THE EFFECTS OF FORMALDEHYDE VAPOUR ON THE MORPHOLOGY OF THE RESPIRATORY EPITHELIUM OF THE PRE- AND POST-HATCHED CHICK.

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by

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ABSTRACT

Disinfecting hatching eggs by the use of formaldehyde vapour during the last three days of incubation is common practice in commercial hatcheries to minimise the presence of potential pathogenic microorganisms and so produce high hatchability and healthy chicks. This study was designed to investigate by the use of scanning electron microscopy (SEM), transmission electron microscopy (TEM) and light microscopy (LM), the effects of exposure to low levels of formaldehyde vapour (10.9 ppm) on the epithelial lining of the respiratory tract of hatching chicks in a commercial situation. As a prelude to this study, a control study on the development of the respiratory tract was carried out using similar techniques and it was established that by 19th to 20th day of incubation, the mucociliated cells of the entire respiratory tract of chicks were well developed. Formaldehyde fumigation however, caused destruction to the entire respiratory tract of the chicks, inducing pathological changes including clumping of cilia and microvilli, development of blebs or balloon-like structures on the cilial and microvillal walls, deciliation and desquamation of the epithelium. In addition, mucus production was also seen to be affected, with increased mucus production and changes in both the nature of the mucosubstances and distribution of the mucous cells and intraepithelial mucous glands. The morphological changes in the lining respiratory tract appeared to last until about the fourth week post-hatching, when regeneration of the lining epithelium appeared to be completed.

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This thesis is dedicated to my late mother and father, Allahyarhamah Azizah Baba and Allahyarham Othman Abdul Samad.
"Al-Fatihah"

DECLARATION

THIS IS TO DECLARE THAT ALL THE WORK DESCRIBED IN THIS THESIS WAS CARRIED OUT BY FAUZIAH OTHMAN. WHERE ASSISTANCE WAS SOUGHT, IT HAS BEEN ACKNOWLEDGED ACCORDINGLY

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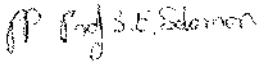

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LIST OF ABBREVIATIONS

AB/PAS	Alcian Blue/Periodic Acid Schiff
AB	Alcian Blue
CRT	Cranial Trachea
CLT	Caudal Trachea
H & E	Haematoxylin and Eosin
IPB	Intrapulmonary Primary Bronchus
L	Larynx
LM	Light Microscope (or Microscopy)
MNC	Middle Nasal Concha
PAS	Periodic Acid Schiff
PPM	Part Per Million
SEM	Scanning Electron Microscope (or Microscopy)
SB	Secondary Bronchus
TEM	Transmission Electron Microscope (or Microscopy)

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

The anatomy of the avian respiratory system was first described by William Harvey, in 1651 (cited by Mayor, 1967). However, only in the early twentieth century, did McLeod and Wagers (1939) study in detail the complexity of the respiratory system of the fowl (*Gallus domesticus*). Since then, much work has been carried out on the anatomy of the avian respiratory system (Rigdon, 1959; Bradley and Grahame, 1960; Salt and Zeuthen, 1960; Akester, 1960; King and Farner, 1964; McLeod *et al.*, 1964; King, 1966; Mayor, 1967; Duncker, 1971, 1972, 1974, 1978b; King and Molony, 1971; Lasiewski, 1972; King and McLelland, 1975, 1984; Nickel *et al.*, 1977; McLelland, 1990).

Of the work that has been carried out on the morphology of the avian respiratory epithelium, most appears to concentrate on the structure of the adult epithelium, in particular on those areas lining the trachea or lungs, and is confined to relatively few species such as chicken (Tyler *et al.*, 1961), goose (Lambson and Cohn, 1968), penguin (Drescher and Welsch, 1983), budgerigar (Smith *et al*, 1986), Ringed turtle dove (McLelland and MacFarlane, 1986), quail (Nowell *et al.*, 1970), duck (West *et al.*, 1977), pigeon (West *et al.*, 1977), Indian dove (Wetzstein *et al.*, 1980) and turkey (Saif *et al.*, 1981). In contrast, much has been written about the adult respiratory epithelium of mammals, focussing on a wide range of species such as rat (Rhodin and Dalhamn, 1956; Kuhn and Finke, 1972; Andrews, 1974; Alexander *et al.*, 1975; Luchtel,1978; Andrews, 1979), mouse (Okada , 1969; Wang and Thurlbeck, 1970; Greenwood and Holland, 1972; Nakano, 1986; Nakano and Muto, 1987), hamster (Becci *et al.*, 1978; Althoff *et al.*, 1981), guinea pig (Dahlgren *et al.*, 1972; Davis *et al.*, 1984), rabbit (Holma,

1969; Sturgess, 1977; Smith *et al.*, 1979), coyote (Morrison *et al.*, 1983), ferret (Hyde *et al.*, 1979), monkey (Greenwood and Holland, 1973; Leela and Kanagasuntheram, 1973; Castleman *et al.*, 1975; Wilson *et al.*, 1984; Maina, 1988b), dog (Groniowski *et al.*, 1972; Parra *et al.*, 1978; Majid, 1986), cat (Plopper *et al.*, 1983; Tandler *et al.*, 1983a, 1983b;), ox (Mariassy *et al.*, 1975; Smith *et al.*, 1979; Iovannitti *et al.*, 1985), sheep (Mariassy and Plopper, 1984; Chen *et al.*, 1991), goat (Kahwa, 1992), pig (Wang and Thurlbeck, 1970; Mebus and Underdahl, 1977; Adams, 1990), horse (Nowell and Tyler, 1971; Pirie, 1990; Pirie *et al.*, 1991a, 1991b) and man (Ali, 1965; Wang and Thurlbeck, 1970; Biondi and Biondi-Zappala, 1974; Greenwood and Holland, 1975; Bloom and Fawcett, 1976; Smith *et al.*, 1979; Boysen, 1982; Sorokin, 1988).

In addition, very little information appears to be available in the literature on the development of the respiratory epithelium of the incubating chick, with such studies as are available involving the use of scanning electron microscopy (Breipohl and Fernandez, 1977; Duncker, 1978b; Mohammed, 1989) and transmission electron microscopy (Petrik, 1967; Petrik and Riedel, 1968b; Kalnins and Porter, 1969; Kalnins *et al.*,1972; Jones and Radnor, 1972a, 1972b; Walsh & McLelland, 1974b, 1978) to concentrate primarily on the domestic chicken. In contrast, there is a wealth of information available on the development of the respiratory epithelium in mammals. Such studies have focussed on various species such as rat (Engel, 1953; Rhodin and Dalhamn, 1956; Leeson, 1961; Neuhauser, 1962; Neuhauser and Dingler, 1962; Leeson & Leeson, 1964; Balis and Conen, 1964; Dirksen and Crocker, 1966; Cireli, 1966; Weibel, 1967), rabbit (Dingler, 1958; Leeson, 1961; Kanda and Hilding, 1968; Gonzales-Crussi and Boston, 1974; Hage, 1974), hamster (Kikkawa and Spitzer, 1969; McDowell *et al.*, 1985), mouse (Hage,1974), guinea pig (Kikkawa and Spitzer, 1969; Hage, 1974), dog (Boyden and Tompsett, 1961; Wright *et al.*,

1983) and man (Emery and Mithal, 1960, 1961; Boyden, 1965, 1967; Boyden and Tompsett, 1965; Emery and Wilcock, 1966; Reid, 1967; Friedman and Bird, 1971; Greenwood and Holland, 1975; Zeltner and Burri, 1987; Kitaoka *et al.*, 1996).

HISTOLOGY AND HISTOCHEMISTRY OF THE AVIAN RESPIRATORY EPITHELIUM.

The ciliated and mucous cells work in harmony to trap undesirable particles from the air-stream and dispose of them. Due to the importance of these structures, numerous histological studies have been carried out on the entire avian respiratory system (Batt, 1926; McLeod and Wagers, 1939; Trautmann and Fiebiger, 1957; Mayor, 1967; Hodges, 1974; Mohammed, 1989; Bacha and Wood, 1990) as well as localised regions of the tract such as the nasal cavity (Cover, 1953a; Bang, 1961; Bang, 1964; Jungherr, 1943; Midtgard, 1989), larynx (Cover, 1953b; Chandra and Bharadwaj, 1970), syrinx (Myers,1917; Chandra and Bharadwaj, 1971), lung (Malewitz and Calhoun, 1958; Tyler and Pearse, 1966; Chandra and Bharadwaj, 1971) and air sac (Cover, 1953c; Lucas and Denington, 1961). Such studies, allowing as they do the identification and characterisation of a number of cell types populating the pseudostratified respiratory epithelium of the tract, have demonstrated that the conducting airways are lined primarily by ciliated and mucous cells (the latter often aggregated into intraepithelial mucous glands), whilst the syrinx, air sacs and respiratory exchange areas of the lung are lined primarily by either squamous or cuboidal epithelial cells. Although such general histological studies have enabled the identification the mucous cells and intraepithelial mucous glands in the lining respiratory epithelium of adult birds, few details on the distribution of the mucous cells and glands in relation to development, regional

differences, or age differences appear to have been noted (Mohammed, 1989). The use of specialised histochemical techniques allowed information about the mucous cells in the respiratory tract in particular, to be attained.

The characterisation of the carbohydrate moiety of the mucus by the use of the Periodic Acid Schiff (PAS) staining reaction (McManus, 1946), a procedure dependent on the fact that the mucous secretions from the mucous cells and intraepithelial mucous glands are a complex mixture of approximately 95% water and 5% protein, carbohydrate, lipid and inorganic materials (Jeffery, 1978), is a technique now routinely used in the histology and pathology laboratory (Mowry, 1956; Wheeldon *et al.*, 1976; Reid and Clamp, 1978; Spicer *et al.*, 1983). When combined with Alcian blue staining, routinely used to identify the various acid groups, which can be divided into sulphomucins containing sulphated esters and sialomucins containing non-sulphated esters such as sialic acid (Spicer *et al.*, 1965), it allows the further characterization of the carbohydrate-rich molecules by assessing their degree of basophilia, Thus, a combined Alcian Blue/ Periodic Acid Schiff (AB/PAS) stain offers a basis for the identification of a number of types of glycoprotein (Mowry, 1956). Mucus-producing cells stained with AB/PAS can be classified to one of three main colour groups for qualitative and quantitative descriptive purposes:

1. Red: indicative of neutral mucosubstance.
2. Blue: indicative of acidic mucosubstance.
3. Purple: indicative of mixed mucosubstance.

The initial work on the histochemistry of the mucociliary components of the nasal mucosa of avian species and its relation to the mucus flow in the chicken and Herring gull was carried out by Bang (1961). However, since then, and despite the importance of mucous cells, studies on the histochemistry of the mucous apparatus consisting of the mucous cells and glands of the adult respiratory epithelium in avian species have only been

reported in the chicken (Chandra and Bharadwaj, 1970, 1971; Bang and Bang, 1969; Mohammed, 1989; Midtgard,1989) and goose (Jefferey, 1978). In contrast, the histochemistry of this apparatus has been extensively reported in adult mammalian species including dog (Goco *et al.*, 1963; Spicer *et al.*, 1971; Wheeldon *et al.*, 1976; Majid, 1986), pig (Jones *et al.*, 1975), cow (Allan *et al.*, 1977), goat (Kahwa, 1992), horse (Pirie, 1990), monkey (Leela and Kanagasuntheram,1973; St. George *et al.*, 1984, 1986; Harkema *et al.*, 1987a) and man (Tos, 1971; Jones and Reid, 1973; Pastor *et al.*, 1994).

Similarly, although there is a large volume of work available, on the development of the mucociliary clearance mechanism in the mammalian respiratory epithelium, especially in the human embryo, dealing with either the entire respiratory tract (De-Haller, 1969; Tos, 1983) or localised regions such as the nose (Tos, 1975a), rhinopharynx and pharynx (Tos, 1975b, 1977), trachea (Thurlbeck *et al.*, 1961; Emery and Mithal, 1961) and bronchus (Tos, 1968, De-Haller, 1969), there appears to be little information available on the development of the mucous apparatus in the respiratory epithelium of the chick, other than the investigation by Midtgard (1989) into the development of seromucous glands in the middle nasal concha of the post-hatched chick from day-old through to 5-month-old. Alcian blue was used to stain serial sections and the remaining middle nasal concha was prepared as PAS-stained whole mounts. Mucous cells occur abundantly in the epithelium of the newly hatched chick, but tubular seromucous glands are scarce. The seromucous glands form by a process of invagination of the epithelium, which occurs predominantly during the first week after hatching. While the number of glands per mm cross-section of the middle nasal concha is constant after the first week, the gland fraction of the mucosa increases until the chickens are about 2-month-old. The glands continue to grow in size and an increasing fraction of the cells become mucous-

secreting.

In the goose trachea (Jefferey, 1978), Alcian blue (pH 2.5) / Periodic Acid Schiff staining of 6,000 goblet cells demonstrated that most of the latter stain blue and although both acid and neutral glycoproteins were present, the acid variety predominated and was present in more than 95% of goblet cells.

One of the earlier reports on the histochemistry of mucosubstance in the respiratory tract of avian species involved 2 to 12- month-old White Leghorns. Using Alcian blue and Periodic Acid Schiff stain Chandra and Bharadwaj (1970), indicated the presence of acid mucopolysacccharides in the larynx. A year later, Chandra and Bharadwaj (1971), using the same techniques reported the presence of acid mucopolysaccharide in the trachea, syrinx, intrapulmonary primary bronchus and secondary bronchus. Intraepithelial mucous glands, however, were reported to be present only in the proximal half of the trachea.

In the trachea of humans, well-defined mucous cells were first seen in the 13$^{1}/_{2}$-week-old foetus, mucous cells increase in number with age and in the 16-week-old foetus they are well developed and are more frequent (Thurlbeck *et al.*, 1961). Mucous cells were also reported to be present in the trachea and large bronchi of a 13-week-old foetus (De-Haller, 1969). There are two types of mucous cells either a cell distended with acid glycoprotein, almost entirely resistant to sialidase and rich in suiphate and a second narrower cell type containing neutral well-defined granules at the base of the cell and sometimes acid material at the top. In the rhinopharynx and pharynx, mucous cell development is visible in the 12-week-old foetus, that is 3 to 4 weeks later than the development of the ciliated cell, however by 17 weeks both the mucous cell and ciliated cell are present throughout the rhinopharynx and in the upper half of the pharynx (Tos, 1975b). In the nose the mucous cell density is highest in the inferior turbinate and lowest in the

nasal septum (Tos, 1983).

The first sign of mucous gland development is in the 11-week-old human foetus in the rhinopharynx (Tos, 1977), 12-week-old foetus in the trachea (Thurlbeck *et al.*, 1961) and 13-week-old foetus in the nose (Tos, 1975a) and bronchus (Tos, 1968; De-Haller, 1969). Gland formation proceeds in the cranio-caudal direction and it is fully developed in the nose of the 23-week-old foetus but later in the left main bronchus and trachea of the 25-week-old foetus (Tos, 1968). Gland density is highest in the nose and low in the trachea, bronchi and nasopharynx but individual gland mass is 3 to 4 times larger in these regions than in the nose (Tos, 1983). In the study of gland development in the trachea of 186 children from 24 weeks gestation to 9-years-old, there is a great individual variation in the microscopic structure of the respiratory tract tissue even from different children of the same age (Emery and Mithal, 1961) and also there is a great individual variation of the gland numbers at maturity (Thurlbeck *et al.*, 1961).

Quantitative studies on the distribution of the mucous apparatus in the developing embryo has received critical consideration with respect to the respiratory epithelium of human (Thurlbeck *et al.*, 1961; Tos, 1971, 1977, 1983). However, in the avian species, the distribution of mucous cells and intraepithelial mucous glands of the respiratory epithelium of chicken have been rather neglected (Mohammed, 1989). The latter author reported the numbers of mucous cells increased gradually from the nasal septum to the region of highest concentration in the intrapulmonary primary bronchus. However, the numbers of intraepithelial glands decreased gradually from the region of highest concentration in the nasal septum to the region of lowest concentration in the intrapulmonary primary bronchus. Mucous cells and intraepithelial glands produced mixed mucosubstance, however, acid mucosubstances predominate in the upper region of the respiratory tract while neutral mucosubstances are more frequent in the lower regions of the

conducting air-ways. However, there have been no reports on the histochemistry or the distribution of developing mucous cells and intraepithelial mucous glands in the developing respiratory epithelium of the avian species.

Bang and Bang (1969) examined histochemically significant different staining properties with regard to acid, neutral or mixed moieties, these staining characteristics often change during and after infection and during physiological alterations in the chicken, including gnotobiotic rearing and specific nutritional deficiency.

SCANNING ELECTRON MICROSCOPY OF THE AVIAN RESPIRATORY EPITHELIUM.

SEM studies on the avian respiratory epithelium (Table 1) have tended to focus on localised areas such as the trachea of the chicken (Dutta, 1975; Bayer *et al.*, 1977; Hod *et al.*, 1982; Lai and Ibrahim, 1983, 1984 and Robinzon *et al.*, 1983) and turkey (Saif *et al.*, 1981 and Nagaraja *et al.*, 1983), whilst comparative studies of the lung have been carried out in a range of the avian species including the quail (Nowell *et al.*, 1970), duck (West *et al.*, 1977), pigeon (West *et al.*, 1977), Khaki Campbell duck (Duncker, 1978 a), Indian dove (Wetzstein *et al.*, 1980), chicken (Maina, 1982, 1988a and Fujii *et al.*, 1981), and Ringed turtle dove (McLelland and MacFarlane, 1986). The infraorbital sinus of the turkey (Sanger, 1973) and middle nasal concha of the chicken (Breipohl and Fernandez, 1977) have also been examined using this methodology. Some of these published works, dealing primarily with pathological aspects of the avian respiratory system, have also included brief reference to the normal structure of the respiratory epithelium for comparative purposes (Dutta, 1975; Bayer *et al.*, 1977; Saif *et al.*, 1981; Hod *et al.*, 1982; Lai and Ibrahim, 1983; Nagaraja *et al.*, 1983; Robinzon *et al.*, 1983; Sawata *et al.*, 1985). It is only recently, that

the three dimensional morphology of the entire respiratory epithelium of the adult chicken has been studied (Mohammed, 1989). Most of these studies described the surface morphological structure of adult birds, and demonstrated that the respiratory epithelium is composed primarily of ciliated cells dominating a non-ciliated cell population comprised of mature and immature mucous cells. The non-ciliated cells occur either singly or are dispersed in large patches or in small groups among the ciliated cells (Lai and Ibrahim, 1983; Mohammed, 1989). The mature mucous cell has a characteristic typical apical protuberance bulging into the lumen of the respiratory tract. This is the effect of its large content of mucous granules. Sparse microvilli are present on the apical surface of the bulging mucous cells. Much of the work on the bird respiratory epithelium has also dealt with the surface structure of the lung. The primary and secondary bronchi are also lined primarily by ciliated cells interspersed with small areas of microvillous cells, except at the openings of the secondary bronchi, where the mucosal surface is covered by mainly microvillous cells (McLelland and MacFarlane, 1986). The tertiary bronchus, however, is covered by non-ciliated microvillous cells.

Very little information appears to be available on the development of the surface morphology of the respiratory epithelium of the chicken (Breipohl and Fernandez, 1977; Duncker, 1978b; Mohammed, 1989; Fagerland and Arp, 1993). Such studies have shown that in the 7-day-old chick embryo only a single microvillous cell type can be distinguished on the middle nasal concha whereas in the 20-day-old embryo, it is possible to differentiate ciliated cells separated by small areas of microvillous cells (Breipohl and Fernandez ,1977).

Although the larynx and trachea of the 17-day-old embryo are populated by relatively few developed ciliated cells, by the nineteenth or twentieth day, there has been a marked increase in the numbers of such

ciliated cells in these structures, which now resembles that of the adult bird (Mohammed ,1989).

These SEM studies not only allow the characterization of the surface morphological features of those cells populating the respiratory epithelium, but also provide an opportunity to examine other relevant structures. For example, the SEM study by Fagerland and Arp (1993) reported that there is an increase in the number of the bronchus-associated lymphoid tissue (BALT) nodules with age in the primary bronchial epithelium. Bronchus-associated lymphoid tissue in chickens of 1 to 6 weeks of age is recognised as raised rings around the secondary bronchial openings, whilst in older birds (6 to 8-week-old chicken) small-folds, associated with germinal centers of lymphoid tissue in pocket-like regions, are also seen at the secondary bronchial openings.

TRANSMISSION ELECTRON MICROSCOPY OF THE AVIAN RESPIRATORY EPITHELIUM.

As noted earlier, relatively little work has been done on birds. Published studies on the ultrastructure (TEM) of the respiratory epithelium of the bird (Table 2) have resulted in the definition and description of nine cell types populating the lining epithelium of the chicken. The cell types described are the ciliated cell (Purcell, 1971; Walsh and McLelland, 1974a, 1978; Lai and Ibrahim, 1984; Smith *et al.*, 1987; Mohammed, 1989), mucous cell or goblet cell (Purcell, 1971; Walsh and McLelland, 1974a, 1978; Bang and Bang, 1977; Lai and Ibrahim, 1984; Smith *et al*, 1987; Mohammed, 1989), basal cell (Purcell, 1971; Walsh and McLelland, 1974a, 1978; Lai and Ibrahim, 1984; Smith *et al*, 1987; Mohammed, 1989), non-ciliated columnar cell (Walsh and McLelland, 1974a, 1978), granular endocrine cell (King *et al.*, 1974; Wasano and Yamamoto, 1979; Walsh and McLelland, 1974b; McLelland and MacFarlane, 1986; Mohammed, 1989),

Type I pneumocyte or non-granular cell (King, 1979; Smith *et al.*, 1986; Tyler *et al.*, 1961; Tyler and Pangborn, 1964; Petrik and Riedel, 1968a; Drescher and Welsch, 1983; Mohammed, 1989) , Type II pneumocyte or granular cell (Lambson and Cohn, 1968; King, 1979; Smith *et al.*, 1986; Tyler and Pangborn, 1964; Petrik and Riedel, 1968a; Akester, 1970; Jones and Radnor, 1972b; Drescher and Welsch, 1983; Smith *et al.*, 1986; Mohammed, 1989), intermediate cell (Jeffery, 1978; Smith *et al.*, 1987; Mohammed, 1989) and serous cells (Jefferey, 1978; Mohammed, 1989).

Amongst those TEM studies available, investigations focusing on the respiratory epithelium of the developing chick appear to be particularly sparse. Those that are available have tended to concentrate on localised areas of the epithelium in the domestic chicken, such as in the lung (Petrik, 1967; Petrik and Riedel, 1968a; 1968b; Jones and Radnor, 1972a; 1972b; Fagerland and Arp, 1993), and in the larynx and trachea (Walsh and McLelland, 1978). Such numerically limited studies have shown that, in early embryonic life, the respiratory epithelium is composed primarily of undifferentiated epithelial cells (Petrik, 1967; Petrik and Riedel, 1968a; 1968b, Jones and Radnor, 1972a; Walsh and McLelland, 1978), whilst in 10 to 14-day-old embryos the laryngeal and tracheal epithelium consists of 2 to 4 layers of round or oval cells. This latter epithelium differentiates with time to become two cells thick (upper and lower layer) in the 15 to 16-day-old embryo, attaining a simple or pseudostratified columnar arrangement only in the 17 to 21-day-old embryo (Walsh and McLelland, 1978).

EFFECT OF FORMALDEHYDE VAPOUR ON THE RESPIRATORY EPITHELIUM

Formaldehyde is a widely used industrial chemical. Its greatest use is in the manufacture of urea-, phenol-, acetol- and melamine-formaldehyde resins, but it has a variety of other uses in agriculture, and is to be found in

paper, textile and dye stuff manufacturing, medicines, cosmetics and deodorants, disinfectants and fumigants, embalming agents, concrete, plaster, leather and photographic chemicals. Since both the makers and consumers of formaldehyde products are potentially exposed to their dangerous carcinogenic properties (Gamble, 1983), thus effects on human health have been extensively studied, with much of this work concentrating on their effect on the respiratory system.

The relevant literature on controlled human exposure, case reports, epidemiological studies, and animal studies relating to formaldehyde exposure is summarised in Table 3. Formaldehyde potentially affects the respiratory system in three ways: irritation, airflow obstruction or asthma or both, and cancer. Mucous membrane or upper respiratory tract irritation is the most commonly reported effect, with eye, nose and throat irritation being the most sensitive symptoms (Gamble, 1983). Numerous published reports of human case studies and industrial experiences, along with other mammalian studies, suggest that exposure to formaldehyde is associated with adverse effects on respiratory health (Schoenberg and Mitchell, 1975; Gamble *et al.*,1976; Hendrick and Lane, 1977; Cockroft *et al.*,1982; Main and Horgan, 1983; Frigas *et al.*, 1984; Sheppard *et al.*, 1984; Nordman *et al.*, 1985; Burge *et al.*, 1985; Witek and Schachter, 1987; Malaka and Kodama, 1990), causing problems such as decreased airway function and reduced efficiency of the mucociliary clearance mechanisms (Cralley, 1942; Dalhamn, 1956; Amdur, 1960; Morgan *et al.*, 1986a; 1986b; Swiecichowski *et al.*, 1993), deciliation (Zwart *et al.*, 1988; Gerrits, 1990; Gerrits and Dijk, 1991; Sander *et al.*, 1995), degeneration to sloughing of respiratory epithelial cells (Chang *et al.*, 1983; Monticello *et al.*, 1989; Furuta *et al.*, 1989; Gerrits, 1990; Gerrits and Dijk, 1991; Monticello *et al.*, 1991; Sander *et al.*, 1995; Cassee *et al.*, 1996), epithelial cell proliferation and squamous metaplasia (Swenberg *et al.*, 1980; Chang *et al.*, 1983; Zwart *et al.*, 1988;

Maronport *et al.*, 1986; Monticello *et al.*, 1989; Klein-Szanto *et al.*, 1989; Swenberg *et al.*, 1983; 1986; Rusch *et al.*, 1983; Maronpot *et al.*, 1986; Monteiro-Riviere and Popp, 1986; Zwart *et al.*, 1988; Monticello *et al.*, 1989; Klein-Szanto *et al.*, 1989; Monticello *et al.*, 1991; Cassee *et al.*, 1996), cell hyperplasia and hypertrophy (Monteiro-Riviere and Popp, 1986; Monticello *et al.*, 1991), in addition to the recognised carcinogenic effects (Swenberg *et al.*, 1980; Albert *et al.*, 1982; Kerns *et al.*, 1983; Olsen *et al.*, 1984; Starr *et al.*, 1985; Blair *et al.*, 1986; Morgan *et al.*, 1986c; Anonymous, 1988; Bermudez *et al.*, 1994). Such observations have resulted in considerable interest in the biological effects of formaldehyde gas and their relevance to human risk estimation in particular.

