Carbohydrate Research, 104 (1982) 299–308
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

COMPLEXES OF HEPARIN WITH POLY(ALKYLENIMINES): COMPETITIVE BINDING WITH METHYLENE BLUE

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(Received October 29th, 1981; accepted for publication, December 15th, 1981)

ABSTRACT

The binding of heparin (Hep) and Hep fractions with oligo- and poly-(alkylenimines) having the general formula $H_2N(CH_2\text{-}CHR\text{-}NH)_nH$, where R = H or Me, has been investigated by spectroscopy, by evaluating the competition of the amines and Methylene Blue for the anionic sites of Hep. The strongest binding was observed at pH 3.5, with the essentially linear triethylenetetramine and the slightly branched tetraethylenepentamine giving the most stable complexes. For N (number of nitrogen atoms per molecule) >5, a decrease of the binding ability of the amines was observed. The apparent stoichiometry of the complexes was a function of the relative concentration of Hep and the amine, indicating an equilibrium between different types of complexes. Beef-lung Hep and a Hep fraction consisting mainly of trisulphated disaccharide blocks gave stronger complexes than the more heterogeneous, pig-mucosal Hep and a Hep fraction of lower sulphate content. The results are interpreted in terms of polyelectrolyte-type associations involving sulphate groups on adjacent residues of the Hep chain and sequences of charged nitrogen atoms on the polyamine.

INTRODUCTION

Dietrich, McDuffie and co-workers[1–3] have reported that heparin can be fractionated by isoelectric focusing (i.e.f.) into at least 21 components, with "pI values" in the pH range 3.2–4.2. This fractionation was apparently associated with differences in molecular mass, the components of low pI exhibiting the smallest average size (3,000 daltons) and the components of high pI the highest (37,500 daltons). There appeared to be graded intervals of 1,500–2,000 daltons in size, which suggested that the heparin biosynthesis could proceed *via* addition of hexasaccharide units.

As in the case of nucleic acids[4], the "isoelectric fractionation" of heparin is unexpected on theoretical grounds. In fact, i.e.f. is a method of charge fractionation

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and, at constant charge-to-mass ratio, is not affected by polydispersity in molecular mass. Righetti and co-workers[5-7] demonstrated that the apparent isoelectric fractionation of Hep was due to strong interaction between the polysaccharide and different amphoteric species in the Ampholine mixture. The binding is strongly pH-dependent, no significant interaction being detectable at pH 6.7, weak binding being apparent at pH 5.1, and strong complex formation occurring at pH 3.5. This pH-dependence was suggested[5-7] to be due to the oligoamino backbone of carrier ampholytes, whose charge density is enhanced as the pH is progressively lowered [these compounds are synthesised from a mixture of oligo(ethylenimines) by treatment with acrylic acid[8]].

The artefactual character of the i.e.f. of Hep is further stressed by the evidence that the 21 bands where Hep is "focused" actually correspond to zones of the i.e.f. slabs where different Ampholine families concentrate[7]. Hep was shown, by low-voltage electrophoresis in barium acetate buffers, to consist of well-defined families of chains[9]. However, each of these families distributes along an i.e.f. slab among more than one Ampholine "band", the actual zone pattern depending on the total amount of Hep and the relative concentration of the Hep species[10].

Although not as sharply as thought in early studies[1-3], some separation of Hep species takes place along the i.e.f. slabs, partially resolving fractions characterised by different molecular sizes and biological activities[11]. It was therefore of interest to investigate systematically the interaction of Hep and the oligo-amines that constitute the backbone of the Ampholine molecules, with the aim of determining the structural factors affecting this interaction. In the present work, complex formation in solution between Hep (and well-defined Hep fractions) and a number of the above oligo- and poly-(alkylenimines) having the general formula $H_2N(CH_2\text{-}CHR\text{-}NH)_nH$, where R = H or Me, was investigated by spectrophotometry. The approach was based on competitive binding in solutions containing the polyamine (PA) and the metachromatic dye Methylene Blue (MB).

