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Surface Coat Material Associated with the Developing Otic Vesicle

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SURFACE COAT MATERIAL ASSOCIATED WITH THE DEVELOPING OTIC VESICLE

by

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This Dissertation submitted by Allan R. Sinning in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota is hereby approved by the Faculty Advisory Committee under whom the work has been done.

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This Dissertation meets the standards for appearance and conforms to the style and format requirements of the Graduate School of the University of North Dakota, and is hereby approved.

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ABSTRACT

The purpose of the present study was to investigate the development of the otic placode/vesicle and the concurrent synthesis of an associated cell surface coat material (SCM) in the chick embryo. This was accomplished by means of precipitation of the glycoconjugate constituents of the SCM with cetylpyridinium chloride (CPC) and subsequent observation by scanning electron microscopy (SEM). Cryofixation and freeze-substitution were utilized to validate the results of CPC precipitation. In addition, specific sugar moieties present within the SCM were characterized, in part, using fluorescein isothiocyanate (FITC)-conjugated lectins.

Embryos utilized for SEM were incubated for 45-56 hours (stages 12-16), fixed in 2% glutaraldehyde with or without the addition of CPC and processed for conventional SEM. Additional embryos were cryofixed in liquid nitrogen cooled Freon 22 with or without prior aldehyde fixation and freeze-substituted in ethanol. Specimens were then warmed to room temperature, critical point dried and observed by SEM.

Embryos used for light microscopy (LM) were cryofixed and freeze-substituted prior to aldehyde fixation, brought to room temperature and embedded in paraffin. Preselected sections through the otic placode/vesicle were labeled with FITC-conjugated lectins. The latter included: concanavalin A (Con A), wheat-germ agglutinin (WGA) and soybean agglutinin (SBA).

At 45-48 hours (stage 12) the otic placode appeared as a depression within the surface ectoderm. By 48-52 hours (stage 13) the placode had continued to invaginate and formed a distinct pit. Closure of the deepened otic vesicle proceeded between 50-56 hours (stages 15-16) as evidenced by alteration in shape and reduction in size of the associated aperture.

Embryos of all ages revealed a flocculent precipitate over the surface ectoderm which was particularly abundant in association with the otic placode/vesicle when exposed to CPC. Specimens which were processed by freeze-substitution yielded a comparable precipitate. The otic placode/vesicle labeled positively with all lectins, the binding affinity of which followed the decreasing order: WGA>Con A>SBA. Differences in lectin binding between the surface ectoderm and the otic placode/vesicle were not apparent due to the low resolution and diffuse label given by fluorescein. The data clearly indicated that otic placode invagination was accompanied by the synthesis of copious amounts of SCM rich in glycoconjugates.

INTRODUCTION

The early development of the inner ear anlage or otic placode as viewed by light microscopy (LM) has been reviewed extensively by Romanoff (1960). The first detailed studies of the embryonic otic placode by scanning (SEM) and transmission (TEM) electron microscopy were presented by Bancroft and Bellairs (1977) and Meier (1978b), respectively.

These investigators reported that the initial indication of otic placode morphogenesis in the chick was represented by a thickening of the surface ectoderm overlying the developing rhombencephalon at approximately 26-29 hours (stage 8) of development (Meier, 1978a). At 33-38 hours (stage 10) the epithelial placode appeared as a shallow depression within the surface ectoderm which continued to invaginate to form a well defined pit by 48-52 hours (stage 13; Bancroft and Bellairs, 1977). Between 50-64 hours (stages 14-17) the aperture of the invaginating otic vesicle (otocyst) closed and the latter separated from the overlying surface ectoderm. Alteration in the shape of the developing vesicle was first apparent at stage 14 when the aperture became pear-shaped. By stage 16 the latter appeared as a narrow elliptical opening in the surface ectoderm and by stage 17 it was almost completely obliterated. At stage 18 the aperture was no longer visible on the surface of the embryo (Bancroft and Bellairs, 1977), however, the otic vesicle maintained a duct-like connection to the surface ectoderm for up to 104 hours of development (Hamburger and Hamilton, 1951).

Initially, the cells of the otic placode were not readily

differentiated from those of the adjacent surface ectoderm. However, after 23-26 hours (stage 7) the cells forming the otic placode were different ultrastructurally from those of the adjacent surface ectoderm. The apical surfaces of the cells that comprised the surface ectoderm were relatively flat and smooth. In contrast, the cells within the placode were elongated, bulged into the surrounding amniotic cavity, and possessed numerous blebs and other microappendages at their apical surfaces (Bancroft and Bellairs, 1977; Meier, 1978b). The most conspicuous appendages project from one cell to another located some distance away and contained a prominent discoid expansion midway along their length (Meier, 1978b). These projections resembled midbodies or beaded threads which were previously described by Bellairs and Bancroft (1975). Similar apical projections were observed in association with invaginating epithelia of the neural plate (Bancroft and Bellairs, 1974, 1975; Bellairs and Bancroft, 1975; Waterman, 1975, 1976; Mak, 1978; Schoenwolf, 1982), nasal placodes (Waterman and Meller, 1973; Bancroft and Bellairs, 1977), and lens placode (Bancroft and Bellairs, 1977; Van Rybroek and Olson, 1979, 1981).

As the cells within epithelial placodes elongated, their cytoplasm became populated with a large number of microtubules which were oriented parallel to the long axes of the cells (Bancroft and Bellairs, 1977; Meier, 1978b). Cellular elongation was thought originally to be due to the presence of microtubules (Karfunkel, 1974; Piatigorsky, 1975). Recent evidence suggests that this process may result from an increase in cell volume in the absence of microtubules (Beebe et al., 1979). Subsequent invagination of embryonic epithelia

appeared to be mediated by microfilaments which were abundant within the apical cytoplasm of the cells and were positioned perpendicular to their longitudinal axes (Wrenn and Wessels, 1969; Wessels et al., 1971; Karfunkel, 1971, 1972). Although it had been postulated that both microtubules and microfilaments were involved respectively in elongation and invagination of epithelial cells (Karfunkel, 1974; Odell et al., 1981), the exact role of these two organelles has not yet been elucidated (Schoenwolf, 1982).

The presence of a carbohydrate-rich cell surface coat material (SCM) or glycocalyx is thought to play an important role during invagination, adhesion, and fusion of epithelia during morphogenesis. SCM can be demonstrated by the nonspecific staining of polyanions with the cationic stains ruthenium red (Luft, 1971a, b) and alcian blue (Scott and Dorling, 1965), or by precipitation with cetylpyridinium chloride (CPC; Scott, 1955). More specific characterization of the SCM can be accomplished using lectins either radioactively labeled (Burk et al., 1979; Lotan, 1979) or conjugated to horseradish peroxidase (HRP; Bernhard and Avremeas, 1971), ferritin (Nicholson and Singer, 1971), colloidal gold (Horisberger and Rosset, 1977; Horisberger, 1984), biotin (Horisberger and Vonlanthen, 1979; Alroy et al., 1984), hemocyanin (Mak, 1978) or fluorescent dyes (Lotan, 1979). The cytochemical probes listed above have been used to demonstrate SCM associated with apical epithelial surfaces during development of the palate (Greene and Kochhar, 1974; Souchon, 1975; Pratt and Hassell, 1975; Meller and Barton, 1978; Baeckeland et al., 1982; Heinen et al., 1982), neural tube (Moran and Rice, 1975; Lee et al., 1977;

Lee et al., 1978; Mak, 1978; Sadler, 1978; Silver and Kerns, 1978; Rovasio and Monis, 1981; Currie et al., 1984), optic vesicle (Hilfer and Yang, 1980; Yang and Hilfer, 1982), nasal folds (Gaare and Langman, 1977; Smuts, 1977; Burk et al., 1979) and lens vesicle (Van Rybroek and Olson, 1981; Olson and Sinning, 1984).

The SCM is implicated to have a role in cellular recognition (Moscona, 1974), intercellular adhesion in several in vitro systems (Oppenheimer, 1973; Roseman, 1974; Biscoff, 1978) and epithelial adhesion and fusion during morphogenesis (Moran and Rice, 1975; Lee et al., 1977). The latter was supported by the fact that an increase in SCM has been shown to occur prior to fusion of the palatal shelves (Greene and Kochhar, 1974; Pratt and Hassel, 1975; Souchon, 1975), neural folds (Moran and Rice, 1975; Lee et al., 1977; Lee et al., 1978; Sadler, 1978; Silver and Kerns, 1978) and the epithelial margins of the lens vesicle (Van Rybroek and Olson, 1981). Once adhesion and eventual fusion have occurred there was a dramatic decrease in the amount of SCM on the epithelial surface (Moran and Rice, 1975; Souchon, 1975; Sadler, 1978). Adhesion and the subsequent fusion of epithelial surfaces can be inhibited by interfering with SCM synthesis through the administration of: 6-diazo-5-oxo-L-norleucine, a glutamine antagonist; tunicamycin, an antibiotic that inhibits N-glycosylation of glycoproteins; and carrageenan, a food additive that is thought to inhibit synthesis of cell surface components. Exposure to these substances subsequently results in facial clefts (Burk and Sadler, 1983), reduced fusion of palatal shelves in vitro (Greene and Pratt, 1977), abnormal optic cup formation (Yang and Hilfer, 1982) and neural tube

defects (Rovasio and Monis, 1981).

Lectins are sugar-binding proteins or glycoproteins of non-immune origin from plants and animals which agglutinate and/or precipitate glycoconjugates. They have been used increasingly to characterize the carbohydrate moieties present in the SCM (Sharon and Lis, 1972; Lis and Sharon, 1973; Nicholson, 1974; Kornfeld and Kornfeld, 1978; Alroy et al., 1984; Horisberger, 1984). They contain at least two sugar-binding sites, the specificity of which is defined in terms of the monosaccharide or simple oligosaccharide that inhibits lectin-induced reactions using the lowest concentration of sugar (Goldstein et al., 1980). A classification system for lectins has been proposed by Gallagher (1984) in which two major classes of lectins are defined. Class I, or exolectins recognize the complimentary monosaccharide located primarily at the non-reducing end of the carbohydrate chain. Class II, or endolectins recognize only linear or branched oligosaccharide chains. Class I lectins are subdivided into obligate or facultative exolectins depending on whether they bind only end-chain or end-chain and internal sugars, respectively. Class II lectins are likewise subdivided into homotypic or heterotypic endolectins in accordance with their binding of homotypic or heterotypic sugar sequences.

The common lectins concanavalin A (Con A) from Canavalia ensiformis (jack bean), wheat-germ agglutinin (WGA) from Triticum vulgaris and soybean agglutinin (SBA) from Glycine max were utilized in the present study. The structure and function of these and other lectins have been reviewed extensively (Sharon and Lis, 1972; Lis and Sharon,

1973; Nicholson, 1974; Brown and Hunt, 1978; Goldstein and Hayes, 1978; Barondes, 1981; Alroy et al., 1984). Concanavalin A, a facultative exolectin, is one of the few lectins that is not a glycoprotein since it contains no covalently bound sugar (Sharon and Lis, 1972). It is a tetramer with a molecular weight of approximately 104,000. It is specific for α-D-glucosyl and α-D-manosyl residues. It requires Ca^{+2} and Mn^{+2} ions for its activity, the removal of which will destroy its binding capacity (So and Goldstein, 1968).

