The Association of Mucopolysaccharides with Morphogenesis of the Palate and Other Structures in Mouse Embryos

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WITH FOUR PLATES

ACID mucopolysaccharides have been postulated to occur in the palatine shelves of mouse embryos on the basis of metachromatic staining with toluidine blue (Walker & Fraser, 1956), and this idea has since been supported by results from radioautography with S\(^{35}\) (Larsson, Boström, & Carlsson, 1958). It was not known which structural component of the tissue contained this substance, but, since aldehyde fuchsin-positive material existed in the same area, the possibility was raised that the component was a network of elastic fibres (Walker & Fraser, 1956). The first purpose of the present paper is to describe further histochemical and radioautographic investigations of this problem, supplemented by electron microscopic observations of palatine shelf tissue.

Numerous sites of S\(^{35}\) incorporation have been identified in embryos of various ages, including embryos too young to contain cartilage (Dziewaitkowski, 1958). Very little was concluded from such studies concerning the role of sulphated acid mucopolysaccharides other than noting their characteristic presence as a component of cartilage and bone. In view of the apparent association between sulphated acid mucopolysaccharides and palate development (Walker, 1960), it seemed desirable to gain a broader perspective on the possible significance of this substance in morphogenesis, so that the experiments described in the present paper were extended secondarily to include earlier stages of embryonic development.

MATERIALS AND METHODS

Timing of embryos was by vaginal plug initially and was confirmed later by reference to morphological criteria. The details of mating and timing procedures have been described previously (Walker & Fraser, 1956). The histochemical methods used were aldehyde fuchsin (Pearse, 1953), toluidine blue (Pearse, 1953), alcian blue (Mowry, 1956), and orcinol-new fuchsin (Fullmer & Lillie, 1956).

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The fixative used was Bouin's fluid in all cases. Embryos were collected at regular intervals from 7 to 17 days post-conception for staining with aldehyde fuchsin, alcian blue, and toluidine blue, but orcinol-new fuchsin was applied only to heads from 15-day-old embryos. Amniotic fluid was withdrawn from embryos at days 14/8 (14 days and 8 hours post conception), 15/8, 17/8, and 19/8. The fluid was smeared on slides, fixed in methanol or Bouin's fluid, and stained with aldehyde fuchsin.

Palate tissue used for electron microscopy was dissected from the living embryo, placed in cold buffered osmic acid sucrose fixative (Caulfield, 1957) for 45 minutes, and then dehydrated and embedded in methacrylate.

For radioautography of the palatine shelves, 1.5 mc. of $S^{35}$ (as Na$_2$S$_{35}$O$_4$) was injected into a pregnant mouse in doses of 0.5 mc. at days 12/10, 13/8, and 14/8, while another mouse received 1.5 mc. $S^{35}$ (in four smaller doses) at days 14/6, 14/10, 14/14, and 14/18. Both were killed at day 15/9. Also, single doses of 180 µc. $S^{35}$ were given at days 13/14 and 14/10 to two pregnant mice, and these mice were killed at days 14/14 and 15/10 respectively.

For radioautography of young embryos 1.0 to 1.5 mc. of $S^{35}$ were administered to four pregnant mice in one to three doses at various intervals starting at day 9/7. These animals were killed at days 9/16, 10/6, or 10/10. Embryos were fixed in Bouin's fluid and the radioautographs were made by the fluid emulsion dipping method which has been described elsewhere (Walker, 1959). Most of the radioautographs were exposed for 3 months; after development, some were stained lightly with eosin.

Experiments in vivo with palatine shelf movement were based on the selection of embryos at the proper stage of development, which was determined by the vaginal plug method and then confirmed by observing shelf behaviour in a few of the embryos from each litter used. The procedure for in vivo experiments has been described elsewhere (Walker, 1954; Walker & Fraser, 1956). The treatments applied were (a) hyaluronidase (150 turbidity reducing units in 2 c.c. H$_2$O for 10 minutes to 2 hours at 37° C., with water controls), (b) 1/100 N and 1 N solutions of HCl or KOH, and (c) various mixtures of acetic acid and alcohol. The experiments were carried out on relatively small numbers of embryos, because the results were not encouraging enough with any of these agents to warrant a large series.

