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Fixation and staining of granules in mucosal mast cells and intraepithelial lymphocytes in the rat jejunum, with special reference to the relationship between the acid glycosaminoglycans in the two cell types

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Summary

Various fixation and staining procedures have been examined in order to obtain optimal numbers and acceptable morphology of the mucosal mast cells and granular intraepithelial cells in the rat jejunum. For subsequent staining with Alcian Blue, the best fixation of the jejunum was obtained with a methanol–formaldehyde–acetic acid mixture. Specific staining of the granules of these cells has been obtained using Alcian Blue at pH 5.8, at which hydrogen ion concentration more cells stain than in the usual very acid conditions. Specificity is achieved by the use of magnesium chloride concentrations above the critical electrolyte concentrations for staining of protein and nucleic acid by Alcian Blue, and by the use of Safranin O as a competitive counterstain.

The critical electrolyte concentration technique has also been applied to a comparative study of the glycosaminoglycan in the two cell types. Evidence is presented that the glycosaminoglycan in the granular intraepithelial cell has either a lower degree of sulphation or a lower molecular weight or both than the material in mucosal mast cells. This finding may support the possibility that the granular intraepithelial lymphocyte is a precursor of the mucosal mast cell.

Introduction

Although mast cells have been recognized for many years (Michels, 1938), it was not generally accepted that the intestinal lamina propria contained a population of mucosal mast cells (MMC) until the systematic study of tissue fixation by Enerbäck (1966a, b). Only a limited number of the commonly used fixatives preserved the

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affinity of the granules of this particular population of mast cells for dyes such as Toluidine Blue, Alcian Blue and Astra Blue. This was in distinction to the satisfactory preservation of the more familiar connective tissue mast cells by a much larger range of fixatives. The difference in fixation requirements of these two populations of mast cells may lie in the degree of sulphation of the acid glycosaminoglycan contained in the characteristic granules of the cells, which is heparin in connective tissue mast cells (Tas & Geenen, 1975) and a lower sulphated glycosaminoglycan in MMC (Tas & Berndsen, 1977).

More recently, another granulated cell has been described in the rat intestinal mucosa (Collan, 1972). This is a cell found between the epithelial cells of the small intestine, with the general morphology of a lymphocyte but containing a small number of basophilic granules. Intraepithelial lymphocytes or 'theliolymphocytes' have been recognized for many years (reviewed by Collan, 1972) and failure of earlier workers to identify these granulated cells suggests that preservation during fixation may be capricious. Granulated intraepithelial lymphocytes (GIEL) have assumed a new significance with the demonstration in that in the mouse they contain histamine, incorporate radio-labelled sulphate and stain with Alcian Blue at pH 0.3, suggesting that the granules contain a sulphated glycosaminoglycan (Guy-Grand *et al.*, 1978). These findings have led to the suggestion that GIEL may be precursors of MMC.

This study examines the properties of several fixatives that have been reported to preserve the staining of MMC with a view to obtaining optimal preservation of GIEL in sections of paraffin-embedded rat intestine. The histochemical properties of the granules of GIEL and MMC have also been examined and compared, using the critical electrolyte concentration (CEC) method of Scott & Dorling (1965), and evidence is presented which is in support of a precursor relationship of GIEL to MMC.

Materials and methods

Fixatives

Carnoy: 6:3:1 (by vol.) ethanol–chloroform–acetic acid (Romeis, 1948); ice cold.

Modified Carnoy: 6:3:0.5 (by vol.) ethanol–chloroform–acetic acid (Tas & Berndsen, 1977); ice-cold.

Newcomer: 6:3:1:1:1 (by vol.) isopropyl alcohol–propionic acid–petroleum ether–acetone–dioxane (Newcomer, 1953); ice-cold.

Isotonic formaldehyde–acetic acid (IFAA): 0.6% formaldehyde, 0.5% acetic acid in distilled water (Enerbäck, 1966a); ice-cold or room temperature, as indicated.

8.5:1:0.5 (by vol.) methanol–formalin–acetic acid (MFAA) (Tas & Berndsen, 1977); ice-cold. Formalin contains 37–40% formaldehyde.