Wheat-germ agglutinin, a homotypic endolectin, is a dimeric protein with a molecular weight of 36,000. It has a binding affinity for N-acetyl-glucosamine (Glc-Nac) and N-acetyl-neuraminic acid (sialic acid). The binding of WGA to these two substances can be distinguished by means of charge affinity. Normally, WGA is positively charged and will bind to the negatively charged sialic acid and the neutral Glc-Nac. However, upon succinylation WGA is negatively charged and will bind only to Glc-Nac (Monsigny et al., 1980).

Soybean agglutinin, an obligate exolectin, is a tetrameric glycoprotein with a molecular weight of 120,000. It shows binding affinity for α-D-galactose and N-acetyl-galactosamine (Gal-Nac; Lis et al., 1970; Hammarström et al., 1977).

In view of the studies described above concerning the involvement of SCM in epithelial placode morphogenesis it seemed appropriate to examine the developing otic placode/vesicle in order to verify a similar phenomenon. Specifically, this study will attempt to reveal the presence of a carbohydrate-rich surface coat material associated with the developing otic placode/vesicle by means of nonspecific

precipitation of SCM glycoconjugates with CPC, and by cryopreservation and freeze-substitution without prior chemical fixation. All specimens will subsequently be observed by means of scanning electron microscopy. In addition, the current study will attempt, for the first time, to characterize the carbohydrate moieties present within the SCM associated with an epithelial placode. The latter will be accomplished through binding specific sugar receptor sites with the lectins Con A, WGA, and SBA conjugated to fluorescein isothiocyanate (FITC) and viewed by epifluorescence microscopy.

MATERIALS AND METHODS

Fertile chicken eggs were incubated in a forced air incubator at 37°C for 45-56 hours. Eggs were removed from the incubator, opened and the contents were emptied into a 70mm petri dish containing warm Hank's saline. Embryos were excised and removed from the yolk. Following removal of the vitelline membrane and amnion, embryos were washed repeatedly in warm Hank's saline and subsequently staged according to Hamburger and Hamilton (1951). Embryos representing stages 12-16 (45-56 hours) were then processed for either scanning electron microscopy (SEM) or light microscopy (LM).

Scanning Electron Microscopy

Embryos utilized for SEM were fixed for 2 hours in cacodylate buffered 2% glutaraldehyde (pH 7.4) with or without the addition of 0.5% cetylpyridinium chloride (CPC; pH 7.2). The latter is a quarternary ammonium compound which precipitates polyanionic substances, including SCM components, and prevents their extraction during fixation (Spicer et al., 1967; Markwald et al., 1978; Van Rybroek and Olson, 1981). After rinsing in 0.2M cacodylate buffer, embryos were post-fixed in 2% osmium tetroxide (OsO_4) in 0.144M cacodylate buffer. Subsequent to rinses in 0.144M buffer, embryos were dehydrated in a graded series of ethanol and critical point dried in liquid CO_2 in a Samdri PVT-3 critical point drying apparatus.

Additional embryos were placed on aluminum foil strips and frozen in liquid nitrogen (LN) cooled Freon 22 (Plattner and

Bachmann, 1982) with or without prior aldehyde fixation as described above. The frozen embryos were placed into liquid scintillation vials containing a layer of molecular sieves and 5 ml each of 1% OsO_4, and 100% ethanol and 10 ml of LN. The vials were placed into an ultralow freezer set at -60°C for 7 days, then into a standard freezer for 12 hours and finally into a conventional refrigerator for 6 hours (Markwald, personal communication). After rinsing in 100% ethanol in order to remove the excess OsO_4, the embryos were critical point dried as previously mentioned. All specimens were mounted on aluminum stubs with silver paint, coated with gold-palladium (60:40) in a Hummer I sputter coater and viewed in a Hitachi S-800 scanning electron microscope at 15KV.

Light Microscopy

Embryos used for LM were removed from the egg, rinsed in Hank's saline and frozen as described above without prior aldehyde fixation. These embryos were placed in liquid scintillation vials containing a layer of molecular sieves and 10 ml each of 100% ethanol and LN. Vials were placed in an ultralow freezer at -60°C for 4-5 days, in a conventional freezer for 12 hours and then into a standard refrigerator for 6 hours (Markwald, personal communication). Upon removal from the refrigerator, the samples were transfered to embedding bags and placed in 100% chloroform for 2 hours, followed by 2 changes of 100% paraffin. Embryos were then embedded in paraffin-filled beam capsules, sectioned at 8µm on a Sorval JB-4A microtome, and placed on slides that were treated with deglycosylated bovine serum albumin (dBSA) in

phosphate buffered saline (PBS; Glass et al., 1981).

Lectin Binding

Preselected slides with unstained paraffin tissue sections through the developing otic placode or vesicle were deparaffinized and rehydrated to dBSA/PBS. Slides were left in this solution for a minimum of 15 minutes, and then incubated in fluorescein isothiocyanate (FITC)-conjugated lectins (50µg/ml) in PBS containing 0.1mM $CaCl_2$ for 20 minutes at the appropriate pH (Table I). Following rinses in PBS, slides were coverslipped using glycerol.

Control sections were deparaffinized and held in dBSA/PBS as described above. Controls were then incubated in PBS containing the inhibitory sugar (PBS/sugar) for each lectin at the appropriate pH (Table I) for 20 minutes and subsequently incubated in 50µg/ml FITC lectin in PBS/sugar for 20 minutes. Slides were rinsed in PBS/sugar and coverslipped as before. All tissue sections were viewed on an Olympus BH2 light microscope equipped with epifluoresence.

TABLE I

PARAMETERS FOR LECTIN BINDING

LECTIN	INHIBITORY SUGAR	PH
Con A	.3M Methyl-α-D-manoside	6.9
WGA	.2M N-acetyl-glucosamine	6.8
SBA	.2M N-acetyl-galactosamine	6.8

RESULTS

At 45-48 hours (stage 12) the otic placode was visible as a shallow depression within the surface ectoderm overlying the developing rhombencephalon (Fig. 1). The cells within the placode were smaller in diameter and their cell borders were not as well defined as those of the surrounding surface ectoderm (Figs. 2, 3). With the addition of CPC to the fixative, a flocculent precipitate was observed over the surface ectoderm, particularly in association with the otic placode (Fig. 4).

By 48-52 hours (stage 13) the placode was more extensively invaginated and now may be referred to as the otic pit (Fig. 5). The precipitate produced with the addition of CPC to the fixative was heavier than during the previous stage (Fig. 6). The floor of the otic pit revealed cells which had irregularly defined lateral borders, and small apical surface areas with numerous blebs and other micro-appendages (Fig. 7). In embryos treated with CPC, the precipitate obscured the floor of the otic pit (Fig. 8).

The otic pit continued to invaginate to form the otic vesicle by 50-53 hours (stage 14). The aperture of the vesicle was circular in shape (Figs. 9, 10). The dense precipitate observed with the addition of CPC to the fixative was largely confined to the immediate area of the vesicle and was sparsely associated with the surrounding surface ectoderm (Figs. 11, 12).

The aperture of the developing otic vesicle was smaller, indicating that it began to close by 50-55 hours (stage 15). The aperture showed

a sequential alteration in shape, changing from a rounded structure (Figs. 13, 14) to one which was oblong or elliptical at the onset of epithelial adhesion and fusion (Fig. 15). Tissues fixed in glutaraldehyde/CPC revealed a localized precipitate over the area of the developing otic vesicle with greater accumulation of precipitate on one side (Fig. 16).

The aperture of the deepened otic vesicle at 51-56 hours (stage 16) of incubation was noticeably smaller than during the previous stage (compare Fig. 17 with Figs. 15 and 16). The opening had reduced in size from an average diameter of 58μm at stage 14 to an average maximum length and width of 50μm x 11μm, respectively, at stage 16. As in stage 15, the CPC precipitable material was largely localized within the developing vesicle with a greater accumulation toward one side (Fig. 18).

The differing extent of the CPC precipitate at the epithelial margins of the otic vesicle aperture was clearly evident in Figures 6, 11, 12, 16, 18, and 19. The cells which comprised the adjacent normal surface ectoderm were larger in circumference and displayed relatively little precipitate. Proceeding to the margins of the otic vesicle aperture, the cells became smaller and showed a more dense accumulation of CPC precipitable material. The latter was increasingly apparent within the lumen of the forming otic vesicle where the precipitate completely obscured the apical surfaces of the cells (Fig. 19).

Due to the required extensive washing of specimens and the nature of the precipitate following fixation in glutaraldehyde/CPC,

it was possible that the results were artifactually produced. In an attempt to validate the above results, a freeze-substitution technique was employed (Plattner and Bachmann, 1982; Markwald, personal communication). Embryos which were pre-fixed in 2% glutaraldehyde and then frozen in LN cooled Freon-22 gave identical results to those processed conventionally (Fig. 20). With the addition of CPC to the aldehyde fixative prior to freezing, a pattern of precipitate very similar to that seen previously was observed (compare Fig. 21 with Figs. 6, 11, 16, and 18). Moreover, embryos that were frozen without prior aldehyde fixation yielded a precipitate very similar to that seen with glutaraldehyde/CPC fixation with or without subsequent freeze-substitution (Fig. 22).

In order to specifically characterize the sugar moieties present within the SCM, the FITC-conjugated lectins Con A, WGA, and SBA were utilized. The binding of the latter to representative paraffin embedded sections of early (stages 12-13), middle (stages 14-15) and late (stage 16) stages of otic placode/vesicle development are shown in Figures 23-31. Sections labeled for Con A binding during early vesicle development showed a labeling of the apical surface of the epithelium and the associated basal lamina (Fig. 23a). Sections labeled for WGA (Fig. 24a) and SBA binding (Fig. 25a) showed similar results but with different levels of binding affinity. However, the binding of WGA appeared to be more diffuse throughout the thickness of the placode epithelium. Control sections showed virtually no labeling with the exception of the basal lamina in WGA controls (Figs. 23b, 24b, and 25b).

The results from the middle stage of otic vesicle development are shown in Figures 26-28. Labeling was similar to that seen in the previous group. The localization of Con A binding to the apical surface of the otic vesicle epithelium is evident in Figure 26a. The binding of WGA (Fig. 27a) continued to be more diffuse than Con A and extended throughout the entire thickness of the epithelium. The binding of SBA was still relatively low but was more intense than in the previous stage (compare Figs. 28a and 25a). Control sections yielded results which were similar to those observed previously (Figs. 26b, 27b, 28b).