EXPERIMENTAL

Materials. — Unless otherwise stated, the Hep used was a commercial preparation from pig mucosa (Hep-PM I, Terhormon b.018), and was ~95% pure by electrophoretic and ^{13}C-n.m.r. analysis[12]. It contained similar amounts of slow-moving (sm) and fast-moving (fm) components on electrophoresis in barium acetate[9]. The beef-lung (Hep-BL) and pig-mucosal (Hep-PM II) preparations used for comparison purposes were commercial products. The sm- and fm-Hep fractions were the first precipitate and the most-soluble fraction, respectively, obtained by precipitation of barium Hep (PM I) with ethanol, essentially as described by Ayotte *et al.*[13]. The two fractions consisted of only slow-moving and fast-moving species, respectively, on electrophoresis in barium acetate. The average molecular mass ($\overline{M}_r$) of the Hep preparations and fractions, as determined by gel filtration[14] on Ultrogel AcA-44, were: Hep-PM I = 14,000; Hep-PM II = 12,500; Hep-BL = 15,000; sm-Hep = 20,000; and fm-Hep = 8,000 daltons[15].

The oligo-imines were obtained from Aldrich, and poly(ethylenimines) ($\overline{d.p.}$ ~15, ~40, and ~1,800) and poly(propylenimine) (d.p. ~50 by ^{13}C-n.m.r., end-group analysis) from Polysciences, Warrington, U.S.A. The extent of branching of the amines was determined by conductimetric titration[16] and ^{13}C-n.m.r. spectroscopy[17]. In the ethylenimino series, only the trimer (DETA) was exclusively linear. The degree of branching was ~10% for the tetramer (TETA), ~15% for the pentamer (TEPA), and ~22% for the hexamer (PEHA). The polymers (PEI) were extensively and statistically branched. By contrast, poly(propylenimine) (PPI) was essentially linear.

Spectrophotometric titrations. — Methylene Blue (30 nmol) in mM formate buffer (pH 3.5) was titrated in the presence of 10, 20, or 30 nequiv. of the amine, essentially as described by Righetti *et al.*[7]. "Equivalents" of Hep were calculated from the content of sulphate + carboxyl groups per disaccharide unit, as determined by conductimetric titration. These values were 2.9 for Hep-PM I, 3.2 for Hep-PM II, 3.4 for Hep-BL, 3.0 for sm-Hep, and 2.7 for fm-Hep[15]. The spectrophotometric measurements were made with a Zeiss PMQ-II instrument.

RESULTS

As observed[7] for Ampholines, the titration curve MB-Hep was practically unaffected by the presence of PA at pH >6, and was only slightly modified at pH

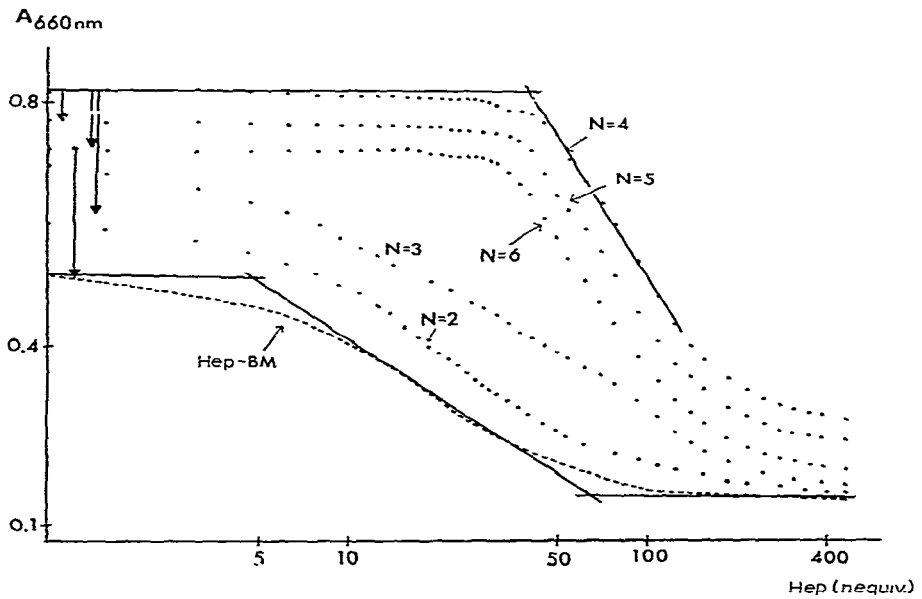

Fig. 1. Spectrophotometric titration curves of Methylene Blue (MB, 30 nmol) with Hep (1–500 nequiv.), in the absence (——) and presence (····) of 30 nequiv. of oligo(ethylenimines) (N = number of nitrogen atoms per molecule of amine). For clarity, curves are vertically displaced as indicated by the arrows.