**RESULTS**

Mesenchyme of the palatine shelves, when viewed with the electron microscope, appears to consist of cells with long processes embedded in an extensive matrix of intercellular ground substance (Plate 1, fig. A). Mitochondria and a small amount of granular endoplasmic reticulum are present within the cytoplasm. At least some of the cytoplasmic processes of each cell come in contact with those of adjoining cells (Plate 1, fig. B). No elastic fibres were found in the
mesenchyme of the palatine shelves. Also, the oral epithelium did not appear to contain any specialized structures (Plate 1, fig. C).

The location and intensity of aldehyde fuchsin staining reactions and of S\textsuperscript{35} incorporation in sections of 15-day-old embryo heads are illustrated in figs. D and E of Plate 2 and in figs. I and J of Plate 3; they are also listed in Table 1.

**Table 1**

*Location and intensity of histochemical reactions in 15-day-old embryo heads*

<table>
<thead>
<tr>
<th>Histochemical agent</th>
<th>Connective Tissue</th>
<th>Other Structures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In palate</td>
<td>In eyelids</td>
</tr>
<tr>
<td>S\textsuperscript{35}</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Aldehyde fuchsin</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Toluidine blue metachromasia</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Alcian blue</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Orcinol-new fuchsin</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 2**

*Aldehyde-fuchsin staining in connective tissue of mouse embryos*

<table>
<thead>
<tr>
<th>Location of connective tissue</th>
<th>Age of embryo (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Bordering neural epithelium</td>
<td>++</td>
</tr>
<tr>
<td>Around lens</td>
<td></td>
</tr>
<tr>
<td>In nasal and maxillary processes</td>
<td>±</td>
</tr>
<tr>
<td>In cardiac jelly</td>
<td>++</td>
</tr>
<tr>
<td>In bulbus</td>
<td>+</td>
</tr>
<tr>
<td>In endocardial cushions</td>
<td>+</td>
</tr>
<tr>
<td>In interatrial septum</td>
<td>+</td>
</tr>
<tr>
<td>In large blood-vessels</td>
<td>+</td>
</tr>
<tr>
<td>In dorsal mesentery</td>
<td>+</td>
</tr>
<tr>
<td>Around U.G. system</td>
<td>+</td>
</tr>
<tr>
<td>In roof of pharynx</td>
<td>++</td>
</tr>
<tr>
<td>In notochord sheath</td>
<td>++</td>
</tr>
<tr>
<td>In limb-buds</td>
<td>-</td>
</tr>
<tr>
<td>Around somites</td>
<td>++</td>
</tr>
</tbody>
</table>

A similar survey was made on sections of both the head and body of younger embryos and the results are illustrated in the remaining figures of Plates 3 and 4 and are summarized in Table 2. Seven-day-old embryos are not listed in Table 2 because these early post-implantation embryos gave essentially negative results. Alcian blue and toluidine blue were also used to stain young embryos and the results were approximately the same as with aldehyde fuchsin, although the weaker reactions were not as easy to identify.
All samples of amniotic fluid collected gave a positive reaction with aldehyde fuchsin. The material was precipitated as strands, loosely associated, to form a network in which cells were embedded. Many of these cells also gave a positive staining reaction. The presence of radioactive or aldehyde fuchsin-positive material on epithelial surfaces (Plates 2, fig. E; 3, fig. G) may be due to the precipitation of this material from the amniotic fluid.