Lectin binding during the late stage of otic vesicle formation is shown in Figures 29-31. Although the binding of Con A (Fig. 29a) and WGA (Fig. 30a) appeared to show a reduction of binding affinity, the results were very similar to the previous stages. Sections labeled with SBA (Fig. 31a) revealed increased labeling of the basal lamina and the apical epithelial surface when compared to previous stages. Control sections showed no labeling above background levels (Figs. 29b, 30b, 31b).

The binding of all lectins revealed the presence of a whispy material at the apical surface of the otic vesicle epithelium which extended into the lumen (Figs. 23a, 24a, 26a, 27a, 29a, 30a, 31a). The surface ectoderm showed no distinct difference in binding from that of the developing vesicle (Figs. 24a, 25a, 31a). The intensity of the binding was different for the various lectins, in that WGA> Con A>SBA. The binding affinity of the tissue for SBA appeared to increase during otic vesicle development (compare Figs. 25a, 28a,

31a), whereas that for Con A and WGA remained constant. The basal lamina was labeled in all instances, moreover, the binding affinity of WGA to the basal lamina was intense enough to not be inhibited at the utilized sugar concentration (Fig. 27b).

DISCUSSION

The overall development of the otic vesicle (otocyst) as reported in the current study was similar to that previously described (Bancroft and Bellairs, 1977; Meier, 1978b). This morphogenetic process began by the establishment of the otic placode, which appeared as a localized thickening of the surface ectoderm overlying the developing rhombencephalon. As development continued, the otic placode proceeded to invaginate and form the otic pit and subsequent otic vesicle. The epithelial margins which surround the aperture of the developing otic vesicle then approached one another and eventually adhered and fused. The vesicle then separated from the overlying surface ectoderm.

The present study demonstrates that the process of epithelial invagination was accompanied by an apparent increase in the amount of CPC precipitable material located within the lumen of the developing otic vesicle. This precipitate was flocculent in appearance, was evident as early as 45-48 hours (stage 12) of development, and is believed to represent SCM components. A similar precipitate was observed following fixation in glutaraldehyde/CPC in association with the developing lens vesicle (Van Rybroek and Olson, 1981; Olson and Sinning, 1984) and during optic cup formation (Hilfer and Yang, 1980), and following alcian blue-lanthanum nitrate staining during amphibian gastrulation (Moran and Mouradian, 1975). Similarly, an increase in SCM has been observed with the utilization of the cationic stain, ruthenium red, associated with the developing palatal shelves (Greene and Kochhar, 1974; Souchon, 1975; Meller and Barton, 1978),

neural folds (Mak, 1978; Sadler, 1978; Rovasio and Monis, 1981), nasal folds (Gaare and Langman, 1977) and lens vesicle (Van Rybroek and Olson, 1981).

The nature of the nonspecific precipitate seen with glutaraldehyde/CPC fixation could be artifactual. Proteins in the amnionic fluid present during fixation, may be precipitated onto the surface ectoderm and accumulate within the lumen of the developing otic vesicle. Two methods were used to reduce the probability of this occurance. The first involved removal of the vitelline membrane and amnion followed by extensive washing of the embryo in saline solution prior to fixation. This process should rinse away most, if not all, extraneous proteins prior to fixation. The second method was to combine the removal of the vitelline membrane and amnion with subsequent cryofixation and freeze-substitution. The ultimate goal of cryofixation was to fix instantly all cell components in their momentary random distribution (Plattner and Bachman, 1982; Plattner and Knoll, 1982). The latter included glycoproteins and glycolipids of the SCM which were precipitated on the cell surface (Feder and Sidman, 1958). Embryos fixed in 2% glutaraldehyde or glutaraldehyde/CPC prior to freezing gave results similar to those processed conventionally. This indicates that the washing procedure was effective in eliminating any amnionic proteins that were present. Since rinsing and manipulation of the tissue during cryofixation and freeze-substitution were kept at a minimum, there was less chance for artifactual accumulation of precipitate within the lumen of the otic vesicle. This was further supported by results from embryos that were frozen without prior

aldehyde fixation. These specimens showed a precipitation pattern very similar to that seen with glutaraldehyde/CPC. Similar results comparing CPC precipitation and freeze-substitution have also been reported by Kitten et al. (1981).

Lectins have been used increasingly to characterize the carbohydrate moieties present within the SCM (Sharon and Lis, 1972; Lis and Sharon, 1973; Nicholson, 1974; Kornfeld and Kornfeld, 1978; Alroy et al., 1984; Horisberger, 1984). The data from the current study indicate that the SCM associated with the developing otic vesicle contains molecules that bind positively with the specific lectins used in the study. This is in agreement with similar studies involving the SCM associated with the developing neural tube (Mak, 1978; Currie et al., 1984), nasal folds (Smuts, 1977; Burk et al., 1979), and palate (Pratt and Hassell, 1975; Baeckeland et al., 1982; Heinen et al., 1982).

The binding intensity for the different lectins was not identical. The pattern of binding affinity revealed that WGA>Con A>SBA. The intense affinity of the SCM for WGA would be expected since the plasmalemma is known to contain large amounts of sialic acid (Wallach and Kamat, 1966). It was not possible in the present study to discern the relative intensity of WGA binding with sialic acid, since WGA binds similarly to both sialic acid and N-acetyl-D-glucosamine (Glc-Nac). The use of succinylated WGA would overcome this problem because it will bind only to Glc-Nac. The binding affinity of the tissue for Con A, which is specific for α-D-glucose and α-D-manose residues, appeared to remain constant during the sequential developmental stages.

The binding affinity of the tissue for SBA, which is specific for galactose and N-acetyl-galactosamine residues, increased as the vesicle developed, so that by later stages it matched or slightly exceeded that of Con A.

The localization of binding for the lectins is also of interest. WGA labeled cells throughout the entire thickness of the placode epithelium, whereas the binding of Con A and SBA was limited to the apical region of the otic vesicle epithelium. The basal lamina was labeled in later stages of development by all three lectins. The binding affinity of WGA and SBA to the basal lamina was so intense that it remained labeled even in some control sections. Similar results of non-inhibited WGA binding has been observed in matrix granules during neural crest cell migration (Brauer; personal communication).

In the present study, the relative thinness of the surface ectoderm combined with the intense positive labeling of the basal lamina made it impossible to resolve differences in lectin binding between the surface ectoderm and the developing otic vesicle, even though previous reports on neural tube formation (Currie et al., 1984) and palatogenesis (Pratt and Hassell, 1975; Baeckeland et al., 1982) have reported such differences. An explanation for this discrepency may be the use of FITC-conjugated lectins in the present study, as opposed to a more localized label such as ferritin. In support of this is the study by Baeckeland et al. (1982) in which both ferritin and FITC-conjugated Con A were utilized. This study reported that a localized ferritin-Con A binding pattern was observed similar to that

seen with ruthenium red staining in the palate (Greene and Pratt, 1974) and neural tube (Sadler, 1978). However, when FITC-conjugated Con A was used, a diffuse labeling of all epithelial cells was observed. Moreover, it was inferred that the differences which were observed were, in part, related to the inherent differences between the two techniques, in that one dealt with whole palatal shelves (ferritin-Con A) while the other invovled sectioned material (FITC-Con A).

Results from both lectin binding and CPC precipitation studies indicate that both methods identify comparable structures. Both are believed to demonstrate carbohydrate moieties, but this has not been conclusively shown. However, recent studies have combined CPC precipitation with either Con A binding (Grote and Fromme, 1984a; Brauer et al., 1985) or silver proteinate staining (Grote and Fromme, 1984b) which have demonstrated labeling of the electron dense precipitate, which suggests the presence of glycoconjugates.

The increased precipitate seen at one edge of the otic vesicle aperture at stages 15 and 16 is interpreted to represent the area of active adhesion and fusion of the epithelial margins which surround the aperture. This is supported by earlier reports which have shown an increase in the SCM prior to and during fusion of the neural folds (Moran and Rice, 1975; Lee et al., 1977; Lee et al., 1978; Sadler, 1978; Silver and Kerns, 1978) and palatal shelves (Greene and Kochhar, 1974; Pratt and Hassell, 1975; Souchon, 1975). This was supported further by studies which have used either Con A (Lee et al., 1977) or neuraminadase treatment (Heinen et al., 1982), which reduced the amount

of SCM, and resulted in reduced fusion of the neural folds and palatal shelves, respectively. Moreover, inhibitors of glycoconjugate synthesis including 6-diazo-5-oxo-L-norleucine, tunicamycin and carrageenan have been shown to result in facial clefts (Burk and Sadler, 1983), neural tube defects (Rovasio and Monis, 1981), reduced fusion of palatal shelves *in vitro* (Greene and Pratt, 1977) and abnormal optic cup formation (Yang and Hilfer, 1982). All of these studies support the association of glycoconjugates with epithelial adhesion and fusion.

The present study has confirmed the presence of SCM at the apical epithelial surface of the developing otic placode and subsequent otic vesicle. This was achieved by the precipitation of SCM components with glutaraldehyde/CPC. In an attempt to verify the CPC results, additional embryos were processed by cryofixation followed by freeze-substitution without prior aldehyde fixation. These embryos yielded results similar to those fixed in glutaraldehyde/CPC. Sugar moieties within the SCM were characterized, in part, using FITC-conjugated lectins. Although all lectins reacted positively with the SCM, it was not possible to detect differences between the surface ectoderm and the developing otic vesicle due to the limited resolution of the technique. It is believed that the use of a more discretely localized labeling technique such as ferritin or colloidal gold would overcome this problem and yield data with improved resolution. In turn, any existing differences in lectin binding affinity for the otic vesicle epithelium and the surrounding surface ectoderm could be readily enhanced.

APPENDIX A

LEGEND TO FIGURES

A - apical surface of otic placode/vesicle epithelium

B - entire thickness of otic placode/vesicle epithelium

LV - lens vesicle

OP - otic placode/pit

OV - otic vesicle

PA - first pharyngeal arch

SE - surface ectoderm

APPENDIX B

PLATES AND FIGURES

PLATE I

Fig. 1. Head region of chick embryo (45-48 hours; stage 12). The otic placodes (OP) are easily distinguished from the surrounding surface ectoderm. x 80.

Fig. 2. Invaginating otic placode (45-48 hours; stage 12). This micrograph represents a higher magnification of the embryo in Figure 1. The cells within the otic placode (OP) appear smaller than those of the adjacent surface ectoderm (SE). x 1,000.

