water content, 13.3%) was dissolved in 20 ml. of water, and run through a mixed resin ion-exchange column containing 125 ml. of IRA-400[12] (OH form) and 85 ml. of IR-120[12] (H form). The anticoagulant was washed out of the column with water, then the effluent was titrated with 0.2161 N sodium hydroxide. The results of this titration are shown in Fig. 2. A total of 2.14 ml. of the 0.2161 N sodium hydroxide was necessary to reach the equivalence point (pH 7.85). This indicates an equivalent weight of 193 for the anhydrous mactin B neutral sodium salt. Essentially the same results were obtained for mactin A employing a similar procedure and a greater quantity of material. Approximately 5 g. of mactin A neutral sodium salt (141 U.S.P. units/mg.) was dissolved in 1000 ml. of water. After clarification of this solution through a bed of mulched filter paper, the filtrate was passed through a monobed consisting of IRA-400[12] (119 ml.) and IR-120[12] (62 ml.) at a rate of 30–50 ml./min. The column was washed with 300 ml. of water, and the two effluents were combined. Titration of this solution of mactin A free acid to pH 7.85 required 22.6 ml. of 1 N sodium hydroxide. The resulting solution was freeze dried to yield 4.8 g. of mactin A neutral sodium salt (water content, 13.0%; 134 U.S.P. units/mg.). Based on the anhydrous weight of mactin A neutral sodium salt actually isolated, an equivalent weight of 185 is obtained.

Further information on the molecular weight of the smallest repeating unit in mactin A was obtained by titration of the acid sodium salt with alkali. Thus, 5.00 g. of mactin A neutral sodium salt (water content, 13.0%, 141 U.S.P. units/mg.) in 100 ml. of water was treated with four volumes of glacial acetic acid, and the mactin A acid sodium salt was removed by centrifugation. The supernatant yielded no solid on addition of more acid and also gave a negative metachromatic test with azure A; this indicated that all of the mactin A was precipitated by the acetic acid. The solid was washed thoroughly with acetone, dried, redissolved in 100 ml. of water and titrated to the equivalence point (pH 7.85) with 1 N sodium hydroxide (7.8 ml.). Assuming that this titration of mactin A acid sodium salt involved neutralization of only the carboxylic acid group, the molecular weight of the repeating unit in mactin A neutral sodium salt was therefore 558. Di- and tri-sodium salts of mucoitin disulfuric acid have molecular weights of 541 and 563, respectively.

Physical measurements were made on mactin A (134 U.S.P. units/mg.), mactin B (176 U.S.P. units/mg.) and a sample of commercially available heparin (146 U.S.P. units/mg.) (Table III).

TABLE III

Sample	$S_{20°}^{H_2O}$	$10^7 \times D_{20}^{H_2O}$	$\bar{M}_w$[a]	$10^7 \times \sigma_D$
Mactin A	2.65	4.41	24,800	1.5
Mactin B	2.57	3.70	28,700	<0.2
Heparin	2.28	6.63	14,200	0.8

[a] $\bar{M}_w$ = weight average molecular weight.

The partial specific volumes of the three samples were calculated from the weights of a pycnometer filled with solutions of different concentrations.[29] Weight average sedimentation constants were measured in a Spinco Model E ultracentrifuge from the second moments of the gradient curves.[30] Electrophoretic experiments were performed in the Spinco Model H electrophoresis-diffusion apparatus. The weight average diffusion constants were calculated by the method of second moments using the Rayleigh interference fringes.[23] The standard deviation of the diffusion constants has been estimated from the reduced second and fourth moments.[31] The diffusion measurements were made at 1° in the Spinco Model H electrophoresis-diffusion apparatus. The comparator used was made by the David W. Mann Precision Instruments Company, Lincoln, Massachusetts. It was equipped with a projection system which gives a magnification factor of 36. With our equipment it is possible to make measurements on patterns containing as many as 35 fringes to a millimeter. The distances between successive fringe minima can be reproduced with a maximum error of 5 μ. Although we have not been able to obtain as high a precision with our apparatus as Longsworth, we find $D^{1°} = 2.414 \times 10^{-6}$ for sucrose, $\bar{C} = 0.35$ and $C = 0.70$ in excellent agreement with Longsworth's value.[32]

The electrophoretic measurements were made in phosphate buffer pH 7.5 and ionic strength 0.15. The partial specific volumes, sedimentation and diffusion constants were measured in phosphate buffer pH 7.5, ionic strength 0.15 containing 0.30 mole of KCl per liter.

Acknowledgment.—The authors are indebted to Mr. L. Brancone for the microanalyses, to Dr. J. Corbett for the anticoagulant assays, to Mr. W. Fulmore for the infrared analyses and determination of optical rotations, and to Dr. Karl Meyer of the Columbia University Medical School for an authentic sample of galactosamine hydrochloride. Samples of purified heparin were obtained from Eli Lilly and Company and Abbott Laboratories.

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