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Insect mucosubstances. II. The mucosubstances of the central nervous system

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Synopsis. The glycosaminoglycans of the glial lacunar system and neural lamella of cockroach and locust ganglia have been characterized histochemically, using primarily Alcian Blue binding methods at various pH levels and salt concentrations, the periodic acid-Schiff test together with recent modifications, the high iron diamine test, and enzymatic digestions. The results suggest the presence of hyaluronic acid in the glial lacunar system and of a mixture of chondroitin and dermatan sulphates, together with keratan sulphate in the neural lamella. The significance of the presence of these substances in the central nervous system of insects is discussed.

Introduction

The first histochemical studies of insect connective tissues were concerned with the neural lamella, an amorphous layer, which surrounds the entire central and peripheral nervous systems. These histochemical studies were designed to discover the nature of this layer, and the results suggested that in cockroaches, grasshoppers and locusts, it contains collagen and neutral mucosubstances (Ashhurst, 1959, 1961*a*; Baccetti, 1955, 1956). The presence of collagen was confirmed by electron microscope studies (Ashhurst & Chapman, 1961; Smith & Wigglesworth, 1959; Baccetti, 1961). An acidic glycosaminoglycan is present in the connective tissue on the dorsal side of the abdominal nerve cord of the moth *Galleria mellonella*, but its exact nature could not be determined histochemically at that time (Ashhurst & Richards, 1964). The apparent absence of glycosaminoglycans from the neural lamellae of cockroaches, grasshoppers and locusts is somewhat peculiar in view of the large number of collagen fibrils in this layer; a very close association between collagen and chondroitin sulphate usually occurs (Mathews, 1967*a*, 1970).

Mucosubstances also occur within the ganglia of many insects. A system of large spaces around the neuropile in cockroach ganglia, named the glial lacunar system, was first described by Wigglesworth (1960). The glial lacunar system was later shown to be an extracellular system of channels containing a glycosaminoglycan (Ashhurst, 1961*b*; Pipa, 1961). This was thought to be hyaluronic acid since it stains metachromatically with Toluidine Blue and is removed by testicular hyaluronidase. This result was confirmed biochemically (Ashhurst & Patel, 1963).

Later studies of the fine structure of cockroach ganglia showed that small channels run from the large peripheral lacunae into the depths of the neuropile (Smith & Treherne, 1963); presumably these channels contain the same substances as the large lacunae.

Originally it was thought that this system was restricted to cockroaches, but later studies using Alcian Blue have indicated that similar systems containing acidic glycosaminoglycans occur in grasshoppers and locusts (Orthoptera), mantids (Dictyoptera) and earwigs (Dermaptera) (Martoja & Cantacuzène, 1966, 1968). Electron microscopical studies have shown the presence of extracellular channels ramifying among the axons of many insect ganglia and connectives (Ashhurst, 1968; Schürmann & Wechsler, 1969; Smith & Treherne, 1963; Treherne & Maddrell, 1967). It is thought that the acidic groups of the mucosubstances in the cockroach glial lacunar system are involved in the maintenance of a favourable ionic environment within the ganglia (Treherne, 1962). This will be discussed later.

None of these histochemical studies has provided unequivocal evidence of the nature of the mucosubstances in the neural lamella and glial lacunar system. Recently developed techniques employing Alcian Blue are more sensitive than those using metachromatic dyes, and hence it was thought possible that acidic glycosaminoglycans might be identified in the neural lamella. In addition, these techniques permit hyaluronic acid to be distinguished from the chondroitin sulphates with some certainty. Thus, it was decided to apply these, together with other techniques, in a re-examination of the mucosubstances of some insect ganglia.

Methods

Thoracic ganglia of adult cockroaches (*Periplaneta americana*) and locusts (*Locusta migratoria*) were fixed in 4% formaldehyde in either 0.1 M phosphate buffer, pH 7.2, or 2% calcium acetate. Fixation was for 18 hr, after which the ganglia were washed, dehydrated and embedded in Paraplast. Sections were cut at 8 μm and mounted on slides without albumen adhesive.

Some ganglia were fixed in a 5:95 v/v mixture of 40% aqueous formaldehyde and absolute ethanol at −20°C. Fixation proceeded for 3 days at −20°C during which time the fluid was agitated from time to time. The fixative was replaced by cold absolute ethanol and the temperature was allowed to rise to room temperature. The ganglia were cleared in xylene and embedded in Paraplast. Sections were cut at 8 μm and attached to slides with albumen adhesive; in some instances, water was used to float out the sections.

The histochemical tests performed are described in the preceding paper (Ashhurst & Costin, 1971). The only modifications to these methods are the following.

