Histochemie 5, 221—233 (1965)

Medical Research Council Rheumatism Research Unit, Canadian Red Cross Memorial Hospital, Taplow, Maidenhead, Berkshire, England

DIFFERENTIAL STAINING OF ACID GLYCOSAMINOGLYCANS (MUCOPOLYSACCHARIDES) BY ALCIAN BLUE IN SALT SOLUTIONS*

By

J. E. SCOTT and J. DORLING

With 14 Figures in the Text, of which 6 in Colour

(Received January 9, 1965)

Summary. The application of the "critical electrolyte concentration" (CEC) concept to the differentiation of acidic glycosaminglycans (mucopolysaccharides) is described. Alcian Blue 8 GX stains with increasing selectivity as increasing amounts of magnesium chloride are incorporated into the dye solution. Model experiments with pure polyanions, or artifically carboxylated, phosphorylated and sulphated liver sections, showed that binding of dye to carboxylate or phosphate groups ceased at low electrolyte concentrations (< 0.3M) whereas dye continued to be held by sulphate ester groups at concentrations five to ten times as high. The similarity to the well established cetylpyridinium system for polyanion fractionation is discussed.

Sections of tissues chosen to contain predominantly or characteristically carboxylated mucins, and/or sulphate ester polyanions showed a staining pattern entirely similar to the model sections. Goblet cell mucin in rat ileum stained at < 0.4M $MgCl_2$, Cartilage at < 0.6M $MgCl_2$, mast cells at < 0.75M, and corneal stroma at < 1.0M. These results are in agreement with the known contents of sialo-mucin, chondroitin sulphate, heparin and keratansulphate, respectively. The conditions in which this principle can be used in a practical technique are described.

About eight members of the group of acid glycosaminglycans are chemically well characterised, and others may exist (see e.g. SCOTT, 1960). They are all densely negatively charged because of the presence of sulphate ester groups and/or carboxyl groups of uronic acids. In consequence they bind, and are precipitated by, cationic dyes. This physico-chemical reaction is the basis of every histological method for their demonstration, since no chemical method is available for the purpose. Some specificity can be introduced by preventing combination of dye with one or more members of the group. Methylation, acetylation, desulphation, and manipulation of pH have been used to distinguish between polysulphates and polycarboxylates, but with small success (QUINTARELLI, SCOTT and DELLOVO, 1964). In theory, the use of enzymes to remove selected compounds provides optimal specificity, but in practice difficulties of penetration to the substrate, lack of adequate controls, and the impure nature of available enzymes limits this approach. Physico-chemical blocking of the staining reaction by colourless cations such as cetylpyridinium, followed by sequential unblocking in salt solutions (KELLY, BLOOM and SCOTT, 1963; QUINTARELLI, SCOTT and DELLOVO, 1964) may be more promising, but yet offers complications, not the least being due to the multistep nature of the technique.

A theoretical treatment (SCOTT, 1961) of the in vitro fractionation procedure for acid glycosaminoglycans using cetylpyridinium chloride (SCOTT, 1960) indi-

* The new and more precise terminology of Jeanloz (1960) is used in preference to the older nomenclature.

cated that any cationic dye could be used instead of cetylpyridinium, provided certain very simple conditions were fulfilled. This paper describes the application of the treatment to the *staining of acid glycosaminoglycans in a simple one-step procedure, using Alcian Blue 8 GX.*

A preliminary account of this work has already appeared (Scott, Dorling and Quintarelli, 1964).

Materials and Methods

Model Experiments on Filter Paper

Polyanions. Sodium hyaluronate and chondroitin sulphate were prepared by the method of Scott (1960) from mesothelioma fluid and horse nasal septa respectively. Rat liver DNA and yeast RNA were gifts from Dr. K. S. Kirby. Keratansulphate was a gift from Dr. Sven Gardell. Sodium Alginate was from Hopkin and Williams, Romford, Essex. Heparin was "Pularin", 1000 units/ml from Evans Biological Products, Runcorn, Cheshire. Sodium polyphosphate was "Phosphate Glass 697" from Albright and Wilson, Birmingham.

Alcian Blue 8 GX (AB) was a gift from ICI, Blackley, Manchester. Commercial specimens were obtained from G. T. Gurr, and reacted similarly.

Magnesium Chloride was A. R., from Hopkin and Williams.

Method. Circles of Whatman No. 1 filter paper were spotted with 2 μl samples of 1% w/v solutions of polyanion and dried in air. They were placed in beakers each containing 50 ml 0.05% w/v AB in 0.05M sodium acetate buffer at pH 5.8 (see below) containing $MgCl_2$ at concentrations shown in Table 1. After 1 hr. the circles were removed, washed rapidly in a large volume of distilled water and dried in air.

Histology

Materials. *Tissues.* Hilar region of human newborn lung, rabbit cornea and glycogen rich rabbit liver, were fixed in neutral phosphate buffered 4% formaldehyde. Rat ileum was fixed in 95% ethanol at 4° C.

Stock Solutions. Chemicals were laboratory grade from B.D.H., Poole, Dorset, except where otherwise indicated.

1% Alcian Blue 8GX (AB) (G.T. Gurr) in distilled water, prepared on the day of use.

5.0M magnesium chloride A.R. (Hopkin and Williams) 0.1M sodium acetate buffer, pH 5.8.

3% cetylpyridinium chloride (C.P.C.)

0.1M methyl isothiouronium sulphate (Lights Organic Chemicals, Colnbrook, Bucks).

Staining Solutions. 0.05% Alcian Blue in pH 5.8 0.025M acetate buffer + $MgCl_2$. (AB—pH 5.8—$MgCl_2$). 0.05% Alcian Blue in 3% acetic acid (pH 2.5) + $MgCl_2$. (AB—pH 2.5—$MgCl_2$).