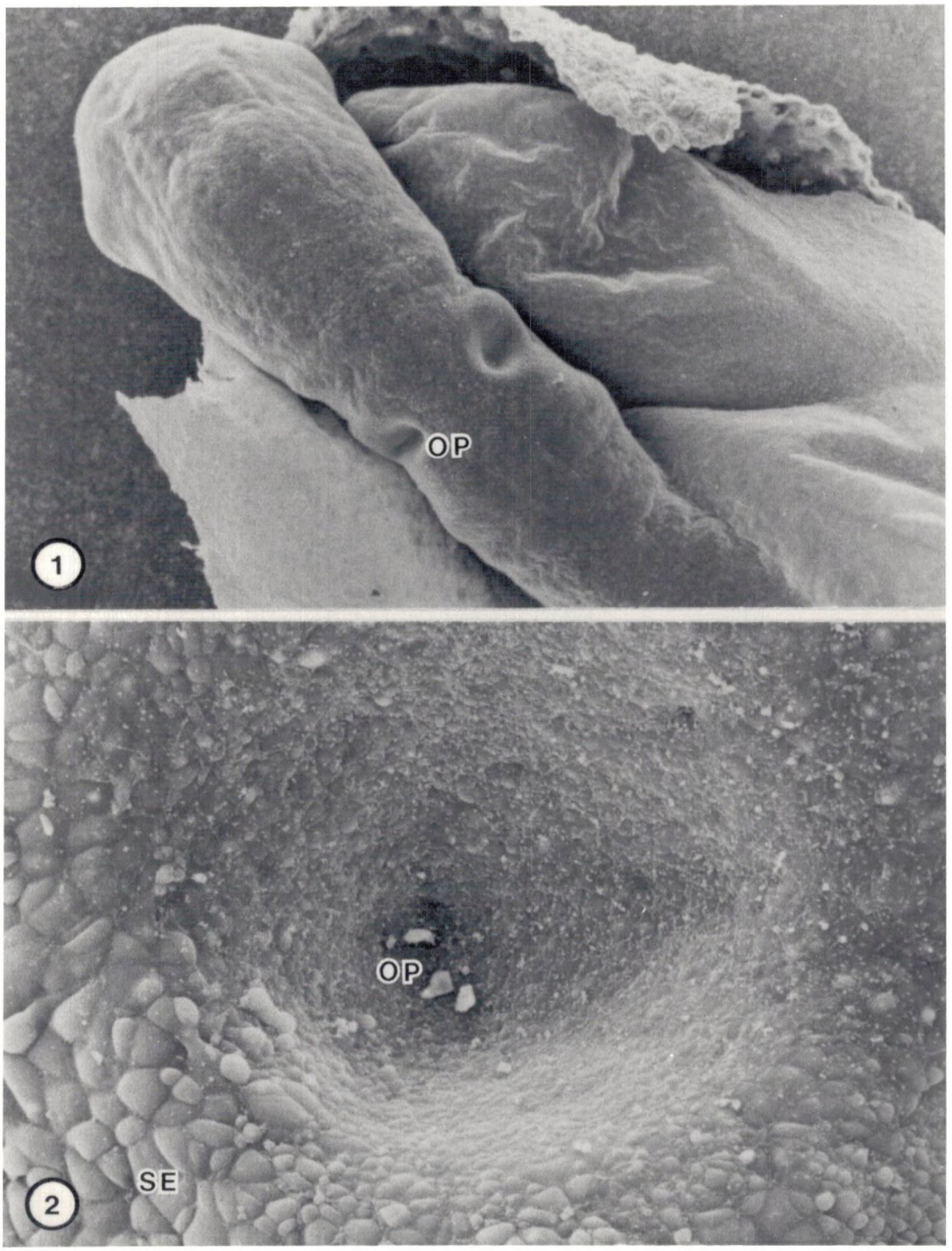
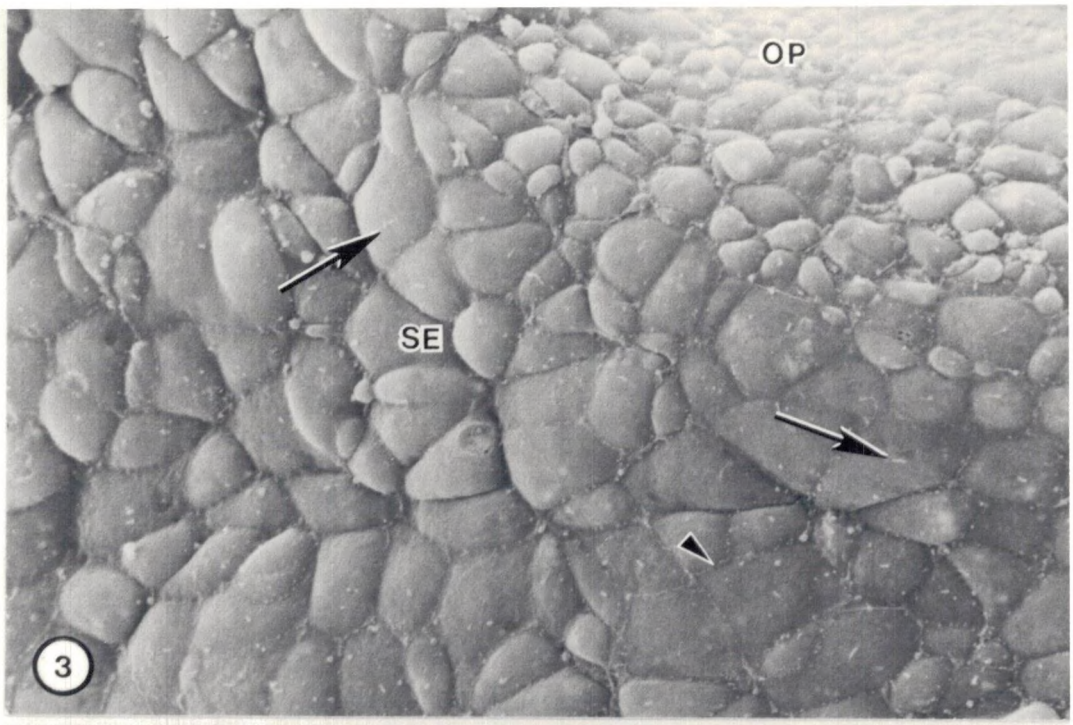

OP
1
OP
SE
2

PLATE II

Fig. 3. Transition zone between the surface ectoderm and otic placode (45-48 hours; stage 12). The cells comprising the surface ectoderm (SE) are larger, possess a single cilium (arrows), and exhibit microvilli along their lateral borders (arrowhead). The cells within the otic placode (OP) are noticeably smaller and protrude extensively from the surface of the embryo. x 1,800.

Fig. 4. Glutaraldehyde/CPC fixation (45-48 hours; stage 12). A flocculent precipitate (arrowheads) is seen overlying the surface ectoderm (SE); it is particularly abundant over the developing otic placode (OP). x 700.


OP
SE
3

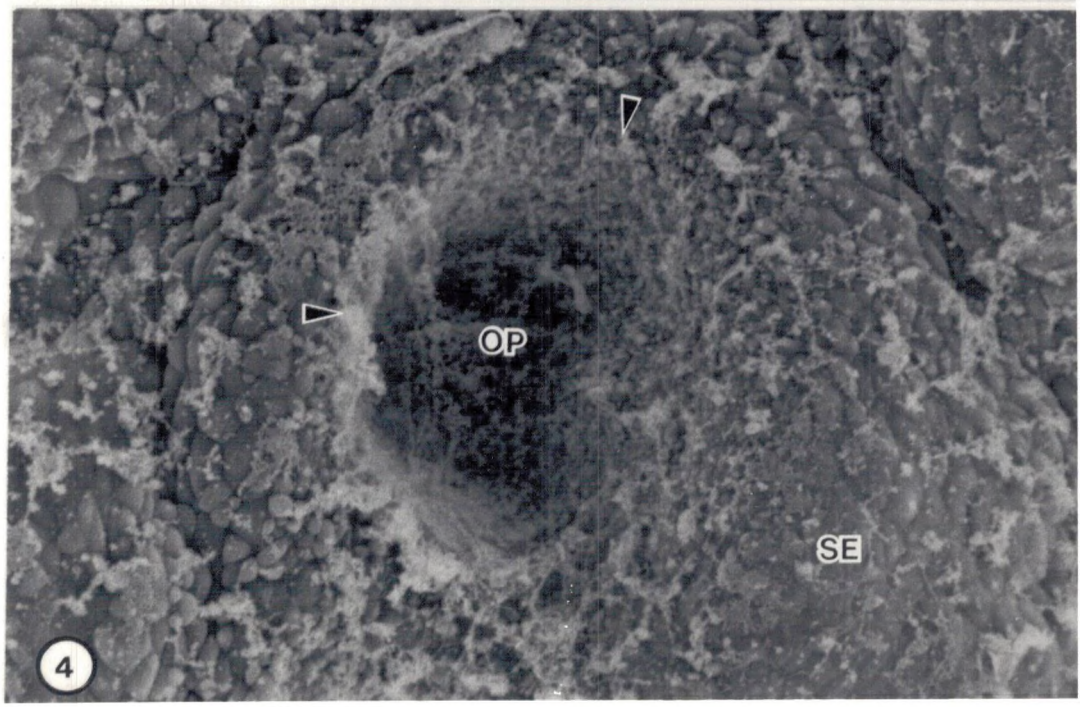
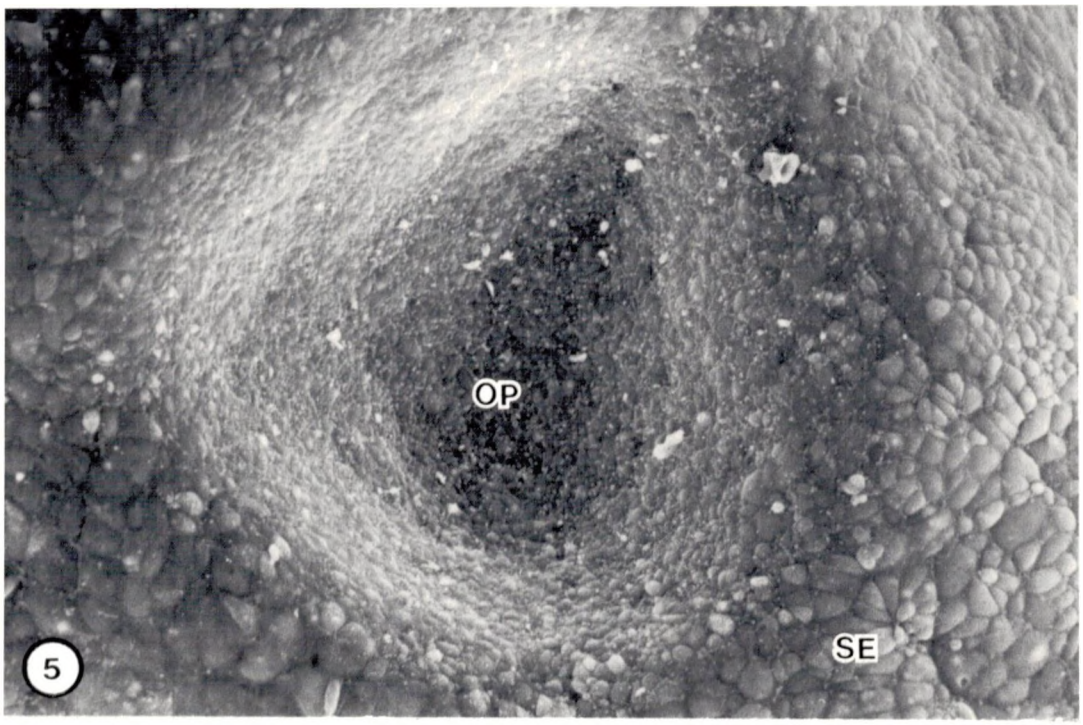

OP
SE
4

PLATE III

Fig. 5. Developing otic pit (48-52 hours; stage 13). At this time the otic placode (OP) is more extensively invaginated and may be referred to as an otic pit. Note significant differences in the size of the cells comprising the surface ectoderm (SE) when compared to those of the otic pit. x 700.

