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SCANNING AND TRANSMISSION ELECTRON MICROSCOPIC STUDIES ON THE OVIDUCTS OF PEKIN DUCKS FED METHYL MERCURY CONTAINING DIETS

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Abstract

This study was undertaken to examine the effects of varying levels of methyl mercury (MeHg) on the ultrastructure of the surface epithelium of the oviduct of ducks. Accordingly, Pekin ducks were maintained on feed containing varying doses of (0.0; 0.5; 5.0; 15.0 ppm) of MeHg (Group I – control to IV) for 12 weeks and sacrificed. Tissue from the magnum and the shell gland regions of the oviduct was processed for scanning electron microscopy (SEM) and transmission electron microscopy (TEM). It was found that the primary and secondary folds of these regions of the oviducts of the control and 0.5 ppm treatment group were densely populated with ciliated cells and that the cilia tend to cover the apical surfaces of the non-ciliated secretory cells. This unchanged ultrastructural morphology of the surface epithelium of 0.5 ppm treatment group was verified with TEM. The ciliated and nonciliated cells in surface epithelium appeared to be equal in frequency. The nuclei of ciliated cells were superficial in location compared to nonciliated secretory cells which had nuclei in the basal part of the cytoplasm. In the oviducal tissues from ducks fed 5.0 ppm MeHg isolated areas of ciliary loss, but minimal disruption of the apical plasma membrane were observed by SEM. In a few birds plasma membrane lesions, condensation of nuclear chromatin and very dilated rough endoplasmic reticulum were seen with TEM. In the oviducal tissues from ducks fed 15.0 ppm MeHg it could be seen that ciliary loss was much more extensive than hitherto observed, and disruption of the apex of cells could be seen. TEM showed degeneration of cytoplasmic organelles, more or less severely damaged ciliated cells, loss of ciliary extensions and formation of compound cilia. These observations indicate that methyl mercury at 5.0 and 15.0 ppm dose levels causes toxic injury to oviducal surface epithelium of Pekin duck that may cause reduced reproductive capability.

Key Words: Oviduct, Methyl mercury, Toxicity, Ultrastructure, Surface Epithelium, Cilia, Scanning Electron Microscopy, Transmission Electron Microscopy.

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Introduction

The overall sublethal effects of alkyl mercury compounds in avian species include a lowering in reproductive efficiency, reduced fertility and hatchability of eggs and impairment of embryonic development.[18,25,46]

A definite association of ovary and oviduct with the concentration and excretion of alkyl mercury compounds, in avian species, has clearly been established in both experimental and natural exposures.[10,17,27,47] It has also been established that in Japanese quail (*Coturnix coturnix*) the uptake and distribution of methyl, phenyl and methoxyethyl mercury compounds are similar. The methyl mercury, despite differences in the route of administration, is readily assimilated and accumulates in high concentrations in liver, kidney, and ovary.[4] One important aspect consistent in most of the above quoted reports was the transfer of mercury from the diets into the eggs. Further, it is now well established that high concentrations of Hg accumulate in oviducts, and that half of the daily intake is deposited in the eggs.[16] The major proportion of Hg in eggs, after the exposure of female birds to alkyl mercury is present in the egg white.[41] Most of this Hg is associated with ovalbumin rather than ovotransferrin, ovoglobulin or ovomucoid.[40] It has been suggested that Hg binds with the albumin protein in the magnum of oviduct and this organic mercurial complex is secreted as a part of albumin to form egg white.[32]

The reports on the effects of Hg on eggshell thinning, although conflicting, indicate that Hg at a relatively high dose level may affect the thickness of the eggshell.[30,36,43] However, thinning of eggshell at very low level of Hg has also been reported.[42]

The information on the transmission electron microscopy[3,22] and scanning electron microscopy[5,7,15,20] of the surface epithelium of the avian oviduct is essentially limited to domestic chicken and quail. In both these species the surface epithelium consists of ciliated cell with subapical nuclei and nonciliated secretory cells with rather basal nuclei. In domestic ducks this information is available only for shell gland region.[19,28]

Information on the morphologic changes in the magnum and the shell gland regions of avian oviducts that correlates them to the possible harmful effects of mercury pollution on waterfowl reproduction is lacking. The present report describes the topographical and ultrastructural alterations in the surface epithelium of the magnum and the shell gland regions of the oviducts of Pekin

ducks (*Anas platyrhynchos*) fed three levels of methyl mercury chloride (MeHgCl) in their diets.

Materials and Methods

Experimental Animals

Twenty-four one-week old female Pekin ducks (*Anas platyrhynchos*) were purchased from a commercial source (Webbfoot Farms, Elora, Ontario) and maintained on a starter diet until the birds reached 10 weeks of age. Ducks were randomly assigned to stainless-steel wire cages and were acclimatized to cages, mash feed and controlled room conditions free of any known contamination, prior to the onset of experimental feeding at 12 weeks of age. Following the acclimatization period the birds were divided into four groups, each consisting of six birds, and were placed on mash diets containing 0.0 ppm (Group I control), 0.5 ppm (Group II), 5.0 ppm (Group III) and 15.0 ppm (Group IV) methyl mercury (MeHgCl) for a subchronic exposure of 12 weeks. MeHgCl was added and thoroughly mixed with the mash ration formulated to contain daily nutritional, vitamin and mineral requirements of a nonfattening type. Experimental mash feed and water were provided *ad libitum*. Feed analysis revealed the Hg content of 0.03, 0.48, 3.76 and 13.43 ppm in the four groups, respectively.

The control ducks were kept in a different room from those on MeHgCl containing diets. The length of the photoperiod, at 14L:10D, and ambient temperatures, at 21°C (70°F), were uniformly maintained in both rooms. Both the control and treated ducks were housed two per cage. Hence individual egg laying records were not maintained.

Sample Collection

After 10 weeks of the experimental diet most of the birds commenced laying, thus indicating the onset of sexual maturity. Laid eggs were regularly observed at the cages of control birds. Whereas the egg laying of treated birds was erratic. The eggshell strength on physical examination appeared normal. At the end of the 12th week on the test diet, all ducks were sacrificed by i/v injection of an overdose of Somnotol (sodium pentobarbital, M.T.C. Pharmaceuticals, Hamilton, Ontario) and the oviducts removed immediately. Regions corresponding to the location of magnum and the shell gland were collected equidistant from the ovary. For magnum the midportion of the region and for shell gland the *Pars major uteri* or shell gland proper was selected and processed for scanning and transmission electron microscopy.

Tissue Preparation

The Osmium-Thiocarbohydrazide method of Kelly et al,[26] modified by Malick and Wilson,[29] was utilized in the specimen preparation for scanning electron microscopy (SEM). One square centimeter size pieces of magnum and shell gland regions of the oviduct were pinned onto a cork and washed in phosphate buffered saline and fixed in 4% glutaraldehyde in 0.05 M phosphate buffer (Osmolality 122.0 mOsm) for 4 h at 20°C and postfixed in 2% OsO_4 in 0.1 M phosphate buffer (pH 7.4) for 2 h. Preparations were washed in phosphate buffer and subsequently treated with a 1% solution of thiocarbohydrazide (TCH, Aldrich Chemicals, Milwaukee, WI) dissolved in distilled water. Tissue samples were gently agitated in TCH solution for 20-30 min and thoroughly rinsed in distilled water over a period of 15 min. After this, specimens were placed in a 2% solution of OsO_4 in phosphate buffer for 2-3 h while being agitated. The tissues were then given another wash treatment. The entire procedure was repeated by placing the specimen in fresh TCH solution followed by washing. The specimens were once again placed in OsO_4 solution and subsequently washed. The final result was an Os-TCH-Os-TCH-Os (O-T-O-T-O) ligand binding. Prepared tissues were dehydrated in a graded series of ethanol solutions, critical point dried in CO_2, mounted on stubs with colloidal silver, and were examined and photographed with a JEOL JSM-35 SEM at 15 kV with tilt angles ranging between 20–45°.