Solutions were prepared by mixing dye, buffer, 5.0M $MgCl_2$ and distilled water to give 50 mls of solution containing the required concentration of $MgCl_2$.

Methods. *Preparation of Sections.* Tissues were dehydrated with alcohol, cleared in benzene and embedded in paraffin wax (57° C M.P) with the exception of the cold alcohol fixed rat ileum which was processed by the technique described by Sainte-Marie (1962).

5 micron sections were mounted from warm water on albumen/glycerol coated slides and dried at 37° C.

Sulphated, carboxylated and phosphorylated sections were deparaffinised as described in the original techniques. In all other cases they were deparaffinised in two changes of xylene and brought to distilled water through one change each of 74 O.P. spirit, 90% spirit and 100% spirit.

Sulphation. Sections were immersed for 5 minutes at room temperature in a 50% v/v mixture of sulphuric acid and ether as described by Mowry (1958).

Phosphorylation. Sections were immersed for 3 hours at 60° C in 1.5% v/v phosphoryl chloride in pyridine as described by Landing and Hall (1956).

Carboxylation. Sections were immersed for 1 hour at 60° C in saturated succinic anhydride in pyridine according to the method of Shackleford (1962).

Blocking of Anionic Sites. Sections were immersed for 24 hours in 500 ml of 3% cetyl pyridinium chloride (C.P.C.) at 37° C (Quintarelli, Scott and Dellovo, 1964) with stirring by magnetic stirrer.

Guandylation. Sections were immersed for 24 hours at room temperature in 0.1 M methyl isothiouronium sulphate.

Standard Staining Proceduse. Sections were immersed overnight at room temperature in 50 mls of dye solution in an upright Coplin jar. They were then individually rinsed in a stream of distilled water and transferred to a fresh distilled water bath. They were dehydrated in 70%, 90% and 100% spirit, cleared in xylene and mounted in D.P.X. (B.D.H.).

Results

Model Experiments on Filter Paper

In the absence of added electrolyte, staining of the more densely charged polyanions (polyphosphate, alginate, heparin) was patchy, and not so intense as that of the others. In the presence of a little electrolyte (0.05 M) all polyanions took up the stain avidly. The increase in staining by AB in the presence of electrolyte has previously been noted (SCOTT, QUINTARELLI and DELLOVO, 1964). As the $MgCl_2$ concentration was raised polyanions ceased to stain in the sequence shown in Table 1.

Table 1. *Staining of spots containing polyanions on filter paper in AB-pH 5.8-$MgCl_2$*

Type of anionic group	Polyanion	Molarity of $MgCl_2$									
		0.0	0.025	0.05	0.1	0.2	0.3	0.45	0.65	0.8	1.0
—COO^-	Hyaluronate	++	++	++	∓	–	–	–	–	–	–
	Alginate	∓	+	++	+++	–	–	–	–	–	–
$=PO_4^-$	RNA	∓	+	++	++	∓	–	–	–	–	–
	Polyphosphate	∓	+	+	+++	∓	–	–	–	–	–
	DNA	∓	++	++	+++	∓	–	–	–	–	–
—COO^- and —OSO_3^-	CSA	++	++	++	+++	+++	+++	+++	±	–	–
	Heparin	∓	++	++	+++	+++	+++	+++	+++	±	–
—OSO_3^-	Keratan sulphate	++	++	++	+++	+++	+++	+++	+++	+++	+

In this and succeeding tables, the following symbols denote strength of staining: +++ Very strong, ++ Strong, + Moderate, ∓ Weak, – None.

A series performed at a four-times greater concentration of AB (0,2%) showed little or no difference in staining pattern. A small increase (not more than 10%) in CEC was the only observable concequence of increasing the AB concentration, but since the background was more difficult to wash free of AB in the concentrated solution, the lower level of AB was preferred.

Histology. Effect of $MgCl_2$ on the Alcian Blue staining of artificially sulphated, phosphorylated and carboxylated liver glycogens

Sulphation. Sulphated sections of rabbit liver, sections treated with ether only and untreated sections were stained in solutions of AB—pH 5.8 containing $MgCl_2$ at concentrations shown in Table 2.

