for treating large numbers of cells. Attempts to remove macrophages by density gradient sedimentation or by formation of rosettes with antibody-coated sheep erythrocytes (EA rosettes) have had only limited success. Although macrophages free of tumor cells can be obtained by culturing mixed cell populations in medium containing low concentrations of trypsin[14], this method is not useful for isolating tumor cells since the 'nonadherent' population from such cultures contains large numbers of less adherent macrophages/monocytes. Moreover, proliferation of intratumoral macrophages has been observed with various tumors[3], resulting in significant inhibition of tumor cell growth in vitro. In the present study we have examined the capacity of intratumoral and alveolar macrophages to inhibit the growth of tumor cells from metastatic murine lung tumors in vitro and the use of mycostatin to suppress the activity of macrophages in vitro.

Mycostatin is a common antifungal antibiotic that has long been used as an antifungal agent in tissue culture, with well-established safety and effectiveness. It is suspended in the medium and, due to its low solubility, is quickly phagocytosed by macrophages, which apparently are unable to digest it. In cultures without mycostatin, tumor cell monolayers were irregular in shape and contained numerous macrophages (figure 1). After prolonged culture periods, the tumor cells were often completely eliminated. Addition of mycostatin early in the culture period quickly and effectively eliminated macrophages and restored the growth of tumor cells (figure 2). Mycostatin-saturated macrophages were apparently unable to affect tumor cells, which began to regrow in the areas where they had been eradicated.

In conclusion, we have found that the addition of mycostatin to tissue culture medium not only prevents fungal contamination but also improves tumor cell growth by inhibiting the cytotoxicity of intra- and extra-tumoral macrophages. This is especially useful for establishing primary cultures of cells from lung tumors or lung metastases.

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Studies on the mechanisms of neurulation in the chick: The intracellular distribution of Ca^{++}[1]

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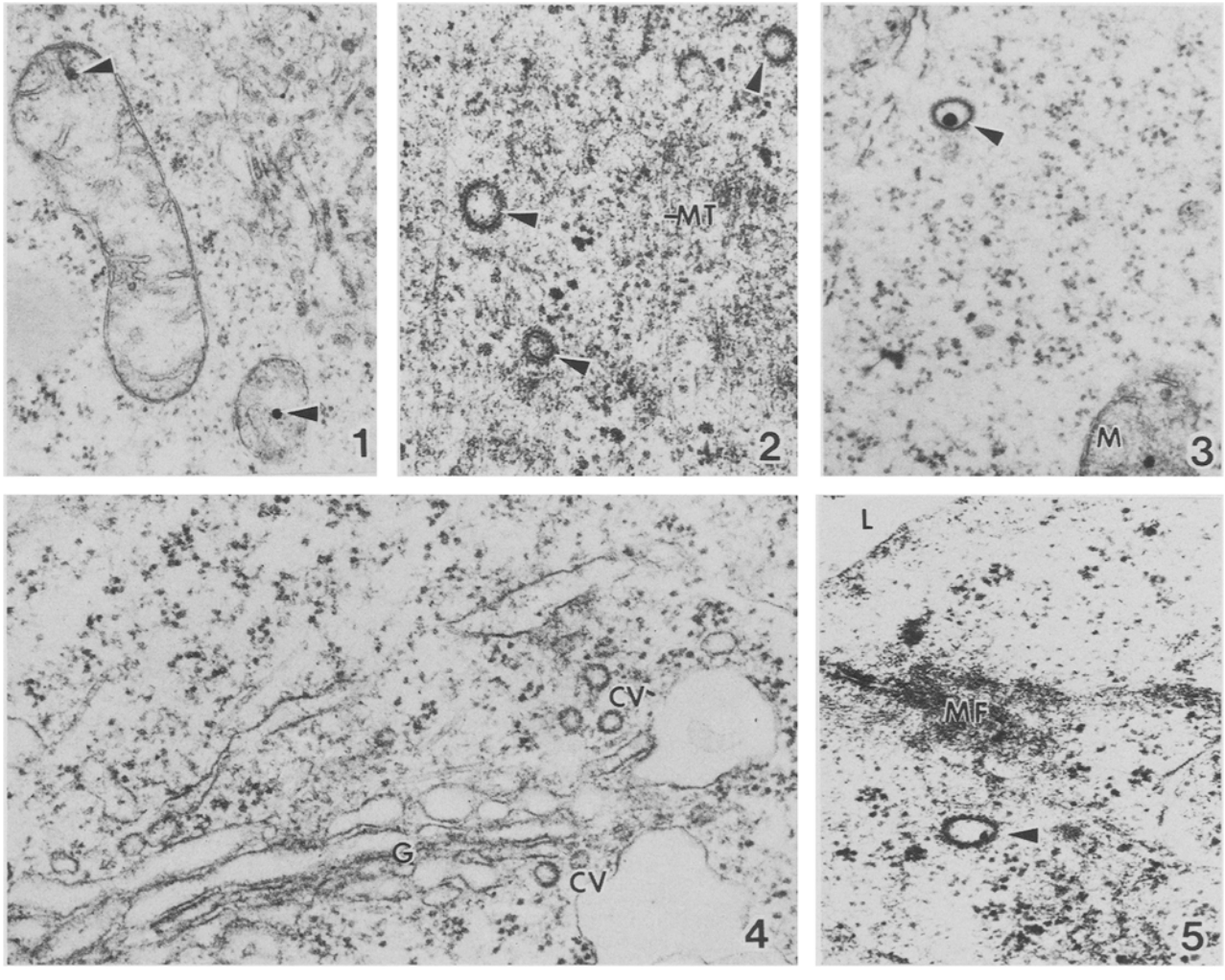
Summary. Coated vesicles were found to accumulate Ca^{++} in neuroepithelial cells and may play a role in regulating the contractile activities of apical microfilament bundles during uplifting of neural folds in the chick.