Modified periodic acid-Schiff (PAS) test for uronic acid-containing glycosaminoglycans (Scott & Dorling, 1969)

Since hyaluronic acid is depolymerized and highly diffusible when exposed to periodate for prolonged periods (Scott & Tigwell, personal communication), some sections were treated for 30 min at 30°C with cetylpyridinium chloride which forms insoluble complexes with glycosaminoglycans. In addition, sodium *meta*-periodate was used as a 1% aqueous (0.05 M) solution, instead of the 2% solution.

Enzyme extractions

Sections were incubated in ovine testicular hyaluronidase for periods of up to 6 hr.

Results

GLIAL LACUNAR SYSTEM

(a) Cockroach

The glial lacunar system is clearly shown after staining with Alcian Blue, pH 2.5, since its contents are strongly stained. At pH 1.0, the contents stain faintly (Table 1; Figs. 1, 2). When the critical electrolyte method is used, dye binding is extinguished at an $MgCl_2$ concentration of 0.2 M (Table 2; Fig. 6). These reactions are not just confined to the large peripheral lacunae; they can be seen within the neuropile as well.

Table 1. Alcian Blue staining of the glial lacunar system and neural lamella at low pH. The intensity of the reactions or staining is indicated as follows. No reaction or staining, 0; increasing intensity of reaction or staining, 1–4+ (1 is weak, 4 is strong); ± very weakly positive reaction or staining

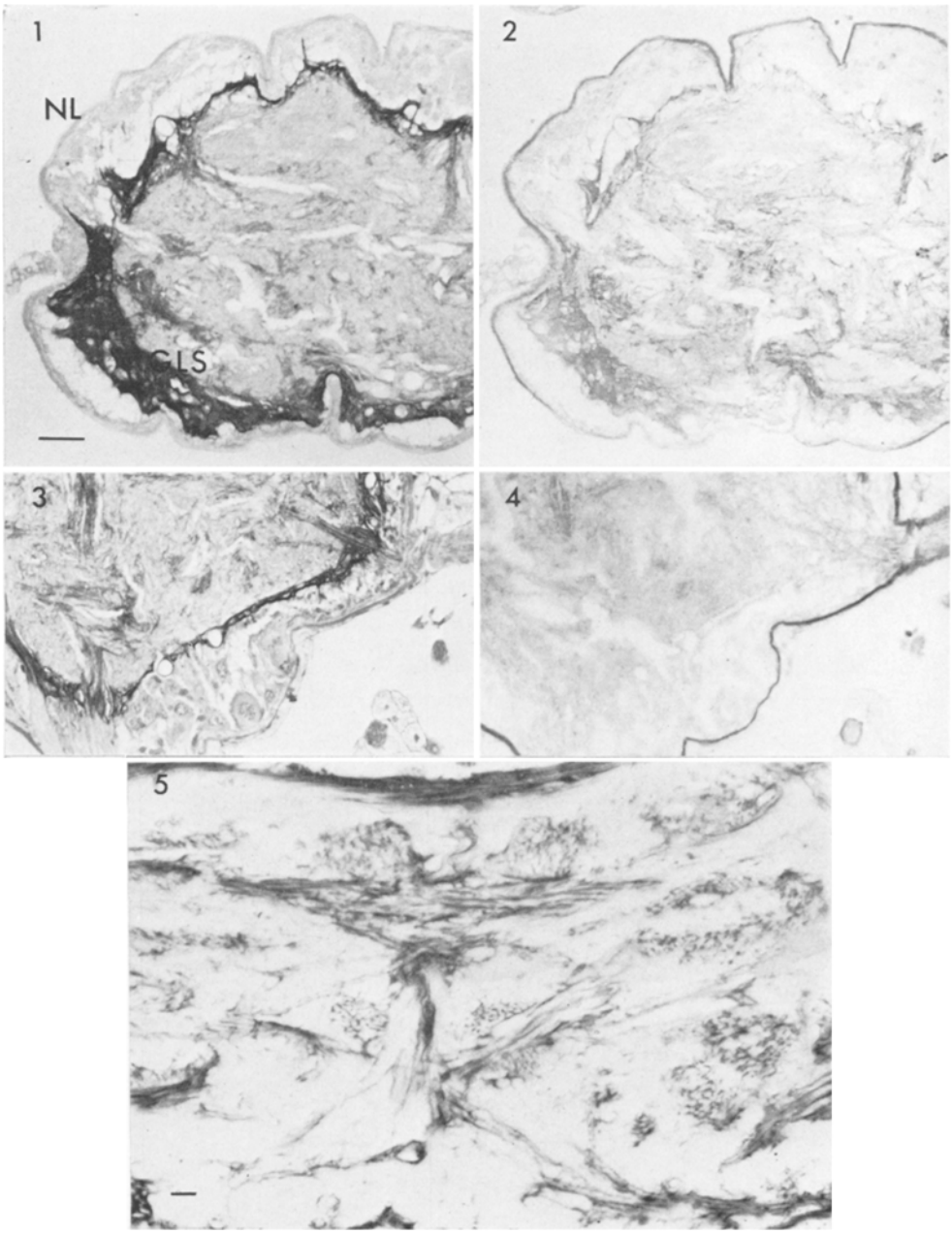
	Cockroach		*Locust*	
Alcian Blue (1%)	*Glial lacunar system*	*Neural lamella*	*Glial lacunar system*	*Neural lamella*
pH 2.5	4+	0–1+	3+	1+
pH 1.0	0–1+	0–3+	0	1–3+