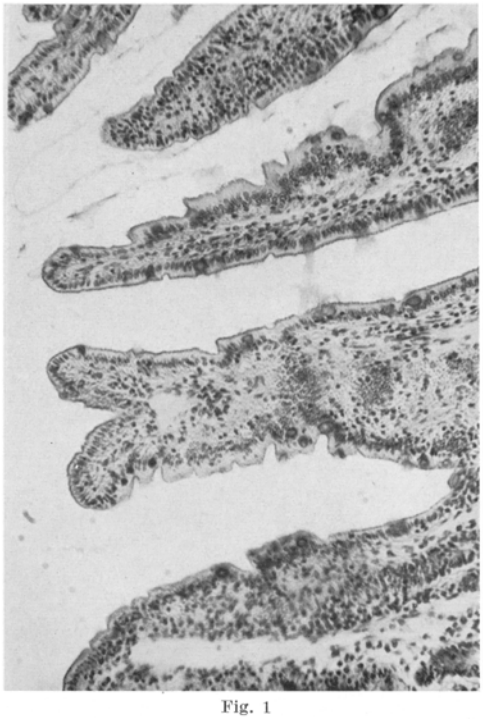

Fig. 1

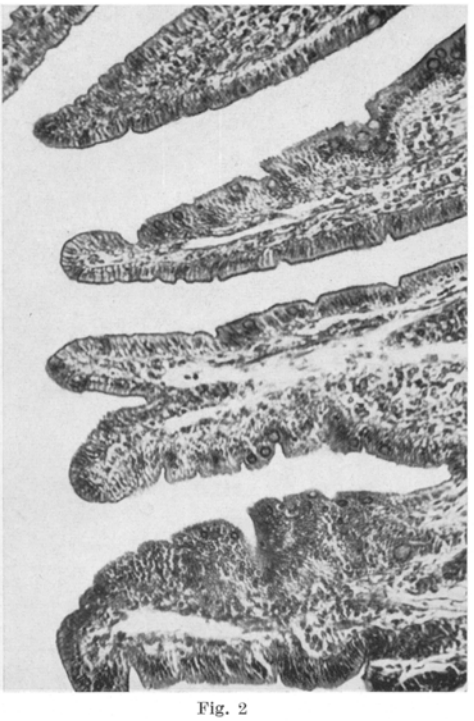

Fig. 2

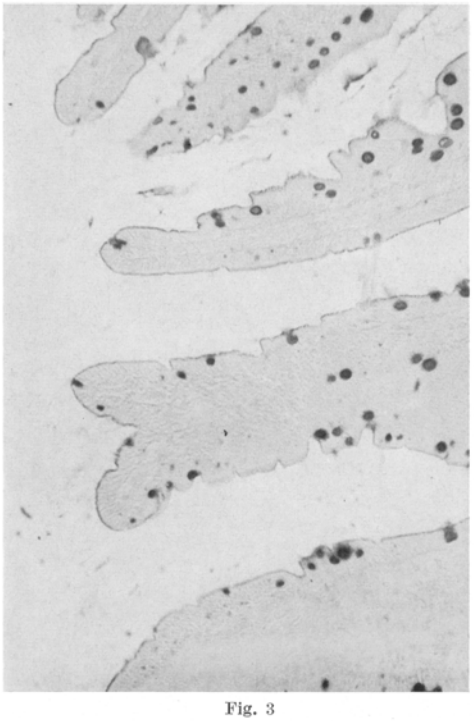

Fig. 3

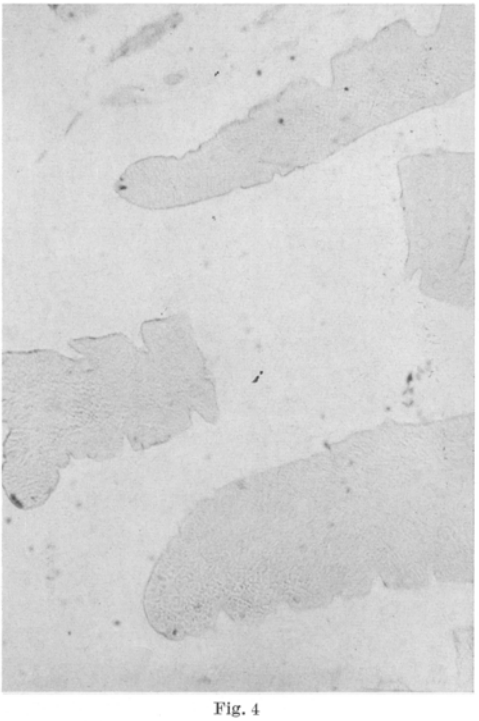

Fig. 4

Figs. 1—4. Rat ileum. $MgCl_2$ concentrations: — 1. 0.1M, 2. 0.0M, 3. 0.2M, 4. 0.5M