During neurulation in the chick the neural plate folds at its midline and eventually rolls itself into a neural tube. The origin of the motive forces for these movements has been the subject of numerous studies over the past decade[3,4]. Microfilament bundles, which encircle the cell apex, are generally thought to play an active role in uplifting of neural folds. Microfilaments forming these bundles are capable of binding heavy meromyosin and therefore contain actin[5]. Indirect immunofluorescence has revealed that myosin is also present in neuroepithelial cells and is most concentrated in the cell apex where the actin-like microfilaments are organized into discrete bundles[6]. These findings along with our previous observation that apical microfilament bundles exhibit substructural features resembling myofibrils[5] strongly suggest that apical microfilament bundles generate the motive forces for uplifting of neural folds by a sliding filament mechanism similar to that of skeletal muscle. This is in line with Burnside's[7] observation of a progressive increase in the thickness of microfilament bundles during apical constriction of amphibian neuroepithelial cells. It is well known that muscle contraction is regulated by the availability of intracellular free Ca^{++}. This raises the possibility that Ca^{++} also serves as a regulator of microfilament contraction in the developing neuroepithelium. This view is supported by the recent findings that chemical agents (e.g., papaverine and ionophore A23187) known to alter intracellular free Ca^{++} levels disrupt the uplifting and alignment of neural folds through their action on microfilaments[8–11]. For Ca^{++} to serve as a regulatory ion, neuroepithelial cells must have a means of controlling local intracellular Ca^{++} levels. Thus, knowledge of the distribution of Ca^{++} in neuroepithelial cells could augment our understanding of the mechanisms which control uplifting of neural folds. As a first part of our interest in this problem, the present study was carried out to localize Ca^{++} in neuroepithelial cells by cation precipitation with pyroantimonate[12,13]. An attempt was also made to identify those structures that have the capacity to serve as intracellular reservoirs from which Ca^{++} may be mobilized to regulate microfilament contraction.