The effect of formaldehyde vapour on the respiratory epithelium is dependent on three factors:

1. The dose-related response (Swenberg *et al.*, 1980; Maronport *et al.*,1986; Cassee *et al.*, 1996)
2. Exposure time (Swenberg *et al.*, 1986)
3. Species sensitivity (Rusch *et al.*, 1983)

The cytotoxic effects of formaldehyde vapour and the regenerative response and cell proliferation involved in cell repair observed by the use of LM, SEM and TEM in the respiratory epithelium of various animal species exposed to formaldehyde vapour are reviewed below and as listed in Table 3. Most of the references are on mammal species, very little work has been carried out in avian species.

Despite these recognised adverse effects of formaldehyde vapour on the lining respiratory epithelium, formaldehyde fumigation is recommended twice in the process of hatching chicks (Buckle *et al.*, 1981; Gerrits, 1990, Gerrits and Dijk, 1991; North and Bell, 1990), once at the onset of the incubation and immediately after transfer to the hatcher (North and Bell, 1990). However, with omphalitis outbreaks in the hatchery, fumigation of

formaldehyde on chicks at pipping and at hatching may be necessary in order to get the disease under control. This treatment causes a pronounced yellowing of the chicks, a feature associated in many minds with good health.

Early studies on the toxic effect of formaldehyde vapour on the embryo of the domestic fowl were very much concerned with the hatchability (Gwatkin, 1926; Insko *et al.*, 1941; Wilson, 1951; Lancaster *et al.*, 1954; Harry and Binstead, 1961). The stage of incubation when formaldehyde vapour was exposed to the developing embryo was also investigated (Gwatkin, 1926). The latter author indicated that 56 ppm formaldehyde vapour did not reduce hatchability. Insko *et al.* (1941) indicated that 12 ppm formaldehyde was harmless to the chick embryo at any stage of incubation except on the second and third day of incubation. However, Wilson (1951) reported that when the 72 hours embryo was exposed to 53 ppm formaldehyde vapour the percentage mortality was 15.9 per cent. Three years later, Lancaster *et al.* (1954), however, found that the harmful effects of formaldehyde may extend to embryos up to 5 days of incubation. However, fumigation of eggs after setting and during incubation (that is at 5 to 9 days of incubation) is not recommended because of the possible toxic effect of formaldehyde on the developing embryos (Harry and Binstead, 1961). In the seventies, the recommended levels of formaldehyde fumigant employed under recommended time and temperature conditions before incubation of the eggs, were reported to be non-toxic to the embryos and thus increase hatchability (Proudfoot and Stewart, 1970; Williams and Gordon, 1970).

More recently, research has focussed on the effect of formaldehyde vapour on the respiratory epithelium of the chick at the second stage of fumigation, when the chicks begin to respire(Furuta *et al.*, 1989; Gerrits, 1990; Gerrits and Dijk, 1991; Sander *et al.*, 1995). Furuta *et al.* (1989) using

both LM and SEM techniques report that hatching chicks exposed to a higher concentration of formaldehyde (16 ppm), exhibited degeneration and desquamation of the tracheal and bronchial epithelium, whereas at a lower concentration (8 ppm), such lesions were absent. Histological studies by Gerrits (1990) and Gerrits and Dijk (1991) demonstrated that treatment with 20-80 ppm formaldehyde vapour during pipping and hatching resulted in the epithelial cells of the upper part of the chick trachea being severely damaged. The observed lesions included deciliation, flattened cells, inflammation, haemorrhages, cell proliferations and reduction in mucous cell numbers. In addition, a scanning electron microscopic study on the respiratory epithelium of formaldehyde-exposed chicks by Sander *et al.* (1995) revealed blunted cilia and blebs occurring on the cilial surface of day-old chicks, and excessive tracheal mucus was observed in 5-day-old chicks. Sloughing of the epithelium was observed by light microscopy and tracheal motility was reduced in the formaldehyde-exposed chicks. It should be noted that the above studies on the effects of formaldehyde vapour on the respiratory epithelium of hatching chicks were experimentally induced, and that there appear to be no similar studies on the effect of formaldehyde vapour on the respiratory epithelium of hatching chicks in the commercial situation. The objective of this study therefore was to investigate, for the first time, the effect of formaldehyde vapour on the respiratory epithelium of hatching chicks in commercial hatcheries.

TABLE 1

SCANNING ELECTRON MICROSCOPY (SEM) OF THE DEVELOPING AND MATURE RESPIRATORY EPITHELIUM OF VARIOUS AVIAN SPECIES.

SPECIES	AGE	ORGAN	REFERENCE
Quail	Not mentioned	Lung	Nowell et al., 1970
Turkey	4-week-old	Infraorbital sinus	Sanger, 1973
Chicken	8-week-old	Trachea	Dutta, 1975
Chicken	20-week-old	Trachea	Bayer et al., 1977
Duck	Adult	Lung	West et al., 1977
Pigeon	Adult	Lung	West et al., 1977
Chicken	7-day-old embryo to day-old chick	Middle nasal concha	Breipohl & Fernandez, 1977
Khaki Campbell duck	6-weeks-old	Lung	Duncker, 1978a
Chicken	19-day-old embryo to 1-year-old chicken	Lung	Duncker, 1978b
Indian dove	Not mentioned	Lung	Wetzstein et al., 1980
Turkey	11-day-old to 35-day-old	Trachea	Saif et al., 1981
Chicken	Adult	Lung	Fujii et al., 1981
Chicken	Adult	Lung	Maina, 1982
Chicken	15-day-old chick	Trachea	Hod et al., 1982
Chicken	10-week-old	Trachea	Lai & Ibrahim, 1983
Chicken	8-week-old	Trachea	Robinzon et al., 1983
Turkey	2 to 8-week-old	Trachea	Nagaraja et al., 1983
Chicken	10-week-old	Trachea	Lai & Ibrahim, 1984
Chicken	7-week-old	Nasal cavity	Sawata et al., 1985

SPECIES	AGE	ORGAN	REFERENCE
Ringed Turtle Dove	4 to 6-week-old	Lung	McLelland & MacFarlane, 1986
Chicken	Adult	Lung	Maina, 1988a
Chicken	17-day-old embryos to 4-day-old chick	Trachea & larynx	Mohammed, 1989
Chicken	Adult	Entire respiratory system	Mohammed, 1989
Chicken	Day-old & 1,2,3,6, 8-week-old	Bronchus-associated lymphoid tissue	Fagerland & Arp, 1993

TABLE 2

TRANSMISSION ELECTRON MICROSCOPY OF THE DEVELOPING AND MATURE RESPIRATORY EPITHELIUM IN VARIOUS AVIAN SPECIES

SPECIES	AGE	SAMPLE SITE	REFERENCE
Chicken	17-week-old	Lung	Tyler et al., 1961
Chicken	75-day-old	Lung	Tyler & Pangborn, 1964
Chicken	9-day-old embryo to day-old chick	Lung	Petrik, 1967
Goose	Adult	Lung	Lambson & Cohn, 1968
Chicken	17-day-old embryo to day-old chick	Air capillaries	Petrik & Riedel, 1968a
Chicken	17-day-old embryo to day-old chick	Air capillaries	Petrik & Riedel, 1968b
Chicken	15 to 19-day-old embryos	Trachea	Kalnins & Porter, 1969
Chicken	15 to 19-day-old embryos	Trachea	Kalnins et al, 1972
Chicken	Adult	Lung	Akester, 1970
Chicken	12-week-old	Trachea	Purcell, 1971
Chicken	13-day-old embryos to day-old chick	Tertiary bronchus	Jones & Radnor, 1972a
Chicken	13-day-old embryo to day-old chick	Tertiary bronchus	Jones & Radnor, 1972b
Chicken	Day-old to adult	Lung	King et al., 1974
Chicken	6-week-old to 12-month-old	Conducting air-way to air sac	Walsh & McLelland, 1974a

SPECIES	AGE	SAMPLE SITE	REFERENCE
Chicken	15-day-old embryos	Conducting air-way	Walsh & McLelland, 1974b
Chicken	6 to 12-week-old	Trachea	Walsh & McLelland, 1974c
Chicken	5 to 36-week-old	Lung	King et al., 1977
Goose	Not mentioned	Trachea	Jeffery, 1978
Chicken	10-day-old to 21-day-old	Larynx & trachea	Walsh & McLelland, 1978
Chicken	2 to 34-day-old	Nasal mucosa	Reissig et al., 1978
Chicken	3 to 5-day-old	Lung	Wasano & Yamamoto, 1979
Chicken	Adult	Lung	Abdalla et al., 1982
Adelie Penguin	Adult	Lung	Drescher & Welsch, 1983
Chicken	Adult	Lung	Kazachka, 1984
Chicken	10-week-old	Trachea	Lai & Ibrahim, 1984
Budgerigar	Adult	Lung & air sac	Smith et al., 1987
Chicken	1-day-old & adult	Abdominal air sac	Cook et al., 1986
Collared Dove	4 to 6-week-old	Lung	McLelland & MacFarlane, 1986
Budgerigar	Adult	Parabronchi & air sac	Smith et al., 1986
Budgerigar	Adult	Trachea & bronchi	Smith et al., 1987
Chicken	2-month-old	Respiratory system	Mohammed, 1989

TABLE 3

EFFECTS OF FORMALDEHYDE VAPOUR ON THE RESPIRATORY SYSTEM OF VARIOUS ANIMAL SPECIES

SPECIES	SAMPLE SITE	TECHNIQUES	REFERENCE
Rabbit	Trachea	Cilia function (LM)	Cralley (1942)
Rats	Trachea	Cilia function (LM)	Dalhamn (1956)
	Nasal cavity	LM	Swenberg et al. (1980)
	Trachea	TEM	Klein-Szanto et al. (1981)
	Nasal cavity	LM	Albert et al. (1982)
	Nasal cavity	LM, SEM &TEM	Swenberg et al. (1983)
	Nasal cavity	LM	Kerns et al. (1983)
	Nasal cavity	LM	Chang et al. (1983)
	Nasal cavity	LM & TEM	Rusch et al. (1983)
	Nasal cavity	Cilia function (LM)	Morgan et al. (1986a)
	Nasal cavity	Cilia function (LM)	Morgan et al. (1986b)
	Nasal cavity	LM	Morgan et al. (1986c)
	Nasal cavity	TEM	Monteiro-Riviere & Popp (1986)
	Nasal cavity	LM & TEM	Zwart et al. (1988)
	Nasal cavity	LM	Monticello et al. (1991)
	Nasal cavity	LM	Bermudez et al.(1994)
	Nasal cavity	LM	Cassee et al. (1996)
Mice	Nasal cavity	LM	Chang et al. (1983)
	Nasal cavity	LM	Kerns et al. (1983)
	Nasal cavity	LM	Jiang et al. (1986)
	Respiratory tract	LM	Maronpot et al. (1986)
Guinea pig	Lung	Physiological technique	Amdur (1960)
	Lung	Airway reactivity (LM)	Swiecichowski et al. (1993)
Hamster	Nasal cavity	LM &TEM	Rusch et al. (1983)
Monkey	Nasal cavity	LM & TEM	Rusch et al. (1983)
	Respiratory tract	LM	Monticello et al. (1989)
Chicken	Respiratory tract	LM & SEM	Furuta et al. (1989)
	Respiratory tract	LM	Gerrits (1990)
	Respiratory tract	LM	Gerrits & Dijk (1991)
	Trachea	LM & SEM	Sander et al. (1995)
Human	Respiratory system	Clinical observation	Schoenberg & Mitchell (1975)
	Respiratory system	Clinical observation	Gamble et al. (1976)
	Respiratory system	Clinical observation	Hendrick & Lane (1977)
	Respiratory system	Clinical observation	Cockcroft et al. (1982)

SPECIES	SAMPLE SITE	TECHNIQUES	REFERENCE
Human	Respiratory system	Clinical observation	Main & Horgan (1983)
	Nasal cavity	LM	Olsen et al. (1984)
	Respiratory system	Clinical observation	Frigas et al. (1984)
	Respiratory system	Clinical observation	Sheppard et al. (1984)
	Respiratory system	Clinical observation	Starr et al. (1985)
	Respiratory system	Clinical observation	Nordman et al. (1985)
	Respiratory system	Clinical observation	Burge et al. (1985)
	Respiratory system	Clinical observation	Blair et al. (1986)
	Respiratory system	Clinical observation	Anonymous (1988)
	Respiratory system	Clinical observation	Witek & Schacter (1987)
	Respiratory system	Clinical observation	Malaka & Kodama (1990)

CHAPTER 2

GENERAL MATERIALS AND METHODS

Control chicks

One hundred and thirty fertilized eggs from breeder hens which have not been exposed to formaldehyde were obtained from a commercial supplier and incubated at 37.5^{o}C (Brinsea Multihatch incubator). Nine embryos per day were randomly selected on days 15, 16, 17, 18, 19 and 20 of incubation. At hatching, nine day-old chicks were randomly selected and the remainder of the hatched chicks were kept in brooder trays and provided with heat, chick starter mash and water *ad-libitum*. Subsequently, nine chicks per day were randomly selected on days 3, 5, 7, 11 and 13 post-hatching. All chicks were sacrificed and preserved accordingly for normal control specimens, from each group of 9, 3 chicks were sampled for light microscopy (LM), 3 for scanning electron microscopy (SEM) and 3 for transmission electron microscopy (TEM) as in Table 4.

Formaldehyde-exposed chicks

Chicks were collected from a commercial hatchery which practised formaldehyde fumigation of chicks at pipping. Nineteen thousand fertile eggs were transferred from the setter to the hatcher (Petersime 192) on the 18th day of incubation. 18 hours after transferring the eggs to the hatcher, a single dose of formaldehyde vapour was liberated from plastic containers containing 400 ml of concentrated formalin with 400 ml of water per hatcher of 14. 7 m^3 (this resulted in a formaldehyde concentration of 10.9 ppm). The humidity of the hatcher was set at 80 percent on the 18th day of incubation and it was increased to 90 percent from day 19 to day 21 of incubation, whilst the temperature was set at 98^{o}C on day 18 of incubation and then

lowered to 97°C from day 20 until day 21 of incubation.

TABLE 4

CHICKS USED, AND TECHNIQUES INVOLVED, IN THE INVESTIGATION OF THE RESPIRATORY EPITHELIUM OF CHICKS.

Age	Number of chicks	Techniques Involved Number of samples		
		LM	SEM	TEM
15-day-old embryo	9	3	3	3
16-day-old embryo	9	3	3	3
17-day-old embryo	9	3	3	3
18-day-old embryo	9	3	3	3
19-day-old embryo	9	3	3	3
20-day-old embryo	9	3	3	3
1-day-old chick	9	3	3	3
3-day-old chick	9	3	3	3
5-day-old chick	9	3	3	3
7-day-old chick	9	3	3	3
11-day-old chick	9	3	3	3
13-day-old chick	9	3	3	3

Thirty minutes after the formaldehyde had been released in the hatcher, the concentration of formaldehyde vapour was detected to be 9.4 ppm using a Lion Formaldemeter II. Twelve eggs were randomly selected from the hatcher each day, this meant that samples collected on day 18 were from chicks unexposed to the formaldehyde vapour, whilst samples collected on day 19 and day 20 were from chicks exposed to formaldehyde for 6 and 30 hours respectively. Twelve additional chicks that hatched on the 19th day of incubation were marked and retained in the hatcher until day 21 of incubation, thus remaining exposed to the formaldehyde vapour for 54 hours, a situation which the early hatched chicks would normally face in the commercial hatchery. Samples were collected from these chicks and were considered in this study to be day-old chicks. At hatching 100 of the hatched

chicks were kept in brooder trays and provided with heat, chick starter mash and water ad-libitum. Subsequently, twelve chicks per day were randomly selected on days 3, 5, 7, 11 and 13 post-hatching and a further six chicken per day randomly selected on day 22, 29, 35 and 43 post-hatching. All chicks were sacrificed and were sampled for light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as in Table 5.

TABLE 5

CHICKS USED, AND TECHNIQUES INVOLVED, IN THE INVESTIGATION OF THE EFFECT OF FORMALDEHYDE VAPOUR ON THE DEVELOPING RESPIRATORY EPITHELIUM OF CHICKS.

Age	No. of birds	Techniques involved		
		LM	SEM	TEM
18-day-old embryo	12	3	6	3
19-day-old embryo	12	3	6	3
20-day-old embryo	12	3	6	3
1-day-old chick	12	3	6	3
3-day-old chick	12	3	6	3
5-day-old chick	12	3	6	3
7-day-old chick	12	3	6	3
11-day-old chick	12	3	6	3
13-day-old chick	12	3	6	3
22-day-old chicken	6	3	6	3
29-day-old chicken	6	3	6	3
35-day-old chicken	6	3	6	3
43-day-old chicken	6	3	6	3

Sample collection

Each chick was euthanised using an overdose intracardiac injection of sodium pentobarbitone (Euthatal; May & Baker). The respiratory system was removed and tissue samples collected from preselected sites as detailed below and as illustrated in Figure 1.1:

1. Middle nasal concha
2. Larynx
3. Cranial trachea
4. Caudal trachea

5. Lungs : a. Intrapulmonary primary bronchus

b. Secondary bronchus

c. Tertiary bronchus

Processing of samples for histology

The tissues were fixed in 10% buffered formalin for at least 5 days. They were then post-fixed with mercuric chloride formol for two days. After fixation, tissues were dehydrated through an ascending alcohol series, cleared in two changes of Histoclear and infiltrated in two changes of polywax (a mixture of paraffin wax and plastic polymer). Then the tissue blocks were sectioned to a ribbon of 3-4 um thickness using a Leitz rotary microtome, and mounted on glass slides. Mounted sections were stained with Haematoxylin and Eosin (H & E), Periodic Acid Schiff (PAS) and Alcian Blue/Periodic Acid Schiff (AB/PAS) (pH 2.5) according to a modification of the method of Mowry (1956). When staining with Alcian Blue/Periodic Acid Schiff (AB/PAS), the mucous cells will stain red for neutral mucopolysaccharides, blue for acidic and purple for mixed, according to the modification of the method of Mowry (1956) as detailed below:

1. Sections in Histoclear and wax for 5 minutes.
2. Hydrate sections.
3. Rinse in running tap water for 1 minute.
4. Lugol's iodine for 1 minute.
5. Rinse in water for 10 seconds.
6. Hypo for 1 minute.
7. Rinse in running tap water for 10 seconds.
8. 1% alcian blue in 3% acetic acid (pH 2.5) for 5 minutes.
9. Rinse in running water for 10 seconds.
10. 1% freshly made aquous periodic acid for 2 minutes,

11. Rinse in running water for 10 seconds.
12. Schiff's reagent for 8 minutes.
13. Rinse in running water for 10 seconds.
14. Mayer's haematoxylin for 5 minutes.
15. Rinse in running water for 10 seconds.
16. Differentiate in acid alcohol for 1 second.
17. Rinse in running water for 10 seconds.
18. Blue nuclei in Scott's tap water substitute for 3 minutes.
19. Rinse in running water.
20. Dehydrate, clean and mount.

Counting of mucous cells and intraepithelial mucous glands.

All histological sections were stained with PAS and AB/PAS (pH 2.5) to demonstrate the mucous cells and to differentiate the cells producing either neutral (red), acidic (blue) and mixed (purple) mucosubstance respectively. The total number of mucous cells and glands were assessed by choosing at random six areas of the sampled region in each bird. Each region was examined with a light microscope fitted with a graticule in the X10 eyepiece. Observation was carried out with a X25 objective, which gave a unit length of 0.46 mm for each selected length of respiratory epithelium. This gave a measurement of 2.76 mm to a standardized length of respiratory epithelium. To provide consistency in identifying individual mucous cells and mucous glands, a gland was identified at its simplest level when there was an invagination of the luminal epithelium lined with at least two stained mucous cells. The total number of mucous cells and mucous glands from the pre-selected standard length of the respiratory epithelium were noted, along with the nature of the contained mucosubstances.

Photography for light microscopy

A Leitz Laborlux 12 microscope with a Wild MPS45 photoautomat unit attached was used for light microscopy photography. Agfa PAN 35 mm film (12 ASA) was used for colour transparencies. For black and white prints, Agfa-Gevaert Rapitome photographic paper (P1-P4) was used and processed in an Agfa-Gevaert Radioprint PD 3700 automatic processor.

Processing of samples for scanning electron microscopy.

The tissues were gently washed in 0.1 M sodium cacodylate buffer to remove surface mucus and blood, and fixed in chilled Karnovsky's for 2 hours. The tissues were then rinsed in 0.1 M sodium cacodylate buffer for 4 hours. Subsequently, dehydration was carried out using a graded series of acetones; 70% for 4 hours, 90% for 2 hours, 100% for 2 hours and another 100% overnight. The tissues were then dried using a critical point dryer (Polaron: Watford, UK) for 2 hours. The dried specimens were orientated, carefully mounted on aluminium stubs using colloidal silver, and coated with gold-palladium using a sputter coater (EMSCOPE SC 500).

Photography for scanning electron microscopy.

Viewing of the specimens was carried out on a Philips 501B SEM. Examination of samples was done at an accelerating voltage of 15Kv using a range of spot sizes from 1000 - 200 depending on the magnification. An attached Rolliflex camera with Ilford FP4 120 (125 ASA) film was used for photography. Black and white prints were processed as for light microscopy.

Processing of samples for transmission electron microscopy (TEM).

The tissues were trimmed to 1 mm^3 and fixed in Karnovsky's fixative for 2 hours at 4^{o} C. The samples were then rinsed in 0.1 M sodium cacodylate buffer for 1 hour. Post-fixation of the tissues was then carried out

using 1% osmium tetroxide for 1 hour. The tissues were then rinsed three times with distilled water, and dehydration was carried out using a graded ethanol series: 25%, 50%, 70%, 90% for 10 minutes each. Finally dehydration was carried out in three changes of 100% ethanol for 15 minutes each change. Before infiltration of the specimens, the tissues were immersed in a mixture of 1:1 ethanol and propylene oxide for 10 minutes followed by 2 changes of propylene oxide (10 minutes, each change). Infiltration of tissues was carried out initially in two different mixtures of propylene oxide and Araldite, 1:1 for 4 hours, and 1:3 overnight. Infiltration of the tissues was then carried out in 100% Araldite for at least 6 hours. The tissues were then embedded in fresh Araldite resin in Beem capsule moulds. Polymerisation of the embedded blocks was carried out for 48 hours in an oven at 60°C. The polymerised blocks were trimmed into semi-thin sections, approximately 1um thick, using an ultramicrotome (LKB Pyramitome 11800). The sections were mounted on glass slides, stained with toluidine blue, dried and viewed under the light microscope. From light microscopic observation, the area of interest was selected and the block was retrimmed accordingly. Ultrathin sections were prepared using an LKB MK III ultramicrotome. Ribbons of ultrathin sections of silver to gold interference colour (about 600-900 Å thick), were mounted on clean 200-mesh size copper grids. They were stained with uranyl acetate for 5 minutes and then washed thoroughly in 3 changes of distilled water. Double staining was carried out using lead citrate for 10 minutes, followed by rinsing in 3 changes of distilled water. The grids were then examined under a Jeol JEM-100 CX 11 transmission electron microscope. Chemicals used for electron microscopy are as listed below:

<u>0.2 M Cacodylate Buffer (500 ml).</u>

0.4 M sodium cacodylate	250 ml
0.2 M hydrochloric acid	40 ml

Distilled water	210 ml

Karnovsky's Solution.

Paraformaldehyde	10 gms
Distilled water	100 ml
NaOH	10 drops

dissolved at 60°C then add to the stock solution .

Stock Solution

0.2 M cacodylate buffer	250 ml
2.5 % glutaraldehyde	50 ml
Distilled water	100 ml

Toluidine blue stain.

1% borax (sodium tetraborate)	1 gm
1% toluidine blue	1 gm
Distilled water	100 ml

Uranyl acetate.

0.2 gm of uranyl acetate was dissolved in 10 ml of distilled water, providing a saturated solution.

Lead citrate.

1.33 gm of lead citrate and 1.76 gm of sodium citrate were dissolved in 30 ml of distilled water and shaken for 30 minutes. 8 ml of 0.1 M NaOH were added, followed by distilled water to give a final volume of 50 ml.

Photography for transmission electron microscopy.

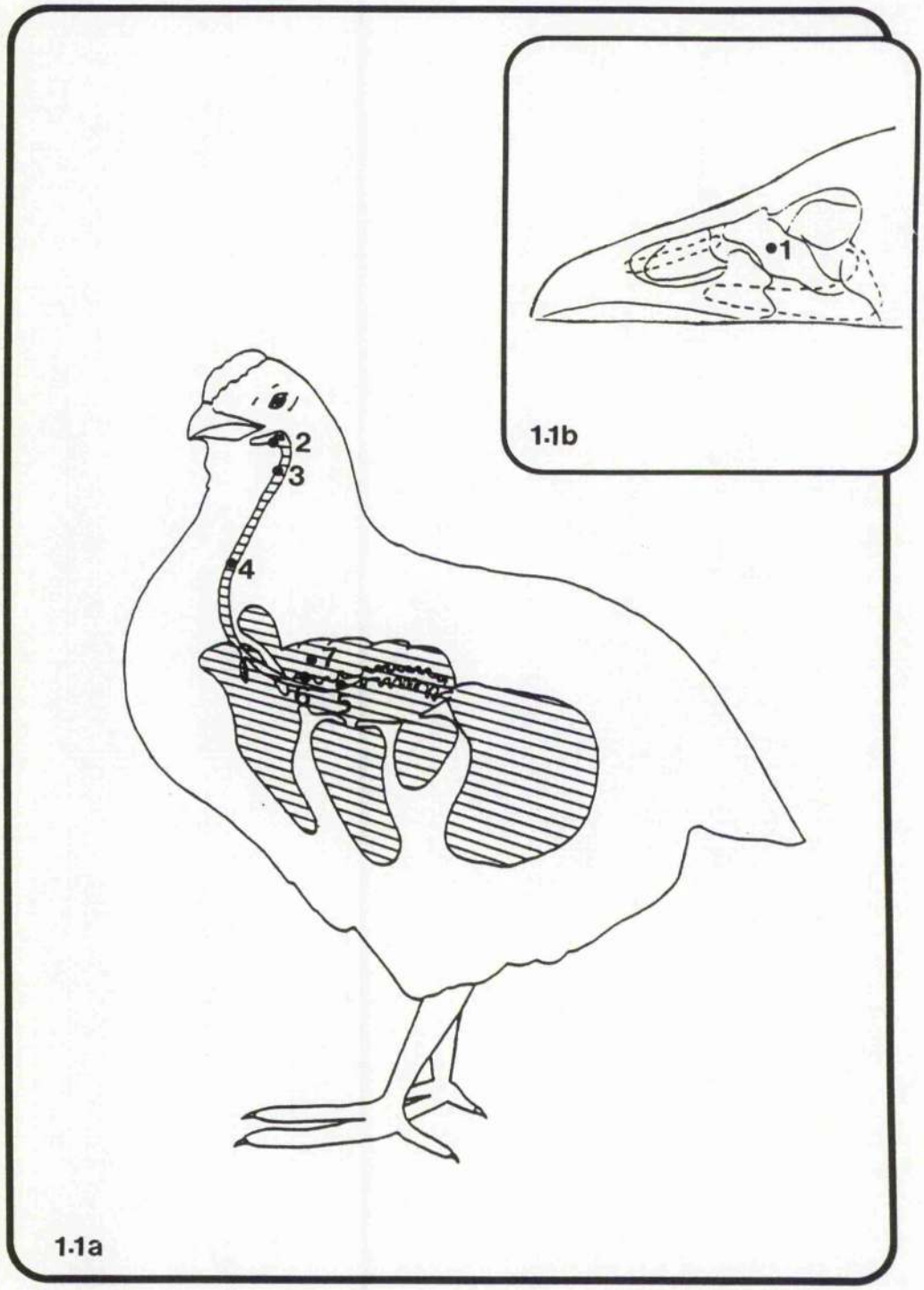
Transmission electron micrographs were taken using Ilford Technical EM plates ($3^1/_4$ X $4^3/_4$ inches), developed in PQ Universal and fixed in Ilford fixer. Black and white prints were prepared as for light micrographs and scanning electron micrographs.