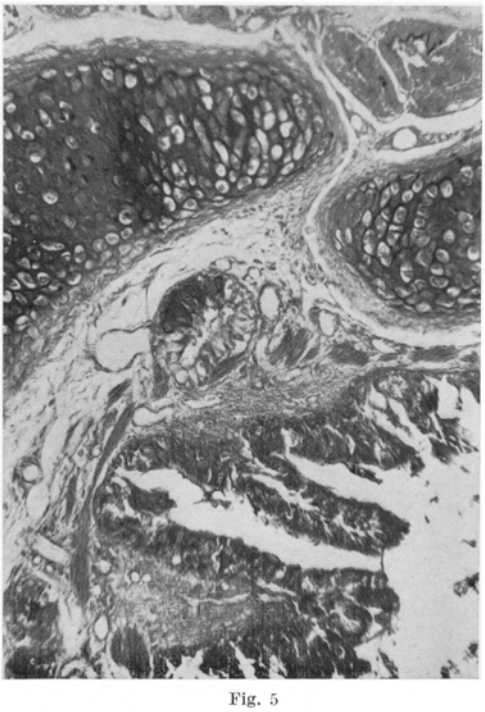

Fig. 5

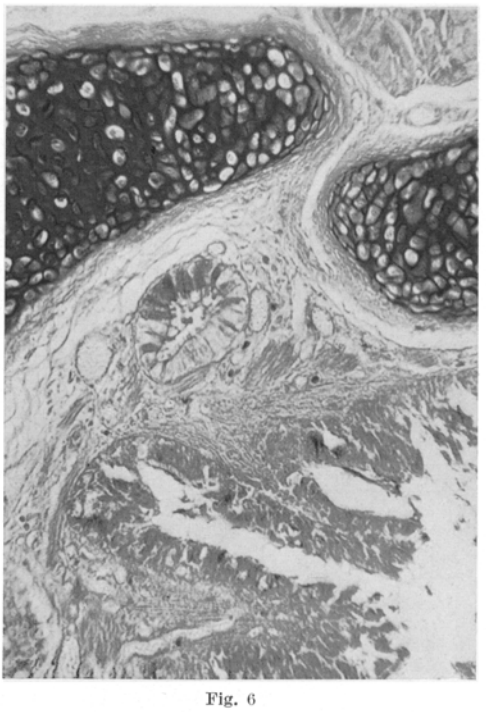

Fig. 6

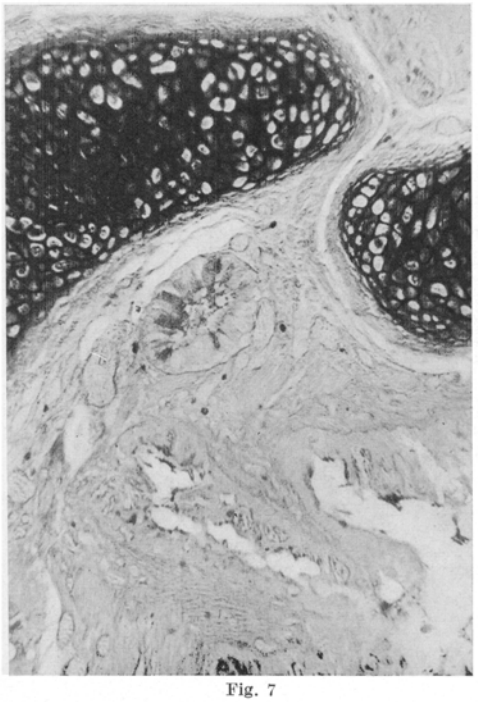

Fig. 7

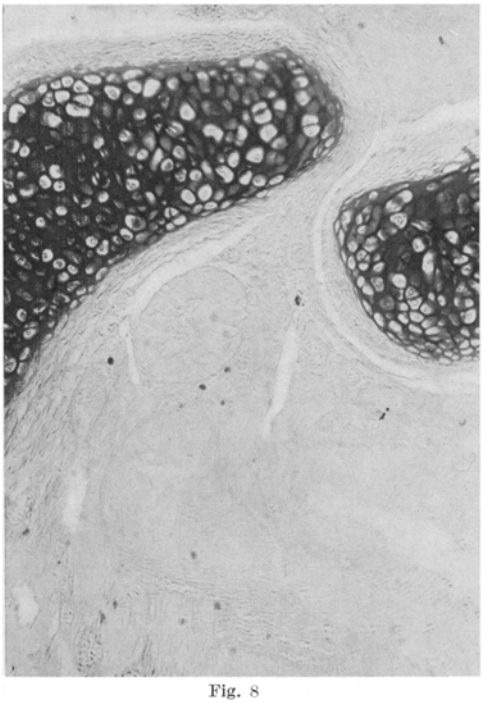

Fig. 8

Figs. 5—8. Human new born lung. $MgCl_2$ concentrations: — 5. 0.0 M, 6. 0.1 M, 7. 0.2 M, 8. 0.5 M

The ether treated and untreated sections stained identically. The uptake of dye by the sulphated and untreated sections was as shown in Table 2.

Phosphorylation. Phosphorylated sections of rabbit liver, sections treated with pyridine only, and untreated sections were stained in solutions of AB—pH 5.8 containing $MgCl_2$ at concentrations shown in Table 2. Results are shown in Table 2.

Carboxylation. Succinylated sections of rabbit liver and untreated sections were stained in solutions of AB—pH 5.8 containing $MgCl_2$ at concentrations shown in Table 2. Results are shown in Table 2.

Comparison of staining pattern of tissues. Sections of rat ileum and rabbit cornea were stained by the standard procedure for 16 hrs in AB—pH 5.8—$MgCl_2$. In

Table 2. *Staining of untreated, sulphated, carboxylated and phosphorylated liver sections by AB-pH 5.8-$MgCl_2$*

	Molarity $MgCl_2$									
	0	0.1	0.2	0.3	0.4	1.0	1.4	1.8	2.2	2.6
Untreated . . .	++	+	∓	∓	−	−	−	−	−	−
Sulphated . . .	+	++	++	++	+++	+++	++	+	∓	−
Carboxylated . .	+++	+++	+	∓	−					
Phosphorylated .	++	+	−	−	−					

Degree of staining recorded as in Table 1.

Table 3. *Staining of rat ileum AB-pH 5.8-$MgCl_2$ (see Fig. 1)*

	Molarity $MgCl_2$									
	0	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Nuclei	++	++	++	−	−	−	−	−	−	−
Goblet cell mucin	++	++	++	++	++	+	−	−	−	−
Striated border .	++	++	++	++	++	+	+	+	∓	−

Degree of staining recorded as in Table 1.

Table 4.
Staining of human newborn lung in AB-pH 5.8-$MgCl_2$ and AB-pH 2.5-$MgCl_2$ (see Fig. 1)

		Molarity $MgCl_2$													
		0	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.2	1.5
Nuclei	pH 5.8	++	++	+	−	−	−	−	−	−	−	−	−	−	−
	pH 2.5	+	∓	−	−	−	−	−	−	−	−	−	−	−	−
Mucin	pH 5.8	++	++	++	++	+	−	−	−	−	−	−	−	−	−
	pH 2.5	++	++	++	+	+	+	+	∓	∓	∓	∓	∓	±	∓
Carti-	pH 5.8	++	+++	+++	+++	+++	+++	+++	+	∓	−	−	−	−	−
lage	pH 2.5	++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	∓	−	−
Mast	pH 5.8	?+	?+	+++	+++	+++	+++	+++	++	+	±	−	−	−	−
cells	pH 2.5	?∓	?∓	+	++	+++	+++	+++	+++	+++	+++	++	++	++	−
Col-	pH 5.8	++	+	+	+	+	+	+	+	+	+	+	+	−	∓
lagen	pH 2.5	∓	+	+	+	+	+	+	+	+	+	+	+	+	∓

Degree of staining recorded as in Table 1.

addition lung sections were stained in AB—pH 2.5—$MgCl_2$. The complete results are shown in Tables 3—5. Those results obtained at pH 5.8 are shown in Figs. 1—14.