Preparation of tissues

Upper jejunum was obtained 2–4 cm distal to the ligament of Treitz from an 8-month-old conventional female WAG rat under ether anaesthesia. The segment was opened along its mesenteric border and laid serosal side down on filter paper moistened with

phosphate-buffered saline (PBS, Dulbecco solution A; Oxoid, England). Strips approximately 1 mm × 4 mm were cut with a scalpel blade, with the long axis in the orientation of the axis of the gut lumen, and four strips were placed into each of six containers. After briefly washing in ice-cold PBS, the PBS was replaced with one of the above fixatives for 2 min, at which time it was replaced by further ice-cold fixative. Fixation was continued at 4° C for 24 h, with the exception of a duplicate sample fixed in IFAA, which was allowed to fix at room temperature. IFAA-fixed material was dehydrated by passage through 70% ethanol and 95% ethanol into absolute ethanol, while tissue fixed by other methods was dehydrated by two changes of absolute ethanol. Tissues were then cleared in xylene and embedded in paraffin. With the exception of the duplicate fixed at room temperature in IFAA, all processing up to embedding was carried out at 4° C. Five micrometre sections were de-waxed in xylene and rehydrated through 70% ethanol into distilled water prior to staining.

Staining

The stains used were Alcian Blue 8GX (Gurr Michrome No. 24, Searle Diagnostic, High Wycombe, England) and Safranin O (Michrome No. 405, Gurr, London). Stains were freshly prepared each day. Two staining procedures were used.

(A) After brief rehydration in distilled water, sections were stained with 0.1% Alcian Blue in 0.7 N HCl for 30 min, rinsed in 0.7 N HCl and further stained for 2 min in 0.5% Safranin O in 0.125 N HCl (modified from Bloom & Kelly, 1960). They were then rinsed briefly in distilled water and taken rapidly through 70% ethanol and absolute ethanol into xylene, to be mounted in Slidemount (Australian Laboratory Services).

(B) Rehydrated sections were stained essentially as described by Scott & Dorling (1965). In most instances, staining was for 4 h in a 0.05% solution of Alcian Blue containing 0.02 M acetate buffer (pH 5.8) and specified concentrations of $MgCl_2$, followed by a brief rinse in distilled water and 5 min in a 0.25% solution of Safranin O in the same buffer but at pH 5.0. Rinse, dehydration and mounting as in (A).

Examination of sections

Sections were examined using a Leitz Dialux 20 microscope fitted with NPL Fluotar lenses. Areas for cell counts were selected on the basis of regular general morphology in areas where the section was cut parallel to the axes of the villi and crypts. Counts were then performed on seven or eight consecutive crypt-villus units (Miller & Jarrett, 1971) in each of the four strips, until 30 crypt-villus units had been counted. In the case of MMC, counts are expressed as the mean (±1 S.D.) cells per crypt-villus unit, while in the case of GIEL they are expressed as cells per villus. Kodak Technical Pan Film (Estar-AH Base) SO-115, Eastman Kodak, Rochester, N.Y.) was used for photographs, with illumination through a red K565 filter from a 12-volt quartz–halogen lamp.

Statistical analysis

The probability that differences observed between groups of observations were significant was tested by Student's *t*-method.

Results

Effect of fixative on numbers of MMC and GIEL

Assessment of the performance of the various fixation procedures was made on sections stained with Alcian Blue in 0.7 N HCl (approx. pH 0.3, method A). These

conditions rigorously exclude staining of all tissue elements except the sulphated glycosaminoglycans (Quintarelli *et al.*, 1964). IFAA, whether used cold or at room temperature, was immediately excluded from further study on the grounds of poor preservation of tissue architecture and because staining of both MMC and GIEL was extremely light.

On a qualitative assessment, tissue fixed with MFAA had the best appearance. Of particular importance to the present study, cell boundaries of MMC were well preserved, granules were distinct and well stained, and the basement membrane of the epithelium was easily visible (important to classify a cell as intraepithelial) and interfaces between structures of different densities (for example, nuclear–cytoplasmic boundaries) were less refractile. However, staining with Safranin O was light.