TABLE I

APPARENT STOICHIOMETRY OF THE PA-HEP COMPLEXES

	PA (nequiv.)	*Hep/PA (nequiv.)*[a]					
		1st end-point			*2nd end-point*		
EDA (N = 2)	10	0.6			<1		
	20		0.2			<1	
	30			0.1			<1
DETA (N = 3)	30			0.2			0.3
TETA (N = 4)	10	1.6			2.0		
	20		1.5			1.9	
	30			1.3			1.8
TEPA (N = 5)	10	2.2			2.3		
	20		1.7			1.8	
	30			1.2			1.5
PEHA (N = 6)	10	1.3			1.4		
	20		1.0			1.1	
	30			1.0			1.2
PEI (N ~15)	10	1.4			1.6		
	20		1.0			1.3	
	30			1.0			1.2
PEI (N ~40)	30			0.9			1.0
PEI (N ~1800)	30			0.9			1.0
PPI (N >50)	10	1.6			1.8		
	20		1.0			1.2	
	30			1.1			1.0

[a]Corrected for the amount of Hep (30 nequiv.) bound by Methylene Blue.

5.5 (curves not shown). The largest modifications were observed at pH 3.5, which was chosen in the present study.

Fig. 1 shows the spectrophotometric titration curves of MB (30 nmol) with up to 500 nequiv. of Hep, in the absence and presence of 30 nequiv. of oligo(ethylenimines) (N = 2–6, where N is the number of nitrogen atoms per molecule). In the absence of amines, the absorbance of the dye at 660 nm decreases following the first additions of Hep. The absorbance also decreases, though with a different slope, in the presence of EDA (N = 2) and DETA (N = 3). In contrast, in the presence of TETA (N = 4), TEPA (N = 5), and PEHA (N = 6), the absorbance remains constant until an excess of Hep is added. This point (first inflection in the titration curve) occurs for 40 nequiv. of Hep for N = 4, and for somewhat lower concentrations of Hep for N = 5 and N = 6. An inflection at much lower concentrations (~7 nequiv.) of Hep is also apparent in the curve for N = 3. Since the plateau region corresponds to concentration ranges for which Hep is completely bound to the amine, and thus not available[7] for complexing with MB, the first inflection point indicates the amount of Hep bound by the amine.

A second inflection point, corresponding to concentrations at which both the

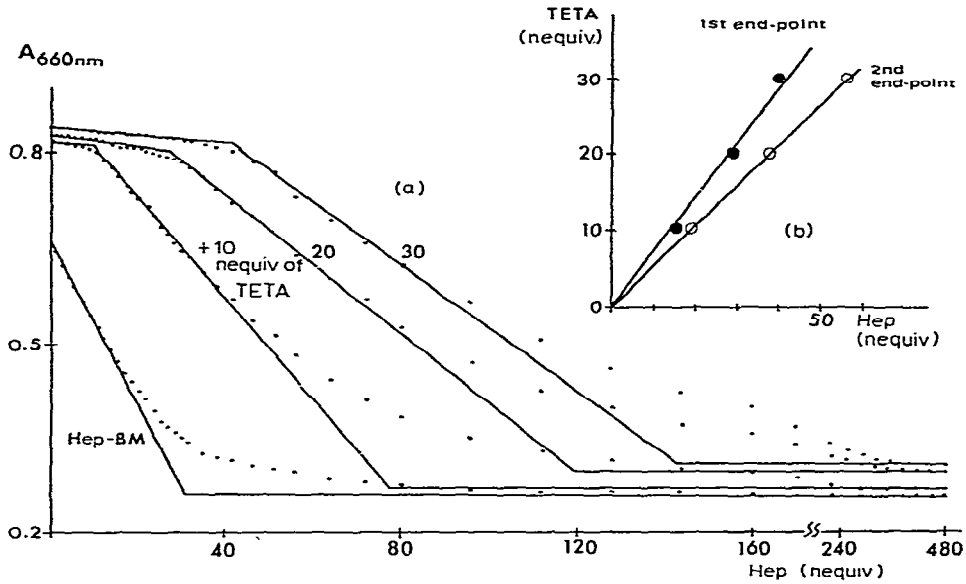

Fig. 2. (*a*) Spectrophotometric titration curves (linear plots) of MB (30 nmol) with Hep (1–500 nequiv.) in the presence of 10, 20, and 30 nequiv. of TETA. (*b*) Concentrations of Hep (nequiv.) corresponding to the first and second end-points of the titration curves as a function of the molar concentration (nequiv.) of TETA. Values at the second end-point are corrected for the Hep complexed with MB.