Samples for light microscopy (LM) and transmission electron microscopy (TEM) were chopped into small 1 mm^3 pieces and immersion fixed with 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4). The buffer osmolality was 122.0 mOsm and the final osmolality ranged between 580–600 mOsm. These tissue pieces were processed for TEM using standard procedures. Areas of interest chosen from 1 μm toluidine blue stained sections, were retrimmed and ultrathin sections were contrasted with uranyl acetate and lead citrate and examined and photographed in a JEOL JEM 100S operating at 80 kV.

Results

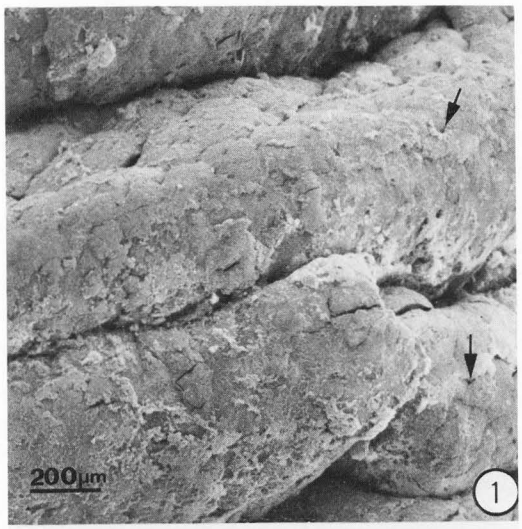
General Observations

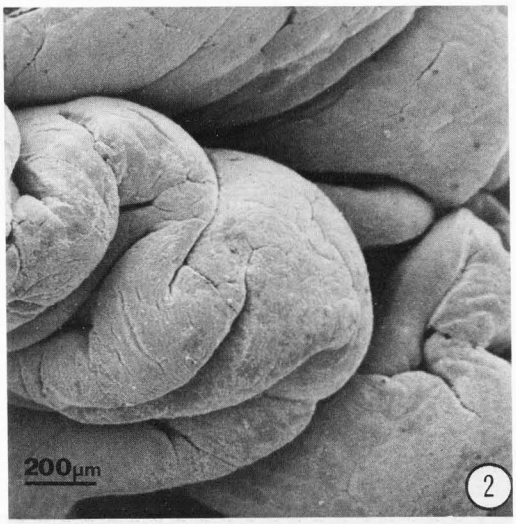
Since the ducks were housed two per cage the exact egg laying status of individual ducks was not determined. However, most of the ducks in control and 0.5 ppm MeHgCl treatment group had started laying. The egg laying in 5.0 and 15.0 ppm treatment group was erratic. No apparent difference was noted in the eggshell strength between the control and treated group as determined by simple physical pressure. The gross examination of the oviducts at postmortem revealed no discernible difference between the control and treated ducks. The ducks in 15.0 ppm MeHgCl group had dull and ruffled feathers. The overt neurological symptoms in this group appeared first at week eight postinitiation of experimental feeding. By week 12 all birds in the group were paralyzed.

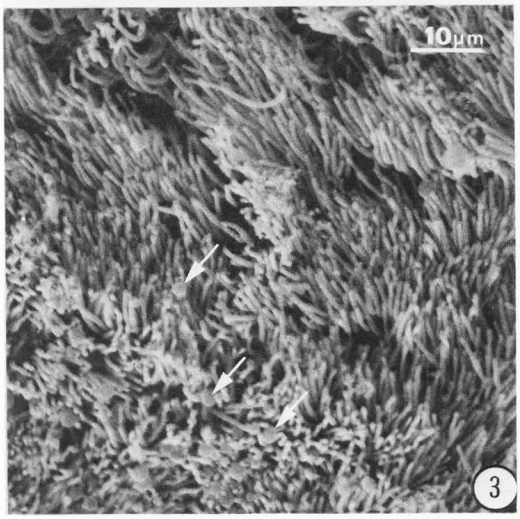
Ultrastructure of Surface Epithelium

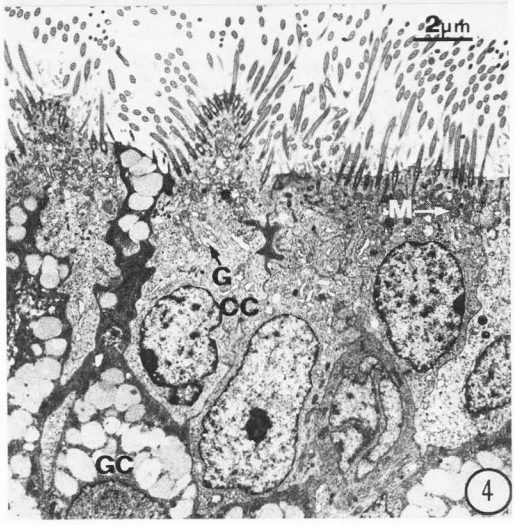
Group I (Control – 0.0 ppm MeHgCl) and Group II (0.5 ppm MeHgCl) Scanning electron microscopic observations on the mucous membrane of magnum and shell-gland regions of the oviducts of this group showed longitudinally oriented primary folds and a limited amount of secondary folding. Secondary folds appeared as shallow grooves at approximate right angles or obliquely along the long axis of the oviduct (Fig. 1). The openings of proprial glands were more numerous in the magnum and appeared as small pores between the interdigitations of secondary folds on the luminal surface of the epithelium (Fig. 1). The mucosa of the shell-gland region appeared as longitudinally oriented elevated folds, which were more tortuous than the folds in the magnum (Fig. 2). Ciliated cells were abundant in the surface epithelium of both magnum (Fig. 3) and shell gland regions. The slender cilia overlapped the entire surface and concealed the apical surfaces of nonciliated secretory cells. Because of this the relative proportion of ciliated and nonciliated secretory cells could not be observed under SEM.

The transmission electron microscopic examination of the surface epithelium revealed that the surface epithelial layer contains two types of well differentiated cells in nearly equal proportion. The ciliated cells, with little evidence of secretory activity, and









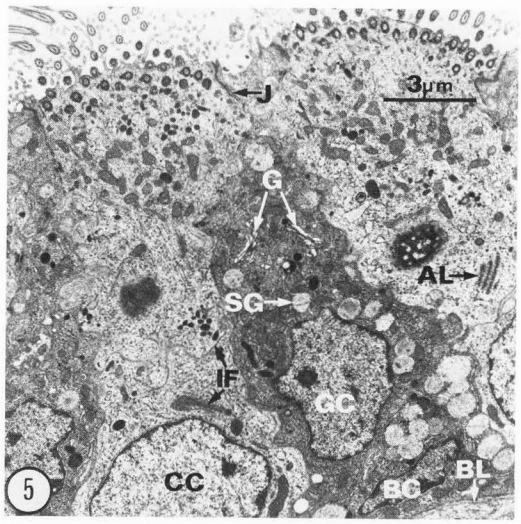
nonciliated secretory cells, with an intracellular fine structure of typical mucoprotein secreting cells (Fig. 4). In general the nuclei of ciliated cells were subapical whereas the nuclei of nonciliated secretory cells were rather basal in location. This arrangement of nuclei gave the surface epithelium a pseudostratified appearance. The ciliated cells were wider at the apex and narrow at the basal aspect. The mitochondria were primarily localized in the apical region close to ciliary basal bodies. The rough endoplasmic reticulum (rER) appeared as small stacks of two to four cisternae. The Golgi complex was small and supranuclear in position. The free ribosomes were dispersed sparsely in the cytoplasm either singly or in polyribosome configuration. Occasionally, lysosome-like dense bodies were also observed in the subapical cytoplasm. The lateral borders of these cells showed junctional complexes containing tight and adherent junctions. Such junctional complexes occurred both between two adjacent ciliated cells or between ciliated and nonciliated secretory cells. The cytoplasm of the nonciliated secretory cells contained membrane bound droplets filled with low electron density fibrillar material. The relatively few rER profiles and oval mitochondria were scattered throughout the cytoplasm. A prominent Golgi complex was located in supranuclear position. The apical