Materials and methods. Fertile White Leghorn eggs were incubated at 37.5 °C to obtain embryos at stage 8 or 8+ of development[14]. These stages were chosen because the forming neural tube exhibits a gradual variation in the degree of

openness along the length of the embryo axis and offers an excellent opportunity to study the intermediate stages of uplifting of neural folds in a single specimen[15]. Embryos were isolated from the yolk and separated from the vitelline membrane prior to fixation. Some embryos were processed for routine transmission electron microscopy as previously described[15]. Others were fixed for Ca^{++} localization according to the method of Weakly[13] with modifications as follows: Embryos were fixed for 4 h with 2% glutaraldehyde containing 1.7% potassium pyroantimonate (Fisher Scientific Co.) (adjusted to pH 8.0 using 0.1% acetic acid) at room temperature. They were rinsed briefly in distilled water, postfixed with 1% osmium tetroxide in Millonig's buffer for 20 min at room temperature, and treated with either a saturated solution of ethylene-glycol-bis (p-aminoethyl ether) N,N′-tetraacetic acid (EGTA) adjusted to pH 7.4 using 0.1 N KOH or deionized water at 4 °C for 2 h. All specimens were rapidly dehydrated in a graded series of ethanol, embedded in Epon 826, and sectioned. Thin sections (silver/pale gold) were mounted on uncoated copper grids. Some were stained with uranyl acetate and/or lead citrate, while others were left unstained. All were examined with an RCA EMU-4 electron microscope.

Results and discussion. The potassium pyroantimonate technique is now widely used for studies of Ca^{++} distribution in muscle[17,18] and other tissues[13,18–20]. The present study showed that the ultrastructural preservation in antimonate-fixed specimens was comparable to that in routinely fixed material. Staining of thin sections with uranyl acetate and/or lead citrate did not appear to alter the distribution or amount of electron-opaque reaction product (= precipitate). This precipitate was not found in specimens treated with EGTA, indicating that it contained Ca^{++} [13,21]. In general, the precipitate was lightly scattered throughout the cytoplasm, but was concentrated in mitochondria (figure 1) and coated vesicles (figures 2 and 3) found in the cell neck region. Coated vesicles were more numerous in those cells forming the region of uplifted neural folds than in cells of the neural plate. They appeared to originate from the Golgi complex in the lower cell neck region (figure 4). Newly formed vesicles were small and usually contained an amorphous material. They subsequently moved along the paraxially oriented microtubules toward the cell apex (figure 2).



All micrographs were taken from cells forming the uplifted neural folds in the midbrain region. Fig. 1. Lower neck region of a neuroepithelial cell. Large divalent cation storage granules (arrows) within mitochondria are stained darkly due to uptake of antimonate. × 27,000. Fig. 2. Lower neck region of a neuroepithelial cell. Coated vesicles (arrows) contain small, yet well-defined, Ca^{++}-antimonate granules. MT, microtubules. × 36,000. Fig. 3. Upper neck region of a neuroepithelial cell showing a coated vesicle (arrow) with a large Ca^{++}-antimonate granule in its lumen. M, mitochondrion. × 33,000. Fig. 4. Lower neck region of neuroepithelial cell showing saccules of the Golgi complex (G) and coated vesicles (CV). × 48,000. Fig. 5. Apical region of neuroepithelial cell showing a coated vesicle (arrow) with a small amount of Ca^{++}-antimonate perecipitate in the vicinity of a microfilament bundle (MF). L, lumen. × 41,000.

Those in the upper cell neck region often contained large Ca^{++}-antimonate granules (figure 3), suggesting that they accumulated Ca^{++} during their migration through the cell neck region where numerous mitochondria were located[15]. Since mitochondria are known to sequester intracellular Ca^{++} in various non-muscle cells[22], they may serve as a source of Ca^{++} for coated vesicles during their transit through the cell neck region. The fact that coated vesicles found in the vicinity of apical microfilament bundles contained less Ca^{++}-antimonate precipitate (figure 5) than those in the cell neck region suggests that they release most of their stored Ca^{++} in the apical region. Although precise mechanisms of Ca^{++} accumulation and release are largely unknown, coated vesicles isolated from brain and other tissues have been shown to both biochemically and functionally resemble the sarcoplasmic reticulum[23-25]. Thus, the results of the present study are interpreted to indicate that coated vesicles in neuroepithelial cells are capable of sequestering Ca^{++} and, by functioning as a mobile source of intracellular Ca^{++}, may play a role in regulating contraction of apical microfilament bundles during uplifting of neural folds in the chick.