Table 2. Critical electrolyte concentrations; Alcian Blue staining of the glial lacunar system (GLS) and neural lamella (NL) at pH 2.5 and pH 5.7 in solutions containing $MgCl_2$. Also see legend of Table 1

Concentration of $MgCl_2$ (M)	*Alcian Blue pH 2.5*				*Alcian Blue pH 5.7*			
	Cockroach		*Locust*		*Cockroach*		*Locust*	
	GLS	NL	GLS	NL	GLS	NL	GLS	NL
0.05	1–3+	1–2+	1–2+	2+	4+	3+	4+	4+
0.1	1+	1–2+	±	2+	4+	3+	3+	4+
0.2	0	1–3+	0	3+	0	3+	0	3–4+
0.4	0	1–2+	0	2+	0	1–2+	0	2–3+
0.6	0	1–2+	0	1–2+	0	1+	0	1–3+
0.8	0	0–1+	0	1–2+	0	1+	0	1–2+
1.0	0	±	0	0–1+	0	±	0	1+

The contents of the glial lacunar system are stained metachromatically by Azure A at pH 4.0 and by Toluidine Blue.

When the standard PAS test is applied, there is no reaction in the glial lacunar system (Table 3). That the substance in this system is PAS negative is clearly demonstrated, since if Alcian Blue (pH 2.5) staining is followed by the PAS test, the two reactions clearly do not overlap. When the modified PAS test, with the additional modifications described in the *Methods* section is employed, a strong reaction is seen in the glial lacunar system, which provides con-



Figures 1–4. Thoracic ganglia of locust and cockroach stained with Alcian Blue at pH 2.5 and pH 1.0. In both instances the glial lacunar system (GLS) is stained strongly at pH 2.5, weakly or not at all at pH 1.0. The neural lamella (NL) is most strongly stained at pH 1.0.

Fig. 1, cockroach ganglion, Alcian Blue, pH 2.5; Fig. 2, Alcian Blue, pH 1.0; Fig. 3, locust ganglion, Alcian Blue, pH 2.5; Fig. 4, Alcian Blue, pH 1.0.

Figures 1–4 are at the same magnification; bar on Fig. 1 equals 50 μm.

Figure 5. Thoracic ganglion of locust fixed by freeze substitution and stained with Alcian Blue, pH 2.5. Rings of Alcian Blue staining can be seen around the axons in the neuropile. Magnification bar equals 10 μm.

firmatory evidence for the presence of uronic acid (Table 3; Figs. 11, 12). There is no reaction with the high iron diamine test for substances possessing sulphate groups (Table 3).

The material in the glial lacunar system is partially removed by hyaluronidase digestion; the reason for this partial removal is not clear (Table 4).

Table 3. Reactions of glial lacunar system and neural lamella to some histochemical texts.

	Cockroach		*Locust*	
Procedure	*Glial lacunar system*	*Neural lamella*	*Glial lacunar system*	*Neural lamella*
PAS (standard)	0	3+	0	3+
PAS (diastase control)	0	3+	0	3+
PAS (modified)	4+	2+	3+	2+
HID	0	2+	0	2+

Abbreviations: AB = Alcian Blue. Also see legend of Table 1.

Table 4. Alcian Blue staining of glial lacunar system and neural lamella after enzymatic digestions.

	Cockroach		*Locust*	
Procedure	*Glial lacunar system*	*Neural lamella*	*Glial Lacunar system*	*Neural lamella*
Hyaluronidase-AB, pH 2.5	1–2+	±	2+	0–1+
Control-AB, pH 2.5	4+	±	3+	0–1+
Hyaluronidase-AB, pH 1.0	0	1+	0	1+
Control-AB, pH 1.0	0	1+	0	2+
Neuraminidase-AB, pH 2.5	4+	±	3+	0–1+
Control-AB, pH 2.5	4+	±	3+	0–1+

Abbreviations: PAS = periodic acid-Schiff. HID = high iron diamine. Also see legend of Table 1.