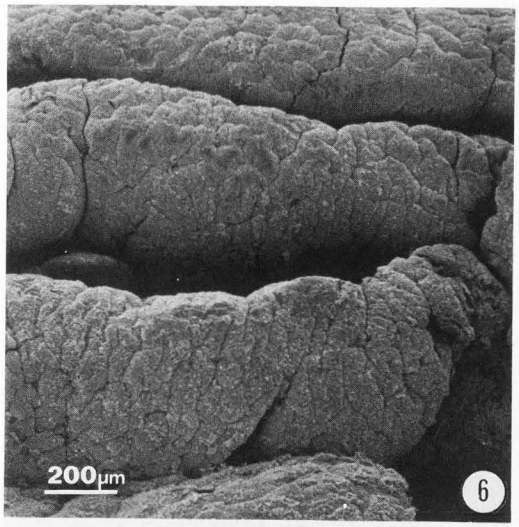
Fig. 1. SEM of the mucosal folds of the magnum from a duck belonging to control group. Note the longitudinal primary folds are completely covered by fuzzy surface. The arrows show the opening of the proprial glands.

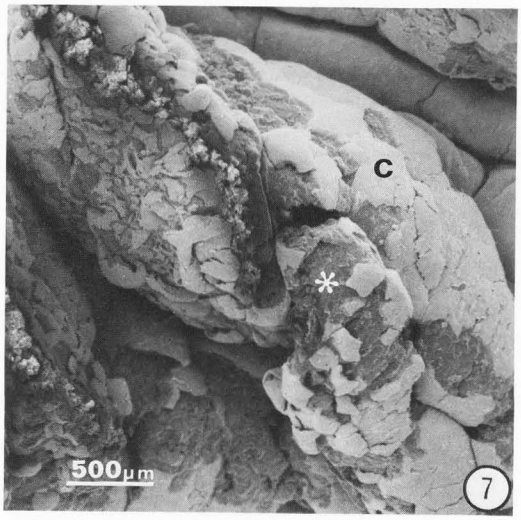
Fig. 2. SEM of the mucosal folds of shell gland region from a control duck showing uniformly fuzzy luminal surface. Note the primary and secondary folds are highly corrugated.

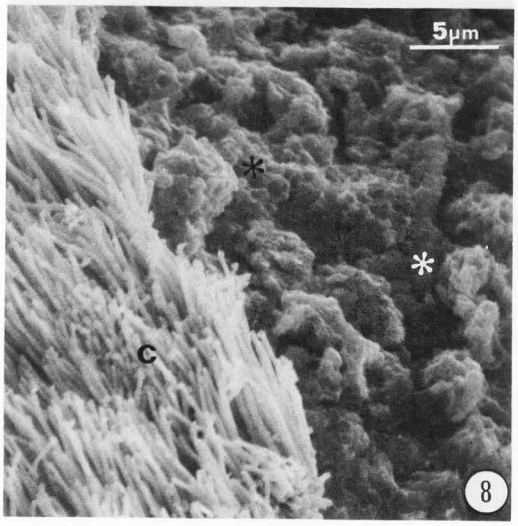
Fig. 3. High magnification SEM of the surface epithelium of shell gland region of a control duck. Note the luminal surface is completely carpeted with cilia masking the existence of nonciliated secretory cells. Small globular bodies are secretory droplets (arrows).

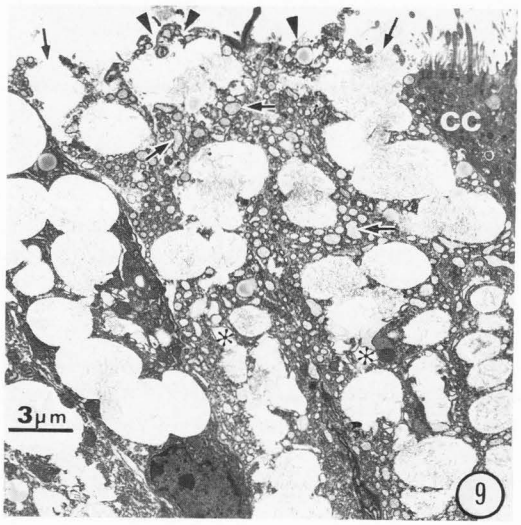
Fig. 4. TEM of the surface epithelium of the magnum from a control duck showing the fine structural organization of ciliated (CC) and nonciliated secretory cells (GC) in a pseudostratified arrangement. The cilia tend to mask the apecies of nonciliated secretory cells. G: Golgi complex, M: Mitochondria.











cell membrane carried short thin microvilli, and the lateral border had junctional complex similar to those of ciliated cells. The ciliated cells of the magnum often showed protrusion of apical cytoplasm containing cilia interspersed with microvilli (Fig. 4). Such protrusions were not prominent in the ciliated cells of the shell-gland region but these cells showed bundles of intermediate filaments and occasional annulate lamellae and electron dense granules. The nonciliated secretory cells of this region appeared not to contain as many secretory droplets (Fig. 5) as those in magnum probably reflecting the functional differences of the two regions of the oviduct.

At the dose level of 0.5 ppm,MeHgCl failed to significantly alter the fine structure of either the magnum or the shell gland surface epithelium (Fig. 6).

Group III (5.0 ppm MeHgCl) Interspersed with normal surface epithelium were patches of disrupted epithelial cells. Here the cells were devoid of cilia and in some cases appeared to lack an apical membrane. Debris of either cellular or secretory origin was abundant (Fig. 7). Other denuded areas were totally covered with mucous debris, leaving no room for recognition, by scanning electron microscopy, as to the type or types of cells involved. Cilia surrounding these areas appeared free of any noticeable changes (Fig. 8).

Fig. 5. Electron micrograph of the surface epithelium of the shell gland region from a control duck. Note the ciliated (CC), nonciliated secretory (GC) and basal (BC) cells rest on a basal lamina (BL). In the ciliated cells mitochondria with dense matrix are located close to basal bodies. Nonciliated secretory cells show secretory droplets (SG) of medium electron density and well developed Golgi complex (G) in the supranuclear cytoplasm. IF: intermediate filament bundles, AL: annulate lamellae, J: junctional complex.