Observations on shelf movement in ‘living’ embryos were made under a variety of experimental conditions. Incubation at 37° C. for 2 hours in distilled water did not inhibit palatine shelf movement. Sections made after an hour of such treatment showed swelling of the nuclei and a marked reduction in the density of their staining. Average diameters ($\sqrt{\text{longest axis} \times \text{maximum diameter at } 90^\circ}$) were calculated for 40 nuclei in the palate mesenchyme and they averaged 6.5 $\mu$, whereas the corresponding figure in a control embryo was 5.4 $\mu$. Hyaluronidase may have had a slight inhibitory influence under these same conditions of incubation, but in most cases the shelves were still able to move after exposure to this enzyme. HCl at a concentration of 1/100 N did not inhibit shelf movement, whereas 1 N HCl not only stopped shelf movement, but tended to harden the whole embryo; the action of this agent on shelf movement is therefore not necessarily different from its action on tissues in general. The same was true for the other reagents used. Thus, no agent specifically inhibitory to shelf movement was demonstrated by these experiments.

**DISCUSSION**

At the time of normal palate closure, the palatine shelves are able to move from a vertical to a horizontal plane due to some force residing within the shelves (Walker & Fraser, 1956). In a search for the physical basis of this force with the electron microscope, no unique structural specialization was found in the thin epithelium or loose mesenchyme of the palatine shelves (Plate 1, figs. A–C). Accordingly, the force would seem most likely to reside in the ground substance, or in the network of mesenchyme cells, or in the vascular system. The latter has already been ruled out by demonstrating that shelf force is independent of blood-pressure and that it even is resistant to immersion in 70 per cent. alcohol for 15 minutes (Walker & Fraser, 1956). The in vivo experiments reported here were not described in detail because they did not lead to the identification of reagents affecting palatine shelves specifically. They were included just to emphasize the resistance to noxious agents exhibited by the force which caused shelf movement. Since the palatine shelves can still move after exposure to weak acid solutions or after immersion in distilled water at 37° C. for 2 hours, it seems probable that the final release of this force is not immediately dependent on cytoplasmic activities, and that it should be attributed to some material produced by the cells prior to these treatments. The extensive network of ground substance in the mesenchyme is the most likely site for this
material, considering the morphology of the shelves as seen with the electron microscope.

The orcinol-new fuchsin stain introduced by Fullmer & Lillie (1956) is specific for elastic fibres. When used on sections from 15-day-old embryo heads, it did not stain any of the tissues present (Table 1). Since the electron microscope also did not reveal any elastic fibres, the hypothesis that these latter structures are responsible for the shelf force must be discarded. This hypothesis was originally based on the presence of aldehyde-fuchsin positive material in the palate mesenchyme, so that the problem which now remains is to determine what is being stained by this reagent. A comparison of figs. D and E in Plate 2 shows the exceptionally close parallel between aldehyde fuchsin staining and $^{35}$S incorporation. There is an extensive literature (e.g. Boström, 1958; Dziewiatkowski, 1958; Friberg, 1958) associating $^{35}$S incorporation with sulphated acid mucopolysaccharide synthesis when the $^{35}$S is administered as sodium sulphate. Furthermore, there is a close parallel between the reactions produced by these reagents and the reactions of two stains widely used for acid mucopolysaccharides, namely, toluidine blue (metachromasia) and alcian blue (Table 1). Thus it seems reasonable to conclude that the ground substance of the palatine shelves contains a considerable amount of sulphated acid mucopolysaccharide. There is probably more than one type of mucopolysaccharide present in the ground substance, as it is believed that these various histochemical stains cannot all react to the same mucopolysaccharide (Spicer, 1960; Fullmer, 1960).

The considerably greater intensity of palatine tissue radioactivity in radioautographs derived from embryos receiving $^{35}$S from day 14/8 to 14/18 (Plate 2, fig. B) than from embryos receiving $^{35}$S from day 12/10 to 14/8 can be considered as evidence associating sulphated mucopolysaccharide synthesis with the build up of palatine shelf force, since the latter phenomenon is known to take place mainly from day 14/8 to 14/20 (Walker, 1954).