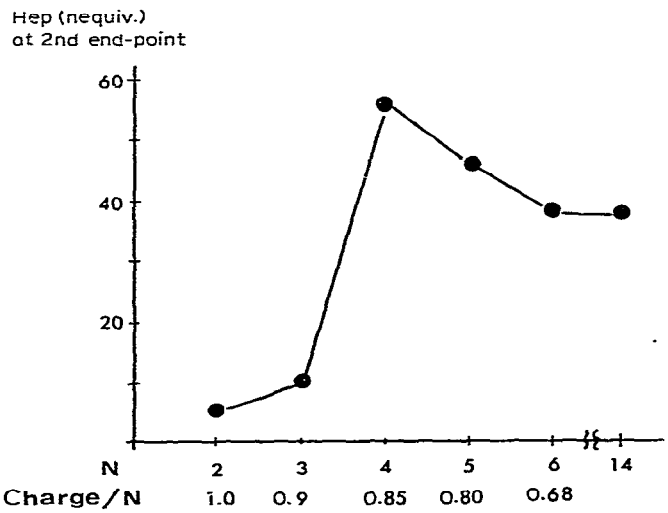

Fig. 3. Hep concentrations (nequiv.) corresponding to the second end-point of the titration curves (after subtraction of the Hep needed for complexing MB), for different oligoamines (30 nequiv.), as a function of the number of nitrogen atoms (N) and the charge/N ratios of the amines.

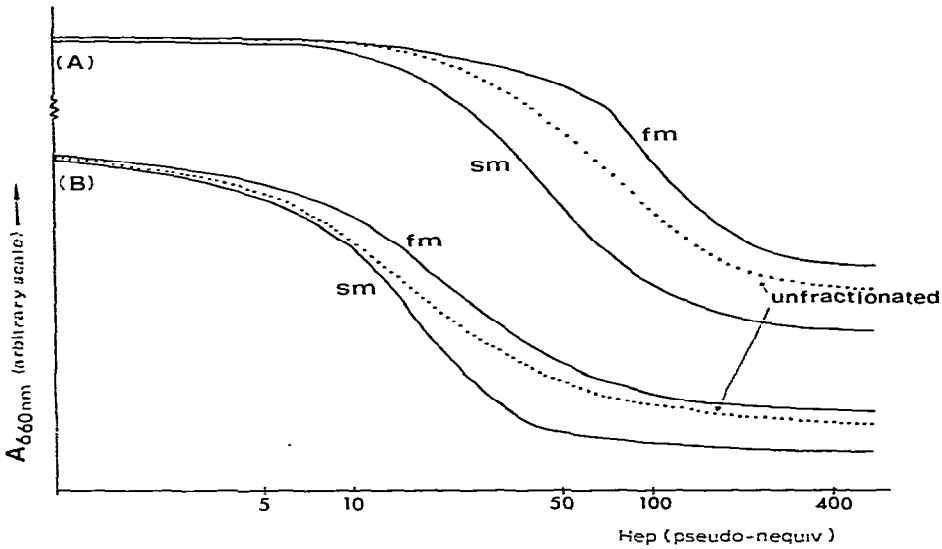

Fig. 4. Spectrophotometric titration curves of MB (30 nmol) with the sm- and fm-Hep fractions (——) and of the parent Hep (····), in the presence (A) and absence (B) of TETA (30 nequiv.). For clarity of comparison, the concentrations of the Hep fractions are expressed in "pseudo-nequiv.", conventionally assuming the same content of sulphate + carboxyl groups as for the parent Hep).

amine and MB are fully complexed, occurs at ~10 nequiv. of Hep for N = 3, 56 nequiv. for N = 4, 46 nequiv. for N = 5, and at somewhat lower concentrations for N > 6. The Hep/PA ratios at the two inflection points are given in Table I for solutions containing 10, 20, and 30 nequiv. of the various oligo- and poly-amines. These data show that the Hep/amine concentration ratios at the inflection points are dependent, to some extent, on the total content of amine in solution. At the lowest concentration of the amine (10 nequiv.), the above ratio is higher for TEPA than for TETA.