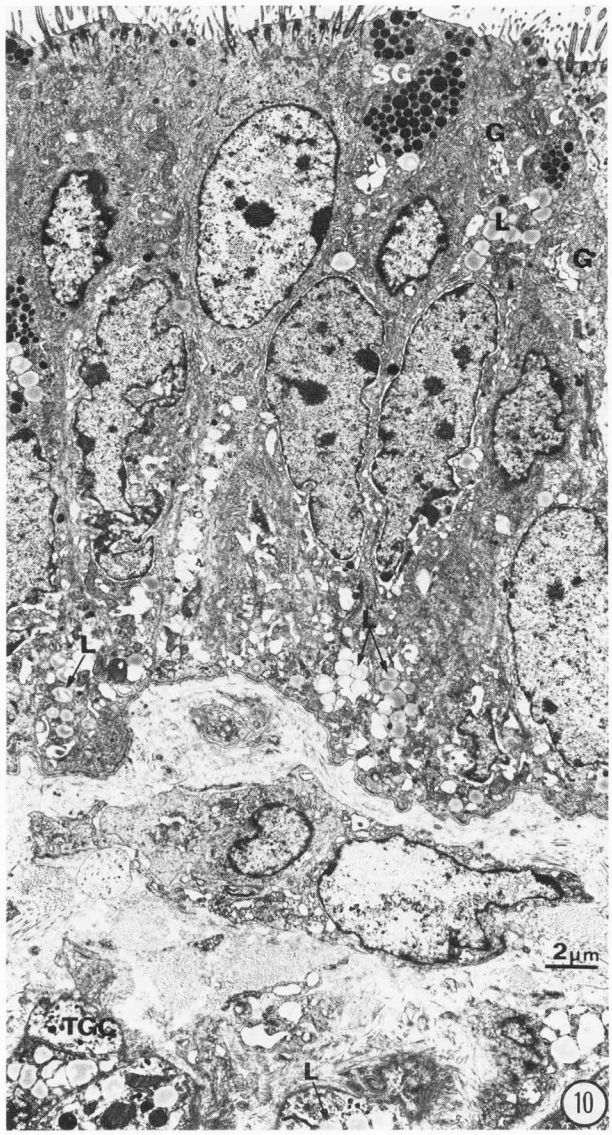
Fig. 6. SEM of the mucosal folds of the magnum from a duck fed with 0.5 ppm MeHgCl in the diet. Note the luminal surface shows no apparent change compared to control group.

Fig. 7. SEM of the mucosa of the magnum from a duck fed with 5.0 ppm MeHgCl in the diet. Primary folds show a few denuded areas (asterisk) in the mucosa carpeted with cilia (c).

Fig. 8. High magnification SEM of luminal mucosa of magnum from a duck fed 5.0 ppm MeHgCl. Note the denuded area (asterisks) show apical bulging probably representing mucus droplets and debris. The adjoining area (c) shows healthy cilia.

Fig. 9. Electron micrograph of the surface epithelium of the magnum from a duck fed with 5.0 ppm MeHgCl showing hypertrophy of nonciliated secretory cells with damaged apical membranes (arrowheads). Condensed nuclei, dilated vesicles of endoplasmic reticulum (white and black arrows) and cytoplasmic vacuoles (asterisks) are seen in the cells showing apical membrane damage. The ciliated cells (CC) and nonciliated secretory cells in the neighbouring area are relatively unaltered.

Fig. 10. Electron micrograph of the surface of shell gland region from a duck fed with 5.0 ppm MeHgCl showing accumulation of lipid droplets (L) in nonciliated secretory cells. Note presence of lipid droplets in tubular gland cells (TGC) in the propria. Dilated Golgi complex (G), SG: secretory droplets.

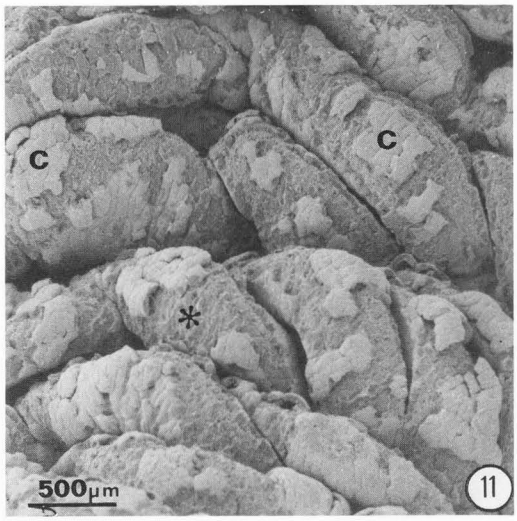


The fine structure of the surface epithelium of the magnum in most of the birds of this group was similar to that of control group. However, in a few specimens from two birds, the apical plasma membrane showed lesions. Such affected areas presumably corresponded to SEM images, in which they appeared as intermittent patches devoid of cilia and other surface modifications. Cells in these areas of denudation appeared enlarged and were packed with large secretory droplets of medium electron density, some of which were being secreted by exocytosis. Some plasma membrane disruption was also noted at the sites of exocytosis. However, nuclei of these cells were distorted and contained large heterochromatic patches. Most of the endoplasmic reticulum was in vesicular form. The ciliated cells which were wedged in between the secretory cells showed no overt changes, but their number appeared to be reduced (Fig. 9).

SEM observations of the mucosal surface of the shell gland showed no signs of alteration in the distribution and structure of cilia. TEM investigations revealed a mild accumulation of fat droplets in the epithelial cells (Fig. 10).

Group IV (15 ppm MeHgCl) The SEM examination of the primary folds of the magnum of all birds of this group showed that about 70% of the surface epithelium was severely disrupted (Fig. 11). The debris was abundant and obscured features of intact epithelium. At higher magnification (Fig. 12), clumps of cilia, small microvilli, cellular or mucous debris were often encountered intermingled with morphologically intact cilia. The denuded surfaces presented no further information, as to the nature of the underlying cell or the depth of the lesion, due to the severe disruption of the apical membranes (Fig. 12).