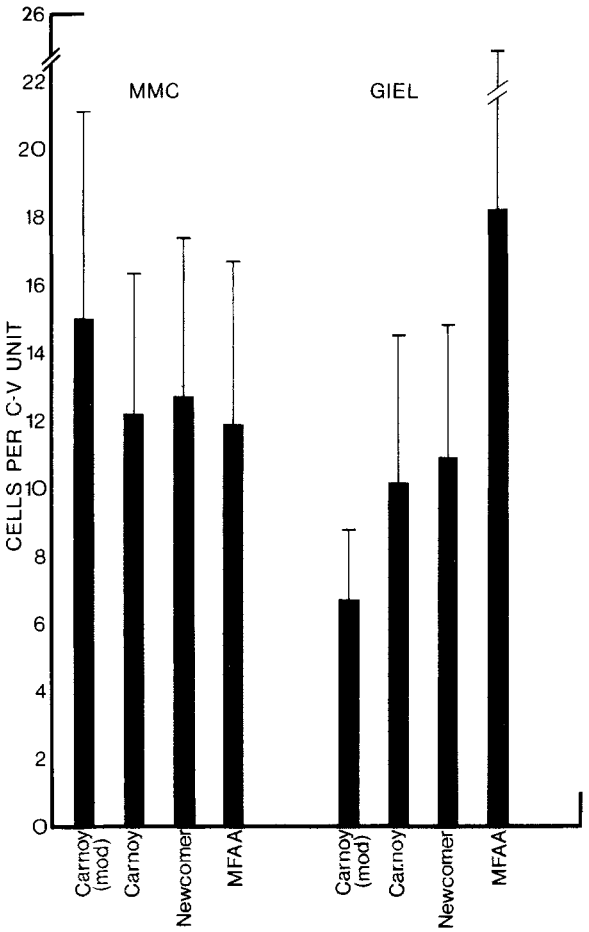

Fig. 1. Counts of MMC and GIEL in sections of jejunum. Results are shown for tissue from blocks prepared with four of the fixatives under study. Counts are expressed per crypt-villus (C-V) unit, although GIEL are present only in the villus epithelium.

Carnoy's and Newcomer's fixatives allowed ready counting of MMC, but cell boundaries were blurred by diffused cell contents, granules were often not individually distinguishable, the basement membrane of the epithelium was often not visible and refractile nuclear–cytoplasmic borders were sometimes difficult to distinguish from granules lying close to the nuclear membrane in GIEL. The MMC appeared somewhat more 'washed-out' in tissue fixed with the modified Carnoy's fixative.

Fig. 1 represents counts of MMC and GIEL in sections from tissue prepared with each of the remaining fixatives. For MMC, the choice between the four fixatives lies in the appearance of the cells rather than the preservation of cell numbers. Counts in Newcomer-fixed and MFAA-fixed material were not significantly different from counts in Carnoy-fixed material. Although counts in modified Carnoy-fixed material were significantly higher ($P = 0.025$), this may represent a regional variation in MMC numbers rather than better fixation, because in fact the appearance of MMC was worst with this fixative.

It is clear that GIEL numbers are best preserved in tissue fixed with MFAA. There was no significant difference in numbers between Carnoy-fixed and Newcomer-fixed tissues, a highly significant difference between Carnoy-fixed and MFAA-fixed material ($P < 0.001$) while modified Carnoy-fixed material had significantly fewer GIEL than Carnoy-fixed material ($P < 0.001$). MFAA was chosen for further study.

The effect of staining at higher pH

Use of Alcian Blue at a low pH lends specificity to the molecules stained by the dye, in practice restricting staining to the highly acidic sulphated glycosaminoglycans (Quintarelli *et al.*, 1964). However, it also seems possible that in such acid solutions sufficient neutralization of charge on lower sulphated glycosaminoglycans might occur as to render them unstainable by the cationic dye. More stained cells might be detected by staining at a higher pH, but at the expense of loss of specificity as binding of dye can occur through carboxyl groups to proteins and less acidic glycosaminoglycans and through phosphate groups to nucleic acids (Scott & Dorling, 1965).

Sections of MFAA-fixed jejunum were stained according to method (B), in the absence of added ions. This resulted in dark staining of most structures in the tissue, to the extent that MMC were difficult to identify and GIEL could not be distinguished.

The effect of added divalent cation

The CEC technique of Scott & Dorling (1965) was applied to staining at pH 5.8 in an attempt to block unwanted staining of protein, nucleic acids and non-sulphated glycosaminoglycans. Serial sections of MFAA-fixed jejunum were stained by method (B) in dye solutions containing graded concentrations of $MgCl_2$. A qualitative assessment of staining of structures such as MMC and GIEL granules, cell nuclei

Table 1. Effect of $MgCl_2$ concentration on staining of tissue components by Alcian Blue. The notation attempts to show the relative intensity of staining of a given structure at various concentrations of $MgCl_2$ and also to indicate the intensities of staining of tissue components relative to each other.

	$MgCl_2$ concentration (M)								
Tissue component	0	0.1	0.2	0.4	0.6	0.8	1.0	1.2	1.4
Deoxyribonucleoprotein (nuclei)	++	+	±	−	−	−	−	−	−
Ribonucleoprotein (plasma cell cytoplasm)	+++	+	±	−	−	−	−	−	−
Intercellular ground substance	++	+	+	+	−	−	−	−	−
Granules of GIEL	−	+++	+++	+	−	−	−	−	−
Granules of MMC	±	+++	+++	+++	++	+	±	−	−
Epithelial basement membrane	++	++	++	++	+	±	−	−	−
Goblet cell mucus	+++	++	+	+	+	+	+	+	+

(DNA), plasma cell cytoplasm (RNA), goblet cell mucus, epithelium basement membrane and amorphous ground substance is shown in Table 1. It is apparent that useful reduction in staining of most structures can be obtained without reduction in staining of the granules of MMC in the concentration range of 0.1 M to 0.4 M $MgCl_2$ and in the staining of the granules of GIEL in the concentration range 0.1 M to 0.2 M $MgCl_2$.