Fig. 1.1

1. Middle nasal concha
2. Larynx
3. Cranial trachea
4. Caudal trachea
5. Intrapulmonary primary bronchus
6. Secondary bronchus
7. Tertiary bronchus

Figure 1.1

1.1a. Schematic diagram of the respiratory system of a chicken to show sample site (solid black circles). 1.1b. Schematic diagram of nasal cavity to show middle nasal concha (solid black circle)



CHAPTER 3

SCANNING ELECTRON MICROSCOPY OF THE NORMAL RESPIRATORY EPITHELIUM OF THE DEVELOPING CHICK

INTRODUCTION

The respiratory epithelium of chicks, through mechanical, cellular and humoral actions, plays an important role in the defence of the respiratory tract against irritants and infectious agents (Breeze and Wheeldon, 1977; Toth and Siegel, 1986; Toth *et al.*, 1987). The state of development of the respiratory epithelial lining at hatching is therefore of prime importance, since respiratory-related diseases have been shown to be the principal cause of chick mortality, especially within the first three weeks of life (Hofstad, 1984; Morris, 1992; Alexander, 1994; Bekker *et al.*, 1995; Jones, 1996), and are of significant economic importance within the poultry industry (Leong *et al.*, 1994; Cook, 1996).

In recent years there has been an increase in the use of scanning electron microscopy for examining the surface morphology of the normal and pathological respiratory epithelium in the chicken. Most of the information available, however, concentrates on the adult respiratory epithelium, with very little information apparently being available on the development of the respiratory epithelium of the incubating chick. Such studies as are available appear limited to either selected regions of the respiratory tract, such as the middle nasal concha (Breipohl and Fernandez, 1977), larynx and trachea (Mohammed, 1989) or lung (Duncker, 1978b).

It is the purpose of this section of the study to provide, for the first time, a scanning electron microscopic account of the development of the lining epithelium of the entire respiratory tract of the incubating chick.

MATERIALS AND METHODS

Source of chicks

Control chicks for this study were obtained as mentioned in Chapter 2. The number and age of chicks involved in this study are as tabulated below:

TABLE 6

CHICKS USED FOR SEM IN THE INVESTIGATION OF THE DEVELOPING RESPIRATORY EPITHELIUM OF THE CHICK.

Age of chicks	Number of chicks involved in SEM
15-day-old embryo	3
16-day-old embryo	3
17-day-old embryo	3
18-day-old embryo	3
19-day-old embryo	3
20-day-old embryo	3
1-day-old chick	3
3-day-old chick	3

Sample collection, processing of samples and photography for scanning electron microscopy.

As in Chapter 2.

RESULTS

The distribution and nature of the cells lining developing respiratory epithelium are summarised in Table 7.

Middle Nasal Concha

15-day-old Embryo

Scanning electron microscopy of the middle nasal concha of the 15-day-old embryo demonstrated that the surface epithelium, which presented a typical cobblestone appearance, was populated predominantly by either flattened or dome-shaped non-ciliated microvillous cells (Fig.3.1). The individual raised, dome-shaped microvillous cells were well demarcated, although the sizes and shapes varied considerably. In addition, the microvilli of the dome-shaped cells were very sparsely distributed compared to those of the flattened microvillous cells. Occasionally, microplicae were found covering the raised dome-shaped cells. Easily identifiable developing ciliated cells characterised by the presence of a few short surface cilia, were observed, usually as single isolated cells, but sometimes in small groups. Other cell types, superficially resembling the non-ciliated microvillous cell but carrying usually a single cilium, were often seen and were considered to be a very early developing stage of the ciliated cell. Pits were frequently seen on the surface of some of the epithelial cells.

16 to 17-day-old Embryo

In this age group, the mucosal surface of the middle nasal concha still presented the typical cobblestone appearance as seen in the 15-day-old embryo. Although numerous developing ciliated cells, characterised by a microvillous surface carrying a single cilium, were still observed at this stage, patches of more developed but still immature ciliated cells with a few

short cilia were also seen on the epithelial surface, in addition to occasional groups of fully mature ciliated cells (Fig. 3.2). Many of the surface microvillous cells presented a markedly raised dome-shaped surface covered with regularly distributed microvilli, suggestive of maturing 'mucus-producing' cells (Fig.3.3). Occasionally, mature mucous cells were noted characterised by sparsely distributed microvilli on their bulging surface, which also demonstrated numerous surface pits. On many occasions mucous granules were seen near the openings of the pits. In some cases, coalesced mucous granules were observed beneath the apical plasmalemma of the mature mucous cells (Fig.3.4).

18-day-old Embryo

By this stage of development, extensive patches of mature ciliated cells were observed at the surface of the middle nasal concha, with smaller patches of microvillous cells, carrying varying densities and lengths of surface microvilli, interspersed amongst the well ciliated areas (Fig. 3.5). Numerous mucous granules gathered beneath the apical surface of these microvillous cells. Pits were frequently seen on the surface of some microvillous cells, and may be the site of release of mucous granules (Fig.3.6). Indeed, the presence of mucus at the luminal surface indicated that these developing mucous cells were already active at this stage. In addition, mucous strands were occasionally seen to be extruded from the relatively increased numbers of intraepithelial gland orifices at this stage of development (Fig.3.7).

TABLE 7

DISTRIBUTION OF CILIATED AND MICROVILLOUS CELLS IN SELECTED REGIONS OF THE RESPIRATORY TRACT OF THE DEVELOPING CHICK.

Regions	Age							
	Day-old embryo						Day-old chick	
	15	16	17	18	19	20	1	3
Middle nasal concha	1+	2+	2+	3+	4+	4+	4+	4+
Larynx	1+	1+	2+	3+	3+	4+	4+	4+
Cranial trachea	1+	2+	2+	3+	3+	4+	4+	4+
Caudal trachea	1+	2+	2+	3+	3+	4+	4+	4+
Lungs: Intrapulmonary primary bronchus	4+	4+	4+	4+	4+	4+	4+	4+
Secondary bronchus	3+	3+	4+	4+	4+	4+	4+	4+

1+ - predominantly microvillous cells, with or without cilia (e.g. Fig. 3.1)

2+ - patches of developing ciliated cells (e.g. Fig. 3.3)

3+ - extensive patches of mature or fully developed ciliated cells (e.g. Fig. 3.5)

4+ - predominantly covered with dense fully mature ciliated cells (e.g. Fig. 3.6)

19-day-old embryo to 3-day-old chick

No significant surface morphological changes were observed between the 19-day-old and the 3-day-old chick, allowing observations in these groups of birds to be presented in unison. The characteristic feature of the mucosal surface of the middle nasal concha in this age group was the presence of a densely ciliated surface epithelium organised into a series of

ridges and gutters (Fig. 3.8a and Fig. 3.8b). The ridges were more densely ciliated than the gutters and occasionally the mucosal surface was covered by a thin sheet of mucus. Numerous mucous cells were frequently seen protruding from the dense ciliated epithelial surface. The gutters were poorly ciliated, with numerous protruding microvillous cells and intraepithelial gland openings being observed. Occasionally, groups of protruding microvillous cells were seen at the periphery of the opening of the intraepithelial glands. The non-ciliated microvillous cells were frequently seen to contain mucous granules, visible beneath the plasmalemma and also releasing mucous globules. Occasionally, the mucus secretion formed a covering mucous sheet on both the microvillous and ciliated cells.

Larynx

No obvious differences in the surface appearance between the dorsal, lateral and ventral walls of the larynx were observed during the developmental stages in this section of the study.

15 to 16-day-old embryo.

In the 15-day-old embryo, scanning electron microscopy revealed the presence of non-ciliated microvillous cells at the luminal surface of the larynx (Fig. 3.9). The polygonal, well-demarcated microvillous cells gave the mucosal surface its characteristic paving-stone appearance. Depressions were also frequently seen at the surface (Fig. 3.10). In the 16-day-old embryo, the surface of the larynx still exhibited a 'paving-stone' appearance similar to that in the 15-day-old embryo. However, it differed from the 15-day-old embryo in that, besides the microvillous cells, isolated developing ciliated cells were also seen (Fig. 3.11). Pores on the mucosal surface appearing to be the sites of intraepithelial gland openings were frequently observed and the microvillous cells often displayed a single cilium.

17-day-old embryo

The epithelial lining of the larynx of the 17-day-old embryo still presented the characteristic 'paving-stone' appearance, although patches of developing ciliated cells and openings of intraepithelial glands were observed more frequently (Fig. 3.13). Though the mucosal surface was predominantly covered by microvillous cells and developing ciliated cells, occasional isolated patches of mature ciliated cells were occasionally seen.

18 to 19-day-old embryo.

The characteristic feature of the laryngeal surface at this stage of development was the presence of numerous patches of mature ciliated cells interrupted by microvillous cells and intraepithelial gland orifices. These patches of ciliated cells varied from early developing ciliated cells to mature ciliated cells, the length and distribution of the cilia varying from cell to cell. Intraepithelial gland orifices and microvillous cells were often seen amongst the patches of ciliated cells, the orifices being lined mainly by microvillous cells.

20-day-old embryo to 3-day-old chick

The characteristic feature of the laryngeal lining in the 20-day-old embryo to the 3-day-old chick was the organisation of the epithelium into a series of epithelial gutters and ridges, the latter being covered by a dense carpet of fully elongated cilia with non-ciliated microvillous cells and intraepithelial gland orifices localised to the gutters (Fig. 3.14). Microvillous cells were also seen to be grouped on these ridges, where occasional gland openings were also encountered. Mucous granules were seen at the periphery of the gland orifices (Fig. 3.15) and on the microvillous cells near a pit (Fig. 3.16). When presented with the opportunity to examine fractured specimens, the aggregations of mucous granules within the non-ciliated

microvillous cells constituting the intraepithelial mucous gland were clearly demonstrated (Fig. 3.17).

Trachea

On all the samples examined from the 15-day-old embryos to 3-day-old chicks, the ventral, lateral and dorsal walls of the cranial and caudal trachea seemed to follow the same pattern of surface morphological development.

15-day-old embryos.

In the 15-day-old embryo the mucosal surface of the cranial and caudal trachea was corrugated and covered mainly with microvillous cells. Single cilia were frequently seen arising from individual stubby microvillous cells; such cells were considered to be ciliated cells at a very early stage of development. Occasionally, an individual isolated ciliated cell with short cilia was also seen. On rare occasions, pits on the luminal surface of the microvillous cells were also encountered.

16 to 17-day-old embryo.

In this age group, the epithelial surface was beginning to differentiate into patches of particularly stubby microvillous cells; such cells were considered to be groups of ciliated cells at a very early stage of development and frequently a single cilium was seen arising from the stubby microvillous cells. Though microvillous cells dominated the mucosal surface, mature ciliated cells and ciliated cells of various stage of development were seen on the tracheal surface in this age group. Often the patches of ciliated cells varied in the density and length of the cilia both within a cell and between neighbouring cells.

18 to 19-day-old embryo

At this stage, there was an obvious increase in the numbers and distribution of mature ciliated cells. The luminal surface showed extensive patches of mature ciliated cells interrupted by non-ciliated microvillous cells. Mucus was first seen to be extruded from the gland orifices in the 19-day-old embryo. Mucous cells were frequently seen either protruding singly from amongst the ciliated cells or in groups at the tracheal luminal surface.

20-day-old embryo to 3-day-old chick.

By the 20th day of incubation the mucosal lining of the trachea presented a dense carpet of cilia interrupted by microvillous cells (Fig. 3.18). Within the microvillous areas, pits on the surface of the non-ciliated microvillous cells, possibly the sites of mucus release, were often seen (Fig. 3. 19), along with intraepithelial gland openings. Mucous was occasionally observed being extruded from the latter. Mature mucous cells appeared to present a number of recognisable features, the accumulation of varying numbers of mucous granules beneath the plasmalemma including the sparse distribution of apical surface microvilli, and the active discharge of mucus.

Lungs: Intrapulmonary Primary Bronchus

15-day-old Embryo to 3-day-old Chick

In marked contrast to the epithelial lining of the more proximal parts of the respiratory tract previously described, the intrapulmonary primary bronchus, even at 15 days of incubation, exhibited a folded lining epithelium composed primarily of ciliated cells which provided the lining surface with a dense cilial carpet covering (Fig. 3.20). The mucosal surface of the primary bronchus was observed to be interrupted by intraepithelial gland orifices which were lined by microvillous and ciliated cells (Fig. 3.21). Occasionally,

isolated microvillous cells were seen emerging amongst the densely ciliated mucosal surface. At the origin of the secondary bronchus itself, there was a small transitional region in which the densely ciliated carpet, typical of the primary bronchus, rapidly changed into a non-ciliated microvillous lining epithelium composed of well demarcated dome-shaped or flattened individual cells. Even though this transitional region showed a sharp change in epithelial lining, isolated ciliated cells were still present and intraepithelial gland openings were also seen in this region (Fig. 3.22). However, towards the opening of the secondary bronchus the ciliated folds of the intrapulmonary primary bronchus frequently alternated with microvillous folds where isolated ciliated cells were encounter (Fig. 3.23). No further development was observed beyond the 15 day stage of incubation because the dense ciliated covering laid down by this time was identical to that of the established adult respiratory epithelium.

Lung: Secondary bronchus

15 to 16-day-old embryo

The mucosal surface of the secondary bronchus of the 15 to 16-day-old embryo was covered by a fairly extensive ciliated epithelium, interspersed with patches of non-ciliated microvillous cells. Towards the opening of the tertiary bronchus, the covering cilial carpet began to decrease whilst the patches of non-ciliated microvillous cells became more extensive; the latter appeared to consist of immature ciliated cells in various stages of development, with individual long cilia arising from stubby microvillous cells and a number of short cilia arising from developing ciliated cells, being frequently observed (Fig. 3.24).

17-day-old embryo to 3-day-old chick

By the 17th day of incubation, the mucosal surface of the secondary

bronchus was covered by a dense carpet of mature cilia (Fig. 3.25), a situation also observed up to the 3-day-old chick. At the opening to the tertiary bronchus, there was a sharp change in the epithelial lining (Fig. 3.26); the neck region nearest the opening of the tertiary bronchus was covered mainly by well demarcated non-ciliated microvillous cells amongst which occasional isolated ciliated cells were observed.

Lung: Tertiary bronchus

15 to 16-day-old embryo

In the 15 to 16-day-old embryo, the tertiary bronchus was lined by non-ciliated microvillous cells (Fig. 3.27) exhibiting a cobblestone appearance. The atria were not obvious since the parabronchial lining cells were compacted together at this stage (Fig. 3.28).

17-day-old embryo to 3-day-old chick

In the older embryos (17-day-old embryo to 3-day-old chick), atria became clearly recognisable (Fig. 3.29) and surfactant appeared to be present on the mucosal surface. The formation of the atrium took place when the parabronchial wall expanded radially into the mesenchyme, flattening the well-defined, lining, non-ciliated microvillous cells. With time the epithelium expanded further, resulting in a less obvious demarcation of these cells (Fig. 3.30). Smooth sheets of surfactant were frequently seen on the epithelial surface. In the well expanded parabronchial wall of the 1-day-old chick, atrial openings were prominent, allowing easy identification of the enclosing bands of bronchial muscle (Fig. 3.31). Frequently, openings to the infundibula were seen on the atrium (Fig. 3.32).

Fig. 3.1

Middle nasal concha, 15-day-old embryo.

Note the typical cobblestone appearance of the mucosal surface, and the well demarcated borders (---) of the individual microvillous cells both raised and flattened. Pits on the microvillous cell (arrow) and occasional appearance of microplicae (open arrow).

x 5,500

Fig 3.2

Middle nasal concha, 17-day-old embryo.

All three stages of developing ciliated cells can be seen in this scanning electron micrograph. Note

1. Non-ciliated microvillous cell
2. Early stage; microvillous cell with single cilium
3. Immature stage; numerous cilia are appearing
4. Mature stage; fully developed ciliated cells

X5,500


1
2
3
4

Fig. 3.3

Middle nasal concha, 17-day-old embryo.

Note the numerous rounded apical surfaces of the protruding microvillous cells (MC), and the presence of short microvilli at the apical surface. Small patches of mature ciliated cells (CC) are also visible.

X 5,500

Fig. 3.4

Middle nasal concha, 17-day-old embryo.

Numerous mucous granules (arrow) can be seen near the openings of the pits on the cells with sparsely distributed microvilli (*) and coalesced mucous granules are visible through the plasmalemma (open arrow).

X 5,500.


CC
MC

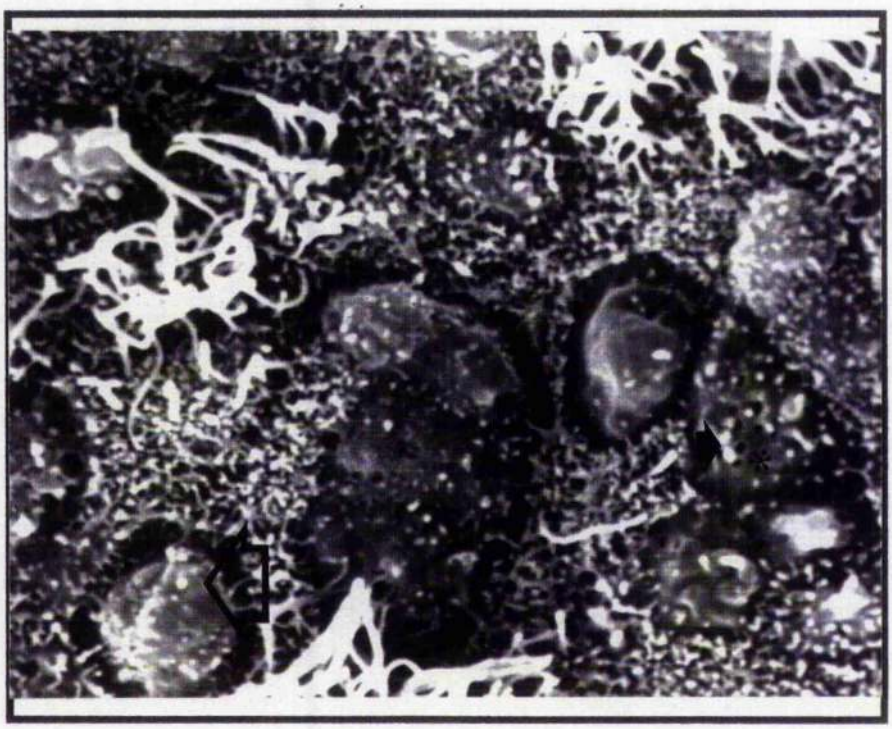


Fig 3.5

Middle nasal concha, 18-day-old embryo.

The ciliated surface is interrupted by a group of microvillous cells. Note the varying density and length of the microvilli on the developing ciliated cell (arrow) and on the raised dome-shaped cells (open arrow).

X 5,500

Fig. 3.6

Middle nasal concha, 3-day-old chick.

Epithelial surface with mucous granules (arrow) at the opening of the intraepithelial gland (open arrow) opening.

X5,500

Fig. 3.7

Middle nasal concha. 3-day-old chick.

A group of protruding microvillous cells with mucous granules (arrow) in the gutters of the mucosal surface.

X5, 500

Fig 3.8a

Ridges of the middle nasal concha. 19-day-old embryo.

Mucous granules seen emerging from the microvillous cells

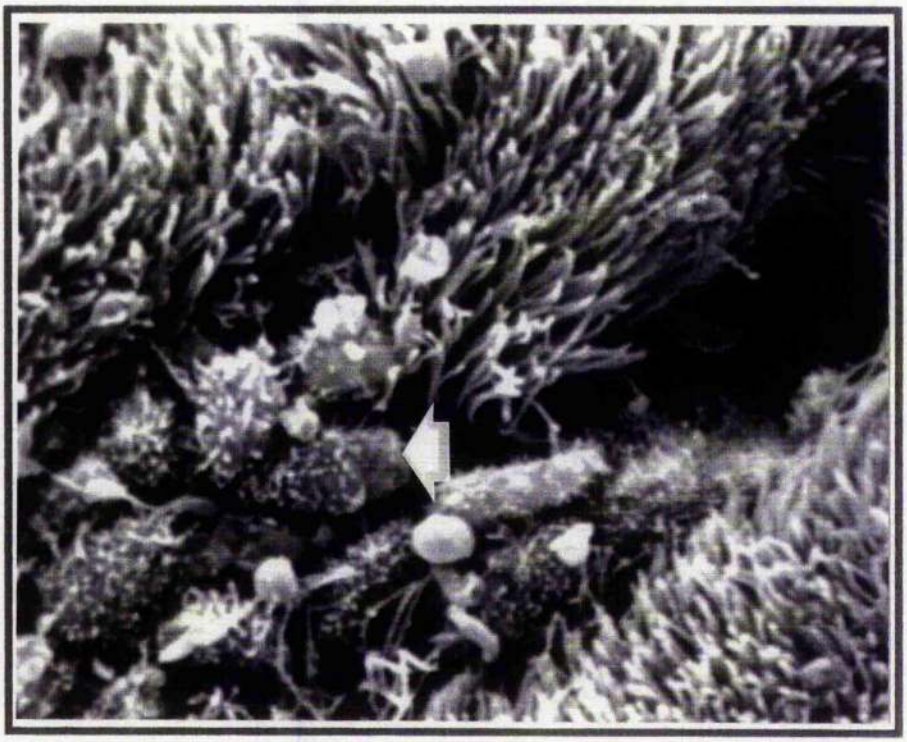
X11,000

Fig 3.8b

Gutters of the middle nasal concha. 18-day-old embryo.

Mucous strand (arrow) secreted from an intraepithelial gland orifice .

X 5,500



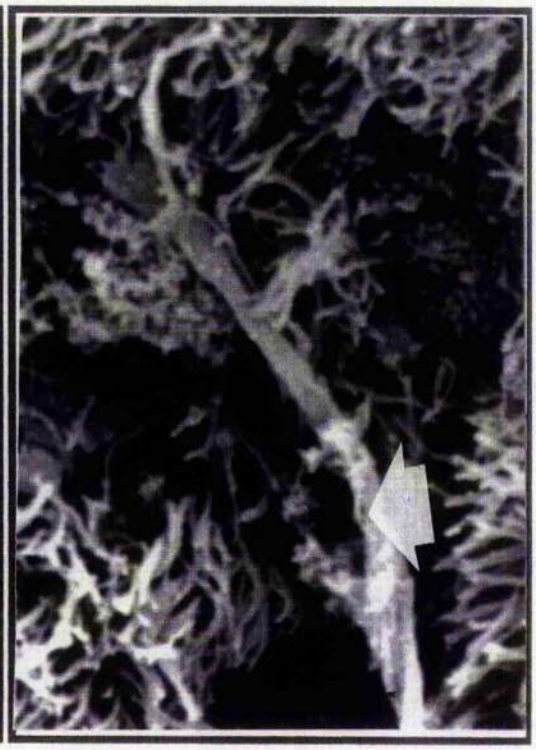


Fig. 3.9

Larynx. 15-day-old embryo.

The mucosal surface is covered with microvillous cells and is devoid of ciliated cells. The cells are polygonal and have a 'paving-stone' appearance. Note that the density and height of the microvilli is regular within an individual cell but it differs from cell to cell.

X5,500

Fig. 3.10 (left)

Larynx. 15-day-old embryo.

Depression (arrow) on the mucosal surface, presumably intraepithelial gland opening.

X 5,500

Fig. 3.11 (right)

Larynx . 16-day-old embryo.

Laryngeal surface, note the isolated ciliated cells projecting amongst the microvillous cells.

X 5,500

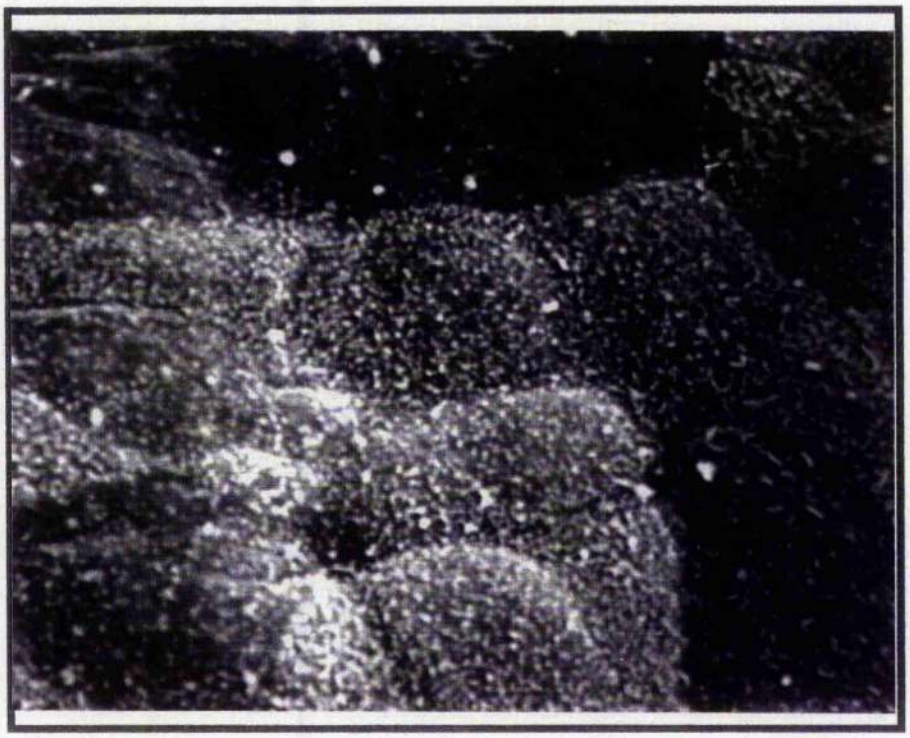


Fig. 3.12

Larynx.17-day-old embryo.

A patch of mature ciliated cells on the mucosal surface.

X 5,500

Fig. 3.13

Larynx. 17-day-old embryo.

Mucosal surface, note the patches of mature ciliated cells (open arrows) interrupted by microvillous cells and intraepithelial gland orifice (arrow).