The TEM examination of ciliated cells of the surface epithelium of magnum showed varying degrees of degenerative changes in the cytoplasm, often associated with alterations in the ciliary structure. Complete disintegration of cytoplasmic organelles (Fig. 13), desquamation of disrupted cells (not shown), aggregated intracytoplasmic inclusions and distorted cilia, were noted. The apical region of the surface epithelium from two birds completely lacked the ciliary shaft while basal bodies and rootlets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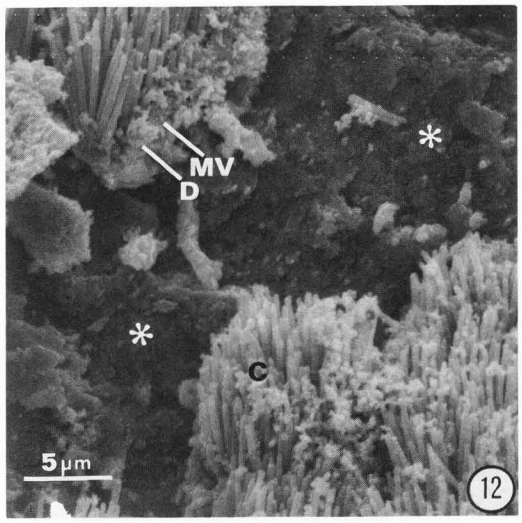
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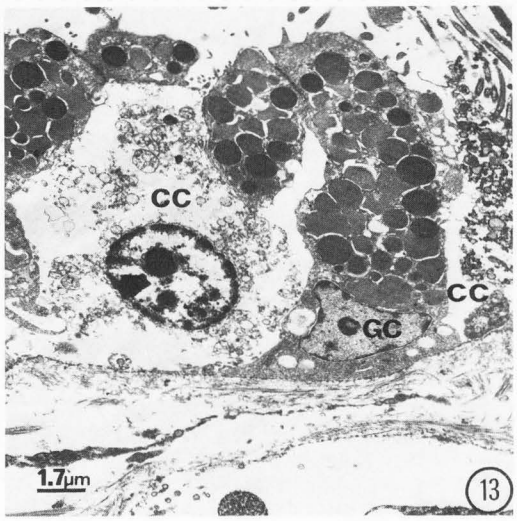
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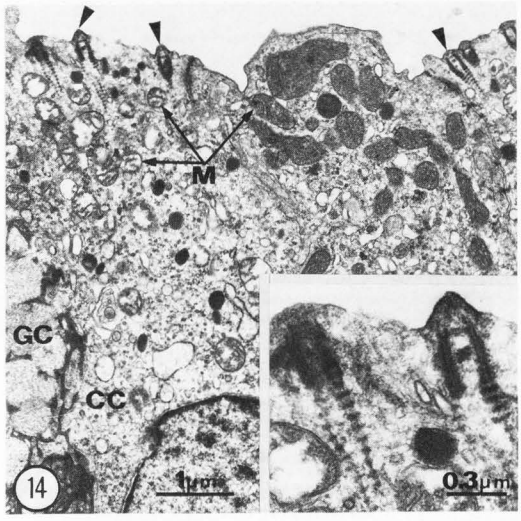
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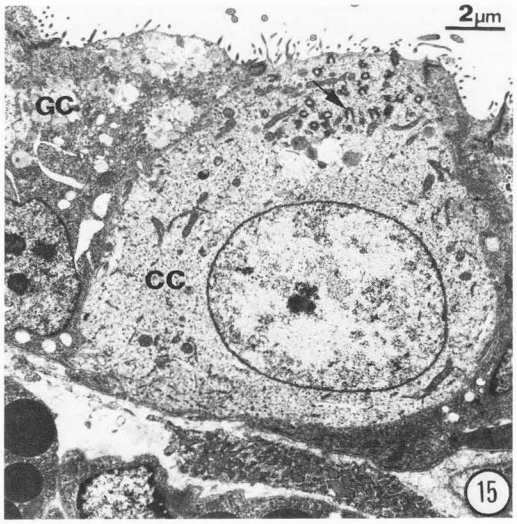
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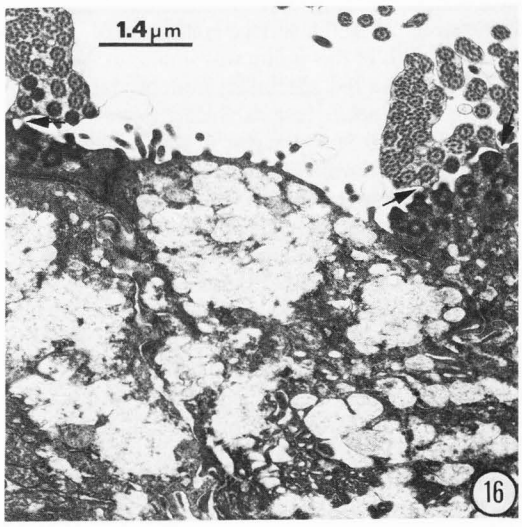
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Fig. 11. SEM of the mucosal folds of the magnum from a duck belonging to the 15 ppm MeHgCl treatment group. Note extensive denudation (asterisk) of the surface epithelium of primary folds with persisting patches of the ciliary carpet (c).

Fig. 12. High magnification SEM of the surface epithelium from the magnum of a duck fed with 15 ppm MeHgCl. Note the deciliated area (asterisks) showing membranous and mucus debris (D). The junction area between deciliated and ciliated (C) patches contains short microvilli (MV).

Fig. 13. Electron micrograph of the surface epithelium of the magnum from a duck fed with 15 ppm MeHgCl. The degenerating ciliated cells (CC) show marked distortion of the cytoplasmic organelles and cytolysis. Nonciliated secretory cells (GC) show electron dense secretion granules.

Fig. 14. Electron micrograph of the surface epithelium of the magnum from a duck fed with 15 ppm MeHgCl showing the nonciliated secretory (GC) and ciliated cells (CC). The ciliated cells show loss of the shafts (arrowheads) of the cilia. Note the distorted as well as unaltered mitochondria (M) in adjacent cells. Inset: shows longitudinal section of basal bodies with rootlets. Note a distinct basal plate and no axonemal microtubules. The plasma membrane forms a conical dome on the apical side of the basal bodies.

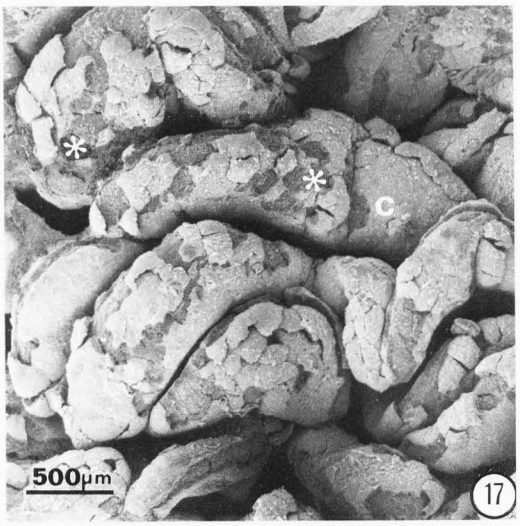
Fig. 15. Electron micrograph of the surface epithelium of the magnum from a duck fed with 15 ppm MeHgCl. Ciliated cell (CC) is devoid of axonemal extensions and basal bodies are dispersed at random subapically (arrow). Nonciliated secretory cells (GC) show mild fatty infiltration in the basal region.

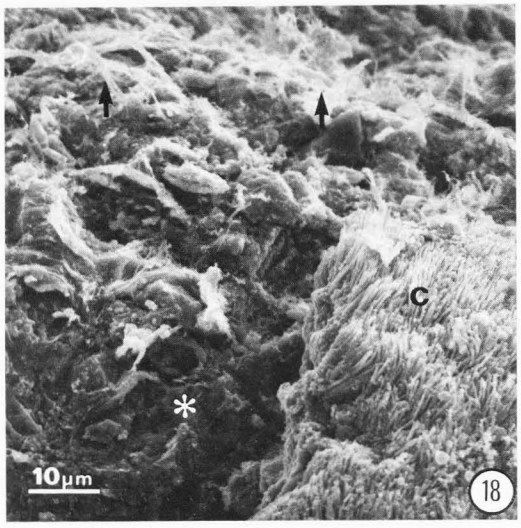
Fig. 16. TEM of the surface epithelium of the magnum from a duck belonging to the 15 ppm MeHgCl treatment group. Note the compound cilia with plasma membrane continuity with the apical cell membrane (arrows).

Fig. 17. SEM of the mucosal folds of the shell gland region from a duck fed with 15 ppm MeHgCl in the diet. The primary folds show patches of deciliation (asterisks) among the ciliated surface (c).

Fig. 18. Scanning electron image of a portion of shell gland region mucosal fold from a duck belonging to 15 ppm treatment group. The denuded area (asterisk) shows apical membrane lesions with surface debris. Intact cilia (C) and collapsed cilia (arrows) with mucous debris are seen in the periphery of the denuded areas.

showed no conspicuous changes (Fig. 14 and inset). Basal bodies lacking rootlets were sometimes observed randomly dispersed in the apical cytoplasm (Fig. 15). The alteration in mitochondrial fine structure included fluffy appearance of the matrix, reduction or loss of matrical density and partial loss of cristae. A compound or multiple ciliary arrangement was seen in some cells, where several ciliary axonemes in a common matrix bounded by a membrane continuous with apical cell surfaces were noted (Fig. 16).