A few studies on young embryos have been reported using $^{35}$S (Dziewiatkowski, 1958) and histochemical stains for mucopolysaccharides (Milaire, 1959). The survey study with $^{35}$S and aldehyde fuchsin reported here is not meant to be comprehensive but has had as its main objective a further test, with these two techniques, of the apparent parallel distribution of positive reactions in embryonic tissues. The aldehyde fuchsin was applied to a greater range of developmental stages than was $^{35}$S, due to the simplicity of the former technique, but in all cases where the two methods were compared, the distribution and intensity of reactions were essentially the same.

A discussion of the significance of this material in morphogenesis is necessarily speculative at this time, but certain features of the embryological events with which the mucopolysaccharides are associated seem worth commenting on in view of the physical properties of these compounds. The distribution of sulphated acid mucopolysaccharide in tissues is mainly in structures having the consistency of a firm gel (Ham, 1957). More specifically, chondroitinsulphuric
acid can be combined with certain proteins in the test tube to produce a 'coherent stringy elastic mass' with an 'appearance not unlike elastic fibres' or 'resembling the ground substance of bone', depending on the protein used (Meyer, Palmer, & Smyth, 1937). The only modification necessary to enable such a substance to bring about movement of the palatine shelves is that it should not be in its conformation of maximum stability prior to the actual movement. This instability might be due to asymmetrical deposition and subsequent growth stresses, to growth pressures from external sources, or to chemical alteration of the material itself. Whether such mechanisms are possible, or whether any of them exist in the embryo, is not known at present. Nevertheless, such an hypothesis is attractive because it could explain not only palatine shelf movement but also a number of other morphogenetic events.

Considering the intense reaction of the notochord sheath and the moderate reactivity of the surrounding mesenchyme (Plate 3, fig. K), the concept of a resilient system under pressure has obvious application to the phenomenon described in the following quotation (Snell, 1941): 'At about 8½ days, and at about the 11 or 12 somite stage, the mid-trunk region turns also. The process is sudden. Transverse sections of the trunk region at about this period show it to be either turned or not turned. It is quite possible that after the growth of the head and tail folds reduces sufficiently the attachment of the trunk region to the yolk sac, this region snaps over like a spring whose tension has come to exceed the forces holding it.'

Closure of the neural tube is another event with an obscure mechanism (Weiss, 1955). Arguments against differential cell-growth or cell proliferation have been reinforced by recent demonstrations of cyclic nuclear migration between the luminal and peripheral areas of the tube (Sauer & Walker, 1959; Sidman, Miale, & Feder, 1959). The force producing a folding of the neural tube was believed to reside within the tube because excised and isolated plates can transform into tubes (Weiss, 1955). Inspection of Plate 4, fig. O will show that the mucopolysaccharide layer enveloping the neural tube (which is also present when the tube is in the process of closing) lies at the epithelial-connective tissue junction and is most unlikely to have been separated off in the experiments referred to by Weiss. The neural tube itself may be contributing to this layer, especially since the epithelial cells of the enamel organ that form the stellate reticulum are apparently able to produce this type of mucopolysaccharide (Plate 3, fig. J).

Another embryological process in which mucopolysaccharides may participate is lip formation. According to Reed (1933), the maxillary process must press tightly against the nasal processes if the lip is to form properly. There is a considerable amount of aldehyde fuchsin-positive (Plate 3, figs. F, G) and S35-containing (Plate 4, fig. O) material in these processes by the time they are ready to fuse.

The progressive development of cardiac jelly into heart-valves proposed by
Patten, Kramer, & Barry (1948) is paralleled by the appearance of mucopolysaccharides in cardiac jelly (Plate 4, fig. Q) and later in septa of the heart (Plate 4, fig. N) and bulbus (Plate 4, fig. M). Also, the property of resiliency suggested here for this type of mucopolysaccharide is consistent with the function attributed to cardiac jelly by Barry (1948).