Fig. 6. Invaginating otic placode (48-52 hours; stage 13). The addition of CPC to the aldehyde fixative results in a heavy flocculent precipitate (arrows) largely confined to the forming otic pit (OP) contrasted with the surface ectoderm which remains relatively clean. x 1,900.


OP
SE
5

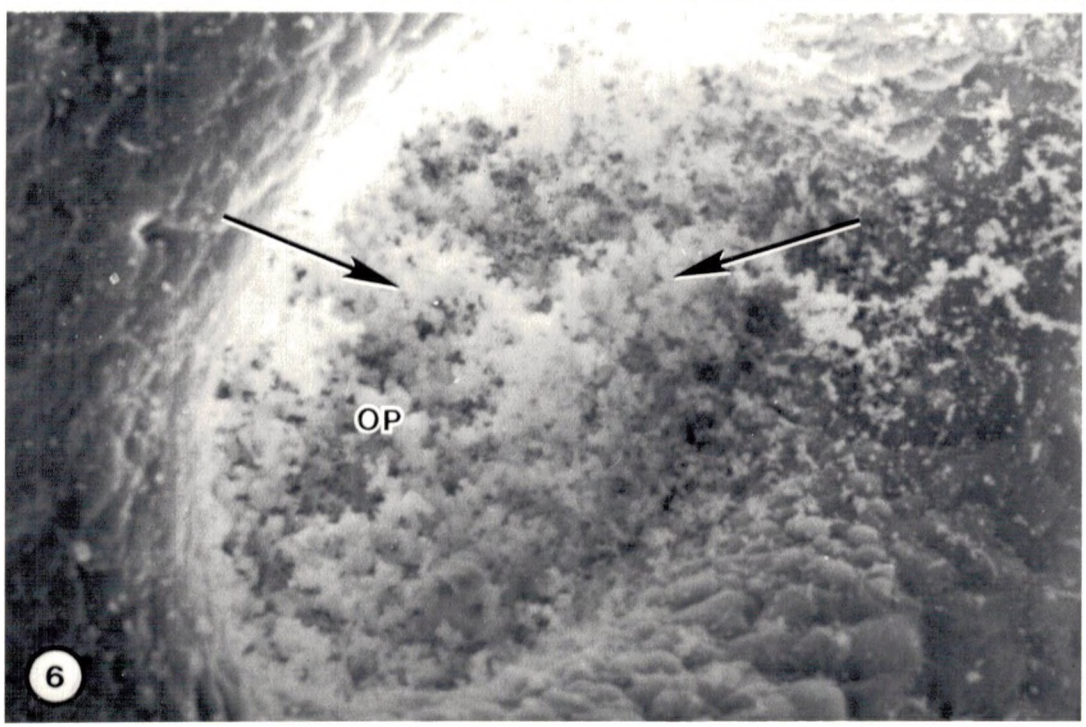
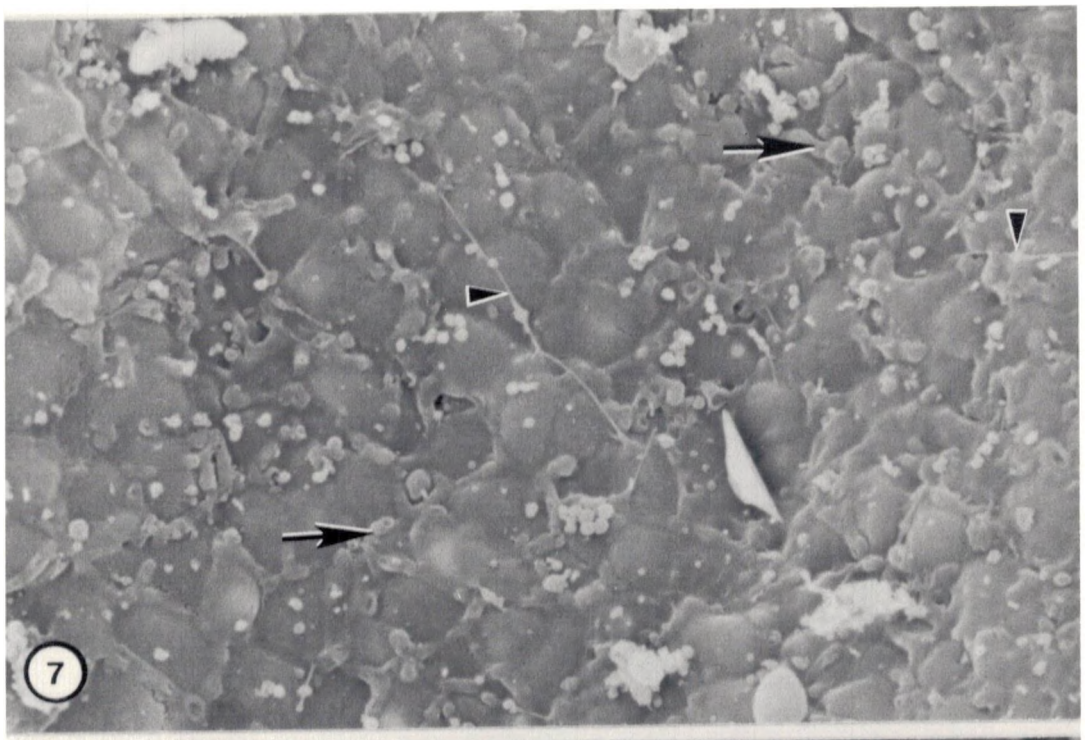

OP
6

PLATE IV

Figs. 7, 8. Floor of otic pit (48-52 hours; stage 13). The floor of the otic pit is characterized by cells with indistinct lateral borders, possessing numerous blebs (arrows) and beaded threads (arrowheads). With the addition of CPC to the fixative (Fig. 8) the precipitate obscures the floor of the pit.
x 5,400, Fig. 7; x 5,800, Fig. 8.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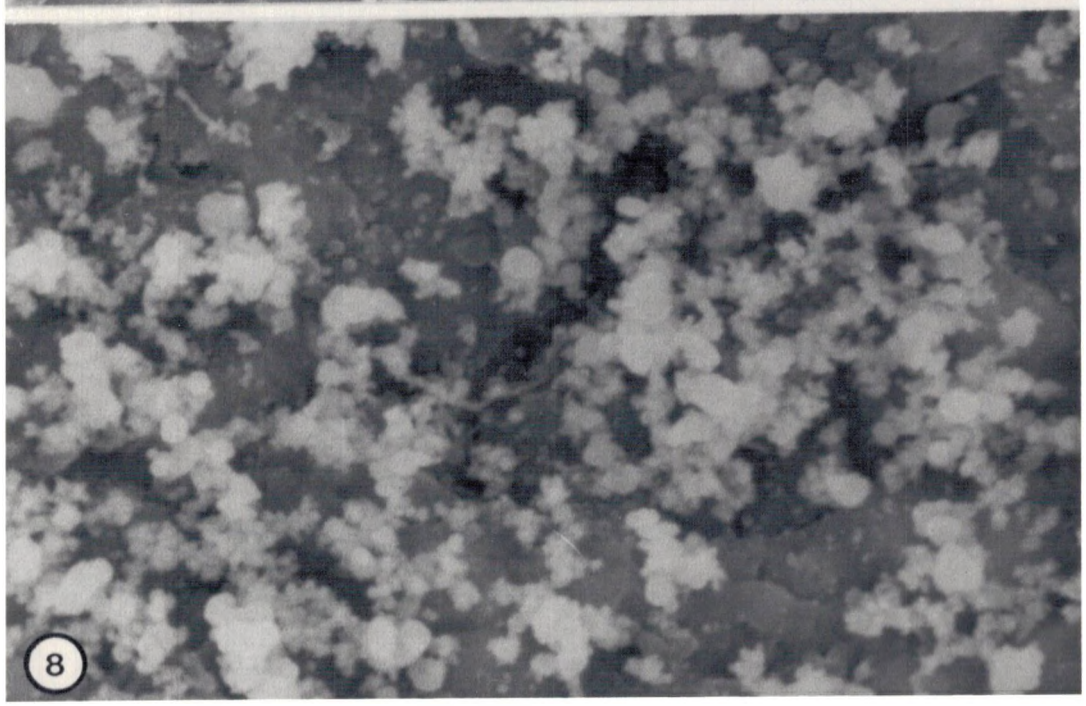

8

PLATE V

Fig. 9. Head region of embryo (50-53 hours; stage 14). The forming otic vesicle (OV) is more extensively invaginated. The head is turned to the right and the lens vesicle (LV) is also present. x 80.

Fig. 10. Developing otic vesicle (50-53 hours; stage 14). The otic vesicle is deeper than in the previous stage and the aperture is rounded in shape. x 675.

Fig. 11. SCM associated with otic vesicle (50-53 hours; stage 14). The precipitate (arrows) formed with glutaraldehyde/CPC is localized to the region of the otic vesicle (OV). The surface ectoderm (SE) possesses little SCM as indicated by the small amount of precipitate. x 1,400.

Fig. 12. Margin of the otic vesicle aperture (50-53 hours; stage 14). The demarcation of CPC precipitatable material is very evident. The larger cells of the surface ectoderm (SE) show relatively little precipitate. The amount of precipitate increases at the margin of the aperture and completely obscures the epithelium which lines the lumen of the otic vesicle (OV). x 3,800.

