These results indicate that aminoacetonitrile can act directly on bone in tissue culture to cause an alteration in the extractability of collagen similar to that observed *in vivo*. Since the hydroxyproline in the salt extracts was labeled equally in the control and aminoacetonitrile samples, while the specific activity of hydroxyproline in the residue was depressed by aminoacetonitrile, it seems likely that the latter exerts its action by blocking the normal maturation process whereby newly synthesized collagen is converted to an insoluble matrix[7]. A similar mechanism has been proposed to account for the action *in vivo* of another lathyrogen β-aminopropionitrile[8–10].

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Macromolecular properties and biological activity of heparin
II. Further electrophoretic studies

In a previous publication on the electrophoresis of heparin[1], it was shown that the number of components observed and their relative concentrations were strongly dependent on the ionic strength. This phenomenon was attributed to the formation of reaction boundaries. In the same publication it was also shown that a correlation existed between the anticoagulant activity and the relative amount of a new intermediate peak formed when heparin was subjected to electrophoresis in the presence of streptomycin. It is the object of this work to examine further the reaction-boundary hypothesis and to use the data available on the heparin–streptomycin complex to attempt further purification of heparin.

All electrophoresis runs were performed in a Spinco Model H Electrophoresis–Diffusion Apparatus. Analytical runs were made in either the 2-ml or the 11-ml cell depending on the amount of material available. The preparative runs were performed in the 80-ml preparative cell using the automatic sampling attachment of the instrument to withdraw fraction.

Analytical moving-boundary electrophoresis on a commercial heparin from pig

tissue at pH 8.9 in Tris buffer (I 0.1) showed 2 peaks as described in our previous publication. This sample under identical electrophoretic conditions was separated in the preparative cell. The lead peak was withdrawn from the ascending limb with repeated use of counter-current compensation to extend the effective length of the electrophoretic cell. At the end of the experiment, the isolated lead peak, the material remaining in the cell, and a control sample not subjected to electrophoresis were dialyzed against running water to remove buffer salts and were then lyophilized. The isolated fractions and control were tested for anticoagulant activity by Standard U.S.P. methods[2] and were subjected to electrophoresis in the Tris buffer. The results obtained on two such separations are shown in Table I. Both the fractions contained the same components in the same ratio as the control sample. Thus, some type of reequilibration has occurred.

TABLE I

ELECTROPHORETIC AND BIOACTIVITY DATA ON HEPARIN FRACTION

Expt.	Sample	Slow peak		Fast peak		Biological activity (units/mg)
		$\mu \times 10^5$ ($cm^2\ sec^{-1}\ V^{-1}$)	Relative amount (%)	$\mu \times 10^5$ ($cm^2\ sec^{-1}\ V^{-1}$)	Relative amount (%)	
1	Control	5.6	[illegible]	12.6	83.8	145
	Lead peak	5.7	[illegible]	12.3	87.0	135
	Remainder	5.7	[illegible]	12.6	85.7	145
2	Control	5.8	[illegible]	12.7	86.0	150
	Lead peak	5.8	[illegible]	12.9	87.0	138
	Remainder	5.5	[illegible]	12.6	90.0	145

The same type of separation was made on the heparin–streptomycin complex using the same electrophoretic conditions. It was hoped that if the peak due to the complex truly contained the active principle then the material remaining in the cell after removal of the lead peak should show an increase in activity since the peak due to complex would have been left behind. After isolation, the streptomycin complex was broken by precipitation with cetylpyridinium chloride. Heparin was resuspended in 2 M NaCl and removed by the method previously described by LAURENT[3]. Each fraction was subjected to analytical electrophoresis to confirm the removal of streptomycin as evidenced by the absence of the intermediate complex peak. If the complex remained, the process of streptomycin removal was repeated. The results at two different levels of streptomycin concentrations are shown in Table II. There was no significant increase in biological activity in either of the fractions.

The observation of an electrophoretic pattern for an isolated peak identical to that of the starting material together with the observation that the anticoagulant activities of lead and slow peaks were identical provides further evidence that multiple peaks in heparin electrophoresis at normal ionic strengths are due to reaction boundaries[4]. It is interesting that JENSEN AND SNELLMAN[5,6] also found two active peaks in the electrophoresis of heparin and attempted to separate the materials in the peaks by fractional precipitation with organic solvents. They offered no explanation for the observation that each of the fractions showed the same two peaks in the same ratio when subjected again to electrophoresis. It appears obvious now that they were

observing the same type of phenomena as described here, peaks produced by heparin interactions with ions leading to formation of complexes.

The results with the streptomycin complex show that more complicated interactions exist in this system. Since both fractions are indistinguishable from the starting material, and both contain anticoagulant activity, this approach to the purification of heparin is not feasible.

While many investigators have shown the heterogeneity of heparin it is of interest that no one as yet has isolated a sample with an anticoagulant potency above

TABLE II

ELECTROPHORETIC DATA AND BIOLOGICAL ACTIVITY OF HEPARIN–STREPTOMYCIN FRACTIONS

Expt.	Sample	Slow peak		Fast peak		Biological-activity data (units/mg)
		$\mu \times 10^5$ ($cm^2\ sec^{-1}\ V^{-1}$)	Relative amount (%)	$\mu \times 10^5$ ($cm^2\ sec^{-1}\ V^{-1}$)	Relative amount (%)	
1	$\frac{\text{Heparin}}{\text{Streptomycin}} = 2$					
	Control	5.8	15.0	12.4	85.0	120
	Lead peak	5.6	14.5	12.7	85.5	125
	Remainder	5.6	16.2	12.7	83.8	145
2	$\frac{\text{Heparin}}{\text{Streptomycin}} = 0.5$					
	Control	5.7	13.0	12.6	87.0	145
	Lead peak	5.6	16.0	12.6	84.0	118
	Remainder	5.7	11.0	12.8	89.0	150

180 units/mg. It may well be that JORPES's[7] contention that heparin is not a single entity is correct. The possibility also exists that the heterogeneity involves polymers of glucosamine with different uronic acids. The isolation of glucuronic acid[8] and iduronic acid[9], and the observation of HELBERT[10] that the uronic acid of heparin was primarily not an aldohexuronic acid seems to confirm this possibility. If then the active heparin is the polymer between glucosamine and a specific uronic acid, it would indeed be extremely difficult to isolate by any of the presently known methods.

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