SEM examination of the primary folds of the shell-gland region revealed prominent patches of epithelial degradation in all the birds of this treatment group. The areas representing denuded cells were clearly distinguished from those of intact ciliated cells of this group (Fig. 17). Here the partial or total loss of cilia and apical membrane were observed. Debris of cellular and possibly secretory origin were associated with intact and degenerated epithelia (Fig. 18).

The ultrastructural alterations seen in the surface epithelial cells of shell-gland region of this group were more extensive than the preceding groups. Loss of cilia and damaged apical cell membrane were common lesions observed. Complete loss of cilia and focal degenerative changes often accompanied a mild increase in fat droplets (Fig. 19). Relatively few specimens exhibited well preserved cilia, but the apical cytoplasm of such ciliated cells was filled with a type of lamellar bodies that contained reticular electron dense material producing wide alternate bands similar to those of "zebra bodies" (Fig. 20). The cytoplasm around these lamellar bodies showed an increased amount of intermediate type of filaments (Fig. 20 inset). Cytoplasmic lucency and abundantly dispersed pleomorphic mitochondria were demonstrable towards the base of these cells.

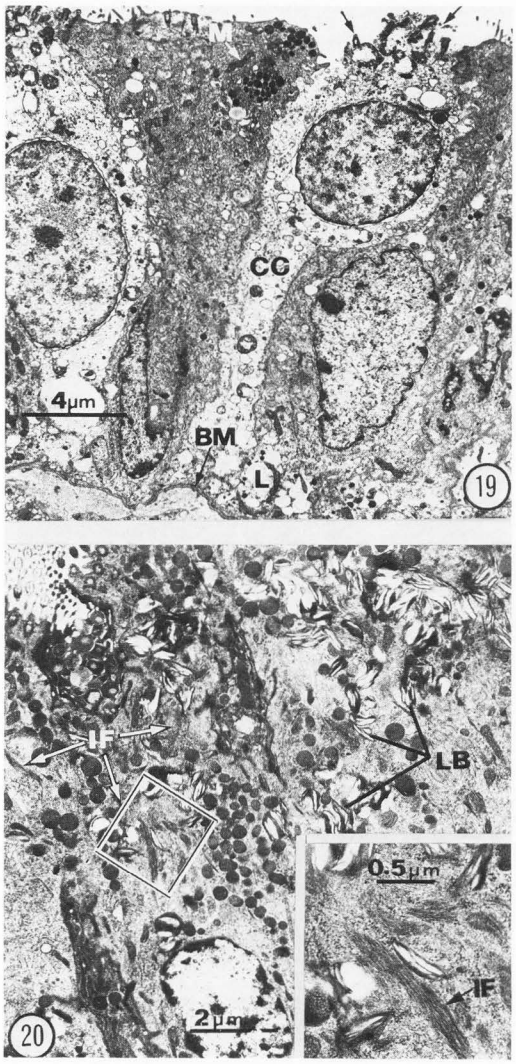


Fig. 19. TEM of the surface epithelium of the shell gland region from a duck fed 15 ppm MeHgCl. The ciliated cell (CC) shows loss of ciliary shafts extensions (arrows), vacuolation of cytoplasm and accumulation of lipid droplets (L) in the basal part of the cells. Basal lamina (BM) appears intact. M: Mitochondrion.

Fig. 20. Electron micrograph of the surface epithelium of the shell gland of a duck from 15 ppm MeHgCl treatment group. The apical cytoplasm of the ciliated cell contains numerous lamellar bodies (LB) and bundles of intermediate filaments (IF). Inset: Higher magnification of the area within the box showing bundles of intermediate filaments (IF).

Discussion

Both theoretical and experimental evidence favours the conclusion that mercury preferentially binds to thiol groups in the tissues.[13] Hence, every cellular component rich in thiol groups in structural and functional proteins is exposed to an irreversible interaction with mercury. Observed changes in the ciliary structure and conformation possibly indicate a complex interaction between ciliary membrane proteins and/or ciliary microtubular tubulin and MeHg. The mercurial effect on microtubules has been indicated under several circumstances. Spindle anomalies and complete inactivation of mitotic apparatus caused by organomercuric compounds have been demonstrated in root mitoses of *Allium cepa*.[35] In HeLa cells alkyl mercury compounds induced an increase of abnormal mitotic cells and the appearance of peculiar polynuclear cells.[48] Interaction of MeHg with microtubular tubulin was more directly demonstrated under *in vitro* conditions, in which 70% of the added MeHg was bound to the sulfhydryl groups in tubulin and caused a complete depolymerization.[1] Loss of the magnum and shell-gland regions of the 15.0 ppm ciliary shaft microtubules and disorganized ciliary basal bodies, in the absence of intermediate elements of ciliogenesis, suggest an interaction between the MeHg and axonemal tubulin that may have resulted in defective or incomplete ciliary nucleation. The conical stubs, with a cell membrane covering over the residual basal bodies, as noted the magnum and shell-gland regions of the 15.0 ppm treatment group of the present study, are similar to those reported by Boisvieux-Ulrich et al.[9] in the deciliation of avian oviduct after ovariectomy and progesterone treatment. Blum[8] has suggested that in several protozoa and some metazoa deciliation occurs by breakage of axoneme at a transition region located near the basal plate of the basal bodies. The breakage of ciliary shaft at this breakage point occurs in response to a wide variety of chemical and physical treatment. In view of the knowledge that deciliation of the surface epithelium of avian oviduct occurs in response to ovariectomy and progesterone and the accumulation of Hg in the pituitary gland in the amount similar to that of brain,[4] it is also possible that deciliation of the magnum and shell gland surface epithelium of the ducks fed 15.0 ppm of MeHg results because of an interference by Hg with the production or release of gonadotrophic hormones. Another possibility for deciliation could be accounted to alteration in the membrane integral proteins of the ciliary necklace that have been suggested to serve as ionic permeability sites.[23] It has been demonstrated that Hg alters the carrier proteins and/or sulfhydryl groups involved in permeability channels.[39]

Various noxious agents that damage the respiratory epithelium also produce a wide spread destruction and loss of cilia.[21] Hilding[24] reported that in calf trachea deciliated cells produced by mechanical trauma were exfoliated and were later replaced by immature cells from the deep layers. Although we did not note overt mitotic activity or intermediate structures involved in ciliogenesis, it is possible that repair of surface epithelium may occur. It has been shown that withdrawal from short mercurial exposures has resulted in the resumption of regular laying in chickens.[42] The desquamation of degenerating ciliated cells observed in the present study may indicate an attempt to repair the damage caused by methyl mercury. However, further work is required to prove that after a subchronic exposure at a relatively high dose of methyl mercury the withdrawal of toxin may result in ciliogenesis and resumption of regular laying, as it has been shown to occur in hen at low dose (<0.8 ppm)[42] of MeHg.

Cilia containing multiple axial microtubular complexes within a single ensheathing membrane were observed in the magnum epithelial cells of birds in the 15 ppm treatment group. Similar

compound cilia have been found in the tracheal epithelium of fowl exposed to infectious laryngotracheitis virus[34] and in carcinoma of the bronchial mucosa.[2] The formation of multiple axial cilia has been shown to be an indirect mechanism of deciliation, in which case previously fused cilia are discharged in groups.[9] The significance and the mechanisms of production of compound cilia are not precisely known. It would appear that such cilia are nonfunctional, since the multiple axonemal complex is unable to move the aggregate mass of cilia effectively.