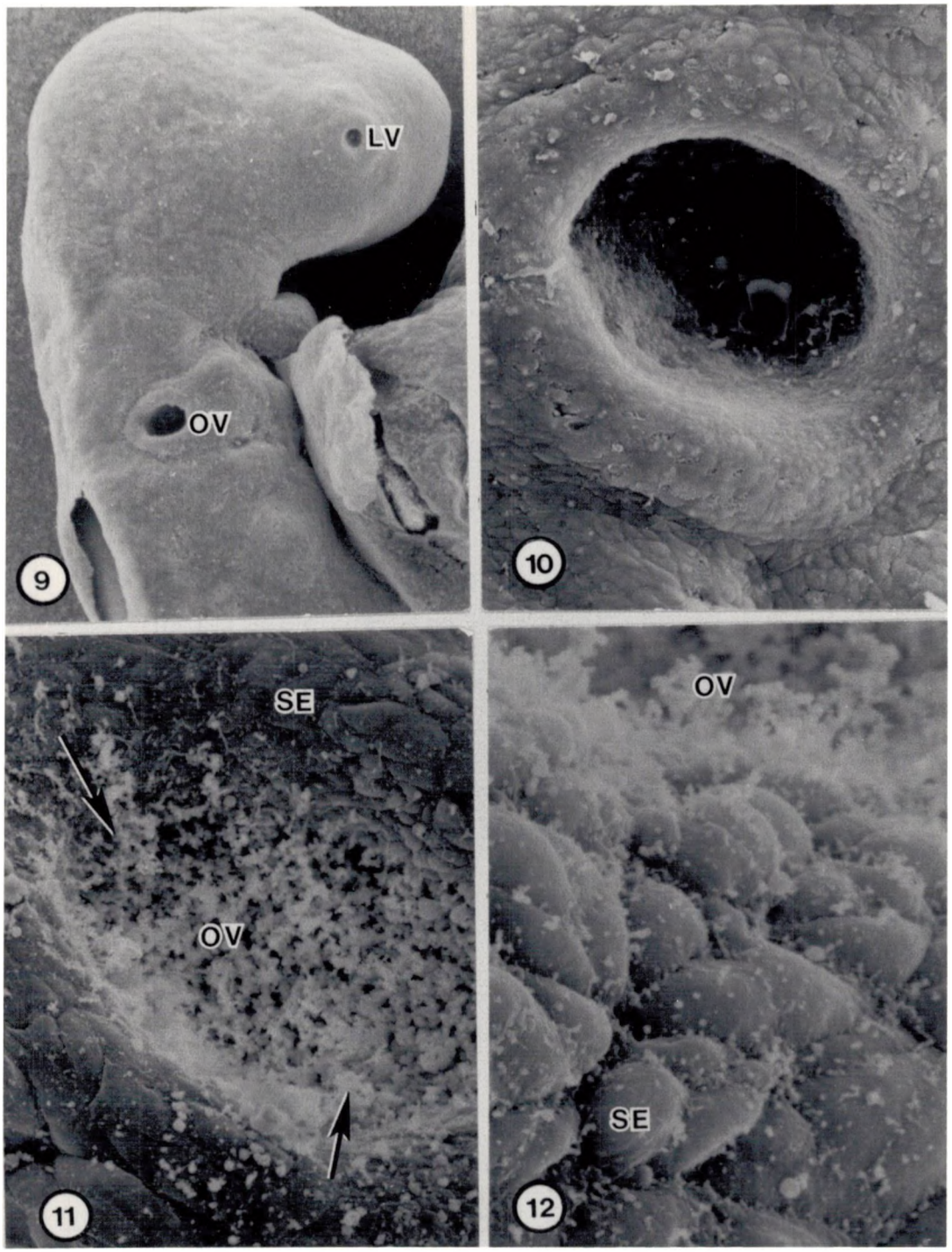

LV
OV
9
10
SE
OV
11
OV
SE
12

PLATE VI

Fig. 13. Head region (50-55 hours; stage 15). The forming otic vesicle (OV) is evident at the same level as the developing heart. The developing lens vesicle (LV) is evident as is the first pharyngeal arch (PA). x 75.

Fig. 14. Aperture of otic vesicle (50-55 hours; stage 15). The aperture of the otic vesicle (OV) is beginning to change to an elliptical shape. The vesicle has continued to invaginate which is apparent since its floor is largely hidden from view. The surface ectodermal cells remain large and possess microvilli (arrowhead) along their lateral borders. x 800.

Fig. 15. Elliptical aperture of otic vesicle (50-55 hours; stage 15). The aperture is now elliptical in shape, indicating closure of the otic vesicle. An area of active fusion of the epithelial margins of the aperture is present in the upper portion of the figure (arrow). x 1,400.

Fig. 16. Epithelial fusion and associated CPC precipitate (50-55 hours; stage 15). The addition of CPC to the fixative again yields a precipitate that is localized within the invaginated vesicle (OV). This material appears heavier at one side of the aperture (arrow) which suggests an underlying area of adhesion and/or fusion. x 700.

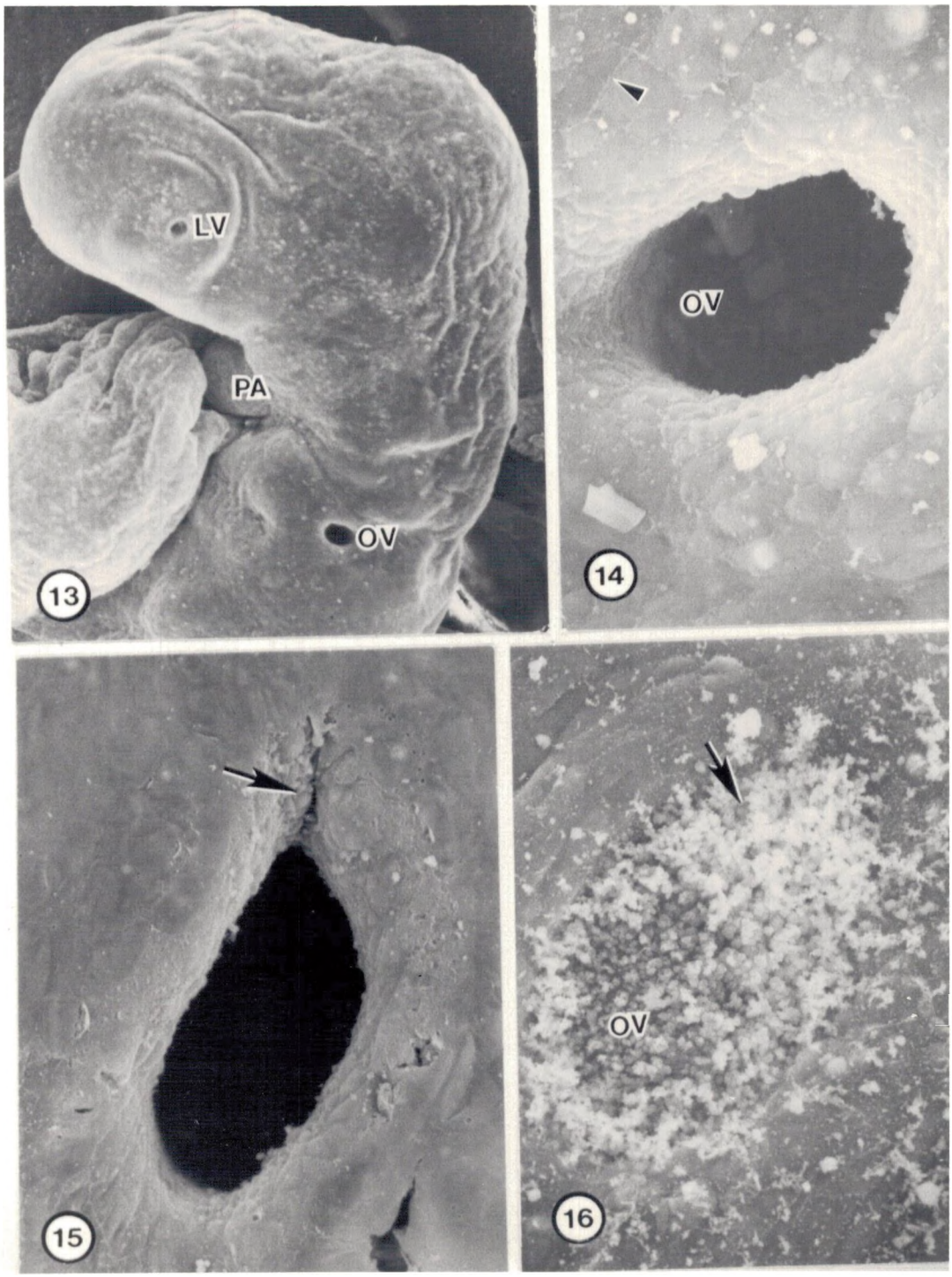
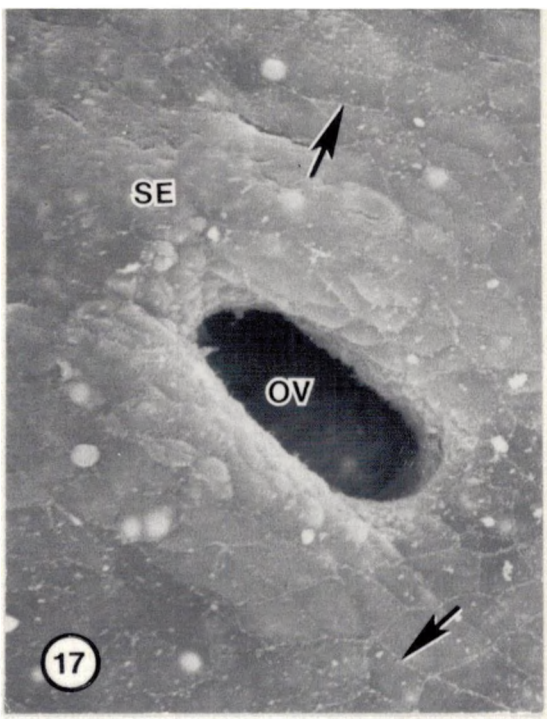
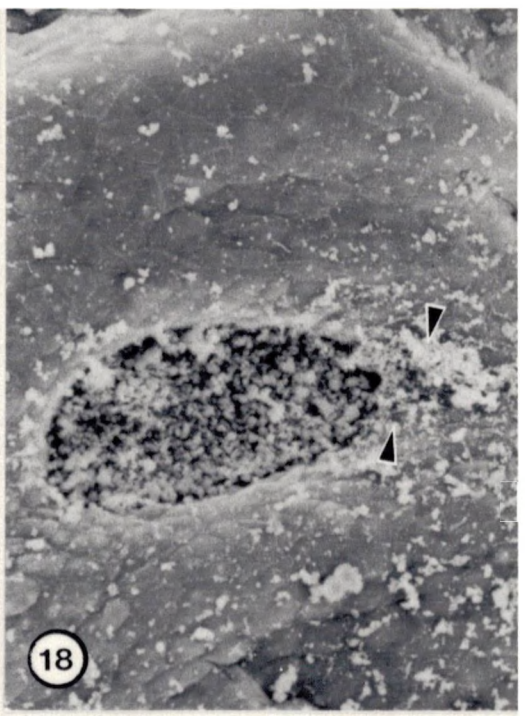

LV
PA
OV
13
OV
14
15
OV
16

PLATE VII

Figs. 17, 18. Closure of otic vesicle aperture (51-56 hours; stage 16). The aperture of the vesicle (OV) is increasingly becoming more elliptical indicative of continued closure. The surface ectoderm (SE) remains comparable to previous stages with microvilli along the lateral borders of cells (arrows). The addition of CPC to the fixative yields a precipitate which is similar to that seen in Figure 16 (arrowheads). x 700, Fig. 17; x 1,500, Fig. 18.

Fig. 19. CPC and otic vesicle (51-55 hours; stage 16). CPC precipitates the glycoconjugate components of the SCM. The precipitate continues to be far more predominant over the otic vesicle (OV) when compared to its sparsity in association with the surface ectoderm (SE). x 3,300.