Table 5. *Staining of rabbit cornea AB-pH 5.8-$MgCl_2$ (see Fig. 1)*

	Molarity $MgCl_2$													
	0	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.2	1.5
Nuclei	+	+	+	−	−	−	−	−	−	−	−	−	−	−
Stroma	+	+	+	++	+	+	+	+	+	∓	∓	∓	−	−
Descemets membrane	+	++	++	∓	−	−	−	−	−	−	−	−	−	−

Degree of staining recorded as in Table 1.

Comparison of differentiation in NaCl and $MgCl_2$. The critical electrolyte concentrations of CPC complexes of polysulphates are more widely separated from those of polycarboxylates in $MgCl_2$ solutions than in NaCl solutions (see Table 6). Experiments (not reported here) confirmed that Alcian Blue complexes behaved similarly to CPC complexes in this respect, giving better differentiation of e.g. keratansulphate from heparin, and heparin from chondroitin sulphate. We have therefore used $MgCl_2$ almost exclusively in this work.

Time dependance of the standard procedure. Sections of lung were stained in AB—pH 5.8—0.2M $MgCl_2$ and AB—pH 5.8—0.7M $MgCl_2$ for $^1/_2$, 1, 2, 4, 8, 24 and 72 hours.

Sections of ileum were stained in AB—pH 5.8—0.2M $MgCl_2$ for the same times.

Microscopic examination showed a progressive increase in staining with time throughout all series, but the increase from 24 to 72 hours was small. There was no alteration with time in the pattern of staining, except that cartilage which stained patchily in the presence of 0.2M $MgCl_2$ in less than 4 hours showed even staining when stained for 4 hours or longer.

Concentration of AB in staining solution. Two series of sections of rat ileum were immersed in AB—pH 5.7—$MgCl_2$ solutions, the first at 0.05% w/v AB and the second at 0.2% w/v AB. Only marginal differences were noted in the pattern of staining, the important factor was considered to be difficulty of removal of the much larger amount of unbound dye from sections in the more concentrated AB solution. We therefore preferred to use 0.05% AB in all other experiments.

Reversibility of staining. A lung section was stained 4 hours in AB—pH 5.8—0.2M $MgCl_2$ and after rinsing in distilled water was immersed in 1.0M $MgCl_2$ for 3 days. Cartilage and mucin were then both unstained, whereas collagen was slightly stained.

Staining of collagen. Sections of lung and cornea were treated with 3% w/v aqueous cetyl pyridinium chloride to block anionic sites (Kelly, Bloom, Scott, 1963; Quintarelli, Scott and Dellovo, 1964) and then stained in AB—pH 5.8—0.8M $MgCl_2$.

Sections of lung and cornea were treated with methyl isothiouronium sulphate to guanidylate amino groups and were then stained in AB—pH 5.8—0.8M $MgCl_2$.

The staining of collagen in the lung was completely abolished by CPC treatment but not affected by prior reaction with isothiouronium salt. The staining of collagen