In the presence of magnesium chloride, it was possible to make counts of MMC and GIEL. The data in Fig. 2 allow important conclusions. Firstly, at pH 5.8 and in the presence of magnesium chloride, more GIEL can be detected than in sections stained at low pH (24.3 ± 8.3 per villus *versus* 18.2 ± 6.7 per villus, $P < 0.01$). Secondly, the presence of low concentrations of magnesium ions (0.2–0.3 M) appears to enhance staining of both MMC and GIEL with Alcian Blue at pH 5.8, and this difference was noted in both intensity of staining as well as in the numbers of cells stained. However, at optimal $MgCl_2$ concentration, numbers of MMC did not exceed those counted in sections stained at low pH (Fig. 1). Thirdly, the CEC for GIEL (0.3–0.4 M), where increasing divalent ion concentration leads to decreased staining, was considerably lower than for MMC (0.6–0.7 M).

The effect of magnesium ions on staining of MMC and GIEL

Scott & Dorling (1965) have suggested that the extinction of dye binding by acidic polymers observed as cation concentration is raised is due to competition between the metallic cations and the cationic dye. However, these workers did not exclude the possibility that loss of staining at high ionic strength might be due to dissolution or disruption of the structures containing the acidic polymers.

A section was, therefore, incubated in buffer containing 1.0 M $MgCl_2$ at pH 5.8 for

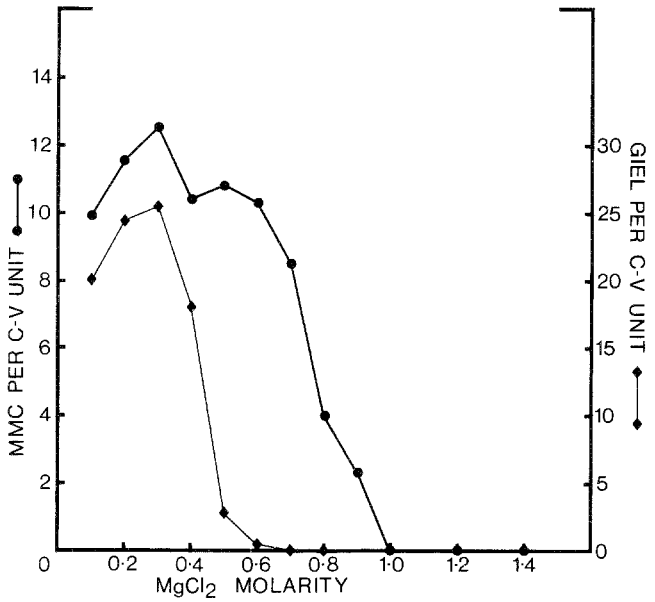

Fig. 2. Effects of $MgCl_2$ concentration on counts of MMC and GIEL in sections of jejunum fixed with MFAA. For both cell types, cells were scored whenever they could be identified, irrespective of the relative intensity of staining. C-V, crypt-villus.

4 h, rinsed briefly in distilled water and transferred to Alcian Blue (method B) containing 0.2 M $MgCl_2$. Exposure to 1.0 M $MgCl_2$ prior to staining at optimal $MgCl_2$ concentration only slightly reduced the counts of MMC (8.1 ± 4.0) and GIEL (19.0 ± 6.0) compared to counts in tissue directly stained in the presence of 0.2 M $MgCl_2$ (11.1 ± 4.2 and 24.3 ± 8.3, respectively), whereas staining in the presence of 1.0 M $MgCl_2$ prevented any staining of either MMC or GIEL (Fig. 2). It is concluded that dissolution or disruption of granules by high ionic strength buffer does not explain the CEC beyond which Alcian Blue staining of MMC and GIEL is extinguished and strengthens the case for competition for binding sites between metallic cations and the dye.