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Use of peroxidase-avidin conjugate for the demonstration of intracellular antigen[1]

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Summary. Horseradish peroxidase was coupled to avidin by periodate oxidation. Using the peroxidase-avidin conjugate together with biotin-labeled anti-chymotrypsinogen antibody, chymotrypsinogen was clearly demonstrated in the zymogen granules of the pancreas. The reaction between avidin and biotin is so rapid and stable that the avidin-biotin system can be effectively applied to immunohistochemical studies.

The strong affinity of avidin for biotin[3] has served as an effective tool for research in the immunohistochemical field. The avidin-biotin (A-B) system has been utilized mostly for the demonstration of antigens and syalyl residues on the cell surface[4-8]. Only the report of Heggeness and Ash[9] on the localization of actin and myosin in cultured muscle cells has delt with the demonstration of intracellular antigens. On the other hand, Guesdon, Ternynck and Avrameas[10] recently introduced a method for coupling enzymes to avidin with the aid of glutaraldehyde, and suggested the possibility of applying the A-B system to immunohistochemical staining. The present study was undertaken to develop a new method of preparing peroxidase-avidin conjugate, and to localize the chymotrypsinogen (CHT) in the pancreas with this conjugate in combination with biotin-labeled (biotinyl) anti-CHT antibody.

Materials and methods. 1 mg of bovine CHT (Sigma Chemical Co.) dissolved in 1 ml of phosphate buffered saline (PBS) was mixed with an equal volume of complete Freund's adjuvant, and injected s.c. into 2 rabbits. 4 weeks after the 1st injection, 0.1 mg of the antigen was administered as a booster injection. 1 week after the booster injection, antisera were harvested from the ear vein. Biotinyl-N-hydroxysuccinimide ester (BOSu) was prepared according to the method of Becker, Wilchek and Katchalski[11] and Bayer and Wilchek[12]. BOSu was coupled to goat IgG directed against rabbit IgG by the method described by Heggeness and Ash[9]. Horseradish peroxidase (HRP) (Sigma, Type VI) was coupled to avidin by periodate oxidation, which was followed by reductive alkylation. That is to say, HRP was oxidized with sodium periodate by the method of Wilson and Nakane[13]. The reactive aldehyde groups so formed react readily with amino groups of avidin.

In practice, the coupling procedure was as follows. 6 mg of HRP was dissolved in 1 ml of distilled water. To this was added 0.3 ml of freshly prepared $NaIO_4$ (21 mg/ml), and the solution was stirred for 20 min at room temperature. The oxidized HRP was dialyzed overnight against 0.001 M sodium acetate buffer, pH 4.0. Then, 20 µl of 0.2 M carbonate-bicarbonate buffer, pH 9.5, was added. Immediately after, 4 mg of avidin dissolved in 1 ml of 0.01 M carbonate-bicarbonate buffer, pH 9.5, was added. The HRP-avidin mixture was stirred gently for 2 h at room temperature in the dark. To this mixture was added 0.15 ml of 4% sodium borohydride ($NaBH_4$) dissolved in distilled water. The mixture was left for 2 h at 4 °C. Finally, the HRP-avidin conjugate was dialyzed against PBS for 2 days.

Application of the A-B system to immunohistochemical studies was made as follows. Mouse pancreas was taken and cut into small blocks in a drop of a mixture of equal volumes of 4% paraformaldehyde and 0.1% glutaraldehyde prepared in 0.1 M phosphate buffer, pH 7.4. The tissue