(b) Locust

The peripheral lacunae of the glial lacunar system of the locust are less extensive than those of the cockroach and hence it is sometimes more difficult to distinguish between the material in the lacunae and surrounding glial cells.

The histochemical reactions are essentially similar to those in the cockroach. The material is stained strongly by Alcian Blue at pH 2.5 and in these preparations one can see that the material is limited to narrow channels; it is unstained at pH 1.0 (Table 1; Figs. 3, 4). It is stained strongly by dye solutions containing less than 0.2 M $MgCl_2$, but at this concentration, staining is completely extinguished (Table 2). The material is partly removed by hyaluronidase digestion (Table 4).

The glial lacunar system is stained metachromatically by Azure A at pH 4.0. This contrasts with the lack of metachromasia after Toluidine Blue staining.

After the usual short oxidation by periodic acid, the material is not PAS positive. There is, however, a strongly positive reaction given by other components of this region of the ganglion. Much of this positivity is due to glycogen in the glial cells, since the reaction in this area is greatly reduced by prior diastase digestion. That the material stained by Alcian Blue is distinct from the PAS-positive substances is shown by a combination of the Alcian Blue, pH 2.5, and PAS tests, since blue and pink staining substances are seen. The modified PAS test, with the additional modifications described earlier, gives a positive reaction in the glial lacunar system (Table 3). There is no reaction with the high iron diamine test (Table 3).

The material in the glial lacunar systems of the cockroach and locust nervous systems is thus similar and it appears to be a glycosaminoglycan with carboxyl as opposed to sulphate groups. Its reactions suggest that it is hyaluronic acid.

Evidence that the narrow channels deep in the neuropile of both the cockroach and locust also contain hyaluronic acid was found in most preparations, especially those of ganglia fixed by freeze-substitution. In regions of the neuropile in which large axons are present, a network of material stained by Alcian Blue at pH 2.5 can be seen. It appears that this material is surrounding these large axons and it seems likely that it is actually in the narrow extracellular channels seen in electron micrographs of such areas (Fig. 5).

NEURAL LAMELLA

(*a*) *Cockroach*

The results of the Alcian Blue tests on this neural lamella are given in Tables 1 and 2 (Figs. 1, 2, 6–8). The lamella is stained weakly, if at all, by Alcian Blue at pH 2.5, but a definite, if somewhat variable, positive reaction occurs at pH 1.0. When the critical electrolyte method with Alcian Blue, pH 5.7, is applied, a strong reaction occurs at $MgCl_2$ concentrations up to 0.2 M which decreases at 0.4 M, and further at 0.6 M, but staining is not extinguished at 1.0 M, though it is weak at this concentration of $MgCl_2$. If Alcian Blue solutions at pH 2.5 are used, it is noticeable that the intensity of staining increases up to 0.2 M $MgCl_2$ and then decreases, although it is still apparent at 1.0 M $MgCl_2$.

The neural lamella is not stained metachromatically by Azure A at any pH used here, or by Toluidine Blue.

The neural lamella is strongly positive with the standard PAS test. It also gives a positive reaction with the Scott & Dorling (1969) modification of the PAS test for uronic-acid-containing glycosaminoglycans, but the staining intensity is weaker than that after the standard procedure (Figs. 12, 13). The presence of sulphate groups is indicated by the positive reaction with the high iron diamine test (Table 3).

Enzymatic digestions with hyaluronidase and neuraminidase revealed that the glycosaminoglycan in the neural lamella is digested only by hyaluronidase. If digestion is followed by staining with Alcian Blue at pH 1.0, a reduction in staining is seen (Table 4).