X5,500

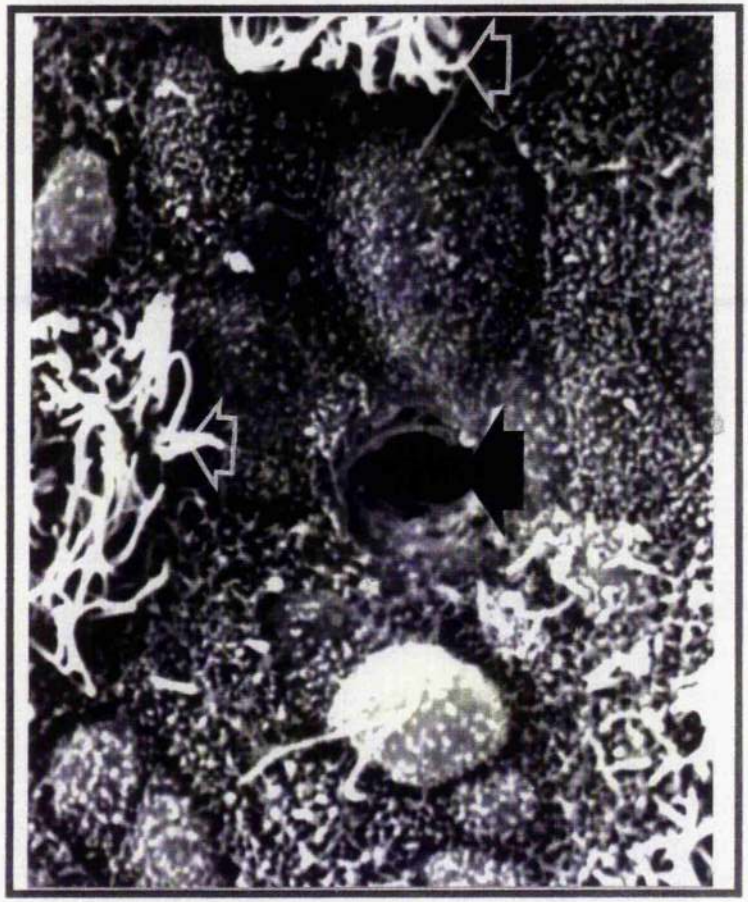


Fig. 3.14

Larynx. 3-day-old chick.

Mucosal surface, note the dense carpet of mature cilia interrupted by microvillous cells and intraepithelial gland orifices (arrow).

X 1,400

Fig. 3.15

Larynx. 3-day-old chick.

Epithelial surface, note the mucous granules around the intraepithelial gland orifice (arrow).

X 5,500

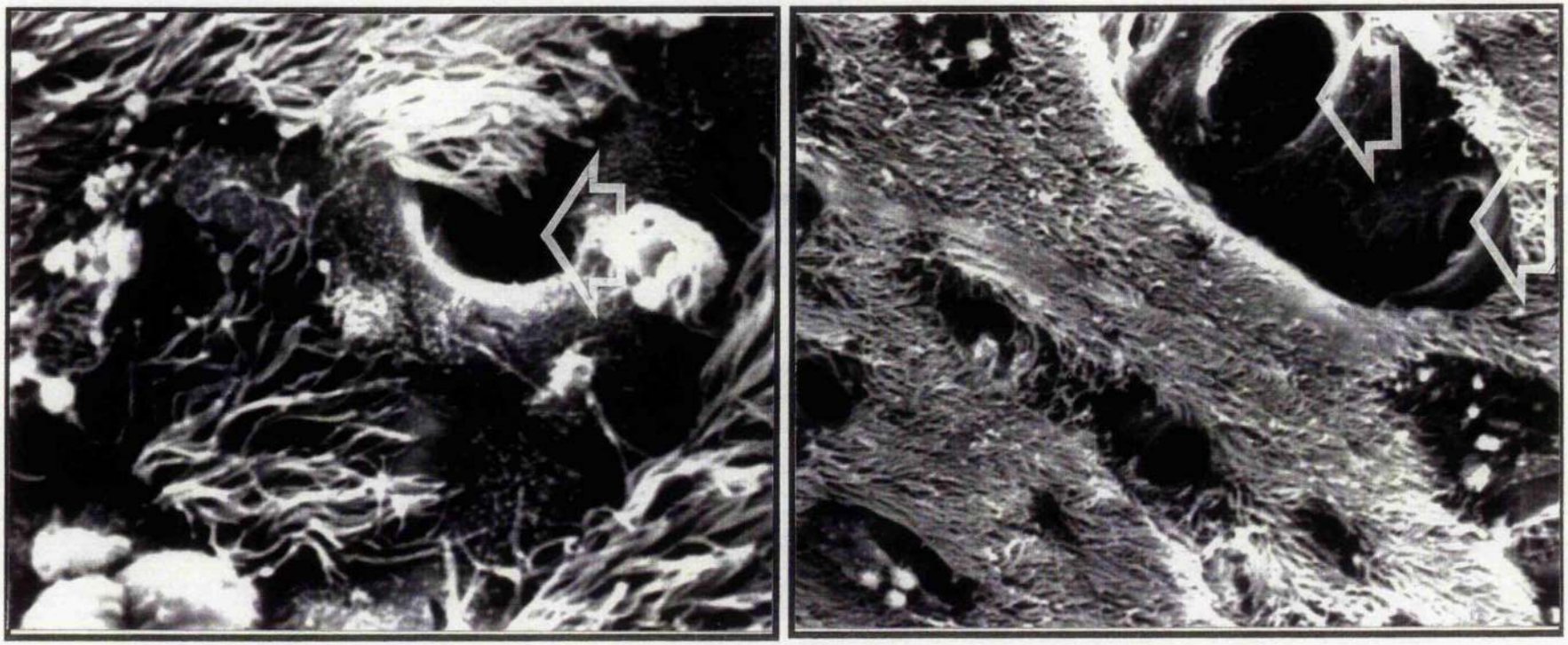


Fig. 3.16

Larynx. 3-day-old chick

Sparsely distributed microvilli on the microvillous cells with mucous granules seen through the plasmalemma (arrow) and mucous granules on the mucosal surface near a pit (open arrow).

X 5, 500

Fig. 3.17

Larynx. 3-day-old chick

Fractured specimen of an intraepithelial gland made up of mucous cells containing numerous mucous granules (arrow). The microvillous surface of some of the component cells of the gland can be seen within the gland lumen (*).

X 5,500

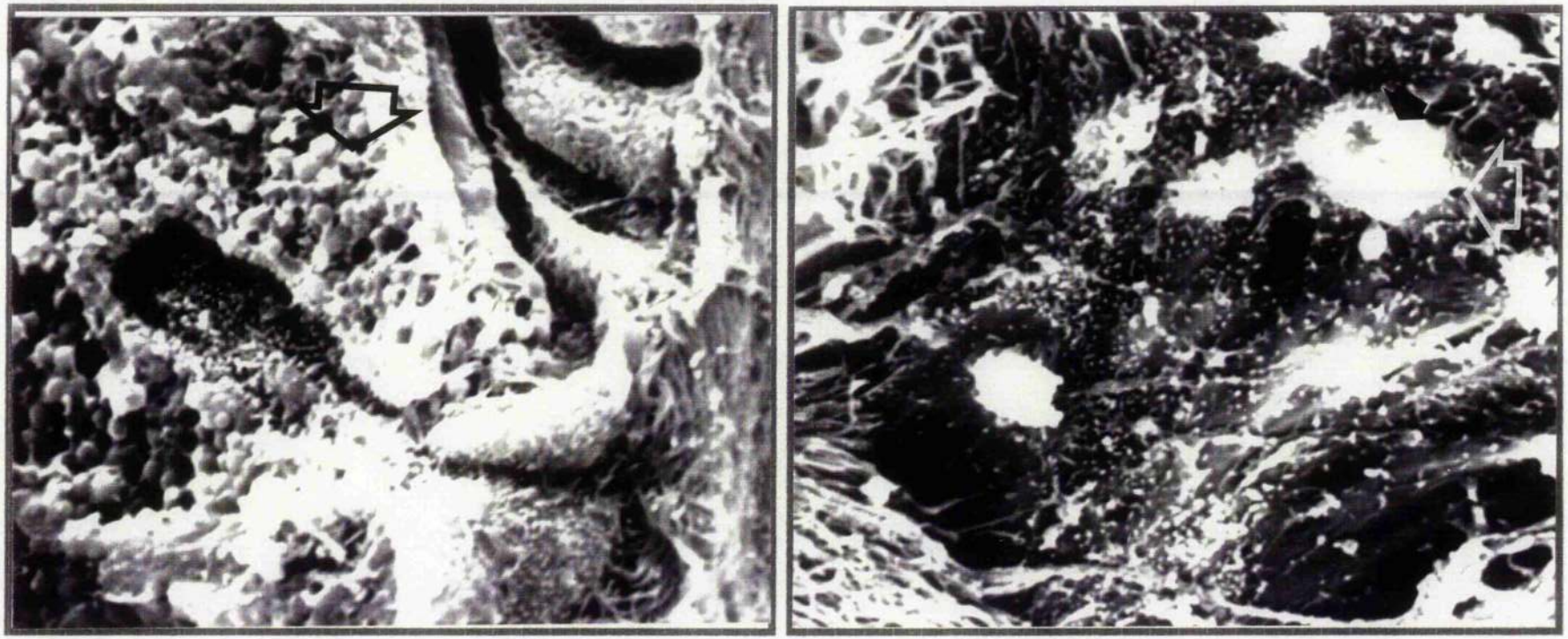


Fig. 3.18

Caudal trachea. 3-day-old chick.

Dense carpet of cilia interrupted by islands of microvillous cells on the mucosal surface.

X 2,750

Fig. 3.19

Cranial trachea. 3-day-old chick.

An island of microvillous cells surrounded by a dense carpet of ciliated cells. Note the mucous granules (arrow) visible through the plasmalemma of the microvillous cells.

X 5, 500

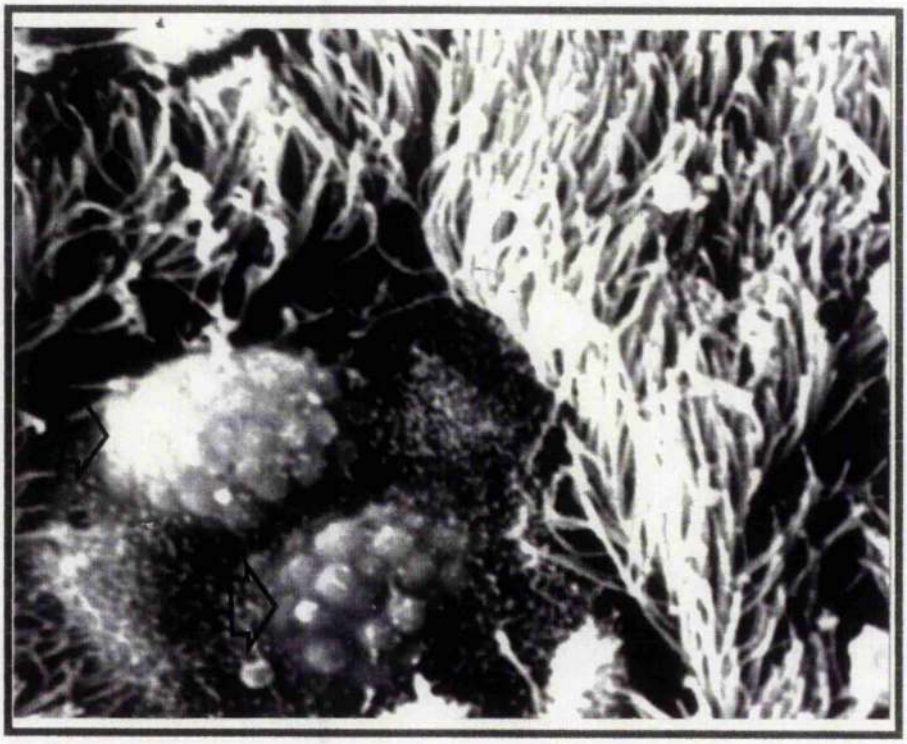


Fig. 3.20

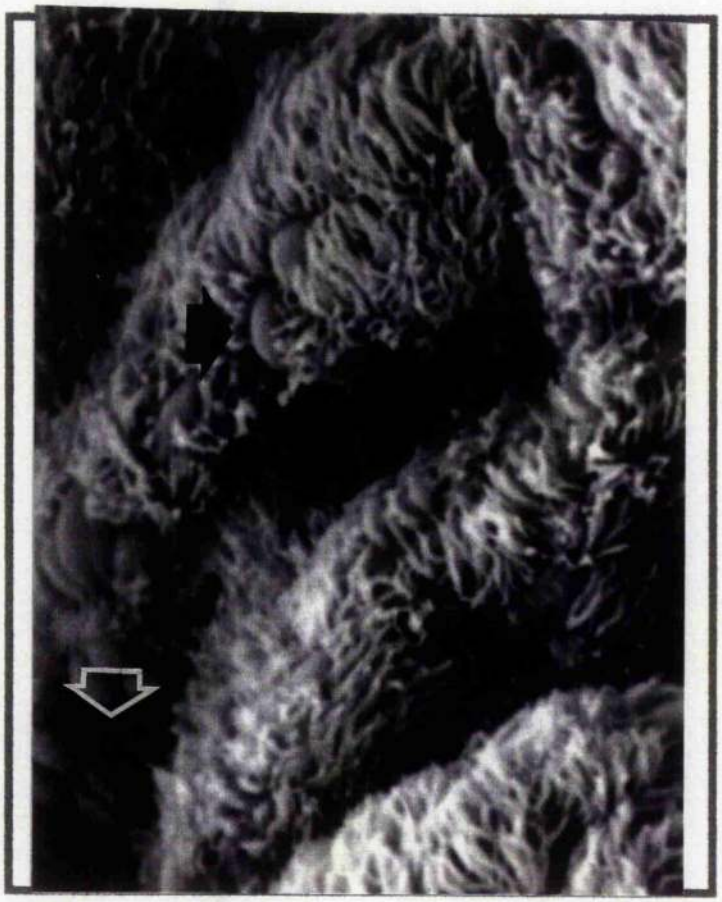
Intrapulmonary primary bronchus (arrow). 19-day-old embryo. The ridges are more heavily ciliated than the gutters. Opening to secondary bronchus (open arrow).

X 2,570

Fig. 3.21

Intrapulmonary primary bronchus. 3-day-old chick. The opening of the intraepithelial mucosal gland surrounded by microvillous and ciliated cells. Note appearance of mucous granules (arrow) beneath the plasmalemma and sparse distribution of the microvilli.

X 5, 500



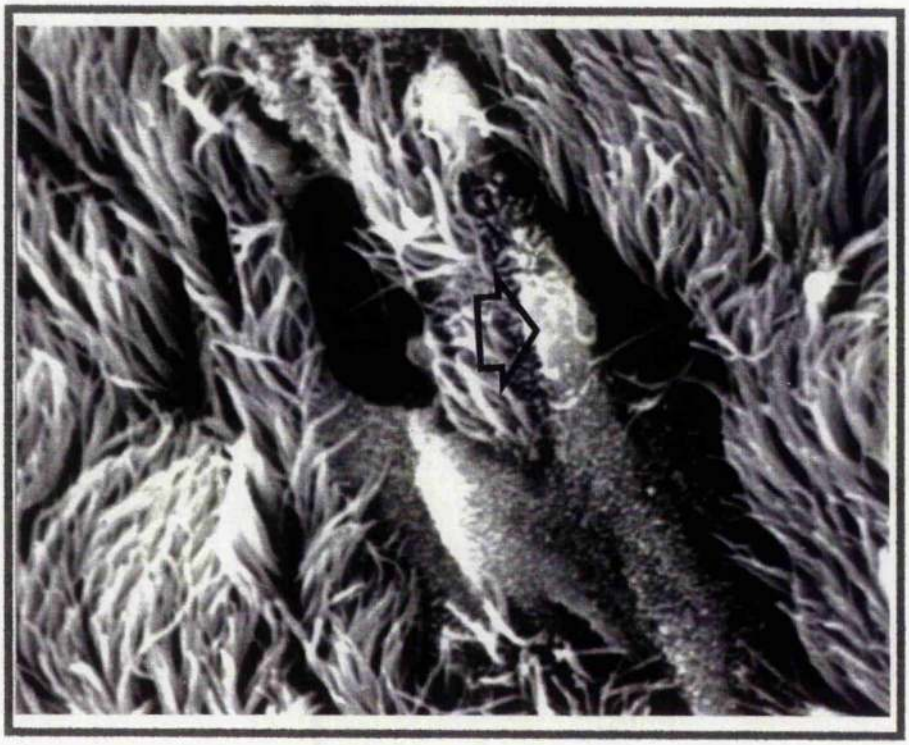


Fig. 3.22

Intrapulmonary primary bronchus. 19-day-old embryo.
The transitional region at the opening of the secondary bronchus mainly covered with microvillous cells and isolated ciliated cells, note also presence of intraepithelial mucous gland opening (arrow).
X 2, 750

Fig. 3.23

Intrapulmonary primary bronchus. 1-day-old chick.
Towards the opening of secondary bronchus, folded ciliated areas alternate with the folded microvillous areas in an apparently haphazard manner. Note presence of isolated ciliated cells (arrow).
X 2, 750

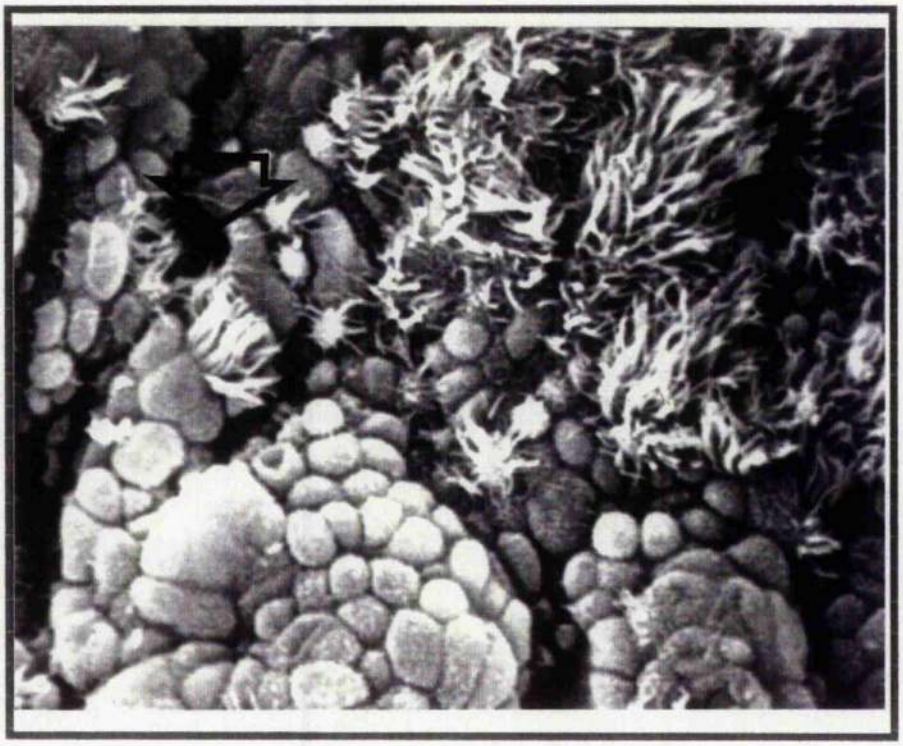


Fig. 3.24

Secondary bronchus. 17-day-old embryo.

At the opening of the tertiary bronchus, note the reduced ciliation of the mucosa and the developing ciliated cells (arrow).

X 2,500

Fig. 3.25

Secondary bronchus. 20-day-old embryo.

Fully covered with mature ciliated cells.

X 5,500

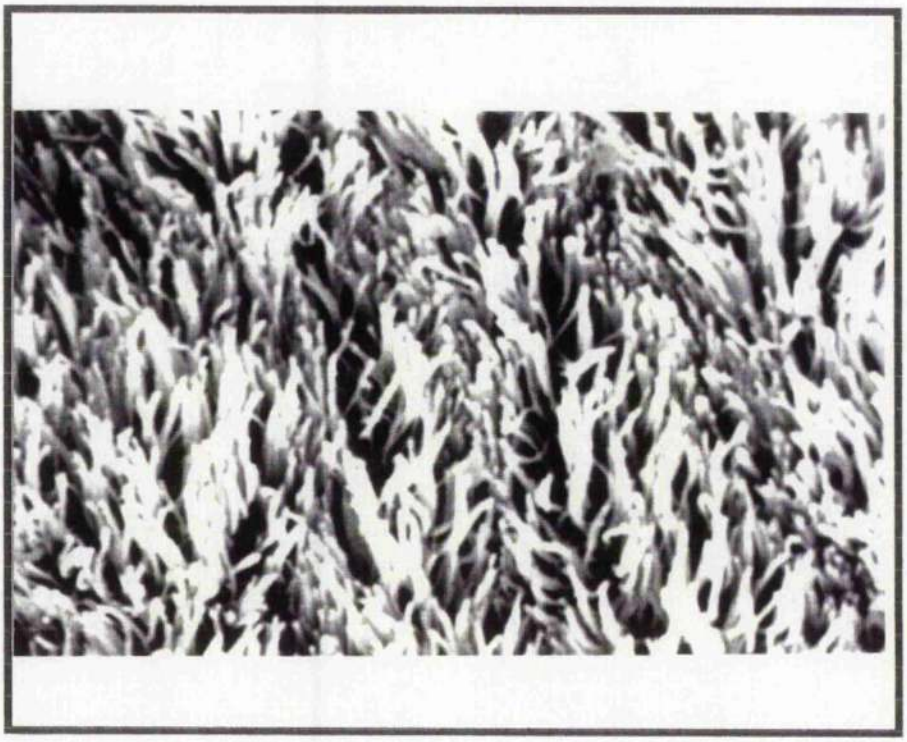


Fig. 3.26

Secondary bronchus at neck region. 19-day-old embryo.

Note the dense nature of the cilia (arrow) on the mucosal surface of the secondary bronchus (S) compared to the less dense nature of the cilia (open arrow) on the mucosal surface near the opening towards the tertiary bronchus (T).

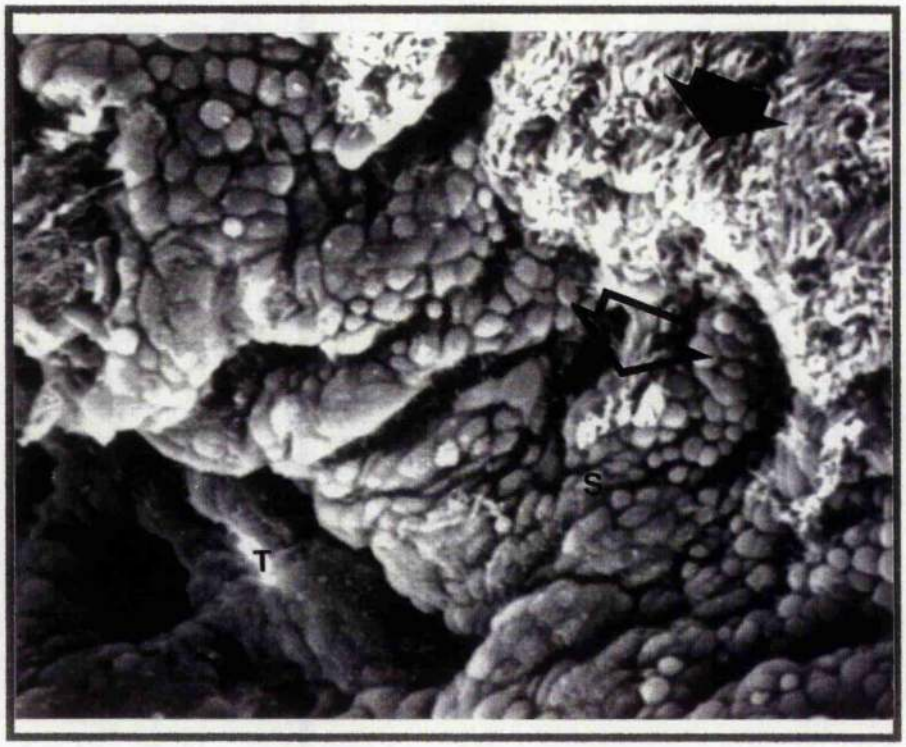
X 640

Fig. 3.27

Tertiary bronchus. 15-day-old embryo

The non-ciliated microvillous cells covering the tertiary bronchus.

X 1, 400


S
T

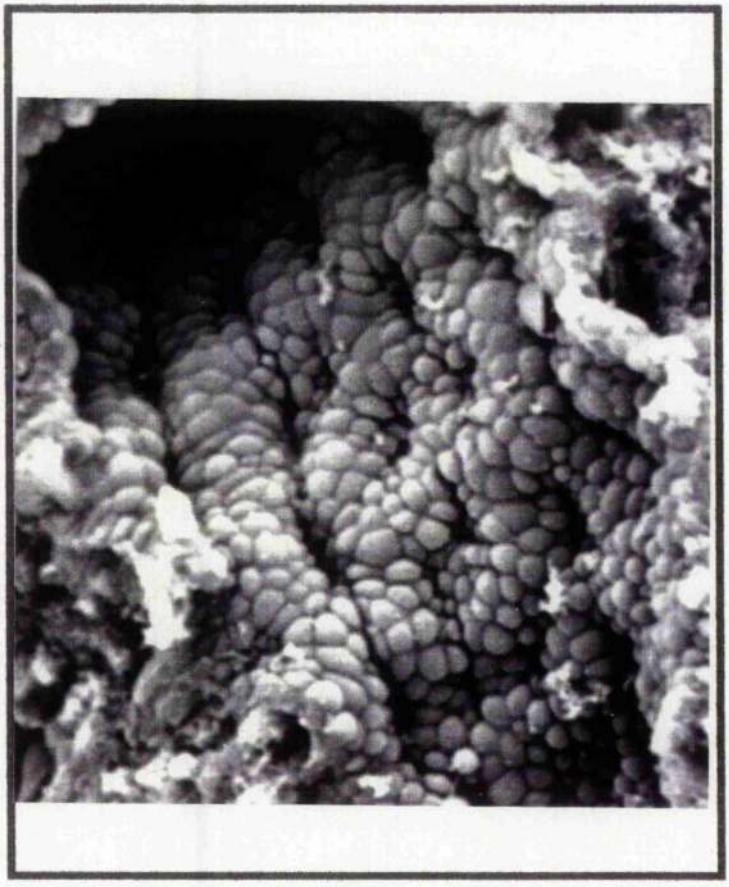


Fig. 3.28

Tertiary bronchus. 15-day-old embryo.

The cobblestone appearance of the parabronchial wall is compact and covered with non-ciliated epithelial cells.

X 2,750

Fig. 3.29

Tertiary bronchus. 17-day-old embryo.