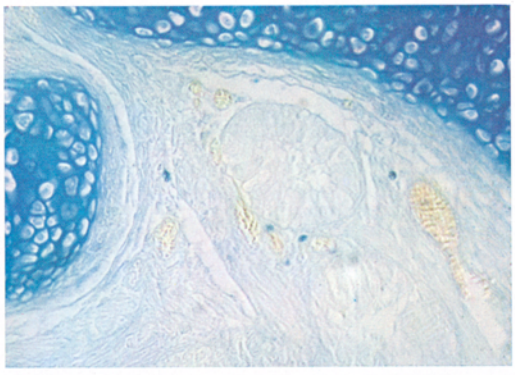

Fig. 9

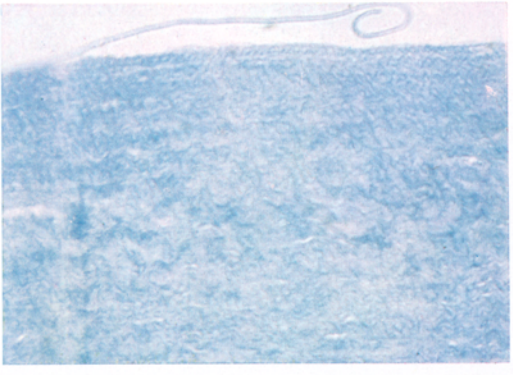

Fig. 12

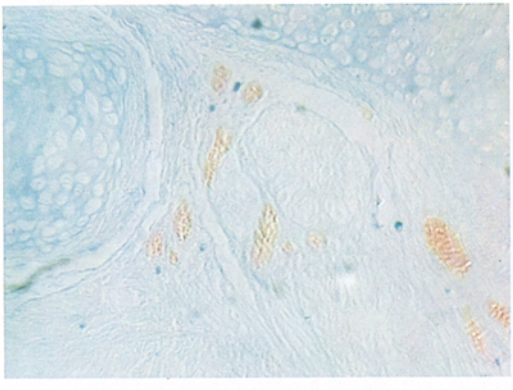

Fig. 10

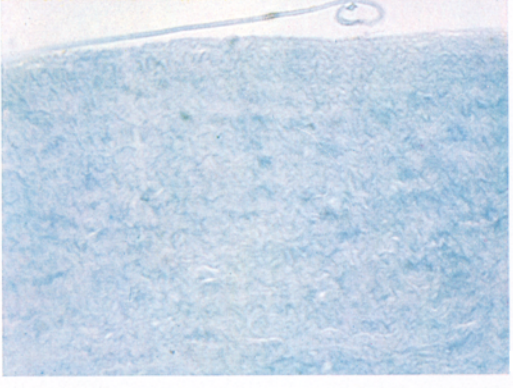

Fig. 13

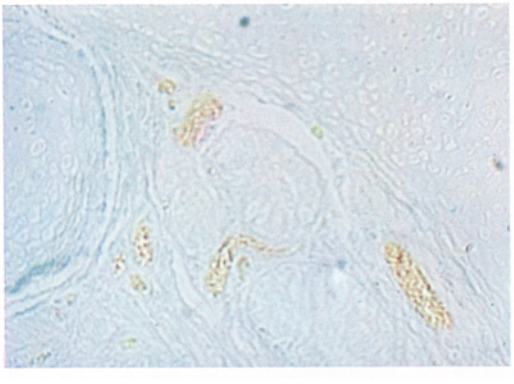

Fig. 11

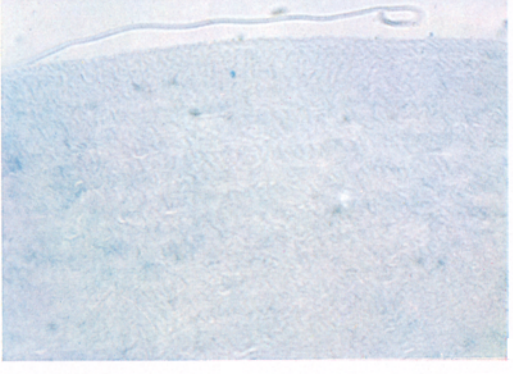

Fig. 14

Figs. 9—11. Human new born lung. $MgCl_2$ concentrations: — 9. 0.5 M, 10. 0.7 M, 11. 1.0 M

Figs. 12—14. Rabbit cornea. $MgCl_2$ concentrations: — 12. 0.5 M, 13. 0.7 M, 14. 1.0 M

in the cornea was almost completely blocked by CPC and only slightly reduced by prior reaction with isothiouronium salt.

Discussion

It has been shown previously that the electrostatic combination with and precipitation of polyanions by organic cations is reversible in the presence of

inorganic electrolyte (SCOTT, 1960) according to a pattern (SCOTT, QUINTARELLI and DELLOVO, 1964)

$$P^{z-}zM^+ + zR^+ \rightleftharpoons P^{z-}zR^+ + zM^+$$

P^{z-} is a polymer P with z negative charges. M^+ is an inorganic counterion (e.g. Na^+, K^+, Ca^{++} etc), R^+ is a precipitating cation (e.g. cetylpyridinium, Azure A, etc.).

Applying the law of mass action,

$$\frac{[P^{z-}\,zR^+]}{[P^{z-}\,zM^+]} \qquad \frac{[M^+]\,z}{[R^+]\,z} = K$$

K is the equilibrium constant. $[M^+]$ is the concentration of M^+, etc.

$\frac{[P^{z-}\,zR^+]}{[P^{z-}\,zM^+]}$ is the ratio of the insoluble dyed product to soluble colourless reactant, and will be replaced by D, the degree of precipitation (staining). The reaction involves an ion-exchange, and in the absence of other, non-electrostatic factors, the value of the equilibrium constant is determined by the relative affinities of M^+ and R^+ for the ionised groups of the polyanion. If M^+ and R^+ are held constant, and the nature of the ionised group on P^{z-} is allowed to vary, then each anionic site will have an associated value of K. There are only 3 anionic groups commonly met with in mammalian tissue, $-COO^-$, $-OSO_3^-$ and $=PO_4^-$. Thus for a given pair of M^+ and R^+, e.g. Na^+ and cetylpyridinium, there different Ks are expected.

If D is plotted graphically against $[R^+]/[M^+]$ for various values of z, it is found that when z is >5, there is a sharp break in the curve. The position of the break on the $[R^+]/[M^+]$ axis is determined by the value of K, the equilibrium constant. Thus if $[R^+]$ was a constant, K could be expressed in terms of $[M^+]$. Since the break is very sharp when z is large, at a critical concentration, K is expressed as a "critical electrolyte concentration" (CEC). z is of the order of 100 or more, in the present context. Thus, each type of anionic site having an associated value of K, also has an associated CEC.

In order that K should be expressible in terms of M^+ concentration, the concentration of R^+ must be constant, both throughout the precipitation (dying) process, and from one experiment to another. Three methods have been used to date to achieve this.

1. An excess of solid may be present throughout, so that the solution is always saturated with regard to R^+.

2. Unreactive micelles of R^+ may be present in solution, which dissociate to replenish the concentration of R^+, as this is removed in the process of precipitation. This is probably the situation in the case of cetylpyridinium and other cationic detergents, and perhaps also with many dyes.

3. The concentration of R^+ may be so high that the amount removed in the form of $P^{z-}\,zR^+$ is negligible in comparison with the total R^+. This is the simplest situation to achieve in practice, and is the one particularly suited to histochemical applications. In our experiments less that 1% of the R^+ is consumed.

In practice, all—COO^- containing polyanions do not have the same CEC, nor do all—OSO_3^- containing polyanions, since the precise value of z (and therefore the molecular weight) has an effect (LAURENT and SCOTT, 1964) as does the distance between anionic sites (the charge density) which affects the binding of neighbouring cations. There is a family of values of K (or CEC) associated with each

type of anionic site, rather than a single discrete value. Table 6 shows the general pattern, in which R^+ is cetylpyridinium, and CECs are in molarity of NaCl or $MgCl_2$.—COO^- containing polyanions have low CECs, —OSO_3^- containing polyanions have high CECs, polyanions with both have values in between, and polyphosphates have slightly higher CECs than polycarboxylates. It has been shown (SCOTT, 1962, and SCOTT, QUINTARELLI and DELLOVO, 1964) that the nature of the cationic group in R^+ can be varied without changing this pattern. The results of the model experiments carried out in this investigation (Tables 1—5) show that Alcian Blue conforms to the pattern.