Surface epithelial cell membrane damage was most prominent in the oviducts of ducks in 15 ppm MeHgCl group. The membrane lesions observed with SEM were confirmed by TEM. The concept of a membrane lesion in the toxicity of mercurial compounds, as a result of the modification in the protein components of the cell membrane, was first advanced by Rothstein.[38] It has also been suggested that Hg ions form cross linkages with membrane proteins.[33] The deposition of Hg in many biological membranes, inducing a change in the permeability has also been shown.[11] Loss of integrity of epithelial cells and membranous organelles noted in this study may be indicative of defective membrane proteins and altered permeability. This view is in agreement with the concept that mercury alters the specific sites in the membrane such as those of the carrier proteins[31] and/or sulfhydryl groups involved in the permeability channels.[39] MeHg is also shown to induce lipoperoxidation and to react with thiols in hydrophobic regions of proteins.[45] From these suggestions, it is clear that sulfhydryl binding agents like MeHg may have a profound general effect on the membrane structure and that it would secondarily change the permeability properties.

Accumulation of lipid droplets in the surface epithelium of shell gland region noted in the ducks in 15.0 ppm MeHg group may be a general reaction of secretory cells to Hg intoxication. Accumulation of lipid droplets in the hepatocytes[6,12,14,44] and the oviducal glandular parenchyma[42] of the chickens has been reported. The exact mechanism of lipid accumulation in the oviducts is not clear. However, the fatty changes seen in the shell-gland region are consistent with those reported in the oviducts of domestic fowl after short and mild exposure to mercurial fungicides.[42] The occurrence of lamellar bodies observed in the epithelial cells of the shell gland region of 15.0 ppm group may indicate an interference by Hg with lysosomal enzymes resulting in incomplete degradation of lipids or glycolipids. The "zebra bodies" having very similar morphology as noted in this study have been shown to occur in "Fabry disease" in which glycolipid deposits occur in kidney epithelial cells.[21] From the known sensitivity of microtubular elements to mercury,[1] it is also reasonable to assume that the depolymerization and denaturation of cytoplasmic microtubular tubulin or tubulin derived from ciliary shaft may also have contributed in accumulation of lamellar bodies.

Mitochondrial changes in the surface epithelial lining were more pronounced in the group of birds treated with 15 ppm MeHg. The observed changes; distortions in the cristae, reduction in the matrical density and membranous disruptions, were similar, though not identical, to many lesions produced in the hepatocyte mitochondria of various species after experimental Hg intoxication.[6,12,14] However, the changes induced by Hg in the epithelial cell mitochondria may suggest, in addition to membrane perturbations, an interaction with calcium deposition and/or interference with calcium binding proteins involved in the mobilization of calcium from the mitochondria during shell formation.

An increase in the cytoplasmic filament content as noted here has been observed in various experimental and pathological situations. Encephalopathy induced by spindle inhibitors caused a prompt disappearance of normal microtubules and a massive replacement of them by cytoplasmic filaments.[49] Likewise, filamentous proliferation was seen in HeLa cells treated with colchicine.[37]

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Discussion with Reviewers

Reviewer 4: Why was there not an effort on your part to locate areas of epithelial disruption in samples from four birds which showed damage by SEM in 5.0 ppm group?

Authors: Since the original experiment was not planned as SEM-TEM correlative study we collected samples for TEM from adjacent areas and did not use the SEM blocks exposed to electron beam for TEM observations. Further, the random and

patchy nature of the surface epithelium lesions made it difficult to get exacting correlation between SEM and TEM in 5.0 ppm treatment group. However the micrograph (Fig. 10) showing fatty changes in the surface epithelium is from a duck belonging to this group.

Reviewer 4: What you may be calling membrane damage in Fig. 9 may be the discharge of secretory material into the lumen by exocytosis?
Authors: Yes, both exocytosis (marked by arrows) and membrane damage (marked by arrowheads) are being shown in Fig. 9. The area of membrane damage shows cytoplasmic condensation, vesiculation, lipid droplets and fragmentation. This point is further supported by Fig. 13 from a duck in 15.0 ppm treatment group where degenerative changes in both the ciliated and nonciliated secretory cells are depicted.

Reviewer 4: Since the major portion of the Hg in eggs is found in the egg white, and more specifically ovalbumin (text ref. 10, 40), what was the appearance of the albumen secreting magnal tubular glands?

Without accompanying micrograph, how can you draw conclusion that "the fatty changes seen in the shell gland region are consistent with those reported in oviducts of domestic fowl after short and mild exposure to mercurial fungicides?
Authors: The magnal tubular glands of both 5.0 and 15.0 ppm treatment groups had altered ultrastructure. In 5.0 ppm group the glandular cell exhibited a mild lipid infiltration in cytoplasm and hypertrophy and cytolysis of Golgi complex cisternae. In 15.0 ppm group the RER was dilated and vesiculated. The mitochondria showed loss of matrix density and cristolysis. The apical membrane was disrupted with dispersion of cytoplasmic organelles in the lumen. In shell gland region extensive lipid infiltration was noted. These changes in the tubular glands are depicted in the Discussion with Reviewers figures (a) and (b) from magnum and (c) from shell gland region.

E. Boisvieux-Ulrich: In my opinion, it is not demonstrated that organomercurials have a depolymerization effect on tubulin of axonemal structures (from TEM examination). Rather than depolymerization of axonemes, membrane lesions and disruptions appear to be implicated in deciliation. Several observations rise to this conclusion: Loss of cilia results of collapsed axonemes in a single membrane. Simultaneously, morphology of the cytoplasmic organelles is damaged, as well as apex of mucous cells.

Reviewer 4: What evidence is there that the tubulin is depolymerized? Axonemes are still observed.
Authors: We agree with Dr. Boisvieux-Ulrich suggestion that the ultrastructural observations in this experiment indicate to membrane lesions and disruption for the patchy deciliation of magnum and shell gland region of the oviducts in 15.0 ppm treatment group (text ref. 23, 39). Nevertheless, the possibility that at this dose level methyl mercury may interact with axoneme tubulin cannot be ruled out especially in the light of its known binding affinity with neuronal tubulin (text ref. 1) and mitotic spindle microtubules (text ref. 35). We would like to concur that further experiments are needed to test the second possibility.

Reviewer 4: Did you note any clinical signs of methyl mercury ingestion that could be helpful to field workers, such as changes in behaviour, shell quality, egg production, weight or feather loss? Were changes noted in the gross appearance of the liver, ovary or oviduct?
Authors: Only the ducks fed 15.0 ppm MeHgCl showed overt clinical symptoms that included dull and ruffled feathers, anorexia, ataxia and leg paralysis. The egg production was erratic (as mentioned in the "Results' section). We did not note any overt alteration in egg shell hardness. The details of body weight data and gross appearance of liver have already been published elsewhere (text ref. 6). At postmortem the ovary and oviduct showed no discernible lesions.

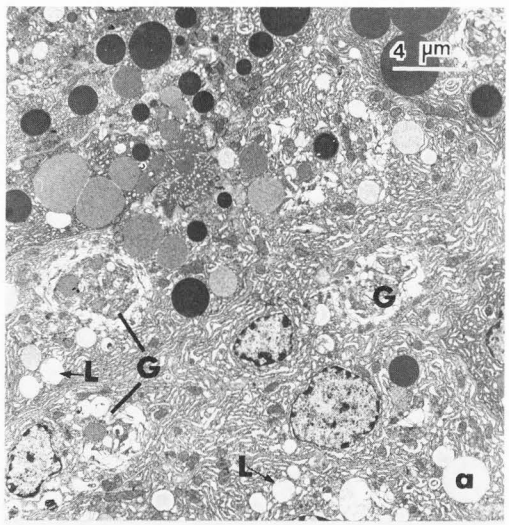
Reviewer 4: What evidence is there to support your statement that degeneration is an attempt by mucosa to repair itself?
Authors: We meant "desquamation of degenerated cells," (Fig. 13) from the surface epithelium of the magnum and shell gland region of the oviducts of the treated ducks, and not the "degeneration" of cells itself, might reflect an attempt to repair. Since there is very little information on the repair of the oviducal surface epithelium after methyl mercury exposure our suggestion was based on the single report available (text ref. 42) in abstract format where resumption of reproductive activity in domestic fowl was noted after the replacement of mercury contaminated diet with clean diet.