Note the formation of the atrium, the parabronchial wall expands radially into the mesenchyme, as a result the microvillous cells are flattened with well demarcated borders. Note presence of surfactant on the mucosal surface (arrow)

X 2, 750

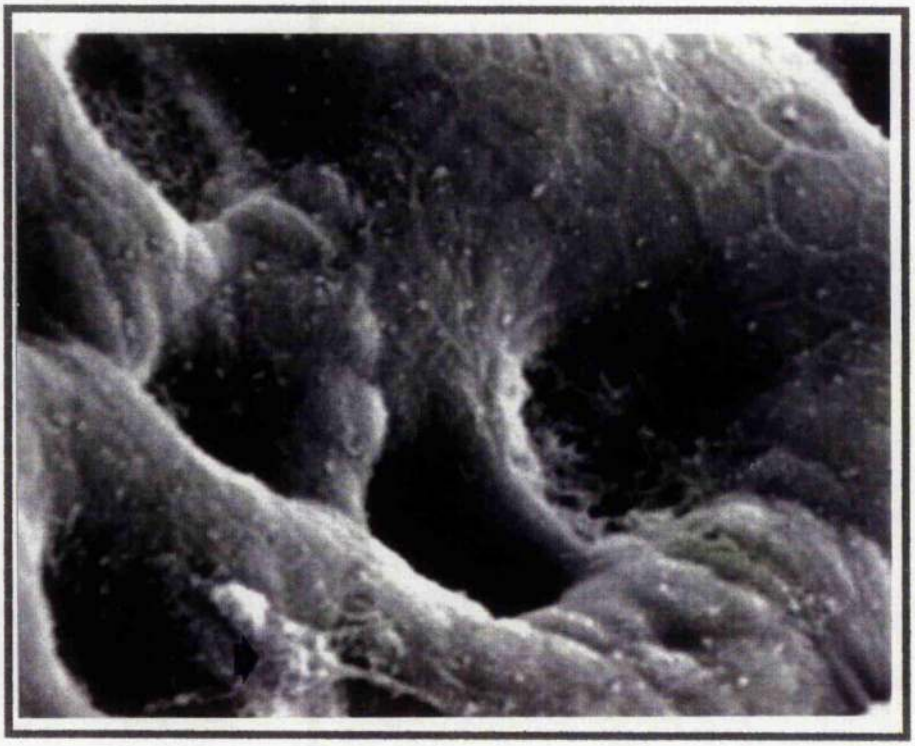


Fig. 3.30

Tertiary bronchus. 20-day-old embryo.

The atria are lined with microvillous cells which are not well demarcated. Note a sheet of surfactant (arrow).

X 2, 750

Fig. 3.31

Tertiary bronchus. 1-day-old chick

A band of bronchial muscle (arrow)

X 700

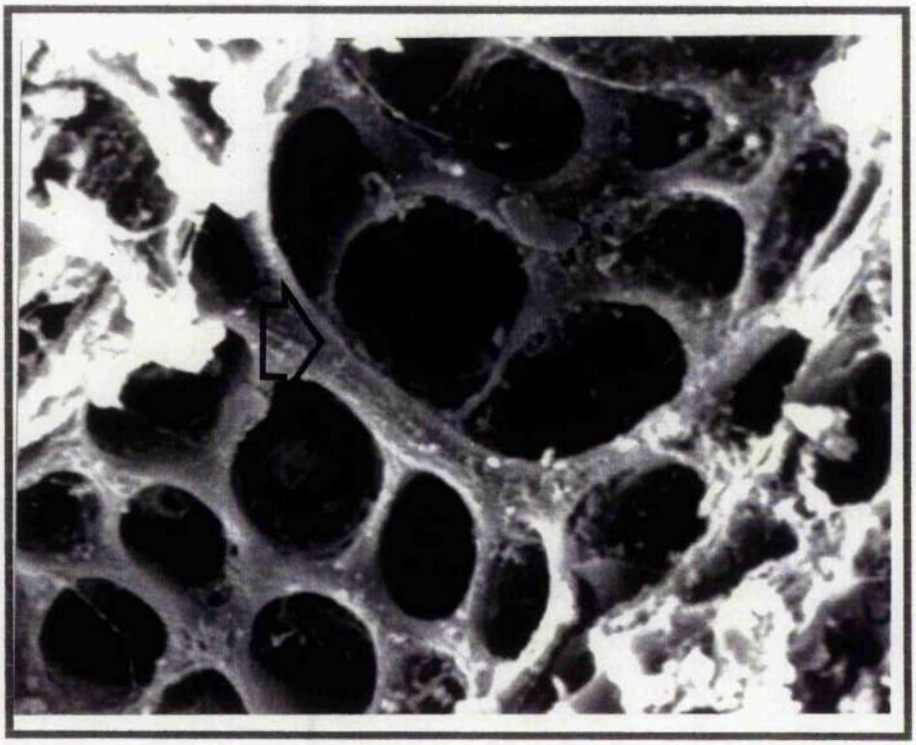


Fig. 3.32

Tertiary bronchus. 19-day-old embryo.

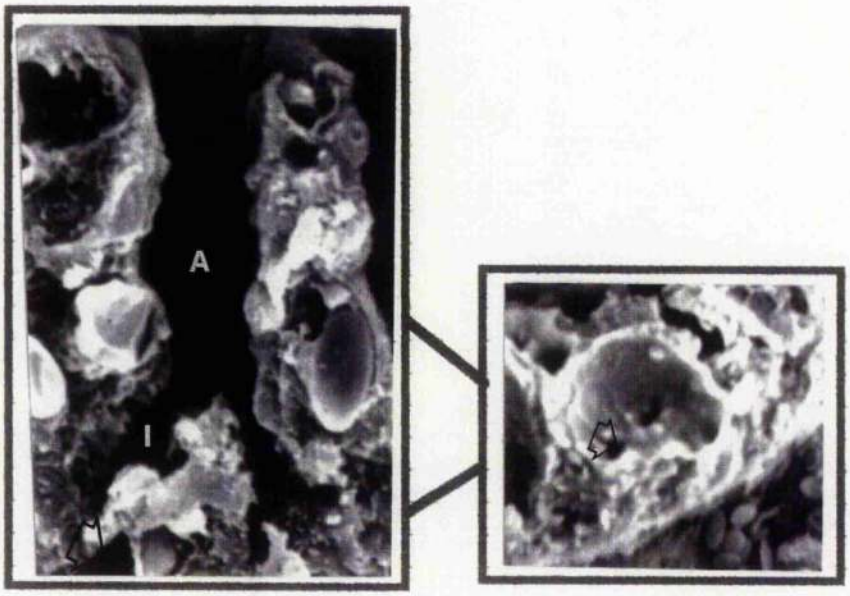
Transverse section of the tertiary bronchus, note presence of atrium (A) and infundibulum (I) leading to the air capillaries (arrow).

X 2, 750

Insert

Fractured sample, openings to the infundibulum on the atrium (arrow)

X 700


A
I

DISCUSSION

In the present study, the luminal surface of the fully differentiated respiratory epithelium demonstrated the presence of two main cell types, the ciliated cell and the microvillous cell. Both cell types, which form important components of the mucociliary defense mechanism of the respiratory tract (Phipps, 1981), were seen to develop from a non-ciliated microvillous cell type characteristic of the developing embryo. The typical cobblestone appearance of such non-ciliated microvillous cells comprising the epithelial lining of the middle nasal concha of 15 to 17-day-old chick embryos seen in the present study, confirms previous observations in the nasal cavity of the chick (Breiphol and Fernandez, 1977), and mammalian species such as the rat (Andrews, 1974; 1979), mouse (Greenwood and Holland, 1972), pig (Adams, 1990) and goat (Kahwa, 1992). The presence of surface microplicae on the dome-shaped, non-ciliated microvillous cells of this lining in the 15-day-old embryo, as observed in this study, does not however, appear to have been noted before in the bird, although such microplicae have been reported to be present on cells in the epiglottis of the mouse (Nakano and Muto, 1987), nasopharynx of the monkey (Leela and Kanagasuntheram, 1973) and mouse (Nakano, 1986), alimentary tract, cornea and conjunctiva of the rat (Andrews, 1976) and nasal vestibule of the goat (Kahwa, 1992). Andrews (1976) has suggested that such microplicae arise from plasmalemmal folds which once provided for intercellular interdigitations and desmosomal adhesions between adjacent cells. He further suggested that, although these microplicae may merely represent the remnants of intercellular interdigitations, they may also serve to hold a layer of lubricating and cushioning mucin in the interplical grooves, protecting the surface plasmalemma from abrasive abuse. The appearance of microplicae in the nasal cavity in particular, might therefore

be expected since it is the first region exposed to air entering the respiratory tract (Swift and Proctor, 1977). However, in the present study, the function of microplicae in the 15-day-old embryo which has not yet started breathing, and which before hatching will be covered by a dense carpet of cilia cannot be explained.

Present observations showed that developing ciliated cells, as seen in the proximal regions of the respiratory epithelium in the 15 to 17-day-old embryos, could be distinguished from surrounding stubby microvillous cells by the presence of a single emerging cilium. The surface appearance of just such a single cilium has also been documented in the respiratory tract of the rabbit foetus (Kanda and Hilding, 1968) and in the nasal cavity of man (Friedman and Bird, 1971).

The present observations, have demonstrated, for the first time, that a fully differentiated, mature, heavily ciliated epithelial lining, similar to that of the adult (Mohammed, 1989), is established in the intrapulmonary primary bronchus of the developing avian embryo by as early as the 15th day of incubation. The secondary bronchus, covered by extensive patches of ciliated cells even in the 16-day-old embryo, attains the mature ciliated epithelial lining, apparent in the intrapulmonary primary bronchus, by the 17th day of incubation, whilst the middle nasal concha, larynx and trachea do not become fully ciliated until later, at between 19 to 20 days of incubation. Such a regional variation in ciliary development, as here described for the first time in the incubating chick embryo, with ciliogenesis progressing along a caudo-rostral axis, contrasts with previous reports of developmental ciliogenesis in mammals, where cilia first appear in the nasal cavity of the rabbit at 22 days of gestation, followed by appearances in the larynx, trachea and finally the bronchi at 28 days of gestation (Kanda and Hilding, 1968). Such ciliary development, similar to that in the rabbit, has also been described in the dog (Wright *et al.*, 1983), where it was

additionally noted that the tracheal and bronchial epithelial linings were not completely ciliated until the puppy was at least 5 days old. Again, such an observation contrasts markedly with the present finding that the entire respiratory epithelium, from the middle nasal concha down to the level of the secondary bronchus of the chick, was covered with densely packed, fully mature, ciliated cells as early as the 19th to 20th day of incubation. The present study demonstrates that a fully developed respiratory epithelium is established throughout the tract by the time the chick hatches. Such present observations are in agreement with earlier SEM studies of the epithelial surface of the middle nasal concha of chicks, which indicated the establishment of the heavily ciliated epithelial lining characteristic of the adult chicken by the 20th day of incubation (Breipohl & Fernandez, 1977). Similarly, Bang and Bang (1977) showed histologically that, with fully differentiated cilia first appearing on the 16th day of incubation at the respiratory-olfactory junction of the nasal cavity of the developing chick embryo, a fully mature mucociliary epithelium was present in the nasal cavity by the 18th day of incubation. Only Mohammed (1989) observed that although by the 19th to 20th day of incubation the major part of the chick larynx and trachea were covered by a fully developed ciliated epithelium, the dorsal laryngeal wall still showed a relatively sparsely ciliated epithelium containing numerous protruding mucous cells.

The present study, which as previously noted has covered the development of the lining epithelium throughout the whole respiratory tract, has then extended and complemented these previous observations, and shown for the first time that such a relatively late development of the mature ciliated mucosal lining of the respiratory tract was also noted in man (Greenwood and Holland, 1975), where a tracheobronchial lining fully covered by well developed ciliated cells did not appear until the 34th week of gestation. However, it contradicts the finding of Thurlbeck *et al.* (1961),

who indicated that goblet and ciliated cells were first seen in the trachea of $13^{1}/_{2}$ weeks-old human foetus but by 15 weeks the mucociliary system was well developed.

Although intracellular mucous granules, along with pits on the surface of the non-ciliated microvillous cells, were seen in the middle nasal concha of 17-day-old embryos, the intrapulmonary primary bronchus and secondary bronchus of 15-day-old embryos, and the larynx, cranial trachea and caudal trachea of 19-day-old embryos, and intraepithelial gland openings were apparent in embryos from 17 days of age, actual mucus secretion onto the epithelial surface was not noted until the 18th day of incubation, and was most active in the 3-day-old chick. It is tempting to speculate that the initiation and increase in mucous cell activity could have been started by the beginning of air breathing as a result of internal pipping (that is the piercing of the chorioallantoic membrane and the entering of the air cell) by the chick. Such an event, which is a prelude to hatching, usually occurs between the 18th to 20th day of incubation (Duncker, 1978b; Burton and Tullet, 1985), a time which also coincides with the full development of a mature ciliary carpet lining the respiratory tract, as previously noted. The combined SEM observations, presented in this study, of the presence of mature ciliated cells and mature mucous cells comprising the major cell populations of the epithelial lining of the respiratory tract of the developing chick embryo from the 18th day onwards, suggest that the chick hatches with a fully functional mucociliary system in place as an integral component of the respiratory defense mechanisms.

The intraepithelial mucous gland openings observed from the 17th day of incubation onwards in the present study were encountered throughout the respiratory tract. Such observations contradict those of Chandra and Bharadwaj (1971), who indicated that the mucous glands of the chicken were restricted to regions proximal to the cranial trachea.

However, intraepithelial mucous glands have been described in the secondary bronchus of the budgerigar (Smith *et al.*, 1987). Although the present study has shown an impressive early development of a fully ciliated epithelial lining throughout the respiratory tract of the chick, such a lining is not observed in the regions of the openings of the secondary and tertiary bronchi. Here the mucosal surface is covered mainly by microvillous cells, with only occasional isolated ciliated cells being observed. In addition, ciliated folds alternate with microvillous folds at the boundary of the intrapulmonary primary bronchus and secondary bronchus. Although these features have been reported previously in the lung of the adult Ringed turtle dove (McLelland and MacFarlane, 1986), this is the first time they appear to have been reported in the domestic chicken. The cobblestone appearance of these microvillous cells at the junction of secondary bronchus and tertiary bronchus is seen to extend into the tertiary bronchi of the younger embryos (15 to 17-day-old) in the present study. A similar appearance of the developing alveolar lining has also been reported in an SEM study of the 26-day-old foetal rabbit lung (Gonzalez-Crussi and Boston, 1974).

In the process of development seen in the present study, the invagination of these non-ciliated microvillous lining cells is seen to form the lumen of the tertiary bronchus. This corresponds to previous SEM observations on the development of the lung in the duck and chicken (Duncker, 1978b), where there is an increase in the luminal diameter of the tertiary bronchus with age, from 3-day-old chicks to 1-year-old chickens. The appearance of atria in the walls of the tertiary bronchus of the 17-day-old embryo as seen in the present study, agrees with similar observations arising from TEM studies of the chick lung (Petrik,1967; Petrik and Riedel, 1968a; 1968b; Jones and Radnor, 1972a). In the present study, surfactant was also observed on the mucosal surface of the tertiary bronchus of 17-day-old embryos to 3-day-old chicks. The presence of surfactant, a feature of

the adult lung in the chicken (Tyler and Pangborn, 1964; Akester, 1970), goose (Lambson and Cohn, 1968), Adelie penguin (Drescher and Welsch, 1983) and budgerigar (Smith *et al.*, 1986, 1987), has also been documented in the atrium of chicken embryos (Jones and Radnor, 1972a; 1972b; Petrik and Riedel, 1968a, b).

CHAPTER 4

TRANSMISSION ELECTRON MICROSCOPY OF THE NORMAL RESPIRATORY EPITHELIUM OF THE DEVELOPING CHICK

INTRODUCTION

Despite the increasing use of avian embryo respiratory epithelium as host tissue cultures in the study of viral (Blaskovic *et al.*, 1972a; Blaskovic *et al.*, 1972b) and mycoplasmal infections (Abu-Zahr and Butler, 1976), such studies have provided little information about the normal ultrastructure of the respiratory epithelium of the developing lung (Jones and Radnor, 1972a, b), larynx and tracheal respiratory epithelium (Walsh and McLelland, 1974a, b, c, 1978; Kalnins and Porter, 1969; Kalnins *et al.*, 1972) in the domestic chicken. There appear to be no reports available on the ultrastructural features of the entire developing respiratory epithelium of any avian species. This contrasts markedly with the transmission electron microscopic studies available in a wide range of various mammalian species on the developing respiratory epithelium; such as in rabbits (Leeson, 1961; Hage , 1974), rats (Leeson and Leeson, 1964; Balis and Conen, 1964; Cireli, 1966; Dirksen and Crocker, 1966), mice (Hage, 1974), guinea pigs (Kikkawa and Spitzer, 1969; Hage, 1974); hamsters (Kikkawa and Spitzer, 1969; McDowell *et al.*, 1985) and men (Sorokin, 1960; Balis and Conen, 1964; Rhodin, 1966; Towers, 1968; Jones, 1972).

The objective of the present study, therefore, was to investigate, at the TEM level, the development of the entire respiratory epithelium of incubating chicks from the 15-day-old embryo through to the 3-day-old post-hatched chick.

MATERIALS AND METHODS

Source of chicks

Control chicks for this study were obtained as detailed in Chapter 2. The number and age of chicks involved in this study are shown in Table 8:

TABLE 8

CHICKS USED FOR TRANSMISSION ELECTRON MICROSCOPY (TEM) IN THE INVESTIGATION OF THE DEVELOPING RESPIRATORY EPITHELIUM.

Age of chicks	Number of chicks involved for TEM
15-day-old embryo	3
16-day-old embryo	3
17-day-old embryo	3
18-day-old embryo	3
19-day-old embryo	3
20-day-old embryo	3
1-day-old chick	3
3-day-old chick	3

Sample collection, processing of samples and photography for transmission electron microscopy.

Chapter 2 gives details on sample collection and processing of samples for transmission electron microscopy.

RESULTS

Middle nasal concha

15 to 16-day-old

Transmission electron microscopy revealed that the respiratory epithelium lining the middle nasal concha of 15 to 16-day-old chick embryos consisted of 2 to 4 cell layers. The upper layer was composed of

cuboidal or columnar cells whilst the lower layer was of oval or cuboidal cells. Intercellular connections were primarily by means of short interdigitating cell processes and desmosomes, although cells in the uppermost layer were connected by tight junctions at their apical poles (Fig. 4.1). The apical surfaces of the cells in the upper layer also carried a population of short microvilli. Most of the epithelial cells were of an undifferentiated type, the cytoplasm containing numerous free ribosomes, Golgi complexes, few mitochondria and little rough endoplasmic reticulum. Some cells in both the upper and lower epithelial layers could be identified as differentiating ciliated cells, as they were characterised by the presence of numerous centrioles, small vesicles and rough endoplasmic reticulum, sparse mitochondria and multivesicular bodies (Fig. 4.1 and Fig. 4.2). Large irregular intercellular spaces were seen between the differentiating cells. Differentiating mucous cells were recognised by the presence, apically, of a few vesicles containing floccular material; these cells also presented a few mitochondria and a well-developed Golgi body with numerous small associated vacuoles (Fig. 4.3).

17-day-old embryo through to 3-day-old chick.

Within this temporal grouping, the epithelium was seen to develop either into a simple or a pseudostratified columnar or cuboidal epithelium. At the same time, cell differentiation took place and established recognisable cell types. The cells in the upper layer became columnar, although some still retained the cuboidal shape, and then developed into either ciliated or mucous cells. The cells in the lower layer retained their oval or cuboidal shape, with increased number of intracytoplasmic filaments and transformed into basal cells.

A few fully differentiated ciliated cells clearly recognisable by the presence of numerous centrioles, in the apical cytoplasm were observed in

the 17-day-old embryo, their numbers increasing with age. Mature ciliated cells were fully developed by the time the chick had reached 3 days of age. Along with the characteristic basal bodies, basally projecting rootlets and projecting surface cilia, numerous vesicles, mitochondria and rough endoplasmic reticulum were observed in the apical regions of these ciliated cells (Fig. 4.4).

Differentiating mucous cells (Fig. 4.5), were characterised by the presence of initially low, and then increasing, numbers of intracytoplasmic granules containing a usually flocculent material. In the hatched chicks the cytoplasm was seen to be packed with numerous mucous granules of varying appearance (Fig. 4.6). Mucous glands, formed by an aggregation of a number of mucous cells at a surface invagination (Fig. 4.7) of the epithelium appeared as early as the 18th day of incubation.

Developing basal cells presented an increase in the number of intracytoplasmic filaments. Contact between adjacent cells was by interdigitation of long cytoplasmic processes and the formation of tight junctions (Fig. 4.8).

Larynx

15 and 16-day-old embryo

The larynx of the 15 and 16-day-old embryos consisted of two layers of undifferentiated cells. Differentiating ciliated and mucous cells were both observed within the upper layer of the epithelium at this stage. The former cell type was again characterised by the presence of numerous centrioles located towards the apical region of the cytoplasm (Fig. 4.9), numerous mitochondria, many free ribosomes, well developed rough endoplasmic reticulum and a sparse Golgi body; numerous microvilli and occasionally, a single cilium were also frequently seen arising from the apical surface of the cell. The latter cell type was characterised by the intracytoplasmic presence

of numerous vesicles, Golgi body, mitochondria, rough endoplasmic reticulum and numerous ribosomes. The cells populating this upper layer were connected to each other by means of tight junctions at their luminal surfaces, numerous short cytoplasmic processes interdigitating with each other laterally, and both laterally and basally placed desmosomes.

Cells of the lower layer consisted of undifferentiated oval or round cells containing a large centrally-placed nucleus, numerous ribosomes, moderate numbers of mitochondria, sparse numbers of intracytoplasmic filaments and rough endoplasmic reticulum (Fig. 4.10). The presence of large, irregular intercellular spaces, noted previously in the middle nasal concha, was also noted in the larynx. Attachment between adjacent cells was by means of the interdigitation of short cytoplasmic processes, and the occurrence of a small number of desmosomes.

17 -day-old embryo through to 3-day-old chick

During this period, differentiation of specific cell types continued to maturity in the larynx. Cells in the upper layer of the developing epithelium became columnar, and the centrioles in the cytoplasm of the differentiating ciliated cells ascended towards the apical margin of the cell, and then transformed into basal bodies, cilia and rootlets (Fig. 4.11). The cilia were intermingled between surface microvilli. Beneath the basal bodies, large numbers of mitochondria and a few small vesicles could be observed.

Differentiating mucous cells transformed into mucous cells by an increase in the numbers of mucous granules. In established mucous cells, the cytoplasm was fully occupied with numerous mucous granules of variable sizes and electron density.

Cells in the lower layer retained their shape and developed into basal cells, exhibiting a recognisable increase in the number of intracytoplasmic filaments. Contact between adjacent cells was more

obvious, due to an observed increase in the number of desmosomes and relatively longer interdigitating cytoplasmic processes.

Trachea

15 and 16-day-old embryo

The trachea of the 15 and 16-day-old embryo was, as in the middle nasal concha and larynx in this age group, lined by a developing epithelium consisting of two layers of stratified undifferentiated cells (Fig. 4.12). The upper layer was seen to contain mainly differentiating ciliated and mucous cells. However, another cell type, characterised by the intracytoplasmic presence of both mucous granules and centrioles (fig. 4.13) was seen within the upper layer of the developing epithelium. The lower layer, comprised undifferentiated oval or round cells.

17-day-old embryo through to 3-day-old chick

Whilst the differentiation of cell types into mature ciliated, mucous and basal cells, continued in this age group as previously described in the middle nasal concha and larynx, the tracheal epithelium was also seen to contain the occasional intermediate cell. This latter cell type was recognised by the presence of numerous rough endoplasmic reticulum, mitochondria and Golgi complex with numerous vesicles in the cytoplasm, and by the absence of either centrioles or mucous granules (Fig. 4.14). The intermediate cell appeared not to reach the luminal surface. Contact between the intermediate cell and adjacent cells was by means of tight junctions at the luminal surface of the cells, the lateral interdigitation of cytoplasmic processes, and desmosomal attachments laterally and basally.

Intrapulmonary primary bronchus

15-day-old embryo through to 3-day-old chick

The intrapulmonary primary bronchus was lined by a pseudostratified

columnar epithelium containing numerous ciliated cells. The latter were recognised by the presence of surface cilia and numerous basal bodies aligned beneath the plasmalemma as early as in the 15-day-old embryo (Fig. 4.15). However, the relatively small numbers of mucous granules present in the mucous cells identifiable in this lining epithelium at this stage suggested that these cells were still in the process of differentiation (Fig. 4.16). Numerous microvilli both single and branched, could be seen at the luminal surface of these differentiating mucous cells. The numbers of mucous granules in the cytoplasm of these differentiating cells had increased in the 17-day-old embryos and continued to increase until the cytoplasm in the 3-day-old chick, was congested with large numbers of mucous granules of varying electron density and size. Mature mucous glands were composed primarily of mucous cells. Ciliated cells were infrequently observed (Fig. 4.17).

Occasionally cells with electron-lucent cytoplasm, and containing electron-dense granules (Fig. 4.18), were observed in the intrapulmonary primary bronchus in all age groups. Such features suggested that these cells were granular endocrine cells. Though the ciliated and granular endocrine cells were well differentiated, a few cells at the basal region were still undergoing mitotic division, recognised by the appearance of chromosomes.

Tertiary bronchus

15-day-old embryo

The tertiary bronchus of the 15-day-old embryo appeared to take the form of a single cord of differentiated columnar cells (Fig. 4.19) surrounded by undifferentiated mesenchyme. The cytoplasm of these columnar cells contained numerous free ribosomes, mitochondria and rough endoplasmic reticulum.

16 to 17-day-old embryo

Canalisation of the cords was seen to begin around the 15th to 16th day of incubation, with an increase in the developing luminal diameters continuing from this point onwards (Fig. 4.20). Continuing growth of the tertiary bronchus was characterised by the appearance and development of outpocketing from the bronchial wall into the surrounding mesenchyme, and a progressive flattening of the lining cells of the tertiary bronchus. The outpocketing which develop to form the atria and infundibula of the tertiary bronchus, will eventually give rise to the developing network of air capillaries. At the same time the height of the columnar cells lining the tertiary bronchus was seen to decrease. In the 16 to 17-day-old embryo, the undifferentiated nature of the tissue lying between the developing tertiary bronchus and its outpouchings and the associated invading blood vessels make it extremely difficult to distinguish between cells lining air capillaries and mesenchymal cells. Numerous blood capillaries and also a few potential air capillaries were seen in embryos of this age group (Fig. 4. 21). In the 17-day-old embryos, the cells nearest the lumen were the potential atrial lining cells (Fig. 4.22), recognised by the presence of a few multivesicular bodies, electron-dense inclusion bodies, Golgi complexes, and mitochondria, an abundance of rough endoplasmic reticulum and vesicles.

18-day-old embryo through to 3-day-old chicks

In the 18-day-old embryo, outpouchings of the tertiary bronchus, leading to the formation of the atria and infundibula continued (Fig. 4.25), with adjacent tertiary bronchi separated by undifferentiated mesenchymal elements (Fig.4.26), along with developing blood and air capillaries, fibroblasts and smooth muscle bundles at the interatrial septa. Two types of

inclusion bodies were observed in the cells lining the atrium of 18-day-old embryos through to 3-day-old chicks: these were considered to represent mature and immature inclusion bodies (Figs. 4.23 and 4.27). Early forms of the latter appeared to be formed by the fusion of small intracytoplasmic vesicles (Fig. 4.24.) The contents of these newly-formed, immature multivesicular inclusion bodies, then appeared to coalesce, leading to the formation of somewhat larger, dense inclusion bodies. The cytoplasmic invagination of the dense inclusion bodies was seen to lead to the formation of the mature, lamellated osmiophilic inclusion bodies, characteristic of the Type II lining cells of the atria and infundibula. The contents of these mature inclusion bodies appeared to be released into the lumina of the atrium (Fig. 4.27) and developing air capillaries (Fig. 4.28) of 18, 19 and 20-day-old embryos. However, relatively larger amounts of surfactant were seen in the atria of day-old and 3-day-old chicks (Fig. 4.29). The surfactant was made up by a non-cellular film of trilaminar material, composed of strongly electron-dense upper and lower layers sandwiching an intermediate layer of lower electron density. In the hatched chicks, the air capillaries were seen to be well developed and clearly associated with adjacent blood capillaries (Fig. 4.30).