Table 6. *Critical electrolyte concentrations (molarities) of cetyl-pyridinium complexes in salt solutions*

Anionic group	Polyanion	NaCl	$MgCl_2$
—COO^-	Pectin	0.13	0.02
	Hyaluronate	0.21	0.10
	Alginate	0.38	0.15
	Polyacrylate	0.43	
	Polyglutamate	0.475	
$=PO_4^-$	RNA	0.45	
	DNA	0.50	0.2
	Polyphosphate	0.4	
—COO^- and —OSO_3^-	Chondroitin sulphate	1.0	0.5
	Heparin	1.6	0.8
—OSO_3^-	Keratansulphate	1.0—2.0	1.0
	Dextran sulphate	1.5	2.0
	Carrageenin	2.5	2.0

This table is complied from SCOTT (1960) and unpublished results.

The tissues (see Figs. 1—14 and Tables 3—5) were chosen to contain predominantly or characteristically members of each main group of polyanions. Ileum is not known to contain highly sulphated polyanions in quantity, its polyanions are characteristically carboxylates. Hilar region of human lung contains chondroitin sulphate, heparin in mast cells, and polycarboxylates in the form of mucin. Because of the youth of our sample we do not expect to find significant quantities of keratansulphate in the cartilage. Cornea is known to contain chondroitin sulphate and keratansulphate in considerable quantity (see e.g. ANSETH and LAURENT, 1961).

In general, the CEC of polyanions on filter paper is about 30—50% lower than in tissues. This could be due to a variety of factors, e.g. the molecular weight of the polyanion in the tissue will certainly be higher, and the tissue concentration is many times greater.

The distinction between polysulphates and polyanions containing carboxyl or phosphate groups is more easily made in magnesium chloride than in sodium chloride (see Results). This is also the case when fractionating polyanions with cetylpyridinium (Table 6), and offers another demonstration of the close similarity between the two systems.

It is fundamental to the argument that the staining should be carried out to equilibrium, and that it should be reversible. The experiments described in the results section demonstrate that staining increases only slightly after 8 hours. Other experiments showed that dye could be removed from stained sections by soaking in concentrated electrolyte solution, although it takes very much longer to remove the stain than to apply it. We conclude that the conditions of equilibrium and reversibility have been met, in this investigation.

Effect of AB concentration. Increasing the concentration of AB from 0.05% w/v to 0.2% in the model experiments on filter paper caused a slight (10%) increase in CEC. In the case of experiments carried out on sections, the change was too small to be recorded. For practical reasons, given in the results section, we prefer 0.05% AB.

„Non-specific Staining" of collagen fibres. Staining does not occur at high salt concentration, in general, in agreement with the postulate that staining is electrostatic. However, collagen fibres in human newborn lung and rabbit cornea retained their stain at salt concentrations of 1.5M, when almost everything else was unstained. This was the sole observation to suggest that staining could occur by a mechanism not involving electrostatic binding. We therefore investigated it.

The cationic sites in Alcian Blue are probably isothiouronium groups (SCOTT *et al.*, 1964) and these might react with some nucleophilic site in collagen, resulting in chemical deposition of dye. Sections were therefore treated with S-methylisothiouronium sulphate prior to staining with AB, to convert any such site by a similar reaction, and therefore to remove reactive centres before AB was applied. No significant difference in affinity for AB was observed, consequent upon this preliminary treatment. Prior treatment of sections with cetylpyridinium chloride (QUINTARELLI, SCOTT and DELLOVO (1964); BLOOM, KELLY and SCOTT (1963)] resulted in a very significant decrease in staining, completely eliminating it in human newborn lung. We conclude that the residual staining of collagen fibres in human newborn lung and rabbit cornea is due to very small amounts of associated polyanion, which is presumably a polysulphate. It is emphasised that the phenomenon is not striking, the staining being faint at best.

Effect of pH. AB is usually used at pH 2.6, in 3% acetic acid (MOWRY, 1956). At this low pH masking of polysaccharide by protein can occur, because of salt links formed between them (SZIRMAI, 1963; QUINTARELLI, SCOTT and DELLOVO, 1964). In this investigation a pH of 5.8 has been chosen to minimise this complication. At 0.0M electrolyte, staining is completely general, and therefore useless for ordinary purposes. This is presumably because many proteins and glycoproteins are weakly negative at pH 5.8. In the presence of a little (0.05M) $MgCl_2$ the "background" staining is drastically reduced, as would be expected, if weakly charged polymers containing $—COO^-$ groups were involved.

In 3% acetic acid the uronic acid $—COO^-$ is largely undissociated. Thus, polyanions containing both $—COO^-$ and $—OSO_3^-$ groups lose the charge associated with the uronic acid, and become essentially polysulphates. In consequence, their CEC becomes characteristic of a polysulphate, i.e. it increases. This phenomenon has been observed previously with cetylpyridinium and chondroitin sulphate (ANTONOPOULOS *et al.*, 1961). The experiments in Table 4 show a similar effect. The CEC of cartilage is higher at pH 2.5 as compared with pH 5.8. This reinforces the conclusion that AB combines electrostatically with the substrate, precisely similarly to other simpler organic cations such as cetylpyridinium. The increase in CEC in 3% acetic acid is diagnostic of the presence of a polyanion containing $—COO^-$ and $—OSO_3^-$. It would not be expected to occur with e.g. keratansulphate, and might serve as a method of differentiating in certain situations. The phenomenon would tend to obscure partially the difference between chondroitin sulphates and keratansulphate, and it is therefore better to stain at pH 5.8, when investigating chondroitin-sulphates.

Blocking of AB staining at pH 2.6, presumably by tissue protein, was apparently completely reversed in the presence of low concentrations of $MgCl_2$ (< 0.1 M).