Effect of counterstaining with Safranin O

Safranin O is commonly used as a counterstain for Alcian Blue (Spicer, 1963; Combs *et al.*, 1965; Enerbäck, 1966b), and in addition the differential affinity of the two dyes for glycosaminoglycans with different degrees of sulphate substitution has formed the basis for a classification of mast cells according to maturity (Combs *et al.*, 1965). The granules of MMC and GIEL have a higher affinity for Alcian Blue than for Safranin O. It has been previously noted (unpublished result) that Alcian Blue is not displaced by Safranin O from polymers such as mucin at pH below 1, but that above this pH, Safranin O has a higher affinity for mucin than does Alcian Blue. It

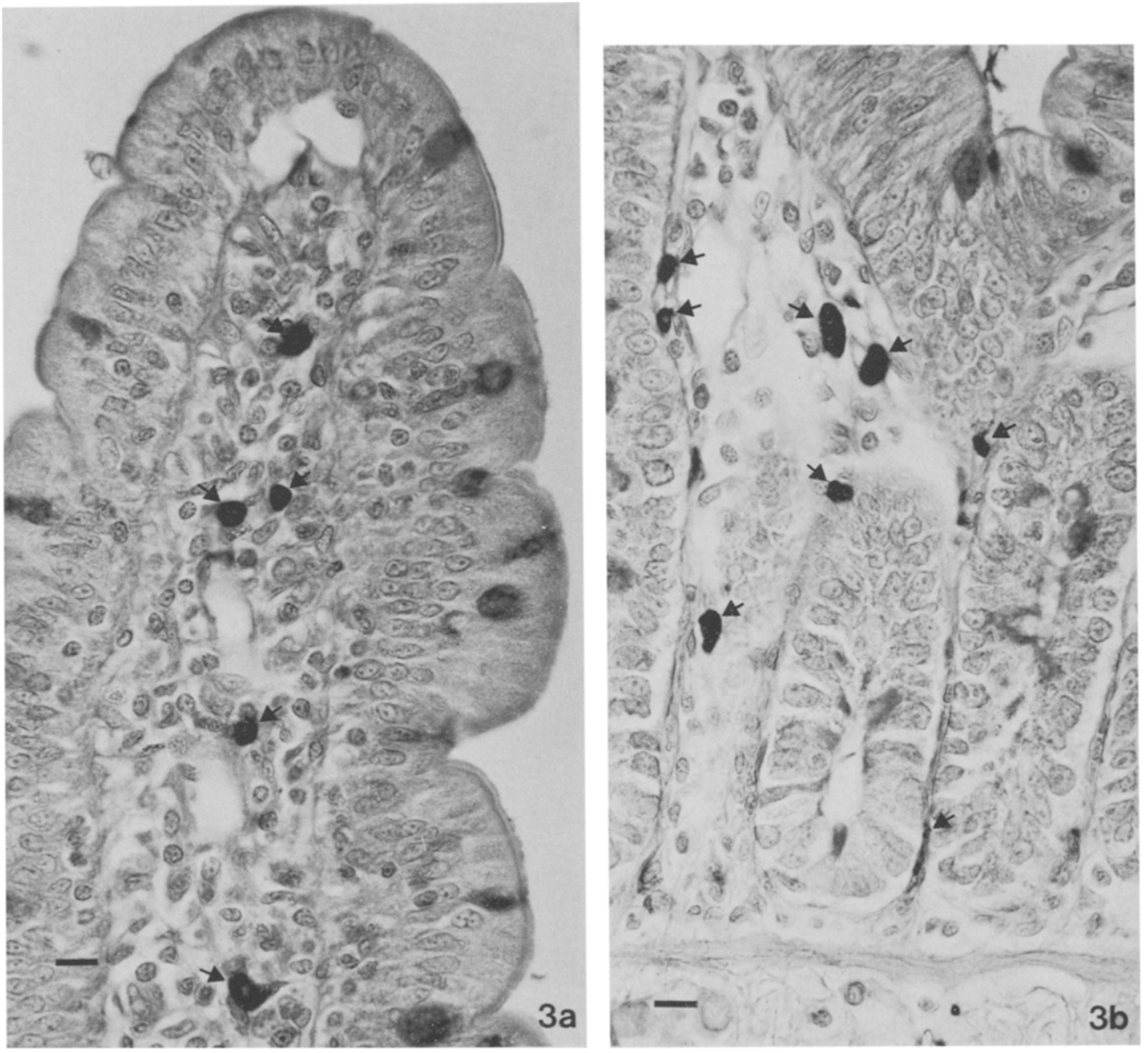

Fig. 3. Sections of jejunum fixed with MFAA, stained with 0.05% Alcian Blue in 0.2 M $MgCl_2$ at pH 5.8 and counterstained with 0.25% Safranin O at pH 5.0. (a) Villus containing five darkly-stained MMC (arrowed). Under the red-filtered illumination used for photography, goblet cells appeared darkly stained, but were grey purple under white light compared to the blue of MMC. (b) MMC (arrowed) in the lamina propria at the base of a villus and between crypts. In tissue fixed with Carnoy and Newcomer, cells at this depth in the mucosa were frequently disrupted and showed diffusion of granule contents. Scale bar: 10 μm.

was, therefore, reasoned that at pH 5.8 Alcian Blue would be displaced from protein, the less acidic mucopolysaccharides and from nucleic acids by counterstaining with Safranin O.