SE
OV
17


18

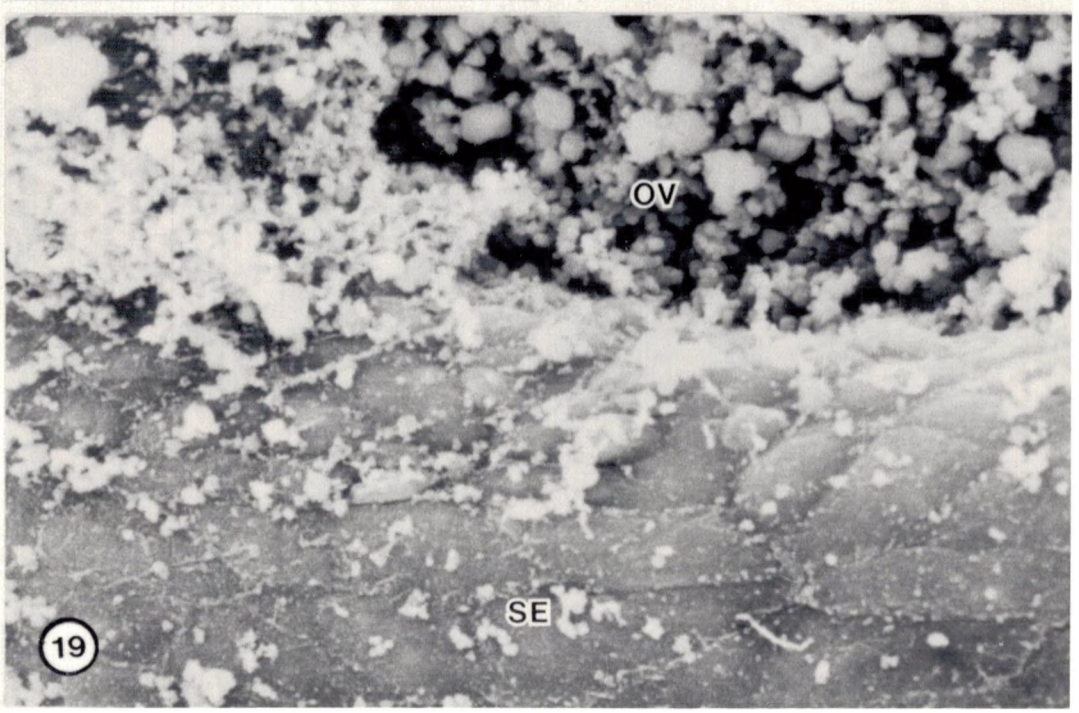

OV
SE
19

PLATE VIII

Fig. 20. Embryo fixed in glutaraldehyde prior to freeze-substitution (48-52 hours; stage 13). The surface ectoderm (SE) and the otic vesicle (OV) are free of any precipitate. x 875.

Fig. 21. Embryo fixed in glutaraldehyde/CPC prior to freeze-substitution (48-52 hours; stage 13). These specimens revealed a precipitate (arrows) similar to that seen in embryos processed by conventional methods. x 1,200.

Fig. 22. Embryo processed by freeze-substitution (48-52 hours; stage 13). Embryos frozen without prior aldehyde fixation reveal disimilar amounts of precipitate when the otic vesicle (OV) and nearby surface ectoderm are compared. This is comparable to specimens fixed in glutaraldehyde/CPC. x 2,400.

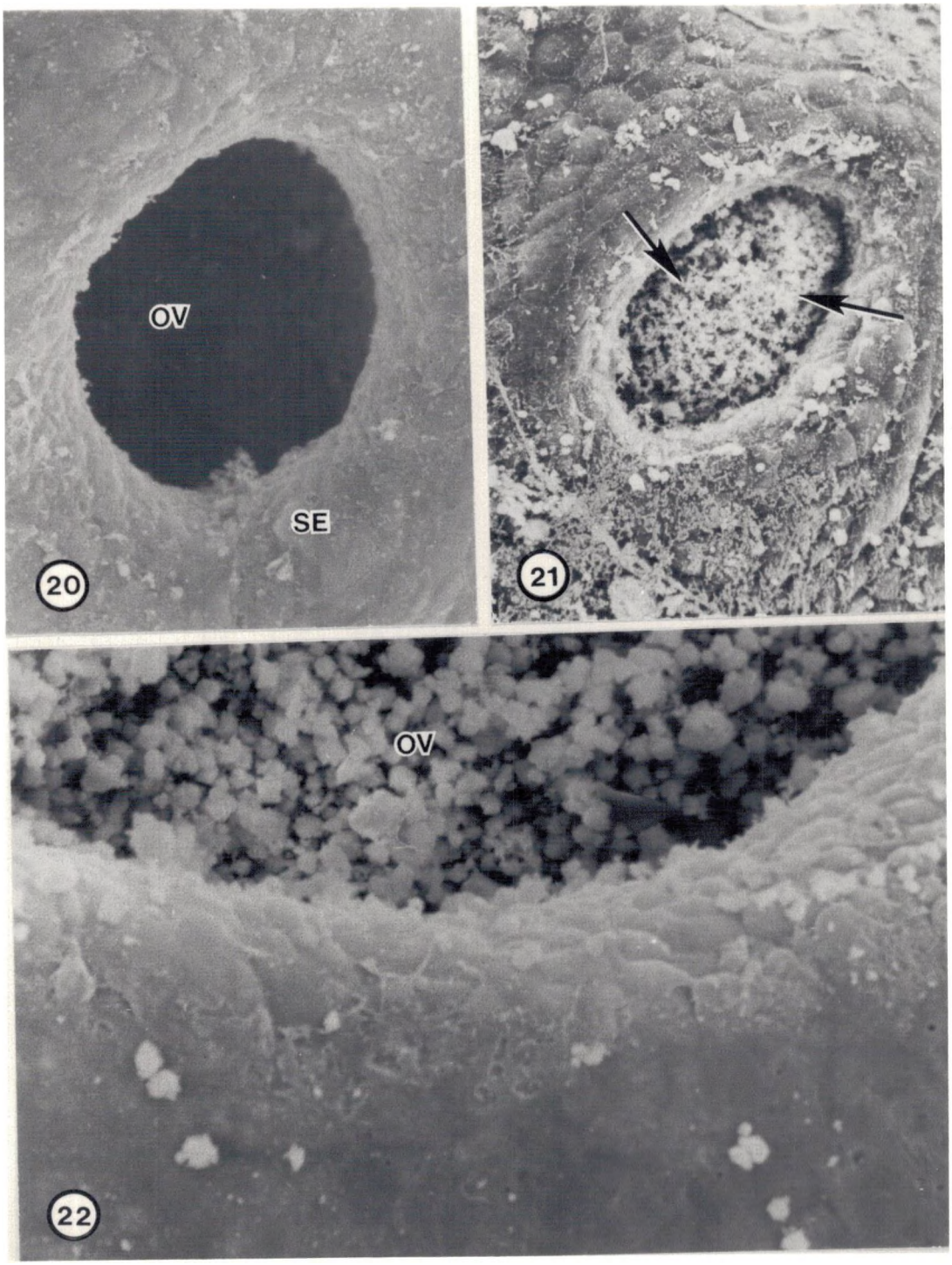

OV
SE
20
21
OV
22

PLATE IX

Fig. 23a, b. Con A binding (45-48 hours; stage 12). The binding of Con A is limited to the apical surface (A) of the placode epithelium and the associated basal lamina (arrow). Control sections (23b) show no labeling. x 450, Fig. 23a; x 450, Fig. 23b.

Fig. 24a, b. WGA binding (48-52 hours; stage 13). The binding of WGA to the developing otic vesicle appears to extend throughout the thickness of the epithelium (B) and the basal lamina (arrow). The surface ectoderm (SE) is also labeled. Control sections (Fig. 24b) show faint labeling of only the basal lamina. x 450, Fig. 24a; x 450, Fig. 24b.

Fig. 25a, b. SBA binding (48-52 hours; stage 13). The binding of SBA to the otic vesicle, like Con A, is limited to the apical surface and the basal lamina (arrows). Control sections (Fig. 25b) reveal no labeling.

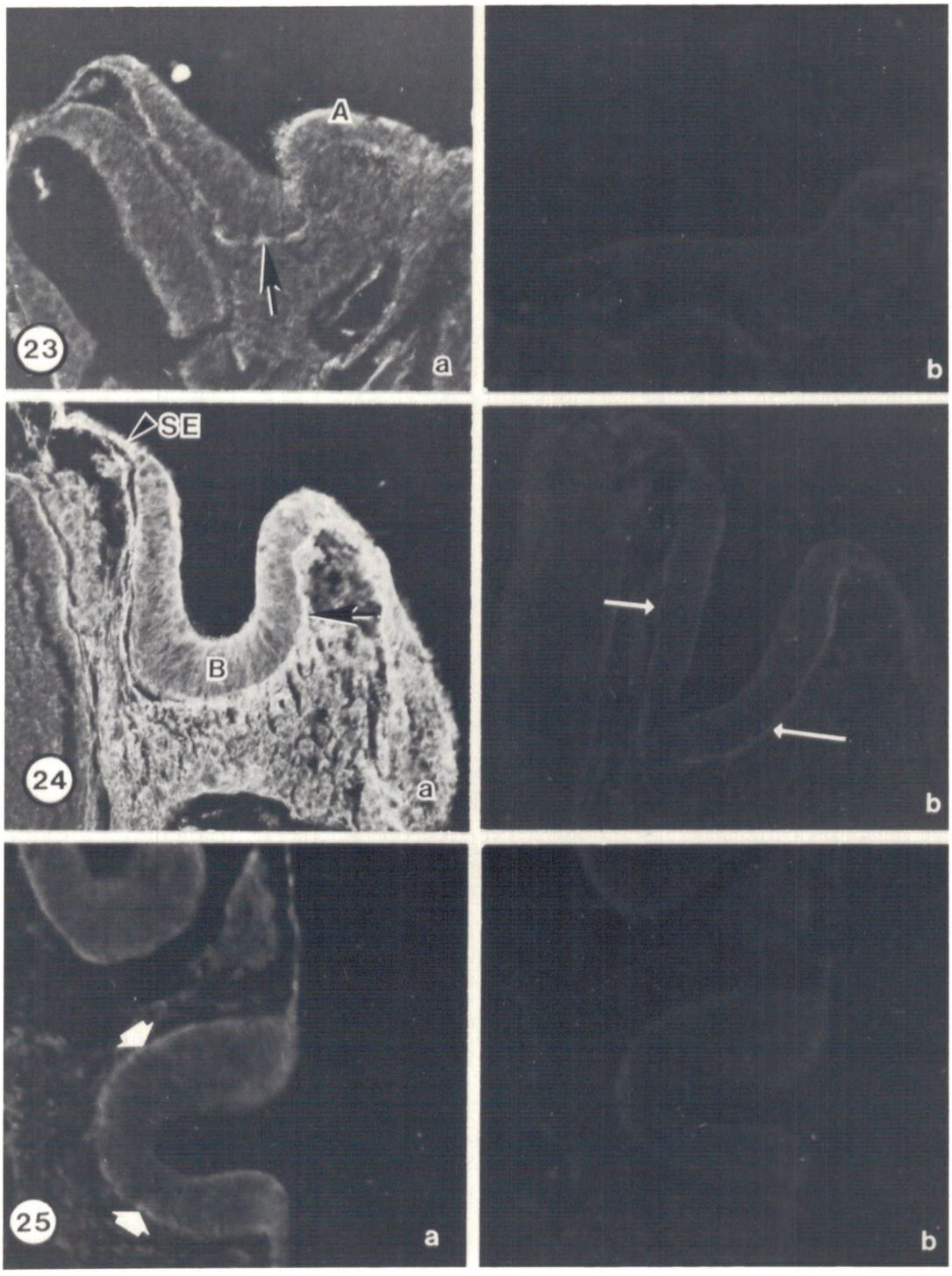

A
23
a
b
SE
B
24
a
b
25
a
b

PLATE X

Fig. 26a, b. Con A binding (50-55 hours; stage 15). The localization of binding to the apical surface epithelium (A) and basal lamina (arrow) of the developing otic vesicle and surface ectoderm (SE) is shown. Control sections (Fig. 26b) show no binding. x 450, Fig. 26a; x 450, Fig. 26b.