The presence of mucopolysaccharide in the dorsal mesentery (Plate 4, fig. L), and especially where it extends into the umbilical cord (Plate 4, fig. P), can be tentatively ascribed to a role of support. Finally, concentrations of reactive material in areas where epithelia have abrupt or elaborate contours (Plate 3, figs. H, I) may be associated with moulding or support, the latter function having been proposed already by Patten et al. (1948) for some gelatinous material like cardiac jelly.

It is perhaps worth emphasizing that the reason for suspecting an important relationship between sulphated mucopolysaccharides and the embryological events referred to above is not simply because this material is present but because the time at which it appears and the locations where it reaches the highest concentration are associated with events which are easiest to explain by assuming the involvement of a firm, elastic gel, such as the gels in which sulphated mucopolysaccharides can be found. Certainly, the exact basis for the movement of palatine shelves remains obscure, but further study of the sulphated mucopolysaccharides appears to be the most promising approach at present.

The potential application of the foregoing to experimental teratology is obvious when one considers the frequency with which the embryological processes just discussed are affected by teratogens like cortisone (Fraser & Fainstat, 1951; Walker & Crain, 1959), hypervitaminosis A (Kalter & Warkany, 1959), and vitamin A deficiency (Wilson & Warkany, 1949), all of which are known to interfere with the metabolism of mucopolysaccharides (Boström, 1958; Dziewitkowski, 1958; Fell, 1956). Similarly, Runner (1959) has related the action of several teratogens (which affected, primarily, the morphogenesis of embryonic neural tube and the differentiation of precartilaginous mesenchyme) to disturbances in carbohydrate metabolism; the latter can then be related to mucopolysaccharide synthesis (Whistler & Olson, 1957).

SUMMARY

1. Palatine shelf tissue was taken from mouse embryos at the stage of palate closure and studied with the electron microscope. The mesenchyme consisted of cells with long cytoplasmic processes embedded in a considerable quantity of ground substance, but no connective-tissue fibres were present. The oral epithelium covering this mesenchyme had no obvious structural specializations.

2. Sections of 15-day-old embryo heads were studied with various histochemical techniques and by radioautography after administration of Na$_2$S$_{35}O_4$. The distribution of aldehyde fuchsin-positive material corresponded to sites of
S$^{35}$ incorporation. No positive reactions were obtained with the orcinol-new fuchsin elastic fibre stain.

3. Earlier stages of embryogenesis were studied with mucopolysaccharide stains and with S$^{35}$ radioautography. Again, the distribution of aldehyde fuchsin-positive material corresponded closely to the areas of S$^{35}$ incorporation.

RESUME

L’Association de mucopolysaccharides avec la morphogenèse du palais et d’autres structures chez l’embryon de Souris

1. Du tissu des lames palatines est prélevé sur des embryons de Souris au stade de la fermeture du palais, et il est étudié au microscope électronique. Le mésenchyme consiste en cellules à longs prolongements cytoplasmiques enrobés dans une quantité considérable de substance fondamentale, mais on ne trouve pas de fibres de tissu conjonctif. L’épithélium oral qui couvre ce mésenchyme ne montre pas de structure spécialisée.

2. Des sections de têtes d’embryon de 15 jours ont été étudiées au moyen de techniques histochimiques variées et par autoradiographie après administration de Na$_2$S$^{35}$O$_4$. La distribution du matériel positif à la fuchsine-aldéhyde correspond aux niveaux d’incorporation de S$^{35}$. Aucune réaction positive n’a été obtenue avec coloration des fibres élastiques à la fuchsine néo-orcinol.


ACKNOWLEDGEMENTS

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REFERENCES


**EXPLANATION OF PLATES**

**PLATE 1**

Figs. A. Electron micrograph of mesenchyme from the palatine shelves. This tissue consists of cells with long processes and an extensive intercellular area. The site of S35 incorporation and aldehyde fuchsin reaction is believed to be the intercellular area. × 3800.