Sections were stained by method (B) in the presence of 0.2 M $MgCl_2$. They were then rinsed and stained for 5 min in 0.5% Safranin O in 0.02 M acetate buffer, pH 5.8. This resulted in specific staining of MMC and GIEL by Alcian Blue, which was displaced from all other structures by the Safranin O. However, GIEL were difficult to distinguish in the heavily Safranin O-stained epithelium. Therefore, further sections were stained as before with Alcian Blue, but counter-stained with Safranin O in the same buffer, at either pH 4.0, 4.5 or 5.0. At all pH levels, MMC and GIEL remained strongly stained by Alcian Blue. At pH 4.0, Safranin O staining of the epithelium was light, while collagen, basement membrane and goblet cell mucus, remained lightly stained by Alcian Blue. At pH 4.5, goblet cell mucus retained affinity for both dyes and stained blue-purple, but Alcian Blue was displaced from all other tissue components except MMC and GIEL. At pH 5.0, Safranin O displaced Alcian Blue from goblet cell mucus, leaving Alcian Blue specific for granules in MMC and GIEL, and staining the epithelium to an intensity allowing relatively easy recognition of GIEL. Recognition of GIEL has been further improved by reducing the concentration of Safranin O to 0.25%. Figs. 3–5 illustrate the staining of MMC and GIEL in sections counterstained with Safranin O at pH 5.0.

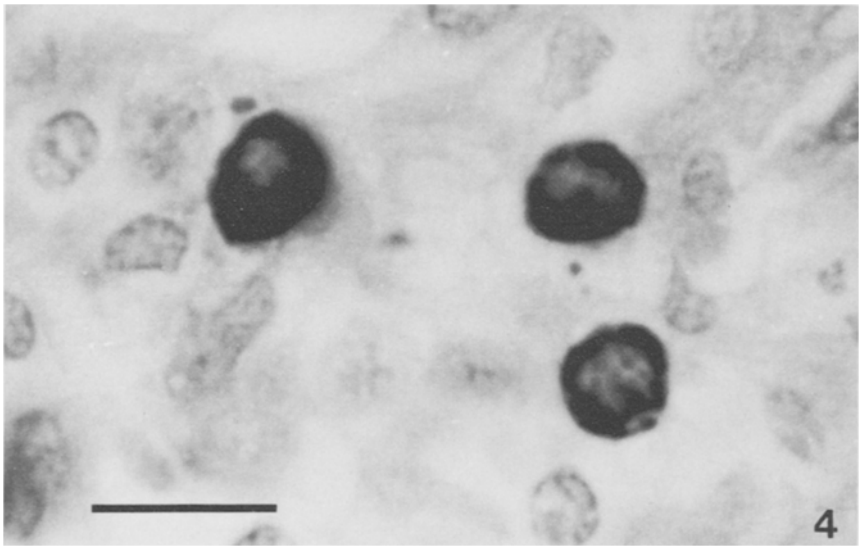

Fig. 4. MMC in the lamina propria of a jejunal villus. Fixation and staining as in Fig. 3. Note the good preservation of cell outline and absence of diffusion of granule substance. Scale bar: 10 μm.