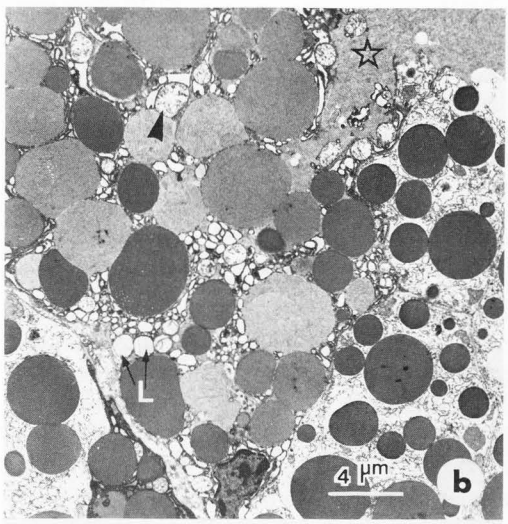
Reviewer 4: What evidence is there of defective synthetic or secretory activity? Secretory granules are present in most TEMs.
Authors: The suggestion of defective synthetic or secretory activity of nonciliated secretory cells of the surface epithelium is based on reduced number and/or altered morphology of the secretory granules in these cells. Moreover, it is now well established that mercury binds with and alters the membrane proteins (text ref. 31, 33, 38). Therefore it is conceivable that glycoproteins of secretory cells may have been affected by methylmercury treatment. However, we acknowledge that in the absence of histochemical study of the secretion granules in the affected cells, the suggestion made in the discussion portion of this report is speculative.

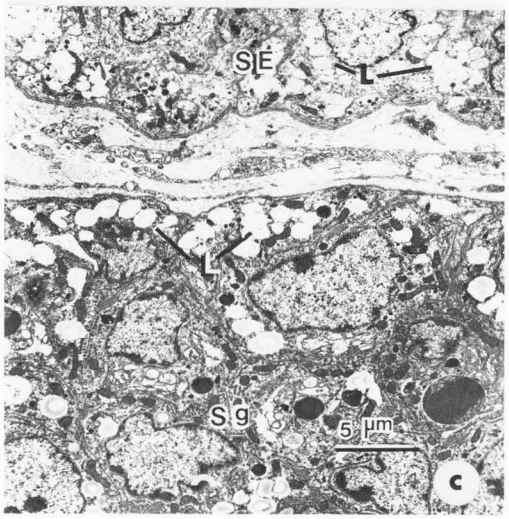
Reviewer 4: What is the origin of the microtubular tubulin? Cilia or free intracellular microtubules?
Authors: We believe that the tubulin in the cytoplasm will be primarily from free intracellular microtubules. However, internationalization of axonemes into the apical cytoplasm of the ciliated cells, as seen in the Discussion with Reviewers micrograph (Fig. d) could also contribute to the cytoplasmic tubulin pool.

E. Boisvieux-Ulrich: Is not the epithelium unistratified in ducks as in chick oviduct? Appearance of pseudostratification could depend on obliquity of section through the tissue.
Authors: The surface epithelium of chicken and quail oviduct is classically identified as pseudostratified columnar ciliated (text ref. 3, 22). The pseudostratification results from differential positioning of nuclei of the ciliated and nonciliated secretory cells forming the epithelium.

S.E. Solomon and J. Reid: The epithelial cells in fig. 15 are distinctly cuboidal. Is this the effect of Hg?
Authors: Yes, it is possible, but most ducks in this treatment group had columnar cells constituting the surface epithelium. The main purpose of Fig. 15 is to demonstrate the loss of ciliary shafts and random dispersion of basal bodies that have no rootlets.







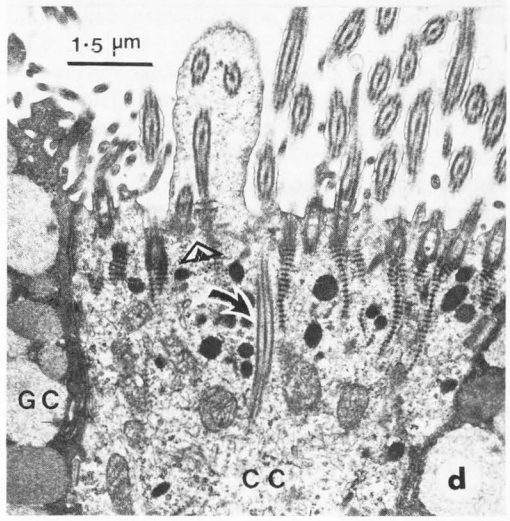


Fig. a. Electron micrograph of magnal tubular gland cells from a duck fed 5.0 ppm of MeHgCl. Note distended RER, dilated and degenerating Golgi complex cisternae (G) and lipid droplets (L). Asterisk marks the lumen of the gland containing microvilli.

Fig. b. TEM image of magnal tubular gland from a duck fed 15.0 ppm of MeHgCl showing vesiculated ER, swollen mitochondria with cristolysis (arrowhead), lipid droplets (L) and disruption of apical cytoplasm of the glandular cells and dispersion of organelles in the lumen (star).

Fig. c. Electron micrograph from shell gland region of the oviduct from a duck fed 15.0 ppm MeHgCl. Both the surface epithelium (SE) and glandular cells (Sg) contain a large number of lipid droplets (L).

Fig. d. TEM image of surface epithelium of the magnum from a duck fed 5.0 ppm MeHgCl. Note a compound cilia with at least two axonemes (arrowhead) projecting into lumen and intracytoplasmic axoneme (arrow) in ciliated cell (cc). Nonciliated secretory cell (GC).

S.E. Solomon and J. Reid: Figs. 8, 9 and 13 could be the mucous region of the magnum. Cf. Fig. 14 which is more characteristic of the epithelium lining the bulk of the magnum.
Authors: Since the tissue samples from magnum were collected from the mid portion and not the terminal portion we believe that Figs. 8, 9 and 13 represent surface epithelium alteration due to methyl mercury treatment. This point is very evident in Fig. 14 where degenerating ciliated cells (cc) alternate with altered nonciliated secretory cells with electron dense secretory droplets.

S.E. Solomon and J. Reid: Were the tissue blocks for SEM analyses coated before viewing?
Authors: In "OTOTO" procedure coating of tissue blocks is not needed (text ref. 26, 29). We used this procedure because of its simplicity as a large number of samples were to be processed from 24 ducks. Nevertheless, we agree that metal coating of specimens would have provided better SEM images.

Reviewer 4: Please comment on microstructure of the oviducal surface epithelia from duck and compare that to the chicken.
Authors: The surface epithelia covering the midportion of the magnum and *Pars major uteri* was lined by pseudostratified columnar epithelium in control ducks. The two types of cells forming the epithelium included ciliated and nonciliated secretory cells in nearly equal proportions. Transmission electron micrographs of magnum (Text Fig. 4) and of shell gland region (Text Fig. 5) resembled the surface epithelium covering the two segments of the oviducts of domestic fowl (Text ref. 3, 22).