Fig. 27a, b. WGA binding (50-53 hours; stage 14). The binding of WGA is similar to that seen in Fig. 24a. Control sections show a distinct binding of the basal lamina (arrows). x 450, Fig. 27a; x 450, Fig. 27b.

Fig. 28a, b. SBA binding (50-55 hours; stage 15). The binding affinity of SBA is greater than in Figure 25a. Binding is present on the apical surface of the forming vesicle and the basal lamina (arrow). Control sections show no labeling except slightly within the basal lamina (28b). x 450, Fig. 28a; x 450, Fig. 28b.

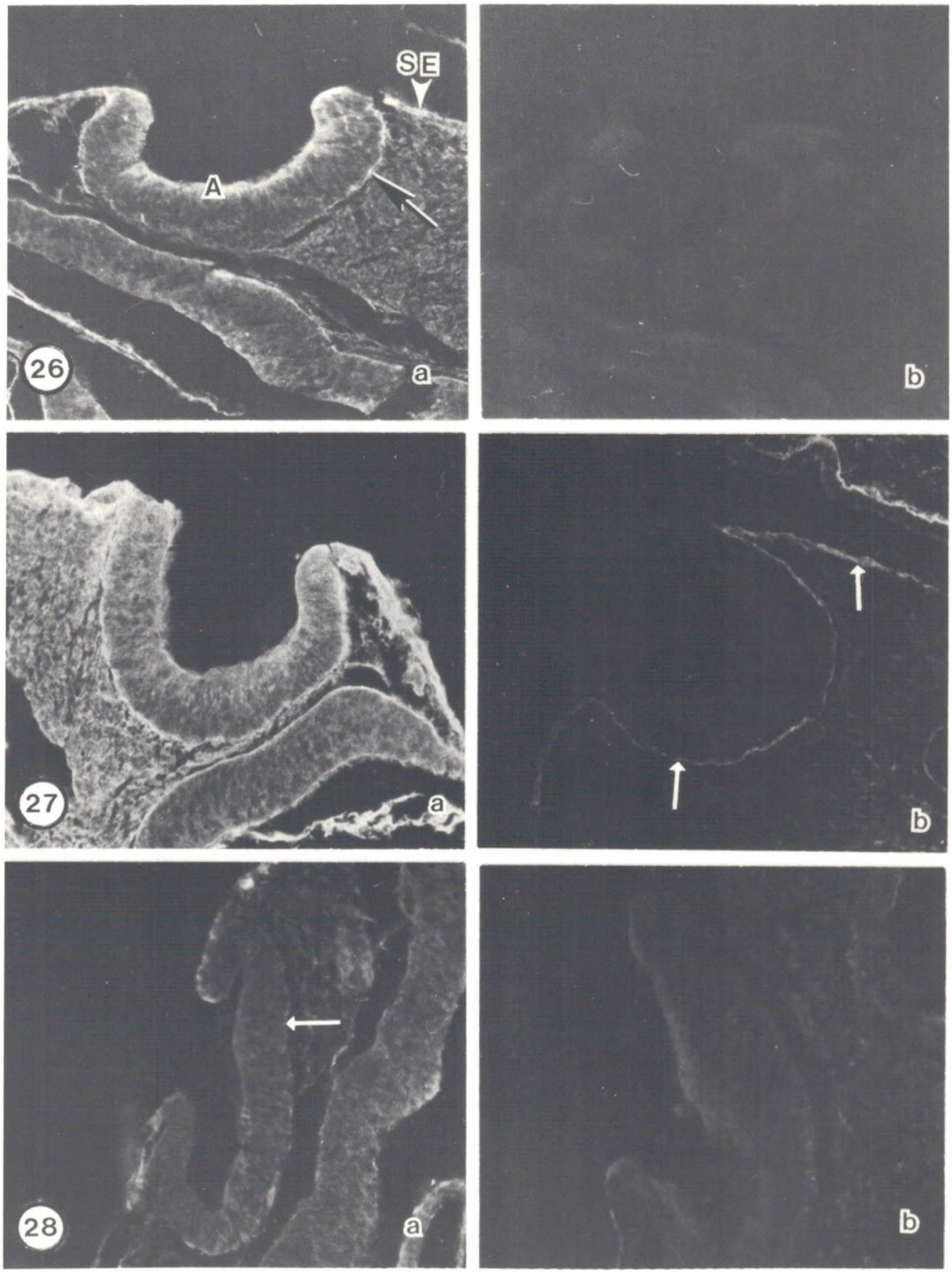
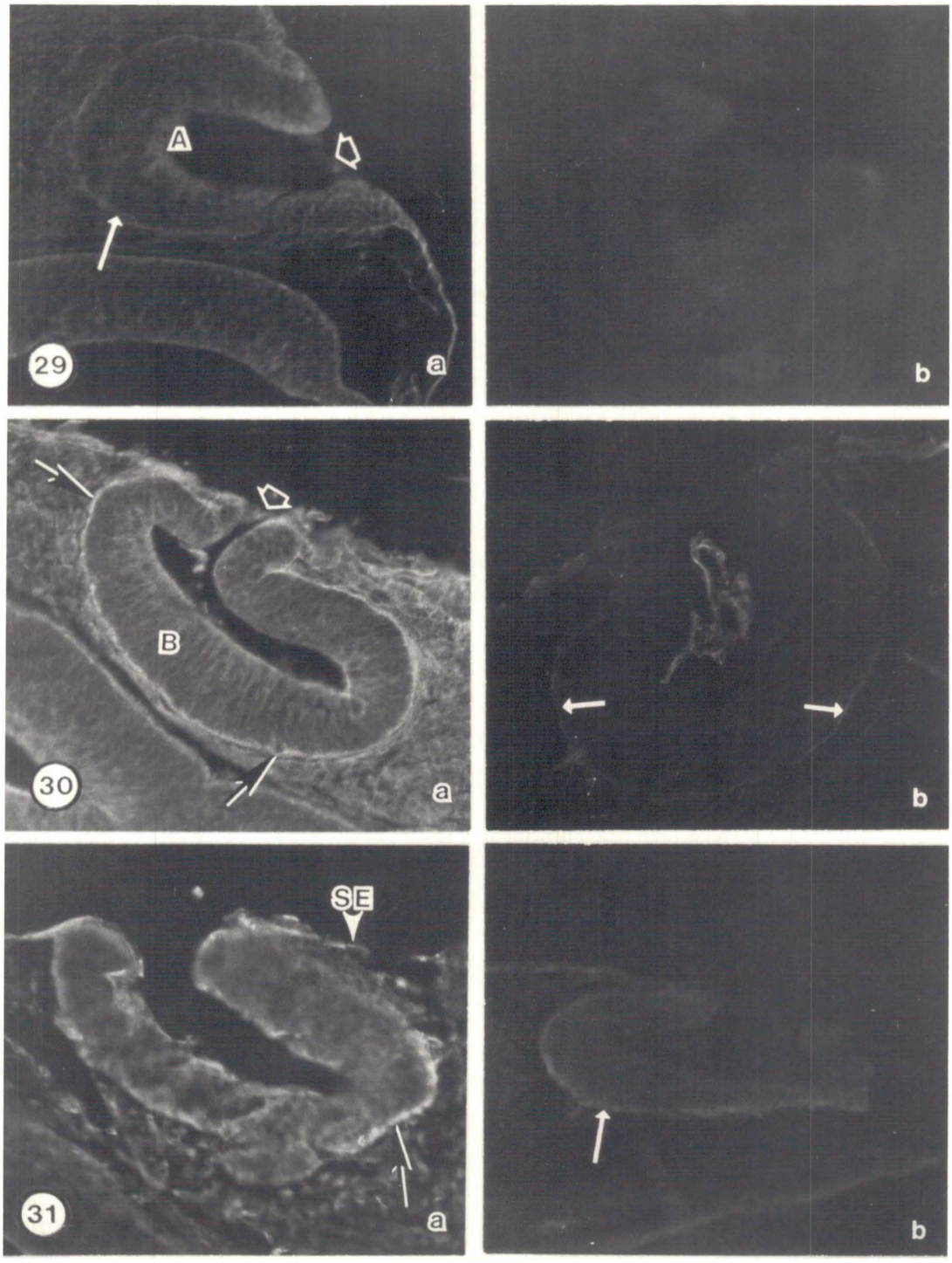

SE
A
26
a
b
27
a
b
28
a
b

PLATE XI

Fig. 29a, b. Con A binding (51-56 hours; stage 16). Labeling of the surface ectoderm (SE), apical surface of the otic vesicle epithelium (A) and basal lamina (arrow) is positive. A whispy material present between the opposed epithelial margins of the aperture is also faintly labeled (open arrow). Control sections (29b) are not labeled. x 450, Fig. 29a; x 450, Fig. 29b.

Fig. 30a, b. WGA binding (51-56 hours; stage 16). The binding of WGA remains throughout the thickness of the otic vesicle epithelium and associated basal lamina (arrows). A whispy material similar to that in Figure 29a is also faintly labeled (open arrow). Control sections are void of label except within the basal lamina (Fig. 30b). x 450, Fig. 30a; x 450, Fig. 30b.

Fig. 31a, b. SBA binding (51-56 hours; stage 16). The binding pattern of SBA is similar to that seen in Figure 28a, but the binding intensity is greater. The surface ectoderm (SE) is also labeled. Control sections show no binding except within the basal lamina (arrow; Fig. 31b). x 450, Fig. 31a; x 450, Fig. 31b.


A
29
a
b
B
30
a
b
SE
31
a
b

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