Fig. 2, which refers to the system Hep-MB in the presence of TETA, shows that the Hep concentrations at the two inflection points increase linearly with increasing concentration of the amine. Fig. 3 shows a plot of the Hep concentrations at the second end-point (corrected for the amount of Hep bound to MB) as a function of the number of N atoms in the oligo-amine molecule (30 nequiv.). The abscissa also shows the corresponding charge/N ratios, *i.e.*, the calculated net charges on oligo(ethylenimines), at pH 3.5, as a function of pK (values for N = 2–5 are from ref. 18, and for N = 6 from ref. 19). A maximum is observed for N = 4, corresponding to a charge/N ratio of 0.85. Fig. 4 shows a comparison of the titration curves of the sm- and fm-Hep fractions with the curves of the parent (unfractionated) Hep, both in the presence and absence of TETA. The curve of the sm-fraction is practically superimposable on that obtained from the Hep preparation from beef lung (Hep-BL, curve not shown), and shows inflections at Hep concentrations significantly lower than for the fm-fraction. The curve of the parent pig-mucosal Hep (Hep-PM I) is intermediate between those of the two fractions. The curve of Hep-PM

II is intermediate between those of Hep-PM I and Hep-BL. The different heights of the curves at the end of the titrations are due to the fact that, with the less-sulphated Hep preparations, full metachromasia with MB is not reached, even in the presence of a large excess of Hep.

DISCUSSION

It has been suggested[7] that the oligo(ethylenimino) backbone of Ampholines can associate with Hep chains in two ways, depending on the molar ratio Ampholine/Hep. With excess of Ampholine, the proposed arrangement is perpendicular, as idealised in Fig. 5A. At lower Ampholine/Hep ratios, the polycationic and polyanionic chains can associate as in Fig. 5B, *i.e.*, in the parallel arrangement typical of polyelectrolyte complexes[7]. The present work conclusively confirms that Hep forms stable complexes with oligo- and poly-(alkylenimines) in solution. The most dramatic evidence of this complex-formation is the plateau region in the first branch of the titration curves of MB in the presence of equimolar concentrations of amines. In the plateau region, Hep is completely bound to the amine, and is therefore not available for complexing with the metachromatic dye until an excess of Hep is present in solution.

A true plateau is not observed until the amine chain is sufficiently long, *i.e.*, corresponding to 4 or 5 N atoms, depending on the relative concentrations of the amine and Hep in solution. The values of Hep concentrations at the first inflection point (Table I), taken as a measure of the plateau length, slightly decrease and level-off for N = 6–15, and significantly decrease for the high polymers (N $\geq$40). From the Hep concentration corresponding to the first inflection point of the titration curves, the apparent stoichiometry of the amine–Hep complexes in the region of "excess amine" can be evaluated (Table I). For N $>$3, this stoichiometry corresponds to

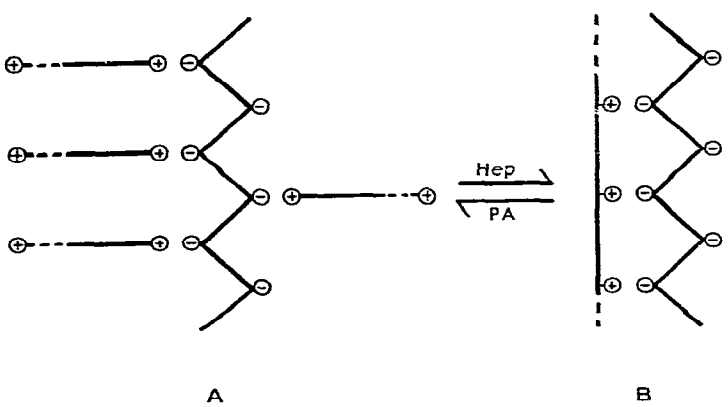

Fig. 5. Possible arrangements of polyamine (PA) and Hep chains in the PA-Hep complexes. The polyelectrolyte-type complexes B are favoured by decreasing concentration and increasing mol. wt. of the amine.

approximately one equiv. of amine per equiv. of Hep. In the presence of excess of Hep. the apparent limit-stoichiometry, as evaluated[7] at the second inflection point after subtraction of the Hep needed for "neutralising" MB, corresponds to somewhat lower amounts of amine bound to Hep.