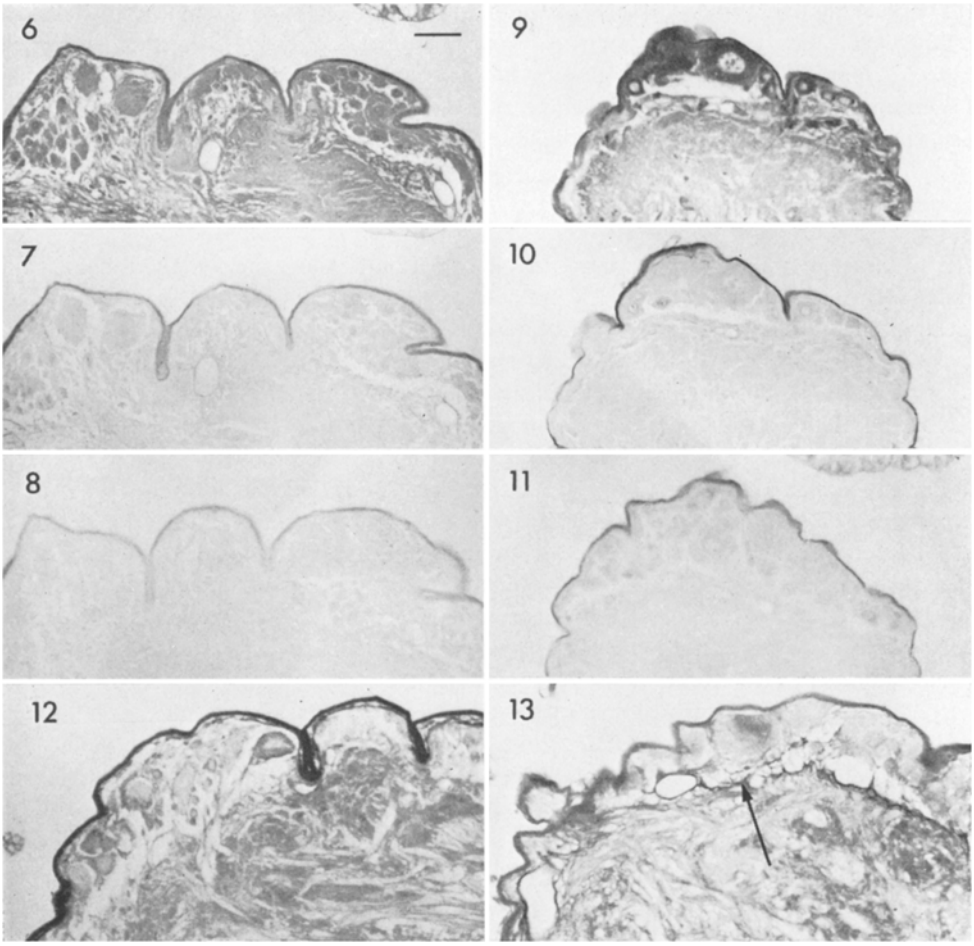
(*d*) *Locusts*

The neural lamella of the locust is stained by Alcian Blue at pH 2.5, but the staining is stronger at pH 1.0 (Table 1; Figs. 3, 4). When $MgCl_2$ is added to an Alcian Blue solution at pH 5.7, staining is intense at low molarities of $MgCl_2$ (Table 2; Figs. 9–11) and while there is some diminution at higher molarities, moderately strong dye binding still occurs at 1.0 M $MgCl_2$; the

colouration is stronger than that seen in the cockroach neural lamella in these conditions. The results with Alcian Blue at pH 2.5 show an increase in dye binding up to 0.2 M $MgCl_2$ and then a progressive decrease, but dye binding still occurs at 1.0 M $MgCl_2$.

Metachromasia is not exhibited by the neural lamella when stained with either Azure A or Toluidine Blue under the conditions used here.

The neural lamella gives a positive reaction with the standard PAS test. If the aldehyde



Figures 6–11. Thoracic ganglia of cockroach and locust stained with Alcian Blue, pH 5.7, containing various concentrations of $MgCl_2$. The glial lacunar system is not apparent when stained in the presence of 0.2 M $MgCl_2$, whereas the neural lamella is still clearly coloured in 1.0 M $MgCl_2$.
Cockroach ganglion: Fig. 6, 0.2 M $MgCl_2$; Fig. 7, 0.4 M $MgCl_2$; Fig. 8, 1.0 M $MgCl_2$.
Locust ganglion: Fig. 9, 0.2 M $MgCl_2$; Fig. 10, 0.4 M $MgCl_2$; Fig. 11, 1.0 M $MgCl_2$.
Figure 12. Thoracic ganglion of cockroach, periodic acid-Schiff. The neural lamella gives a strongly positive reaction, but the glial lacunar system is negative.
Figure 13. Thoracic ganglion of cockroach, Scott & Dorling's modified periodic acid-Schiff reaction. The neural lamella is less strongly positive, but the glial lacunar system is now positive (arrow).
Figures 6–13 are at the same magnification; bar on Fig. 6 equals 50 μm.

groups produced by the initial short oxidation are destroyed by borohydride, and then a secondary, prolonged oxidation occurs, the neural lamella again gives a positive reaction to the PAS test, but it is reduced in strength (Table 3). The neural lamella also gives a positive reaction with the high iron diamine test (Table 3).