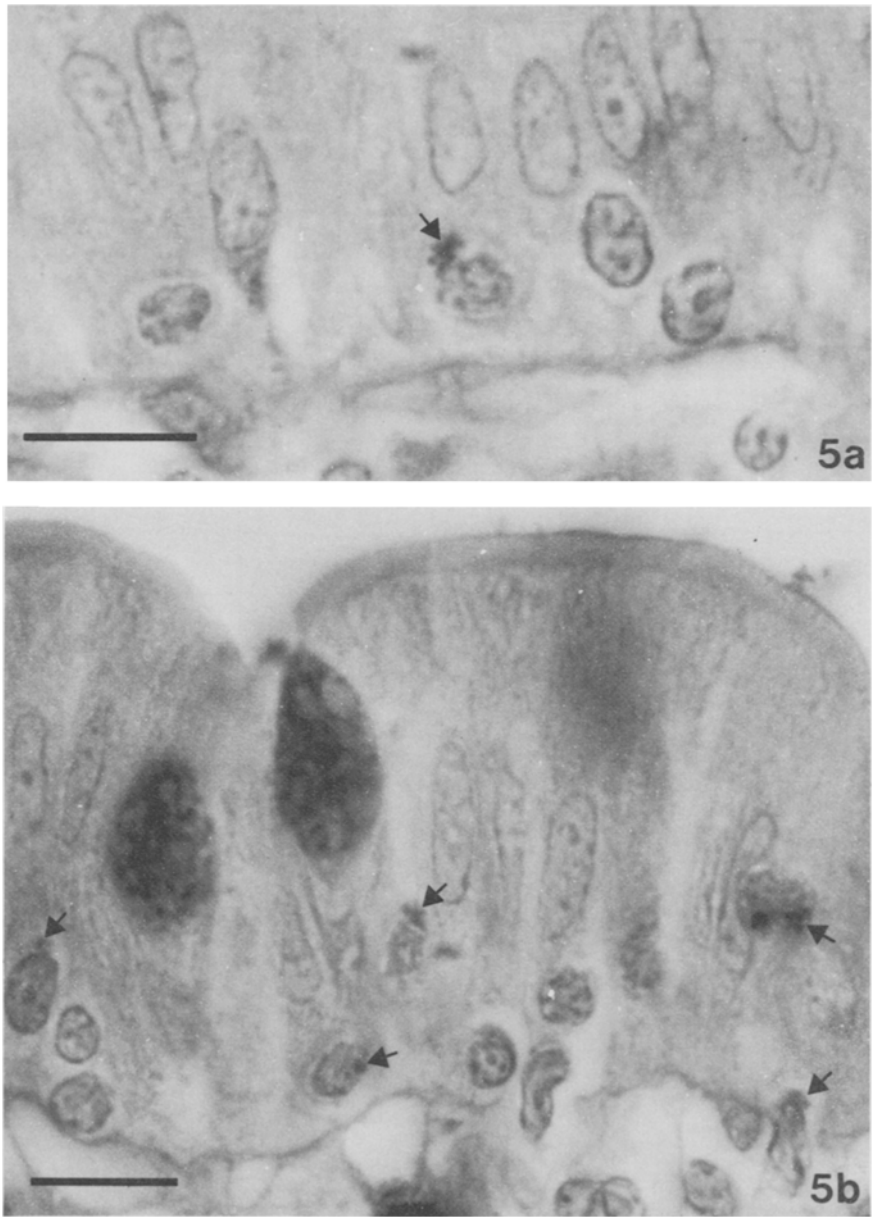

Fig. 5. Lymphocytes in the epithelium of jejunal villus. Fixation and staining as in Fig. 3. Note the clearly visible epithelial basement membranes. (a) One granular (arrowed) and three non-granular intraepithelial lymphocytes. Note the typically polar nature of the cluster of small granules. (b) Five granular (arrowed) and five non-granular intraepithelial lymphocytes. One granular and one non-granular lymphocyte are in the process of passage through the basement membrane. The number of granules per cell depends on the plane of section and the plane of focus, and varies from one to approximately eight. Goblet cells – see comments, Fig. 3a. Scale bar: 10 μm.

Table 2. Effects of staining time with Alcian Blue on counts of MMC and GIEL. Sections of MFAA-fixed jejunum were stained with 0.05% Alcian Blue in pH 5.8 acetate buffer containing 0.2 M $MgCl_2$ for the times indicated and counterstained with 0.25% Safranin 0 in pH 5.0 acetate buffer for 5 min.

	Staining time in Alcian Blue (h)				
	0.5	1	2	3	4
MMC per crypt-villus unit	10.8 ± 4.5	11.1 ± 4.9	12.7 ± 4.2	12.9 ± 4.4	12.1 ± 5.4
GIEL per villus	7.9 ± 3.6	13.8 ± 4.8	16.9 ± 5.6	18.9 ± 4.7	19.3 ± 5.6

Optimal staining time for Alcian Blue

Table 2 lists counts of MMC and GIEL made on sections stained for various times with Alcian Blue. MMC numbers increased only slightly when staining time was extended from 30 min to 4 h. However, intensity of staining of the granules continued to increase throughout the period of observation. The numbers of GIEL increased about three-fold from 30 min to 3 h. Between 3 and 4 h there was no further significant increase in number, but GIEL were more intensely stained after the longer staining period. For both cell types, 4 h appears necessary to achieve optimal staining with Alcian Blue.