In their present form, the titration curves do not yield data on the formation constants of the complexes. In fact, inspection of the Hep-MB titration curve (Fig. 1) suggests that, even in the absence of amine, the equilibrium of the polysaccharide–dye interaction is complex. Moreover, the curves in the presence of amine(s) suggest multiple equilibria. However, the Hep concentrations corresponding to the maximum slope of the titration curves can be taken as relative values for the apparent formation constants of the complexes.

Fig. 3 shows that, for 30 nequiv. of amine, TETA gives the most stable complex with Hep. As deduced from the fact that the strongest complexing of the amines and Hep was obtained at acid pH, a positive charge on the nitrogens of the amine is important for formation of stable complexes. However, since the net charge on the amine corresponding to the most stable complex is <1 (Fig. 3), co-operativity effects associated with a minimum chain-length of the amine are likely to be more important in determining the stability of the complex. The minimum sequence of four nitrogens seems to be necessary for formation of strong complexes. Such a co-operative effect appears to be somewhat decreased for longer chains of the amine, perhaps also due to the fact that, for $N > 4$, branching of the oligoamine is quite extensive. The evaluation of structural factors affecting the complexing ability of the amines used in the present work is complicated by the non-homogeneous comparison between linear and branched amines. In fact, in the ethylenimine series, increasing molecular size (up to $N \sim 15$) was shown to be associated with increasing degrees of branching; the poly(ethylenimines) with high $\overline{M}_r$ have ^{13}C-n.m.r. spectra almost superimposable on those of the highly branched PEI (d.p. ~ 15), whereas the PPI (high molecular mass) was essentially linear[17].

It is conceivable that a larger number of primary amino groups, as made available by increasing degrees of branching, would neutralise anionic sites on Hep more effectively than the internal imino groups of the linear chains. However, the reduced complexing ability observed for both linear and branched amines having high $\overline{M}_r$ can be ascribed essentially to steric factors, *i.e.*, to increasingly difficult matching of the polycationic and polyanionic chains as these chains become long. In both highly branched and high-$\overline{M}_r$ amines, entire segments of the molecule are expected to be excluded from the regions of binding.

The binding sites of Hep are undoubtedly the sulphate groups (at pH 3.5 and at the concentrations used in the present experiments, more than 90% of the carboxyl groups are protonated[7]). The fact that Hep-BL binds significantly larger amounts of TETA than HEP-PM I and -PM II can be ascribed to the fact that highly sulphated regions (essentially consisting of trisulphated disaccharide units) are more numerous in the former[20,21]. Since the structure of the sm-Hep is similar to that of beef-lung Hep[15], it is not surprising that the two preparations gave similar complexes with

TETA. Conversely, the highly heterogeneous fm-Hep fraction[15] gives weaker complexes (Fig. 4).

The stability of the polyimine-Hep complexes is also dependent on the molecular mass of Hep. Assessment of such a dependency cannot be made with the present samples, since they are different not only in molecular size but also in degree of heterogeneity. However, since Hep species of high-$\overline{M}_r$ are also most frequently characterised by higher contents of the regular trisulphated disaccharide blocks than found[15] for low-$\overline{M}_r$ species, it can be inferred that unfractionated heparins of higher, average molecular mass are more easily complexed by polyamines than low-$\overline{M}_r$ heparins. This could explain the observation[1,11] that Hep fractions of different $\overline{M}_r$ have different mobilities in i.e.f. with Ampholines. The Hep species of high-$\overline{M}_r$ have a high affinity for the oligo(ethylenimino) backbone of Ampholines, and are retained in the region of higher pH, whereas the less-sulphated and low-$\overline{M}_r$ species, requiring a pH <4 for formation of the polyelectrolyte complex, migrate further toward the anodic side of the i.e.f. slabs. As a corollary of the present conclusions, it could be predicted that, when used as buffers in electrophoresis, the oligo- and poly-(alkylenimines) should permit separation of different families of Hep chains, and preliminary experiments with TETA have shown that this is so.

ACKNOWLEDGMENTS

The authors thank Mr. G. Zoppetti for the ^{13}C-n.m.r. analysis of the polyamines, and Dr. L. Valentini for helpful discussions. This work was supported, in part, by Crinos Biological Laboratories and by Consiglio Nazionale delle Richerche (CNR).

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