The effect of salts on the metachromatic reaction of e.g. Azure A with its substrate has been investigated by many authors (for a review see Sylven, 1954; Booij, 1958) but almost all the work has been done in dilute solution. Landsmeer (1951), by analogy with the qualitative results of Bungenberg de Jong (see review by Booij, 1958), used a series of electrolytes to prevent staining of mesentery and "nerve tissue" with Azure A in the hope of diagnosing the types of anionic group present in the tissues. He worked at a time when knowledge of the chemistry of the constituents of connective tissue was only in its infancy, and he did no model experiments. He was very successful however in devising conditions in which mast cells only were stained, of all the elements in mesentery. Heath (1959) was able to perform model experiments with sulphated and non-sulphated polysaccarides and a large number of dyes and salts, and although he was able to devise conditions in which only sulphated polysaccharides stained, he was apparently unaware of the results of Bungenberg de Jong, and his explanations, based on complex formation between dyes and cations, are probably erroneous in general. The most complete use of salt differentiation to date is by Saunders (1964) who worked with acridine orange in a fluorescent technique. He proceeded by analogy with the cetylpyridinium fractionation system of Scott (1960) and was able to demonstrate differences in cut-off of staining between various groups of polyanions in NaCl solutions. He assumed that the mechanism was probably associated with the formation of micelles by Acridine Orange, and although this may be so, yet it does not seem a necessary factor, as may be seen from the theoretical treatment given earlier. NaCl is not the best electrolyte to use in the differentiation of keratosulphate from chondroitin sulphate, $MgCl_2$ appears to be superior. We find the simplicity of our technique preferable to the fluorescent microscopy of Saunders.

We conclude from our experiments and from those of Bungenberg de Jong, Landsmeer, Heath and Saunders, inter alia, that the critical electrolyte concentration phenomen is a very general one, which may be applied to a large number of dyes. This is to be expected from the theoretical treatment given earlier, in which the nature of R^+, the precipitating cation (or dye) is quite without restriction, so long as it obeys the reversible equation. We have observed similar phenomena with e.g. methyl green and crystal violet.

References

Antonopoulos, C. A., E. Borelius, S. Gardell, B. Hamnström, and J. E. Scott: The Precipitation of Polyanions by Long-chain Aliphatic Ammonium Compounds. Pt. IV. Elution in Salt Solutions of Mucopolysaccharide-Quaternary Ammonium Complexes adsorbed on a Support. Biochim. biophys. Acta (Amst.) **54**, 213—226 (1961).

Booij, H. L.: Colloid Chemical Aspects of Metachromasia. Acta histochem. (Jena), Suppl. **1**, 37—55 (1958).

Heath, I. D.: Staining of Sulphated Mucopolysaccharides. Nature (Lond.) **191**, 1370—1371 (1961).

Jeanloz, R. W.: The Nomenclature of Mucopolysaccharides. Artrh. and Rheum. **3**, 233—237 (1960).

Kelly, J. W., G. D. Bloom, and J. E. Scott: Quaternary Ammonium Compounds in Connective Tissue Histochemistry. 1. Selective Unblocking. J. Histochem. Cytochem. **11**, 791—798 (1963).

LANDING, H. B., and H. E. HALL: Phosphorylation as a Histochemical Procedure. J. Histochem. Cytochem. **4**, 382—387 (1956).

LANDSMEER, J. M. F.: Some colloid Chemical Aspects of Metachromasia. Influence of pH and Salts on Metachromatic Phenomena evoked by Toluidine Blue in Animal Tissue. Acta physiol. pharmacol. neerl. **2**, 112—128 (1951).

LAURENT, T. C., and A. ANSETH: Studies on Corneal Polysaccharides. II. Characterisation. Exp. Eye Res. **1**, 99—105 (1961).

—, and J. E. SCOTT: Molecular Weight Fractionation of Polyanions by Cetylpyridinium Chloride in Salt Solutions. Nature (Lond.) **202**, 661—662 (1964).

MOWRY, R. W.: Observations on the Use of Sulphuric Acid in Ether for the Sulphation of Hydroxyl Groups in Tissue Sections. J. Histochem. Cytochem. (Abstract) **6**, 82 (1958).

QUINTARELLI, G., J. E. SCOTT, and M. C. DELLOVO: Chemical and Histochemical Properties of Alcian Blue, III. Chemical Blocking and Unblocking. Histochemie **4**, 99—112 (1964).

SAINTE-MARIE, G.: A Paraffin Embedding Technique for Studies employing Immunofluorescence. J. Histochem. Cytochem. **10**, 250—254 (1962).

SAUNDERS, A. M.: Histochemical Identification of Acid Mucopolysaccharides with Acridine Orange. J. Histochem. Cytochem. **12**, 164—170 (1964).

SCOTT, J. E.: Aliphatic Ammonium Salts in the Assay of Acidic Polysaccharides from Tissues. Methods of Biochemical Analysis (ed. D. GLICK), vol. 8, p. 145—197. New York: Interscience Publ. 1960.

— The Fractionation of Polyanions by Long-chain Aliphatic Ammonium Salts. Biochem. J. **78**, 24 P (1961).

— The Precipitation of Polyanions by Long-chain Aliphatic Ammonium Salts, part VI. The Affinity of Substituted Ammon Cations for the Anionic Groups of some Biological Polymers. Biochem. J. **84**, 270—275 (1962).

— J. DORLING, and G. QUINTARELLI: Differential Staining of Acid Glycosaminoglycans by Alcian Blue in Salt Solutions. Biochem. J. **90**, 4—5 P (1964).

SHACKLEFORD, J. M.: Carboxylation of Tissue Sections with the Use of p-Hydrazinbenzoic Acid and Succinic Anhydride. J. Histochem. Cytochem. **10**, 469—472 (1962).

SZIRMAI, J. A.: Quantitative Approaches in the histochemistry of Mucopolysaccharides. J. Histochem. Cytochem. **11**, 24—34 (1963).

Dr. J. E. SCOTT
Canadian Red Cross Memorial Hospital
Taplow, Maidenhead, Berks./England