Fig. 4.1

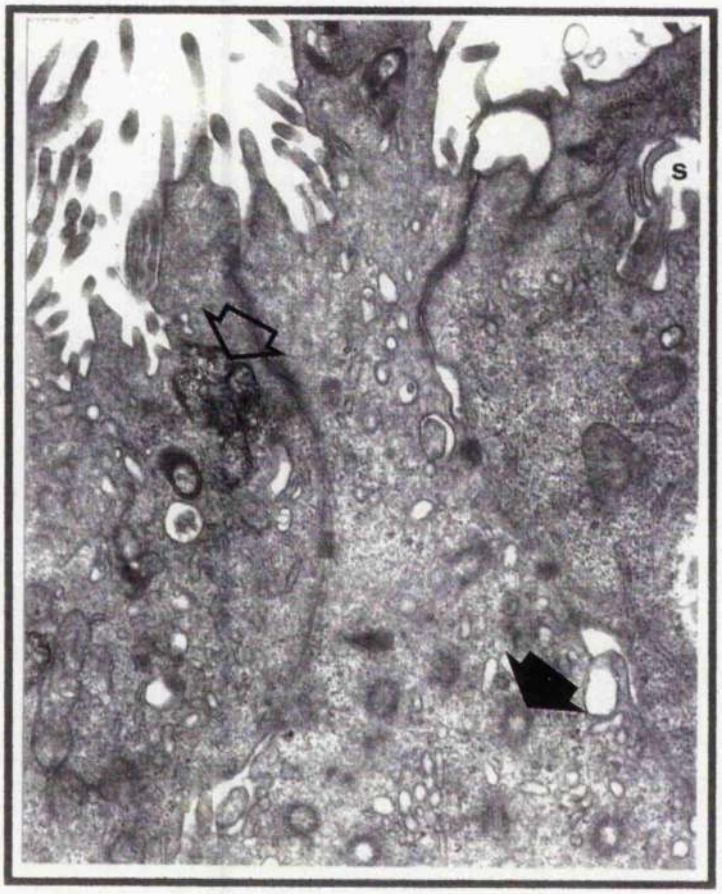
Middle nasal concha. 16-day-old embryo.

Numerous centrioles (arrow) and vesicles are seen in a developing columnar ciliated cell and adjacent to it is a developing mucous cell characterised by presence of floccular material (open arrow) in the vesicle at the apical region with numerous mitochondria and rough endoplasmic reticulum in the cytoplasm. Cells attach by tight junctions at the luminal surface, and a few desmosomes and short interdigitation of cytoplasmic processes. Note also presence of intercellular spaces (s). X 22,500

Fig. 4.2

Middle nasal concha. 15-day-old embryo.

Developing ciliated cell with numerous centrioles (arrow), mitochondria, rough endoplasmic reticulum, a Golgi body and multivesicular bodies are seen emerging toward the luminal surface (L). Note also presence of large intercellular spaces (open arrow). X 15,000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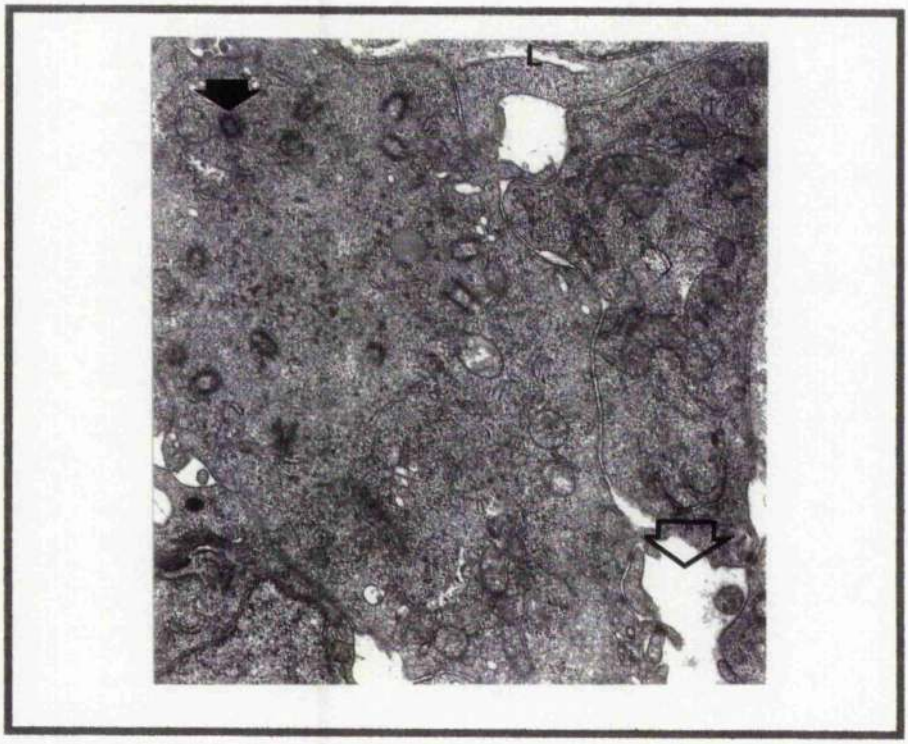


Fig. 4.3

Middle nasal concha. 15-day-old embryo.

Differentiating mucous cell characterised by the presence of few vesicles containing floccular material (arrow) at the apical cytoplasm. Note also the presence of a well-developed Golgi complex, the Golgi lamellae appear to dilate into vesicles at their ends (open arrow). A few short microvilli are also present at the luminal surface. X 22,500

Fig. 4.4

Middle nasal concha. 3-day-old chick.

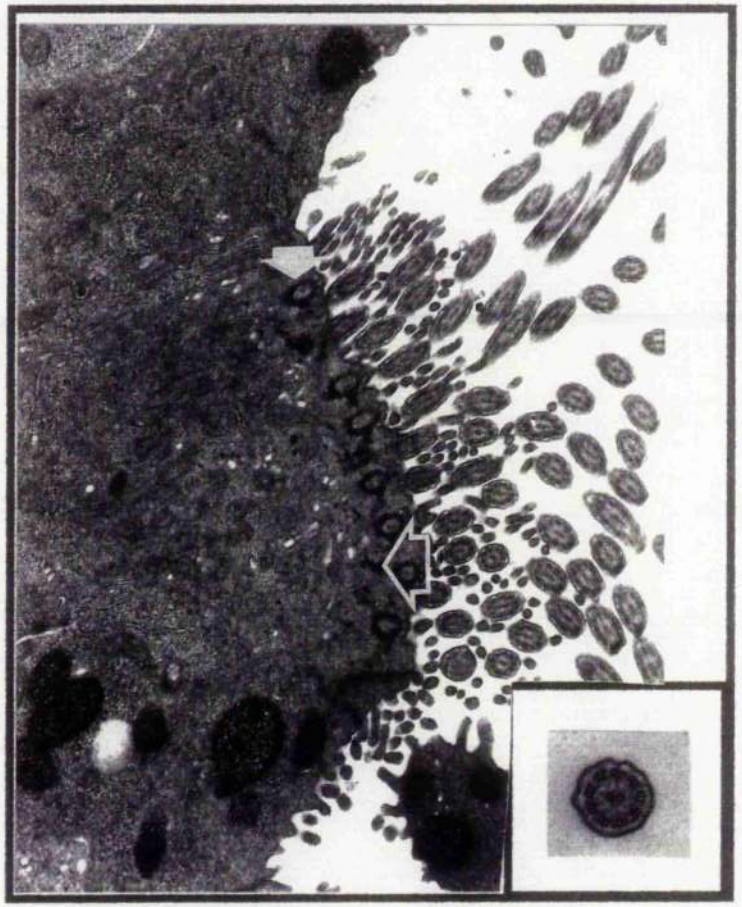
Mature ciliated cells and mucous cell lining the middle nasal concha, note presence of numerous cilia and basal bodies (arrow) and rootlets (open arrow) at the apical region of the cytoplasm of the mature ciliated cell and mucous granules in the cytoplasm of the mucous cell.

X 15,000

Insert

The 9 + 2 arrangement of the microtubules of the cilia.

X 45,000



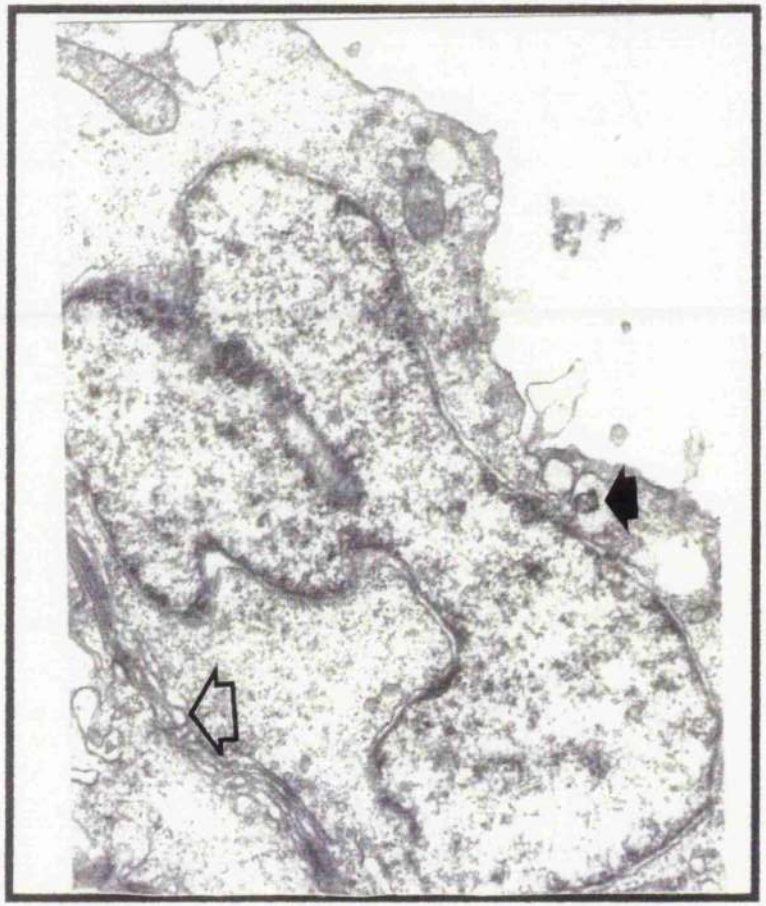


Fig. 4.5

Middle nasal concha. 17-day-old embryo.

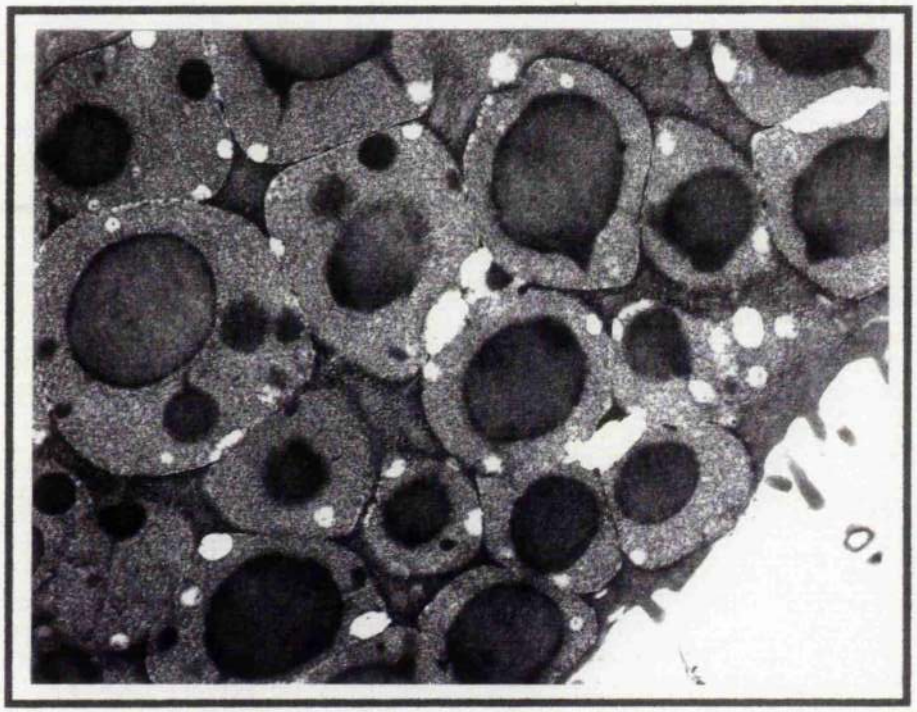
Developing mucous cell contains a number of vesicles (arrow) filled with floccular material.

X 45,000

Fig. 4.6

Middle nasal concha. 3-day-old chick.

Mature mucous cell congested with mucous granules each with an electron-dense core. X 18,000



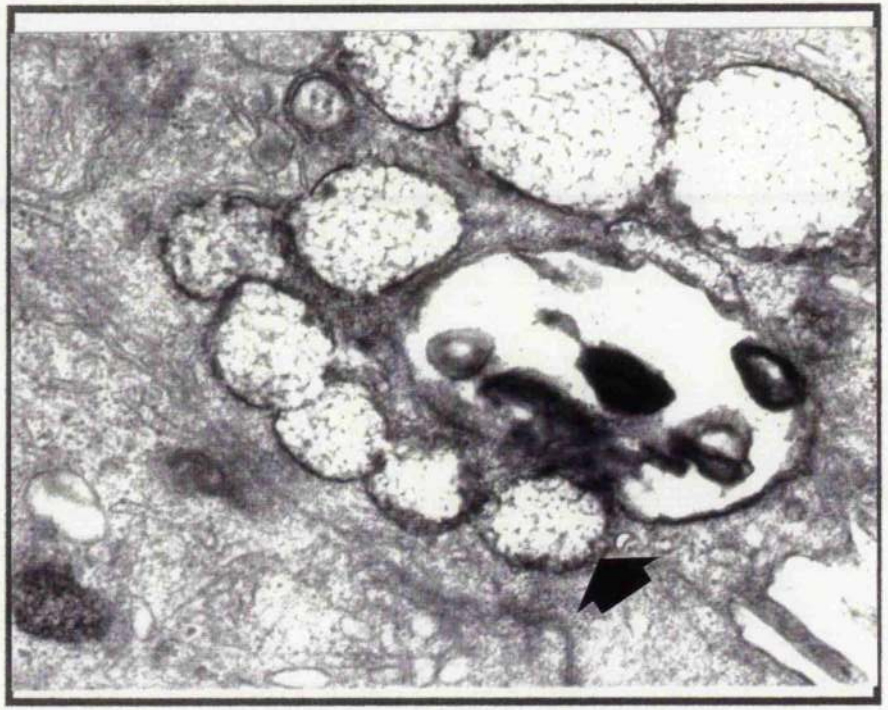


Fig. 4.7

Middle nasal concha. 3-day-old chick.

Mature mucous gland lined by numerous mucous cells.

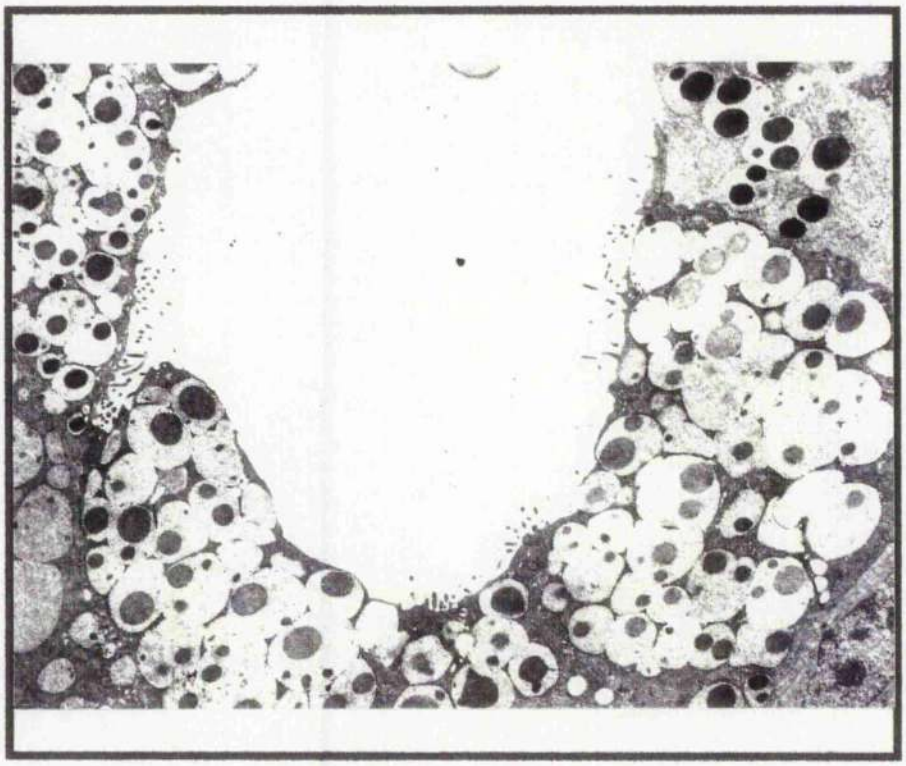
X 6,000

Fig. 4.8

Middle nasal concha. 3-day-old chick.

Basal cells, note increase number of filaments (arrow) and ribosomes in the cytoplasm. Attachment to the adjacent cells is via desmosomes (D). Basement membrane (open arrow).

X 22,500



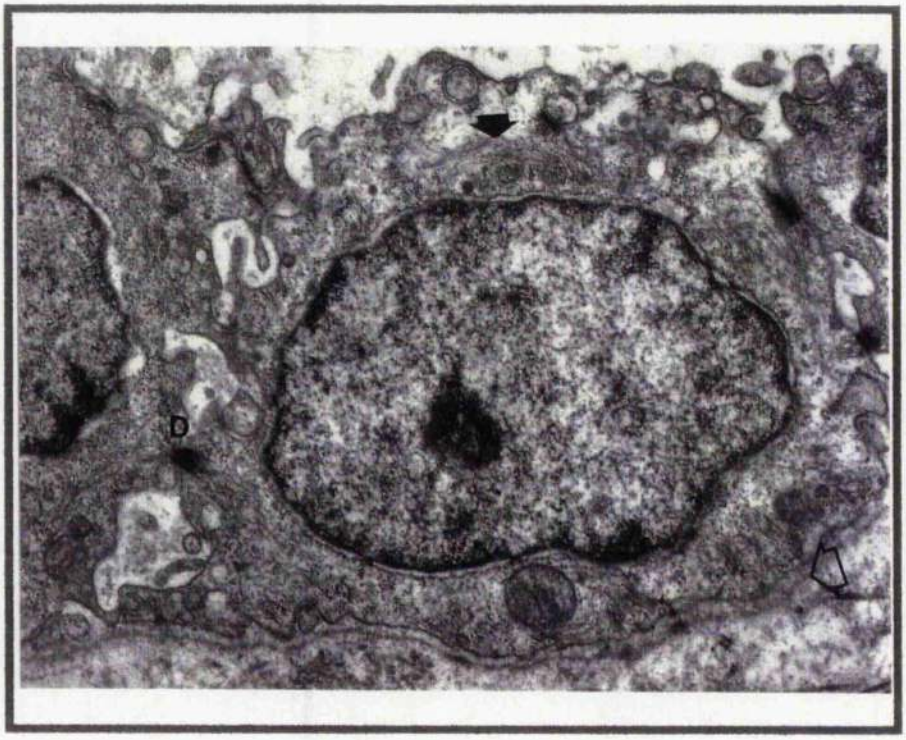

D

Fig. 4.9

Larynx. 15-day-old embryo.

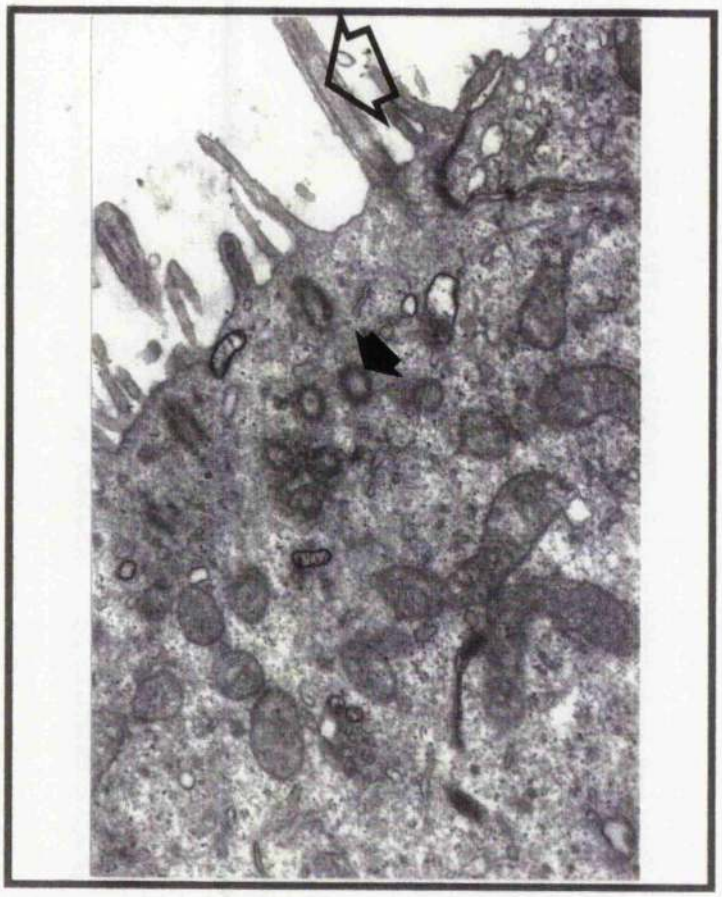
Centrioles at the apical region of the cell (arrow). Single cilium (open arrow) and a few microvilli can be seen at the luminal surface.

X 22,500

Fig. 4.10

Larynx. 15-day-old embryo.

Undifferentiated cell at the basal region. X 15,000



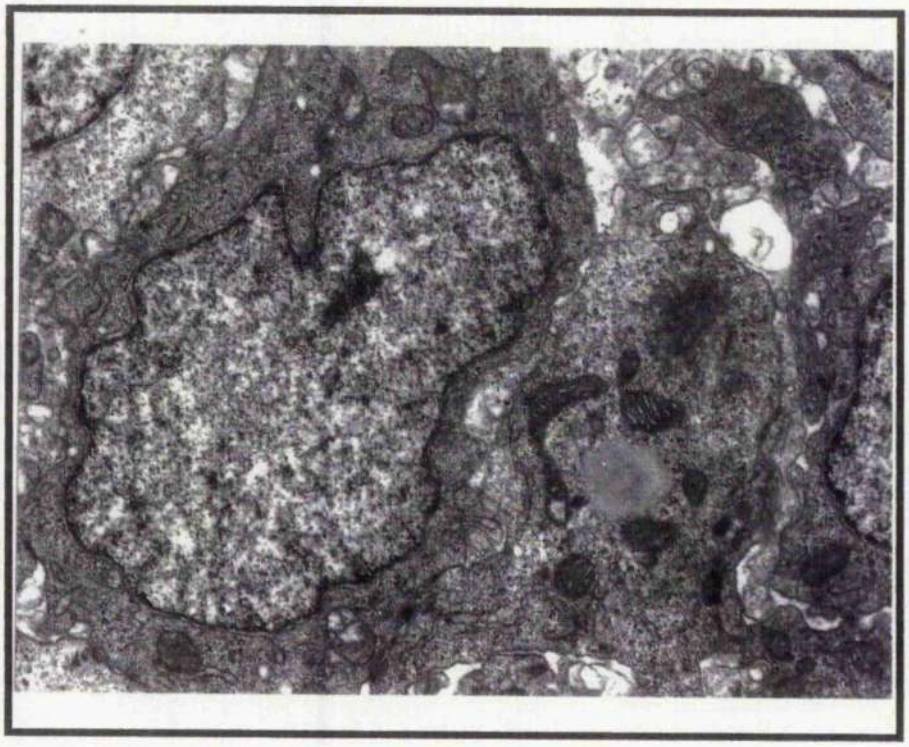


Fig. 4.11

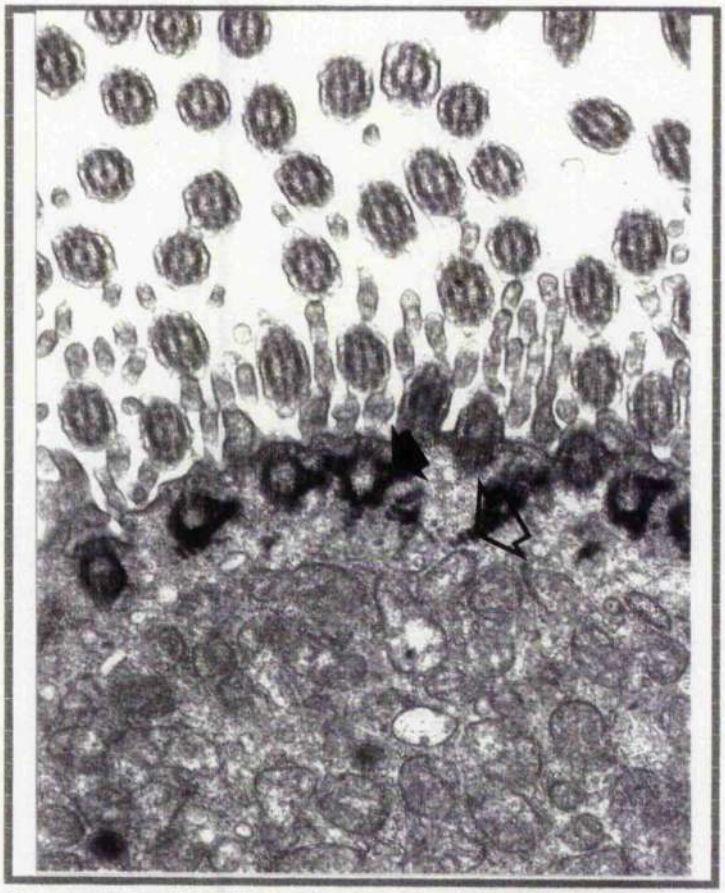
Larynx. 3-day-old chick.

Mature ciliated cell with numerous cilia and basal bodies (arrow), rootlets (open arrow). The apical region also supports a population of mitochondria. X 22,500.

Fig. 4.12

Trachea. 16-day-old embryo.