Discussion

Conditions of fixation and staining have been investigated in order to identify maximum numbers of MMC and GIEL in the rat small intestine. The coagulative fixatives appear best in general for retaining the staining characteristics of the distinctive granules of these cells. However, the best results in terms of general morphology and maximal detection of the cells were achieved by the use of a coagulative fixative containing, in addition, formaldehyde as a cross-linking agent. This fixative has been referred to as MFAA. The success of this fixation appears to lie in maintaining the integrity of the granules and preventing the formation of a halo of diffused granule substance (Fig. 4), as frequently occurs with fixatives such as Carnoy. MMC numbers were less critically dependent on fixation than were GIEL (Fig. 1), but better morphology was obtained with MFAA than with the other fixatives.

When tissues were incubated with Alcian Blue at pH 5.8, most structures were deeply stained (Table 1), probably as a result of salt linkages between the dye and ionized carboxyl groups on protein and phosphate groups on nucleic acids, in addition to sulphate groups on glycosaminoglycans. The staining of polymers other than sulphated glycosaminoglycans was readily suppressed in the presence of relatively low concentrations of $MgCl_2$, as would be predicted from the findings of

Scott & Dorling (1965). As found by these workers and by Miller & Walshaw (1972), low concentrations of $MgCl_2$ actually increased the staining of mast cells and also of GIEL (Fig. 2). This effect has been attributed to the dissociating effect of salt solutions on the electrostatic interactions between the sulphated glycosaminoglycan and its associated protein, thus freeing sulphate groups to bind Alcian Blue (Scott *et al.*, 1968), and also the presence of chloride as a counter-ion for the excess cationic groups on the Alcian Blue molecule (Scott *et al.*, 1964).

In MFAA-fixed sections stained with Alcian Blue at pH 5.8 in the presence of $MgCl_2$, it was possible to make counts of both MMC and GIEL (Fig. 2). While MMC numbers are not significantly different from those in sections stained at pH 0.3, it is clear that the number of GIEL that can be detected is significantly increased (see Results). This may be because at the higher pH there is less interaction between protein and sulphated glycosaminoglycan (Scott *et al.*, 1968) or because there is more ionization of potential binding sites. It is of interest that staining of structures as small as the granules of GIEL is not complete after periods up to 4 h (Table 2), suggesting that the granules have a dense structure offering a considerable diffusion barrier to the large Alcian Blue molecule. Tas (1977) observed slow and incomplete penetration of the granules of connective tissue mast cells by Alcian Blue as compared to penetration by the smaller Toluidine Blue molecule.

Of particular importance is the staining of MMC and GIEL at high $MgCl_2$ concentrations (Fig. 2). As noted by Scott & Dorling (1965), staining of anionic polymers is reduced at high salt concentrations, and the concentration at which significant inhibition of dye binding occurs is referred to as the critical electrolyte concentration (CEC). The present study shows that the extinction of dye-binding at high salt concentrations is not due to dissolution of the granule constituents. The anionic glycosaminoglycan in connective tissue mast cells is heparin, but recent studies have suggested that MMC contain material with a lower degree of sulphation, of the heterogeneous family of molecules referred to as heparan sulphates or heparitin sulphates (Tas & Berndsen, 1977). Miller & Walshaw (1972) have compared the CEC for binding of Alcian Blue to the granules of connective tissue mast cells and MMC, and found a value for MMC similar to that reported in the present study and lower than in the connective tissue cells. These findings, in conjunction with the work of Tas & Berndsen (1977), strongly suggests that the lower CEC of MMC granules is due to the presence of glycosaminoglycan with a relatively low degree of sulphation. The present study clearly shows that the granules of GIEL have a lower CEC than the granules of MMC, suggesting that GIEL contain glycosaminoglycan with either an even lower degree of sulphation or a lower molecular weight (Scott & Stockwell, 1967) or both than the material found in MMC. In conjunction with the observation that GIEL incorporate radio-labelled sulphate, this finding strengthens the suggestion that these cells may be precursors of MMC (Guy-Grand *et al.*, 1978).

Conclusion

The method chosen to obtain optimal fixation and staining of mucosal mast cells and granular intraepithelial lymphocytes in paraffin sections of rat jejunum is as follows.

Fixation – methanol–formalin–acetic acid (MFAA), 8.5:1.0:0.5 by vol.

Staining – (a) 0.05% Alcian Blue in 0.02 M acetate buffer, pH 5.8, containing 0.2 M $MgCl_2$ for 4 h; (b) 0.25% Safranin O in 0.02 M acetate buffer, pH 5.0, for 5 min.

Use of the critical electrolyte concentration (CEC) technique has provided evidence suggestive that the glycosaminoglycan present in granular intraepithelial lymphocytes has a lower degree of sulphation than that present in mucosal mast cells. This finding is consistent with the view that the former may be precursors of mucosal mast cells.

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