Enzymatic digestions with hyaluronidase and neuraminidase show that the reactions with Alcian Blue are affected only by hyaluronidase digestion. This is most clearly demonstrated by staining with Alcian Blue at pH 1.0 (Table 4).

The reactions of both neural lamellae suggest that sulphated glycosaminoglycans are present. It is probable that these include chondroitin or dermatan sulphates, together with some keratan sulphate. The latter would account for the binding of Alcian Blue in 1.0 M $MgCl_2$ and if staining intensity is indicative of concentration, it would appear that there is more in the locust neural lamella than in the cockroach.

Discussion

The arguments put forward to support the validity of the histochemical methods used in this work have been discussed at length in a previous paper (Ashhurst & Costin, 1971). The results of Alcian Blue staining under various conditions give clear indications of the nature of the glycosaminoglycans in the glial lacunar system and neural lamella and which are confirmed by other tests. For instance, the substance in the glial lacunar system of both the cockroach and locust stains with Alcian Blue at pH 2.5, but not at pH 1.0, indicating that carboxyl groups only are involved in the dye binding. This conclusion is supported by the critical electrolyte technique, since Alcian Blue is bound only at $MgCl_2$ concentrations below 0.2 M. When precautions are taken to prevent diffusion, a positive reaction occurs with the modified PAS technique which indicates the presence of uronic acid groups. The high iron diamine test indicates that sulphate groups are not present. The substance is partially labile towards testicular hyaluronidase. A glycosaminoglycan giving these characteristic reactions is hyaluronic acid. The glycosaminoglycans in the neural lamella display quite different characteristics. They are only weakly stained by Alcian Blue at pH 2.5, but more strongly so at pH 1.0. That this increase in dye binding is due largely to unmasking of the glycosaminoglycans by proteins is shown by the great increase in staining at pH 2.5 in the presence of 0.2 M $MgCl_2$. At both pH 2.5 and pH 5.7, dye binding still occurs at 1.0 M $MgCl_2$, although to a considerably decreased extent. The presence of sulphate groups is also indicated by the high iron diamine test and of uronic acid by the modified PAS test. The glycosaminoglycans are partially removed by previous digestion with testicular hyaluronidase. Thus, glycosaminoglycans with uronic acid and sulphate groups, that is, chondroitin or dermatan sulphates are present together with a component having a very high critical electrolyte concentration and which is most probably keratan sulphate.

These results provide more conclusive evidence than was possible in earlier studies for the presence of hyaluronic acid in the glial lacunar system. The presence of hyaluronic acid in cockroach ganglia was suggested from staining with Toluidine Blue, with hyaluronidase controls together with a biochemical investigation (Ashhurst, 1961*b*; Ashhurst & Patel, 1963; Pipa, 1961), but it was later suggested that hyaluronic acid is present in a variety of insect ganglia (Martoja & Cantacuzéne, 1966, 1968). These workers used Alcian Blue at an unspecified pH, and colloidal iron to stain the substance in the glial lacunar system. The absence of sulphate

groups was deduced from the lack of uptake of radioactive sulphate, but no experimental details are given. Thus, the results described here provide the first unequivocal evidence for the presence of hyaluronic acid in the glial lacunar system of the cockroach and locust, though it would appear reasonable to assume its presence in other such systems. Martoja & Cantacuzéne (1966) suggest that a PAS-positive glycoprotein also occurs in the glial lacunar system of the locust. The system in the locust is restricted to very narrow channels and in the peripheral regions these are very close to glial cells containing masses of glycogen. In the present work, the evidence suggests that only hyaluronic acid is present in the lacunae. It is known from many studies of insect ganglia that narrow extracellular channels pass from the large lacunae around the periphery into the neuropile (Ashhurst, 1968; Bloch *et al.*, 1966; Edwards *et al.*, 1958; Nishiitsutsumi-Uwo, 1961; Schürmann & Wechsler, 1969; Smith & Treherne, 1963; Treherne & Maddrell, 1967). It is difficult to detect these in the light microscope, but the dye binding seen in the neuropile did suggest that hyaluronic acid is present throughout the system.