Two cell layers, note electron-opaque mucous granules (arrow) at the apical region of the upper cell layer. X 11,250



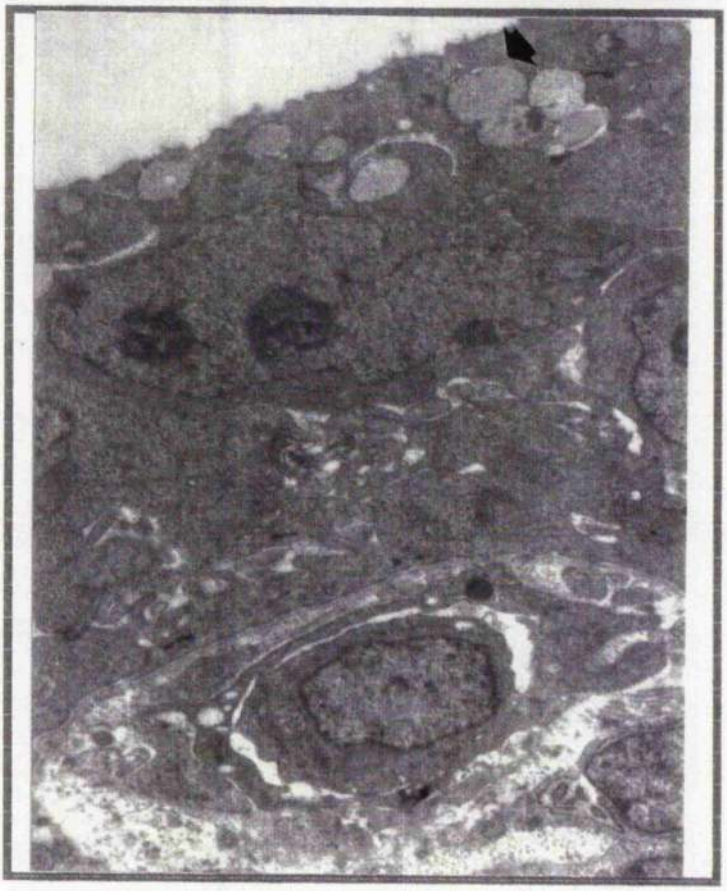


Fig. 4.13

Trachea. 16-day-old embryo.

Numerous centrioles (arrow) and vesicles with floccular material (open arrow) seen in the developing ciliated cell. Junctional complexes (JC), desmosomes (D) and interdigitation of cytoplasmic processes (I) of neighbouring cells serve to maintain contact.

X 18,000

Fig. 4.14

Trachea. 1-day-old chick

Intermediate cell (arrow) emerging towards the lumen of the trachea. X 4,000

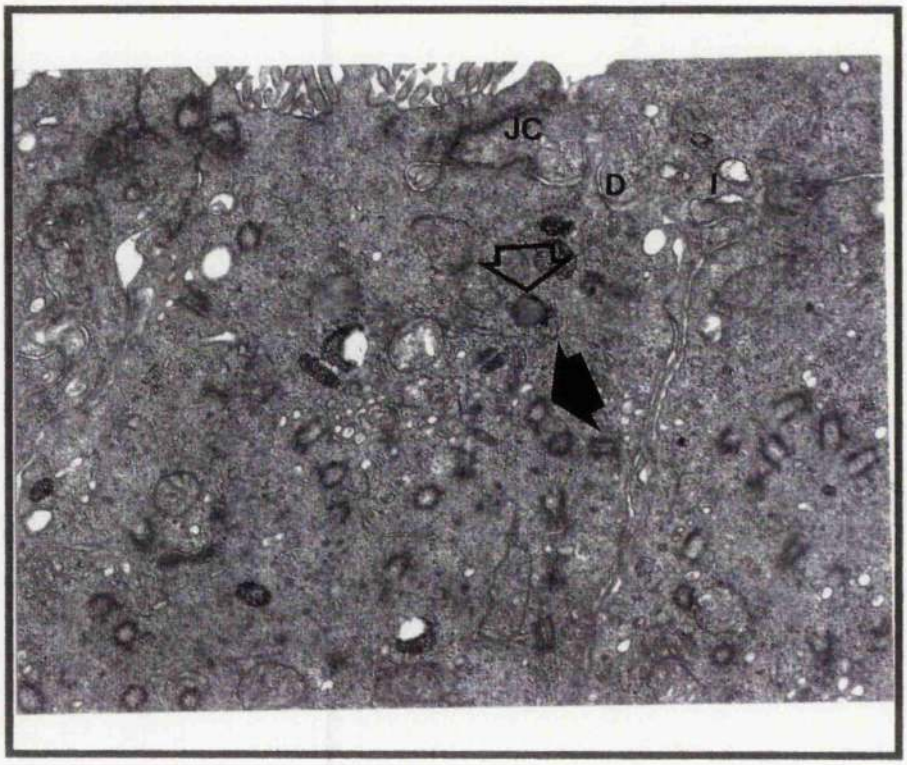

JC
D
I

Fig. 4.15

Intrapulmonary primary bronchus. 16-day-old embryo.

The epithelium is lined with ciliated cells.

Lumen (L).

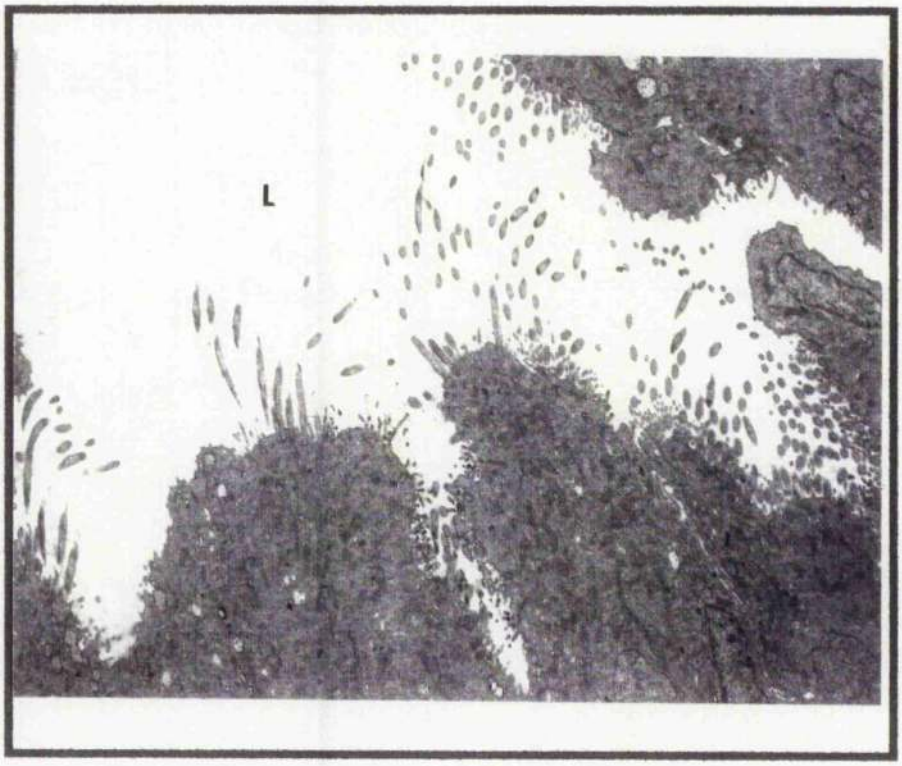
X 4,500

Fig. 4.16

Intrapulmonary primary bronchus. 16-day-old embryo.

A few vesicles filled with flocculent material (arrow) can be seen at the apical region of the cells. Note also the presence of both single and branched microvilli at the lumen.

X15,000


L

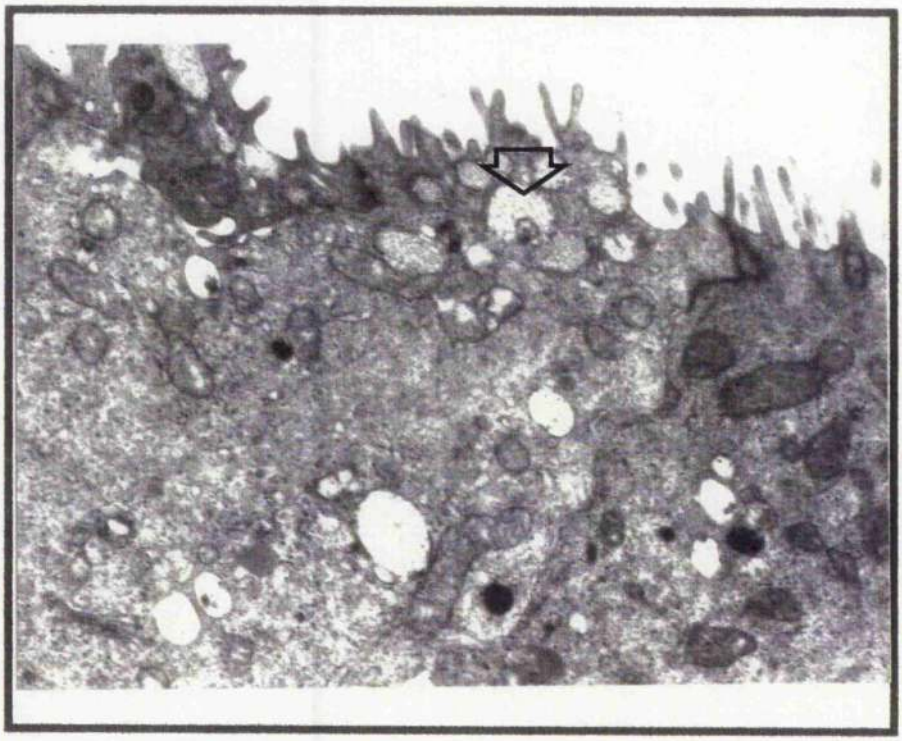


Fig. 4.17

Intrapulmonary primary bronchus. 3-day-old chick.

Mature mucous gland made up of mucous cells and ciliated cell. The cytoplasm of the mucous cells contains numerous granules each with an electron-dense core and mitochondria. The ciliated cell displays numerous basal bodies at the apical region of the cytoplasm and cilia in the lumen (L). Note also the cytoplasmic interdigitation (arrow) of neighbouring mucous cells.

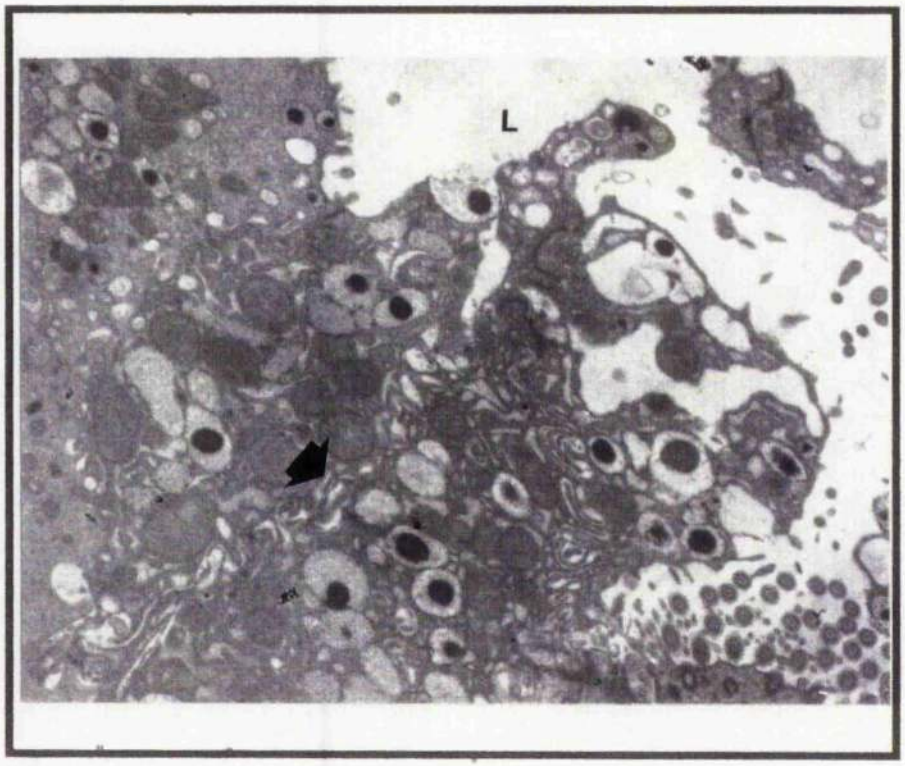
X 9, 000

Fig. 4.18

Intrapulmonary primary bronchus. 16-day-old embryo.

Cells with electron-lucent cytoplasm containing a few mitochondria and electron-dense granules (arrow) at the basal region. Note also cells still undergoing mitosis (open arrow).

X 6,000


L

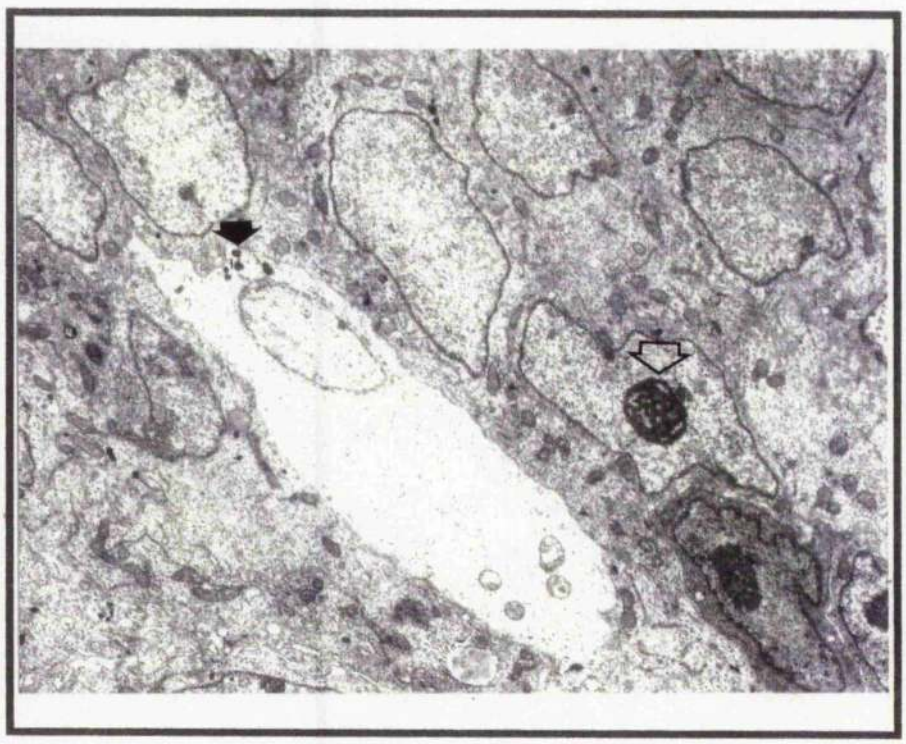


Fig. 4.19

Tertiary bronchus. 15-day-old embryo.

A transverse cut of an early uncanalized tertiary bronchus. The lining cells (columnar) (arrow) are surrounded by undifferentiated cells in the mesenchyme. The cells of the tertiary bronchus contain a number of mitochondria and rough endoplasmic reticulum.

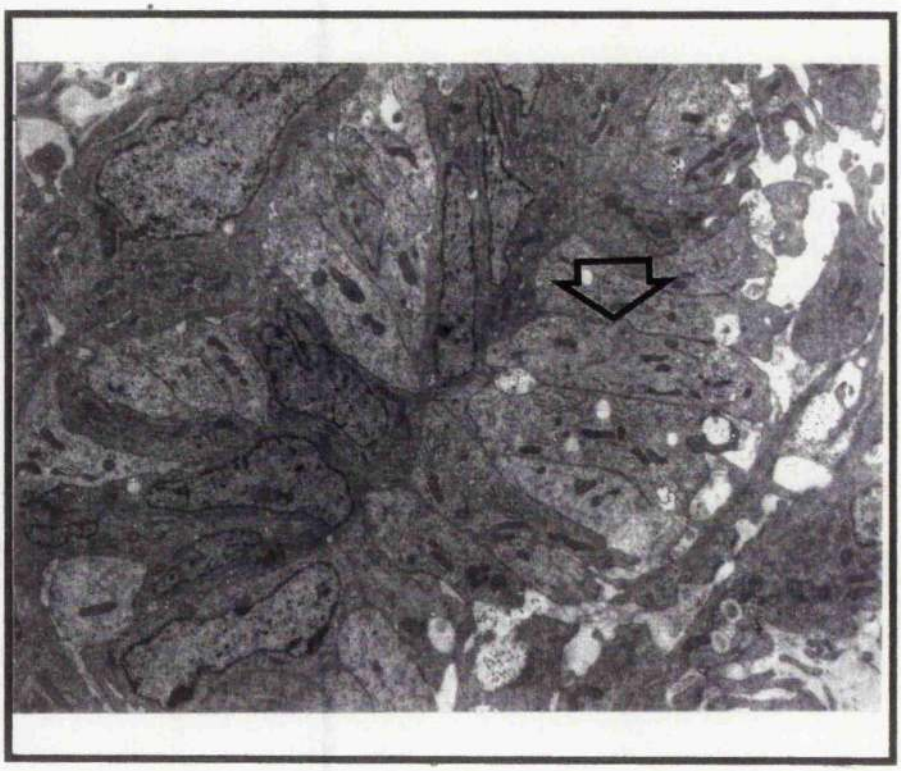
X 6,000

Fig. 4.20

Tertiary Bronchus. 17-day-old embryo.

Cuboidal cells with numerous microvilli in the luminal region (L) of the tertiary bronchus.

X4,500



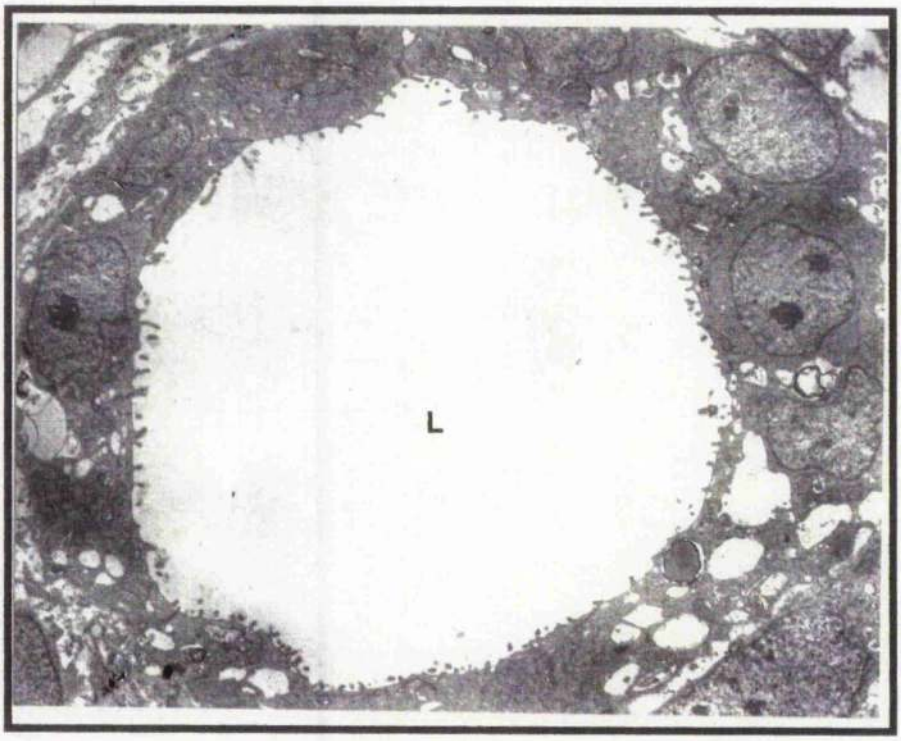

L

Fig. 4.21

Tertiary bronchus. 17-day-old embryo.

Cuboidal cells *viz* the potential atrial lining cells,

contain a variety of inclusion bodies (arrow). The outer zone

consists of cells which are more flattened and

possibly destined to form air capillary lining cells. Undifferentiated (*)

cell types making it difficult to recognise developing air capillaries

(AC). X 9, 000

Fig. 4.22. (Left)

Tertiary bronchus. 17-day-old embryo.

Developing atrial cells. Note presence of multivesicular bodies

(arrow), Golgi apparatus, mitochondria and rough endoplasmic

reticulum and numerous vesicles in the formation of inclusion

bodies (open arrow).

X 22,500

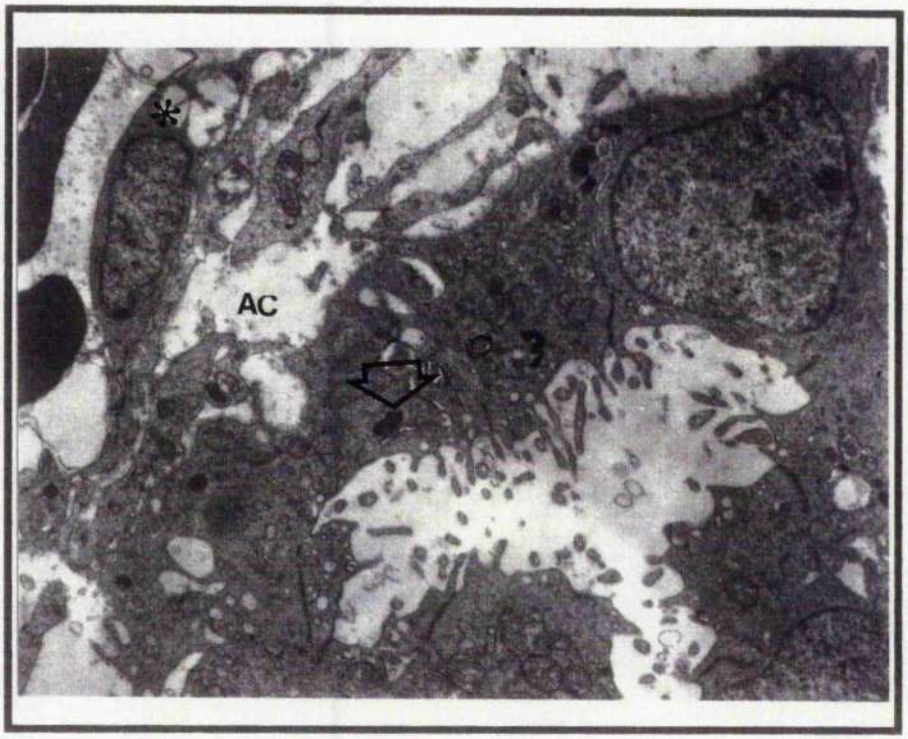
Fig. 4.23 (Right)

Tertiary bronchus. 3-day-old chick.

Immature (arrow) and mature (open arrow) osmiophilic inclusion

bodies in the atrial cells.

X 22,500


AC

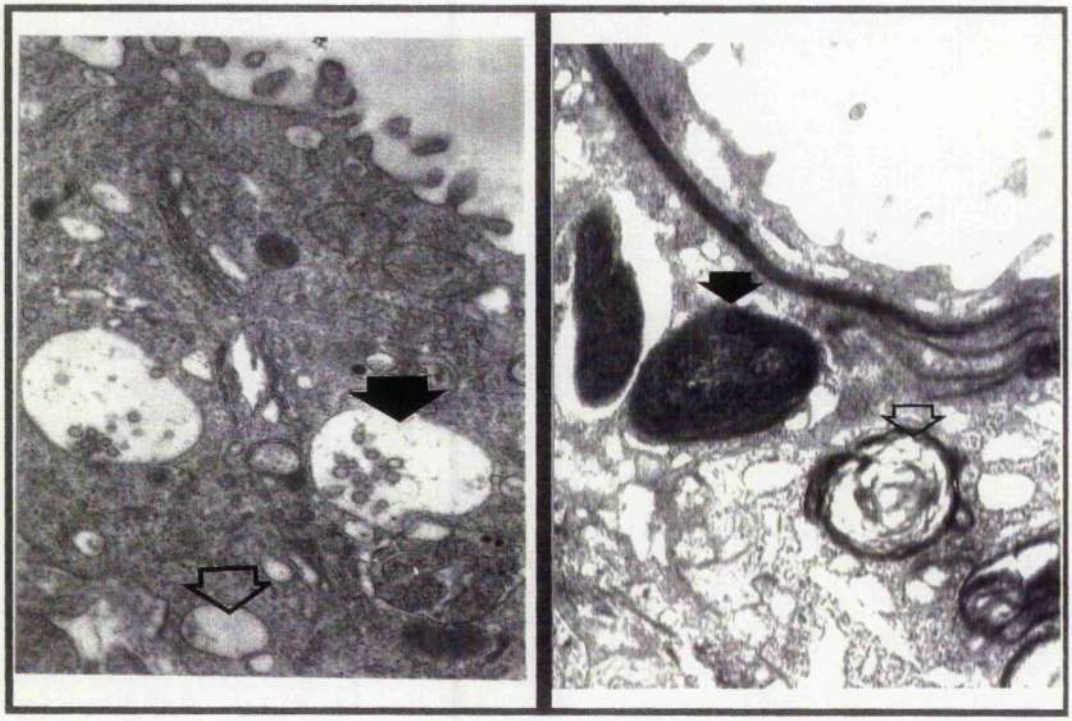


Fig. 4.24

Tertiary bronchus. 1-day-old chick.

Atrial cell with osmiophilic inclusion bodies at various stages of maturation:

1. Fusion of small vesicle into smooth inclusion bodies.
2. Multivesicular bodies.
3. Invagination of the cytoplasm in the dense bodies.
4. Mature lamellated osmiophilic inclusion bodies

X 15,000

Fig. 4.25

Tertiary bronchus. 3-day-old chick

Neighbouring atrial Type II cells with osmiophilic inclusion bodies separated by a newly formed infundibulum (arrow).

X 15,000


1
2
3
4

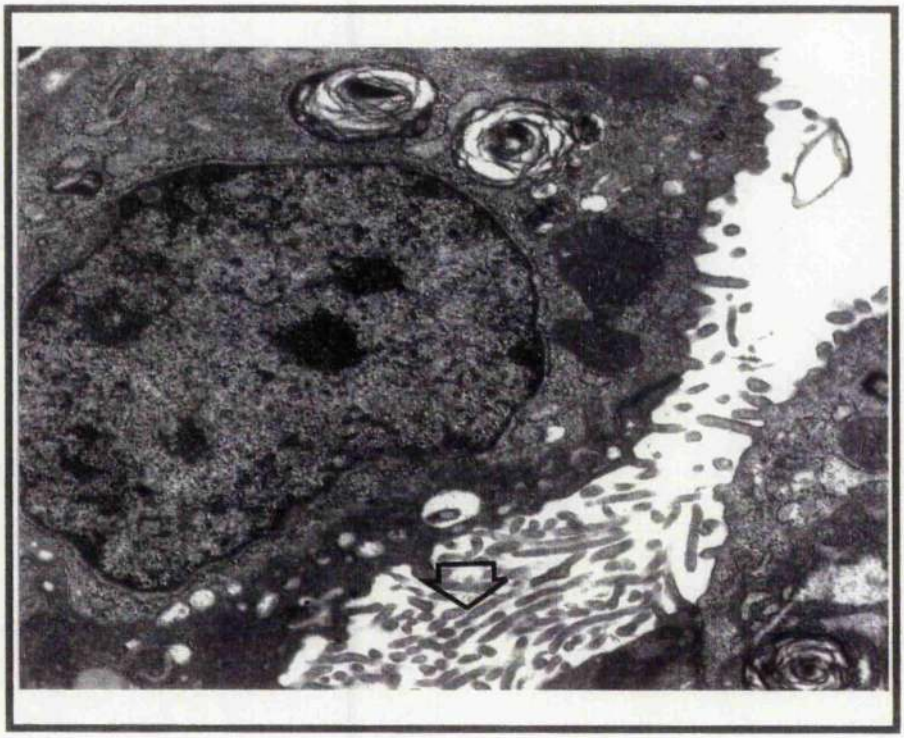


Fig. 4.26

Tertiary bronchus. 3-day-old chick.