Figs. B. Electron micrograph of palate mesenchyme at higher magnification. The mesenchymal cell contains mitochondria and a small amount of granular endoplasmic reticulum. There are no elastic or collagenous fibres in the intercellular substance. × 8600.

Figs. C. Electron micrograph of oral epithelium from the palatine shelves. No special structural elements have been identified in this tissue which could be associated with the mechanism of palate closure. × 5400.

**PLATE 2**

Figs. D. A section through the head in the region of palate and nasal septum, taken from an embryo fixed shortly after closure of the palate. Aldehyde fuchsin-positive material is present in cartilage, at the epithelial-connective tissue boundaries, and in the form of a network throughout the palatine shelves. × 97.

Figs. E. Unstained radioautograph of a section through the head of an embryo taken from the
Plate 1

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Plate 3
same litter as the one represented in fig. D. Notice the exceptionally close parallel between the distribution of aldehyde fuchsin and S\textsuperscript{35}, both qualitatively and quantitatively. The dark reactions at the sides of the photo are over areas of membrane bone formation (which are not included in fig. D although they give a dark reaction with aldehyde fuchsin also). \times 91.

**Plate 3**

Sections from embryos of various ages stained with aldehyde fuchsin only.

**Fig. F.** Maxillary and nasal processes at the time of lip formation. The connective tissue gives a positive reaction. (The aldehyde fuchsin reactions are difficult to demonstrate photographically but the original colour distinctions are clearly defined in the slides.) \times 51.

**Fig. G.** The maxillary process at higher magnification. The distribution of stainable material as a network in the mesenchyme resembles that of the palatine shelves. The layer of stained material on the epithelial surface is probably derived from precipitation of the aldehyde fuchsin-positive material in amniotic fluid. \times 320.

**Fig. H.** A strong reaction outlines the lens and optic cup of the developing eye. \times 260.

**Fig. I.** Sharp contours of epithelium associated with eyelid and hair follicle are outlined by concentrations of aldehyde fuchsin-positive material in the mesenchyme. (Small areas of membrane bone formation to the extreme right give a very dark reaction.) \times 64.

**Fig. J.** The border of the enamel organ and material in the stellate reticulum stain strongly. The stellate reticulum is believed to be produced by cells of epithelial origin. \times 310.

**Fig. K.** The sheath of the notochord (top, centre) stains intensely with aldehyde fuchsin and is surrounded by mesenchyme containing a considerable amount of stainable material (lumen of pharynx is at the bottom of the photo). \times 280.

**Plate 4**

**Fig. L.** Gut (centre) and blood-vessels surrounded by a network of aldehyde fuchsin-positive material in the dorsal mesentery. \times 290.

**Fig. M.** Section through bulbus and aortic arches. The bulbar cushions have a large quantity of aldehyde fuchsin-positive material. \times 67.

**Fig. N.** The septum primum (centre) and endocardial cushion (lower centre) contain dense masses of material stained with aldehyde fuchsin. \times 67.

**Fig. O.** Radioautograph of an unstained section through the brachial arches and neural tube of an embryo which received S\textsuperscript{35}. The epithelial-connective tissue junctions around the neural tube and in the arches are radioactive, as is the connective tissue of the arches. The embryonic membranes are radioactive too. \times 65.

**Fig. P.** Radioautograph of a section through the abdominal region of an embryo which received S\textsuperscript{35}. The dorsal mesentery, extending into the umbilical cord with the gut, contains a large amount of radioactive material. \times 68.

**Fig. Q.** Radioautograph of a portion of the heart-tube at high magnification and stained lightly with eosin. The cardiac jelly between the outer wall and lumen (filled with erythrocytes) is overlaid by silver grains which are distributed in a manner similar to the material precipitated from cardiac jelly that is stained by aldehyde fuchsin. \times 340.

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