Any discussion of the function of the hyaluronic acid in the glial lacunar system must first be concerned with the known properties of this macromolecule (see Laurent, 1970). Hyaluronic acid is a long-chain unbranched polymer of repeating disaccharide units, which consist of D-glucuronic acid and glucosamine (Laurent, 1970). It is of high molecular weight and chains of an average length of 1.64 μm (the maximum length was around 5–6 μm) have been seen by electron microscopy (Fessler & Fessler, 1966). The chain is a flexible structure forming a random coil, so that the coils of adjacent molecules will entangle each other. At concentrations of 0.1%, a continuous chain network exists in solution. The three-dimensional networks thus formed act as molecular sieves; the sedimentation rate of large molecules through hyaluronic acid solutions is retarded, while small molecules are hardly affected. In addition, the three-dimensional network has an exclusion effect, that is, for steric reasons, it limits the amount of space available within the system for large molecules. The chains carry one carboxyl group per disaccharide unit which is ionized at physiological pH. This means that positively charged ions or molecules, such as proteins, can be bound electrostatically to the hyaluronic acid molecule. In addition, the shape of the coil is influenced by the number of charges on the chain. Thus the ionic strength of the surrounding medium affects the conformation of the hyaluronic acid molecules. This also affects the viscosity of the hyaluronic acid, which under most conditions produces a very viscous solution (Laurent, 1970).

Any consideration of the function of the hyaluronic acid in the glial lacunar system must take into account the known properties of the molecule as outlined above. It has been implicated in the maintenance of a favourable ionic environment within the nervous system. Treherne (1962) thought that it might provide the necessary anionic groups within the nervous system of the cockroach to account for the observed Donnan equilibrium between the intercellular fluid in the ganglion and the haemolymph. More recently, Treherne & Moreton (1970) consider that the hyaluronic acid may bind sodium ions, which could then be utilized when the sodium around the axons is depleted.

A trophic role for the hyaluronic acid was suggested by Martoja & Cantacuzéne (1968), but this would appear a novel and highly unlikely role for a glycosaminoglycan. The use of the channels of the glial lacunar system for the transport of nutrients throughout the ganglion was suggested by Wigglesworth (1960). Doubt has, however, been cast on the availability of these channels for the passage of large molecules by some recent experiments which will be discussed later. If this is so, then large molecules such as glycogen might find it difficult to pene-

trate the system. More recently, Smith (1967) has suggested that the glial cells have the major trophic function.

The inclusion of the glial lacunar system as part of the connective tissues of insects (Ashhurst, 1968) has been criticized on the grounds that it does not have a supporting function or contain collagen fibrils (Martoja & Cantacuzéne, 1968). Hyaluronic acid and the sulphated glycosaminoglycans are generally considered to be connective tissue substances, since they occur in the extracellular matrix of connective tissues. Thus any extracellular space containing hyaluronic acid may be included as part of the connective tissue of the animal.

An ion-binding function as suggested by Treherne would appear the most appropriate for the hyaluronic acid in the glial lacunar system. It is now known that glial lacunar systems of varying size and complexity occur in many insect ganglia and it has become apparent that similar intercellular spaces also occur in the central nervous systems of a wide variety of both vertebrates and invertebrates. These include the central nervous system of the leech *Hirudo medicinalis* (Coggeshall & Fawcett, 1964; Kuffler & Potter, 1964), the crabs *Carcinus* and *Uca* (Abbott, 1970; Malzone *et al.*, 1966), squids (Villegas & Villegas, 1968), the scorpion *Androctonus australis* (Lemire & Deloince, 1970) and in the mammalian cerebral cortex (Bondareff, 1965).

Histochemical studies have indicated that the lacunar system in scorpion ganglia contains glycosaminoglycans with both carboxyl and sulphate groups and, in addition, neutral glycoproteins (Lemire & Deloince, 1970), while, in the squid, glycoproteins or glycosaminoglycans appear to be present (Villegas & Villegas, 1968), though the evidence is equivocal. Biochemical studies have identified both chondroitin sulphate and hyaluronic acid in mammalian brains (Clausen & Hansen, 1963; Singh & Bachhawat, 1965; Szabo & Roboz-Einstein, 1962), and it has been suggested that glycosaminoglycans occur in the intercellular spaces since they are penetrated by Ruthenium Red (Bondareff, 1967). In some recent studies, glycosaminoglycans were localized at the nodes of Ranvier by a copper-binding technique (Langley & Landon, 1969); further studies using the Alcian Blue critical electrolyte concentration technique showed that the polyanions are probably carboxyl and sulphate ester groups (Langley, 1970). That the glycosaminoglycans act as cation exchange reservoirs in vertebrate nervous systems has been favoured by several authors (Langley, 1970; Langley & Landon, 1969; Szabo & Roboz-Einstein, 1962); the ability of hyaluronic acid and other glycosaminoglycans to bind cations is well established (Buddecke & Drzeniek, 1962; Dunstone, 1962; Laurent, 1970; Mathews, 1964, 1967*b*).

Some recent observations indicate that hyaluronic acid in the nervous system might exclude other large molecules from these spaces. Lane & Treherne (1970) found that in desheathed cockroach ganglia, previously administered peroxidase passed from one glial cell to another into the neuropile; no peroxidase was localized in the extracellular channels, although they must have been exposed by the desheathing process. A similar situation was observed when locust and cockroach ganglia were fixed in solutions containing lanthanum (Ashhurst, unpublished observations). This phenomenon has also been observed in the mouse cerebral cortex where ferritin passed from the endothelial cells to astrocytes rather than through the extracellular spaces (Bondareff, 1964). Levi *et al.* (1966) suggested that the passage of molecules in earthworm ganglia might be through the glial cells rather than the extracellular channels. Much more information is required before the full significance of the presence of hyaluronic acid, or other glycosaminoglycans, within nervous systems can be fully appreciated.

The presence of acidic glycosaminoglycans in neural lamellae was not indicated by any of the

earlier studies (Ashhurst, 1959, 1961*a*; Baccetti, 1955, 1956), so their identification in this study is of special interest. The increased binding of Alcian Blue at pH 1.0 and in the presence of low concentrations of $MgCl_2$ suggests that under most experimental conditions, the anionic groups of the glycosaminoglycan are blocked by proteins. This would account for the small amount of dye binding at pH 2.5 and, in addition, the lack of metachromasia with Toluidine Blue previously reported (Ashhurst, 1959, 1961*a*). The results suggest that the neural lamellae of both the cockroach and locust contain at least two glycosaminoglycans, possibly chondroitin or dermatan sulphate and keratan sulphate. The glycosaminoglycan content of the neural lamella is thus very similar to that of the connective tissue around the ejaculatory duct of the locust (Ashhurst & Costin, 1971). As the ganglia were from insects of unknown age, it is not possible to comment upon the significance of the presence of keratan sulphate. The identification of acidic glycosaminoglycans in the neural lamella is significant in that insect collagenous tissues were previously peculiar in their apparent lack of these substances, which are known to exert an effect on collagen fibril formation (Mathews, 1967*a* and 1970).

The question arises as to whether the neural lamella has any function other than that of providing a mechanical support around the nervous system. It was originally suggested (Hoyle, 1952) that the neural lamella might form a selective ion barrier around the nervous system which would prevent adverse ionic concentrations in the haemolymph affecting the environment around the axons. Further evidence to support its permeability has been drawn from studies of dye or large molecule penetration; in all instances the neural lamella was penetrated. It is noteworthy, however, that the times allowed for the penetration of the dye or other molecules were prolonged, for example 1.5 hr for Methylene Blue (Eldefrawi *et al.*, 1968), 2 to 13 days for Trypan Blue (Wigglesworth, 1960; Scharrer, 1939), 2 min to 6 hr for peroxidase (Lane & Treherne, 1970), and 5 to 60 min for acetylcholine (Treherne & Smith, 1965). The pertinent question seems to be not whether a molecule will eventually penetrate the neural lamella, but whether it can penetrate rapidly and without hindrance. The neural lamellae of the cockroach and locust contain sulphated glycosaminoglycans and collagen fibrils. There is abundant evidence that these glycosaminoglycans, which are polyanions, bind cations such as Na^+, K^+ and Ca_2^+, or cationically charged molecules (Buddecke & Drzeniek, 1962; Dunstone 1962; Mathews, 1964, 1967*b*). Thus any ion or molecule passing from the haemolymph to the interior of the ganglion must pass through a layer, up to 10 μm thick, which contains a network of fibrils interspersed with negatively charged polymers of glycosaminoglycans. Apart from hindrance caused by the polyanions, large molecules would be expected to experience steric hindrance due to the exclusion factors produced by networks of large polymers (Laurent, 1970). It is interesting that Eldefrawi & O'Brien (1966) found that the permeability of the neural lamella of the cockroach to fatty acids is 13 times less than that to glucose which is unionized, and that Abbott (1970) found that while uncharged molecules up to 10–16 nm diameter passed readily from blood vessels into the nervous system of the crab, negatively charged molecules of the same size were held back; the blood vessels in the crab nervous system are lined by a basement membrane of unknown composition. Kuffler & Potter (1964) thought that the basement membrane in the leech nervous system might impede the diffusion of Trypan Blue. It would appear, therefore, that any evaluation of the physiological significance of the neural lamella should be concerned not so much with whether a large molecule will eventually penetrate it, but instead with whether it can penetrate within a physiological time. In this context, the effect of the neural lamella on the penetration of large insecticide molecules might be significant.

Thus, much further work is needed both to characterize the glycosaminoglycans in nervous systems and to evaluate the role of these substances in the general phenomena associated with nerve conduction.

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