Smooth muscle (arrow) observed lining the interatrial septa.

X 11,250

Fig. 4.27

Tertiary bronchus. 1-day-old chick

Osmiophilic inclusion bodies (arrow) and trilaminar substance (open arrow) seen in the atrial lumen (L).

X 18,000

L

Fig. 4.28

Lung. 1-day-old chick.

This low power micrograph shows the poorly differentiated nature of the compacted lung tissue at this stage of development. Endothelial cells (arrow) demarcating blood capillaries are differentiated while developing air capillary can be seen to be full of surfactant material (BC) blood capillary; (AC) air capillary lumen. X 18, 000

Fig. 4.29

Tertiary bronchus. 1-day-old chick.

Large amount of surfactant (arrow) in the atrium. X 6,000

Insert .

Trilaminar material. The outer layers (open arrows) are electron-dense whilst the middle layer is of low electron density.

X 40,000

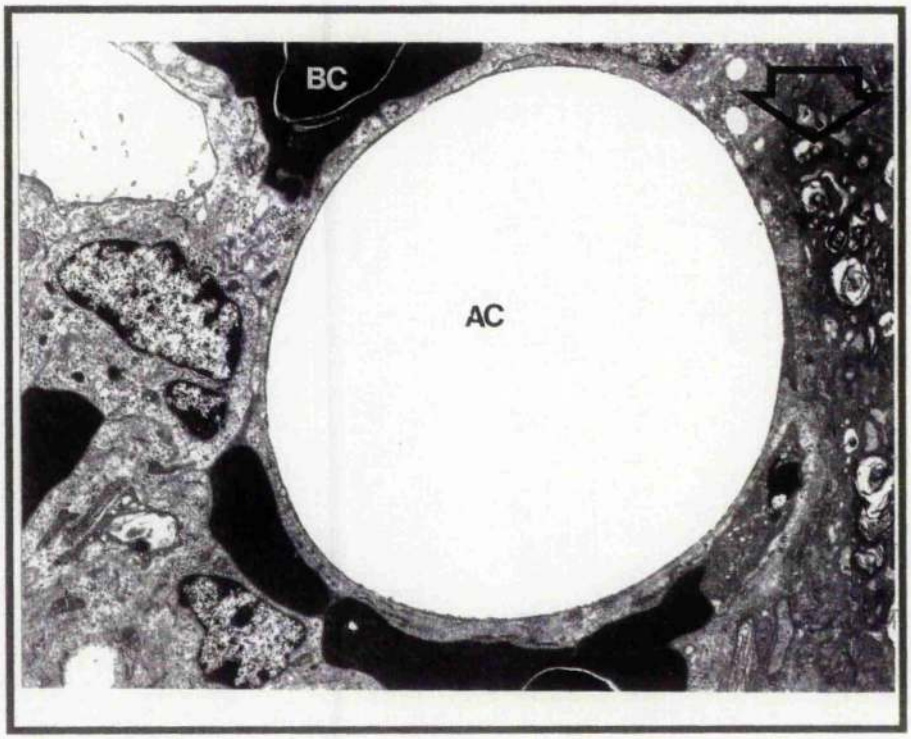

AC
BC

Fig. 4.30

Tertiary bronchus. 1-day-old chick.

Air capillary (AC) and blood capillary (BC) beginning to developed into normal lung picture. Note presence of numerous osmiophilic inclusion bodies (arrow) at this stage of development.

X 22,500


BC
AC

DISCUSSION

The present investigation on the TEM ultrastructural morphology of the developing respiratory epithelium of the chick appears to be the first detailed study carried out on the lining epithelium of the entire respiratory tract in any vertebrate. In the 15 and 16-day-old embryonic chick, the middle nasal concha, larynx, trachea and tertiary bronchus were lined by a stratified epithelium composed of two layers of undifferentiated cells, an observation confirming earlier work in the larynx, trachea and lung of chick (Jones and Radnor, 1972a, b; Walsh and McLelland, 1978; Mohammed, 1989), trachea of rabbit (Leeson, 1961) and lung of the rat (Leeson and Leeson, 1964). An exception to this early appearance of the lining epithelium is noted, however, in the intrapulmonary primary bronchus in which well differentiated ciliated and granular "endocrine" cells can be observed even at the earliest stages examined in the present study. Such an early development of mature ciliated cells does not appear to have been described before. The latter cell type has been reported previously in the trachea, intrapulmonary primary bronchus, secondary bronchus and air sac epithelial lining in the embryos, young chicks and adult chicken (Walsh and McLelland, 1974b, 1978; Wasano and Yamamoto, 1979; King *et al.*, 1974; Cook *et al.*, 1986; Mohammed, 1989).

In the present study, full differentiation of the ciliated cells, mucous cells, intraepithelial mucous glands, basal cells and granular cells was seen to occur between the 17-day-old pre-hatched to 3-day-old post-hatched chick throughout the epithelial lining of the respiratory tract, with the exception of the previously developed lining of the intrapulmonary primary bronchus. Developing ciliated cells could be recognised by the presence of numerous centrioles and small vesicles, rough endoplasmic reticulum, mitochondria and sparse multivesicular bodies, whilst developing mucous

cells were identified by the presence of a few vesicles containing floccular material, mitochondria, Golgi bodies and rough endoplasmic reticulum. However, in the 15 and 16-day-old embryos, basal cells were relatively poorly differentiated compared to the developing ciliated and mucous cells. Such observations confirm earlier findings in both the avian (Walsh and McLelland, 1978) and mammalian respiratory epithelia (Rhodin, 1966).

However, in the present study, such developing ciliated cells, were not seen to contain as many floccular filled vesicles contradicting observations by Walsh and McLelland (1978), who described the frequent occurrence of such vesicles in developing ciliated cells of the laryngeal and bronchial epithelium of the chick. Such vesicles situated primarily at the apical region of the developing ciliated cells, and remaining until the centrioles reach the apical region of the cytoplasm and became transformed into basal bodies, have been noted in the respiratory epithelium of the embryo of the domestic fowl (Kalnins and Porter, 1969; Walsh and McLelland, 1978), the African clawed toad (Steinman, 1968) and the hamster (McDowell *et al.*,1985). Because these vesicles appear to resemble an early form of mucous secretion, as seen in developing mucous cells, it is assumed that the immature ciliated cells have the capability to secrete mucus as well.

The observation of large intercellular spaces between the differentiating epithelial cells of the middle nasal concha, larynx and trachea of the chick in the present study, support similar observations as noted in the developing tracheal epithelium of hamster (McDowell *et al.* 1985). The appearance of such spaces was not observed, however, in the developing chicken respiratory epithelium by Walsh and McLelland (1978).

Centriole replication has been studied in detail (Kalnins and Porter, 1969; Kalnins *et al.*,1972), and ciliated cells, mucous cells and granular "endocrine cells" have been reported (Walsh and McLelland, 1978),

however, in the present study, the Golgi apparatus appears to be the initial site of mucin accumulation. Though it has not been reported in the respiratory epithelium of any animal species, the latter was seen in the digestive system of mammals (Freeman, 1962; 1966; Lane *et al.*, 1964; Merzel and Leblond, 1968; Troughton and Trier, 1969; Cheng, 1974). The present study supports the theory that cilia develop from the centrioles. Such findings confirm earlier observations (Walsh and McLelland, 1978), however, they disagree with Leeson (1961) who indicated that the cilia developed from both the microvilli as well as the centrioles.

The observations made in the present study allow the individual cell types, populating the developing respiratory epithelium to be characterised by those ultrastructural features noted below:

Ciliated cell

In the present study, in the 15 to 16-day-old embryo the cells were of cuboidal shape, and carried a few short microvilli on their apical surface. The rounded nucleus contained finely granular heterochromatin and a prominent nucleolus occupying about half of the nucleus. The cytoplasm was filled with numerous centrioles and free ribosomes, a few small mitochondria, a compact Golgi complex and short cisternae of endoplasmic reticulum regularly studded with ribosomes. Contact with adjacent microvillous and basal cells was by means of junctional complexes at the luminal surface, scattered desmosomes and short lateral interdigitations. A few of the ciliated cells were fully differentiated by the 17th day of incubation, the characteristic developmental feature at this stage being the presence of numerous centrioles, basal bodies and rootlets in the apical cytoplasm, and the appearance of cilia on the apical surface of the cells. When the cilia were fully developed, by day 19 and 20, mitochondria were concentrated in a band immediately below the basal bodies. The features noted in the present study as characteristic of the mature ciliated cells found

throughout the respiratory system, from the nasal cavity down to the secondary bronchi, have also been noted in the respiratory epithelial lining of the adult chicken (Purcell, 1971; Walsh and McLelland, 1974a; Lai and Ibrahim, 1984; Mohammed, 1989) and in the trachea and bronchi of the adult budgerigar (Smith *et al.*, 1987).

Mucous cell

In the present study the columnar mucous cell began differentiating in the 17-day-old embryo. The initial stage of differentiation involved an increase in the number of vesicles containing floccular material. Simultaneously, there was an increase in the number of Golgi complexes in the supranuclear cytoplasm and the appearance of small numbers of long cisternae of rough endoplasmic reticulum in the infranuclear cytoplasm. On the 18th and 19th day of incubation, there was a further increase in the number of cisternae of rough endoplasmic reticulum lying parallel to the side and base of the cell. Mucus production was often seen to involve the presence of a well-developed Golgi complex, displaying dilated vesicles at its maturing phase. By day 19, the central and apical regions of the mucous cell were full of vesicles. And at this stage the mucous cells resembled those of the adult (Purcell, 1971; Lai and Ibrahim, 1984; Mohammed, 1989).

Basal Cell

The differentiation of basal cells in the present study involved a number of changes in the fine structure of these cells located in the outer layer of the two cell thick respiratory epithelium of 15 to 16-day-old embryo. Such cells were round or oval, were positioned along the basal lamina, and contained few ribosomes, mitochondria and cisternae of rough endoplasmic reticulum. Such observations are in agreement with Walsh and McLelland (1978). By day 17 of incubation, there was an increase in the number of free ribosomes, mitochondria and cisternae of rough endoplasmic reticulum.

Between 19 and 20 days of incubation, loose bundles of intracytoplasmic filaments began to appear, along with a simultaneous increase in the number of ribosomes, resulting in the cytoplasm becoming relatively electron-opaque. During this developmental period, the interdigitation of cell processes became more extensive, and numerous desmosomes developed between adjacent basal cells. The appearance of the basal cell at this developmental stage was characteristic of the basal cell found throughout the respiratory tract in species such as the adult chicken (Purcell, 1971; Lai and Ibrahim, 1984; Mohammed, 1989) and adult budgerigar (Smith *et al.*, 1987).

Intermediate cell

In the present study the intermediate cell was identified as a pyramidal or irregularly oval-shaped cell located between adjacent ciliated and mucous cells in the epithelial lining of the trachea in the 1-day-old chick. The cell was characterised by the presence of a large oval nucleus with prominent nucleolus and a relatively electron-lucent cytoplasm. Intracytoplasmic organelles, such as the elongated and rounded mitochondria, sparse granular endoplasmic reticulum and Golgi apparatus appeared concentrated at the apical pole of the cell. Attachment to adjacent cells was by cytoplasmic interdigitations and lateral desmosomes. Such a cell type has been described previously in the nasal septum of the adult chicken (Mohammed, 1989) and the trachea of the adult budgerigar (Smith *et al.*, 1987).

Non-ciliated columnar cell

Although the non-ciliated columnar cell was not seen in the present study of normal respiratory epithelium, it was observed in material examined in Chapter 7, and therefore is discussed, perhaps prematurely, here. As seen in the formaldehyde-exposed chick, and discussed in Chapter 7, the

non-ciliated columnar cell has a relatively electron-lucent cytoplasm, containing few organelles, and carries short microvilli on its luminal surface. The characteristic feature of this cell is the absence of cilia and any membrane-bound secretion droplets in the cytoplasm. This feature was similar to the non-ciliated columnar cells described lining the epithelium of the extrapulmonary airways of the adult chicken (Walsh and McLelland, 1974a). It has also been described in the tracheal epithelium of the 17-day-old chick embryo (Walsh and McLelland, 1978).

Granular endocrine cell

In the present study, well differentiated granular endocrine cells were identified scattered singly in the intrapulmonary primary bronchus, where they were located between differentiating cells in the basal region of the lining epithelium. This cell is usually seen to be associated with an adjacent intraepithelial axon, no such relationship was observed in the present study however. The cell presented a number of characteristic TEM features, including a round or oval nucleus situated near the centre of the cell, and a relatively electron-lucent cytoplasm containing numerous variably-sized granular vesicles, a few free ribosomes, small mitochondria and cisternae of rough endoplasmic reticulum, and a compact Golgi complex. The cell type has been reported to be present throughout the extrapulmonary respiratory tract, although situated primarily in the caudal trachea in the developing embryo and chick (Walsh and McLelland, 1974b, 1978), and also in the secondary and intrapulmonary primary bronchus of the 3 to 5-day-old chick (Wasano and Yamamoto, 1979). The endocrine cells has also been reported to be present in the bronchial epithelium in the developing lung of rabbits, mice and guinea pig (Hage, 1974). In the adult chicken, the granular endocrine cell has been described either singly or in small groups at the base of the epithelium between the ciliated epithelial cells of the intrapulmonary primary bronchus (King *et al.*, 1974; Mohammed, 1989)

especially at the origin of the craniomedial secondary bronchus (King *et al.*, 1974) and at the ostium of the abdominal air sac (Cook *et al.*, 1986; Mohammed, 1989).

Type I (non-granular) and Type II (granular) pneumocyte

In the present study, out-pouchings from the lumen of the tertiary bronchus into the surrounding mesoderm, were seen to lead to the formation of the atria and infundibula of the lungs, such events occurring round about the 17th to 20th days of incubation. The anastomosing of the air capillary system was then seen to develop from the atria and infundibula. In the present study air capillaries start to develop in the 18-day-old embryos and increased in number and size in the older chicks. Such an observation agrees with that of Petrik (1967), who stated that air capillaries develop during the last three days of incubation and ultrastructurally in the process of forming air capillaries, gap formation occurs between epithelial cells as well as development of canaliculi in their cytoplasm. The epithelium lining the air capillaries developed through differentiation of the granular pneumocytes. The epithelium lining the air capillaries was complete and thinnest in areas adjacent to the blood capillaries as had been reported in chick embryos (Petrik, 1967; Petrik and Riedel, 1968a), adult chicken (Tyler *et al.*, 1961; Tyler and Pangborn, 1964), penguin (Drescher and Welsch, 1983) and also in the budgerigar (Smith *et al.*, 1986). Whilst these events were taking place the columnar epithelial lining cells characteristic of the tertiary bronchus were seen to change into flattened attenuated cells in the outer air capillary zone; those cells which lined the atrial area retained a somewhat flattened morphology, intermediate between these columnar and attenuated cell types. Such observations agree with the findings of Jones and Radnor (1972a).

The epithelial cells of the atrium develop into either granular (Type II)

or non-granular (Type I) pneumocytes in the present investigation. The granular (Type II) cells presented a polygonal shape, and many possess surface microvilli, whilst others presented a relatively smooth apical surface. The oval nucleus of the cell was frequently seen to exhibit a corrugated margin, whilst the cytoplasmic organelles were seen to contain numerous oval mitochondria, extensive granular endoplasm reticulum, numerous vacuoles and osmiophilic inclusion bodies. A sequence of events leading to the formation of these mature osmiophilic inclusion bodies was observed in the present study. Such osmiophilic inclusion bodies, were formed in the atrial lining cells by the fusion of small, Golgi-derived, non-osmiophilic or lightly osmiophilic vesicles containing smooth granular dense bodies. Both multivesicular bodies or dense bodies were also found in the vicinity of the Golgi complex. It has been suggested (Jones and Radnor, 1972a) that the contents of the multivesicular bodies (seen in the present study) continue to form these granular dense bodies. The invagination of cell cytoplasm into these bodies then leading to the formation of the mature osmiophilic inclusion bodies, characterised by their circular cross section and content of coarse lamellae. Similar observations have also been noted in the chicken (Jones and Radnor, 1972a), hamster and guinea pig (Kikkawa and Spitzer, 1969). The granular (Type II) cells have also been identified in the lining epithelium of the tertiary bronchus, atrium and infundibulum of embryos and day-old chicks (Petrik, 1967; Petrik and Riedel 1968a; Jones and Radnor, 1972b), adult chicken (Tyler and Pangborn, 1964; Akester, 1970 and Mohammed, 1989), goose (Lambson and Cohn, 1968), penguin (Drescher and Welsch, 1983) and budgerigar (Smith *et al.*, 1986), whilst the early development of such cells has also been noted in the 18-day-old chick lung (Petrik, 1967).

In the present investigation, the osmiophilic inclusion bodies appeared to be active during lung development and discharge their

contained surfactant material onto the atria and air capillary surfaces. Such observations agree with earlier reports that the granular cell, producing lung surfactant from the myelin-like osmiophilic lamellar inclusion bodies (Tyler and Pangborn, 1964; Tyler and Pearse, 1966; Petrik and Riedel, 1968a; Nowell *et al.*, 1970; Akester, 1970; Pattle, 1978), is also active in the developing lung of the chicken (Jones and Radnor, 1972a, b) and of man and rat (Balis and Conen, 1964).

The characteristic features of the mature non-granular (Type I) pneumocyte lining the wall of the air capillaries and infundibula are the elongated and attenuated cytoplasmic processes arising from a cell body containing a large round to ovoid nucleus. The cytoplasmic organelles consist of a number of small rounded mitochondria, sparse granular endoplasmic reticulum, few free ribosomes and occasional Golgi complexes, all these features have been noted in adult avian species (Smith *et al.*, 1986; Mohammed, 1989). In this study, however, it was difficult to separate Type I and Type II cells by their morphological appearance, due to the fact that all atrial cells appeared to contain osmiophilic inclusion bodies (Jones and Radnor, 1972b).

A non-cellular continuous film of trilaminar material was present in this investigation in the atria and the air capillaries of 18-day-old embryo and the amount increased with age in the day-old and 3-day-old chicks. Such observations agree with earlier findings in the air capillaries of embryos and day-old chicks (Petrik and Riedel, 1968 a, b; Jones and Radnor, 1972b). This film has also been reported to be similar to the continuous film on the air capillaries of adult chicken (Tyler and Pangborn, 1964; Akester, 1970; Mohammed, 1989), adult pigeon and sparrow (Petrik and Riedel, 1968b) and goose (Lambson and Cohn, 1968). The thickness of the film is approximately 100-150 Å. The upper and lower layers are strongly osmiophilic, the intermediate layer was of low electron density. In adult

chicken, the production of the trilaminar lining was reported from the atrial osmiophilic inclusion body (Tyler and Pangborn, 1964; Akester, 1970), or in the adult goose, the endoplasmic reticulum of the atrial granular pneumocyte (Lambson and Cohn, 1968). In this present study, observations suggest that the trilaminar surfactant sheet was derived from the osmiophilic inclusion bodies of Type II cells. Such observations agree with those of Jones and Radnor (1972b), who stated that in the last three days of incubation, at the time of expansion of the air capillaries and during the first 7 days of life, surfactant is derived from the same source.

CHAPTER 5

THE MUCUS-PRODUCING APPARATUS OF THE RESPIRATORY TRACT OF THE DEVELOPING AND HATCHED CHICK.

INTRODUCTION

Mucous cells and intraepithelial or sub-mucosal mucous glands are found throughout the respiratory tract of higher vertebrates. Their mucoid secretion, which plays an important role in the defense of the respiratory tract by protecting the underlying epithelium from dehydration, injury due to toxic gases, or invasion by potentially pathogenic microorganisms (Breeze and Wheeldon, 1977), is a complex mixture of approximately 95% water and 5% protein, carbohydrate, lipid and inorganic materials (Jeffery, 1978). Histochemistry of the mucus by the use of Alcian Blue/Periodic Acid Schiff (AB/PAS) staining reaction permits the classification of mucus-producing cells into one of three main colour groups for qualitative and quantitative descriptive purposes (see Chapter 2) *viz*: Red indicating the presence of neutral mucosubstance a PAS positive staining reaction; blue indicating the presence of acidic mucosubstance which is an AB positive reaction and purple indicating the presence of mixed mucosubstance, a positive reaction to both AB and PAS staining.

Quantitative studies on the mucous cells and sub-mucosal glands of the respiratory tract in mammals are numerous (Tos, 1970, 1971,1983; Ellefsen and Tos, 1972b; Harkema *et al*., 1987a), but in the bird, mucous cells and intraepithelial mucous gland numbers have been assessed only in the respiratory epithelium of adult layer and broiler chickens (Mohammed, 1989). This latter study did not provide any information on the development and quantitative analysis of the mucus-producing apparatus in the developing and newly-hatched chick. Similarly, despite the importance of the mucus through its role in the mucociliary clearance system, very little work on the histochemistry of the avian mucous cell has been carried out,

with only limited information available for the chicken (Bang and Bang, 1969; Chandra and Bharadwaj 1970; 1971; Mohammed, 1989; Midtgard, 1989) and goose (Jeffery,1978).

The present study was designed to investigate the distribution of mucous cells and intraepithelial mucous glands throughout the respiratory epithelium of 15-day-old chick embryos to 13-day-old post-hatched chicks, as well as the histochemical changes which take place during this period of development. The information gathered in this study is then used as a baseline for the assessment of the numerical and histochemical changes which occur in the number and chemical properties of the mucous cells and intraepithelial mucous glands of the respiratory tract of chicks exposed to formaldehyde vapour.

MATERIALS AND METHODS

Source of chicks

Control chicks for this study were obtained as described in Chapter 2. The number and age of chicks involved in this study is as shown in table 9:

TABLE 9

CHICKS USED FOR LIGHT MICROSCOPY (LM) IN THE INVESTIGATION OF THE DEVELOPING RESPIRATORY EPITHELIUM

Age of chicks	Number of chicks involved in LM
15-day-old embryo	3
16-day-old embryo	3
17-day-old embryo	3
18-day-old embryo	3
19-day-old embryo	3
20-day-old embryo	3
1-day-old chick	3
3-day-old chick	3
5-day-old chick	3
7-day-old chick	3
11-day-old chick	3
13-day-old chick	3

Sample collection and processing of samples for light microscopy.

Middle nasal concha, larynx, cranial trachea, caudal trachea, intrapulmonary primary bronchus and secondary bronchus were collected, processed and stained with AB/PAS (as detailed in Chapter 2) in order to identify the individual mucous cells and intraepithelial mucous glands contributing to the mucous-producing apparatus of the respiratory tract. The study of these parameters, in the normal developing embryo and chick and the qualitative assessment of mucous cells and intraepithelial mucous gland numbers was carried out as detailed in Chapter 2.

RESULTS

A full statistical analysis was difficult at the present time due to the relatively small sample size, which also resulted in a relatively large standard deviation. This study does however, provide a useful preliminary assessment of mucous cell and intraepithelial mucous gland numbers in the normal developing chick, thus providing a basis for the future assessment of changes in these parameters resulting from exposure to formaldehyde vapour.

15-day-old embryo.

In the 15-day-old embryo, histological sections, when stained with Alcian Blue/Periodic Acid Schiff (AB/PAS), pH2.5, demonstrated the presence of mucous cells throughout the respiratory epithelium from the middle nasal concha down to the level of the secondary bronchus (Table 10). The majority of cells stained red (PAS +) (Fig. 5.13), indicating the presence of predominantly neutral mucopolysaccharides, although occasional evidence of scarce acidic mucosubstances (blue staining) at the apical region of some cells (Fig. 5. 14) was observed. No mucous glands were encountered in this age group.

Quantitative assessment showed that in this age group, the mean total number of mucous cells in a pre-selected standard length of respiratory epithelium was higher in the middle nasal concha, caudal trachea and intrapulmonary primary bronchus than in the larynx, cranial trachea and secondary bronchus (Fig. 5.1).

16-day-old embryo.

In the 16-day-old embryo, the situation is reversed with regard to the histochemical nature of the mucoid secretion. Most of the mucous cells

demonstrated the presence of acidic (blue staining) material at the apical region, and only a few stained positively for neutral mucins. The mean total number of mucous cells appeared appreciably higher in the intrapulmonary primary bronchus than in other regions of the tract. (Fig. 5.2).

17 and 18-day-old embryo.

By 17 to 18 days of incubation, the mucous cells were gradually acquiring an increased complement of mucous granules. One third of the apical region of the majority of the mucous cells demonstrated the presence of acidic mucosubstances, although in the 18-day-old embryo mucous cells with a mixed content of secretory material began to appear (Fig. 5. 15). In the 17 and 18-day-old embryo, rudimentary intraepithelial mucous glands, were identified by the slight invagination of the mucosal surface together with the presence of at least 2 cells revealing the presence of mucus material (Fig. 5.16). In the mucous glands and in the majority of individual surface mucous cells, one third of the apical region of the cell reacted positively to the AB+ and PAS/AB+ stain. Quantitative assessment of the mucous cell and intraepithelial mucous gland numbers in the 17-day-old embryo showed that the mean number of mucous cells was highest in the intrapulmonary primary bronchus and lowest in the secondary bronchus (Fig. 5.3), whilst the mean gland number was higher in both the intrapulmonary primary bronchus and middle nasal concha, but again observed lowest in the secondary bronchus. In the 18-day-old embryo, the number of mucous cells was highest in the intrapulmonary primary bronchus and lowest in the middle nasal concha (Fig. 5.4), whilst the mean mucous gland number was highest in the middle nasal concha and lowest in the secondary bronchus.

19 and 20-day-old embryo.

In the 19 and 20-day-old embryo, the mucous cells were gradually being filled with primarily acidic or mixed secretory granules. Such cells were usually distended although slender mucous cells were also still present (Fig. 5.17). Mucous gland development at this stage was more advanced, the invaginations of the mucosal surface being much deeper, with more cells giving a positive reaction for the presence of mucus. Individual mucous cells within the glands demonstrated two thirds of the apical region gradually being filled with either acid or mixed mucosubstances.

In both the 19 and 20-day-old embryo, the mean number of mucous cells was significantly higher in the intrapulmonary primary bronchial lining compared to other regions of the tract (Figs. 5.5 and 5.6), whilst the mean numbers of mucous glands was highest in the middle nasal concha and lowest in the secondary bronchus.

1, 3, 5, 7, 11 and 13-day-old chick.

In the 1, 3, 5, 7, 11 and 13-day-old chicks, the mucous cells were more distended, appearing entirely filled with mucous granules and frequently recognised as goblet cells. In the post-hatched chick, more established acidic mucous glands were present, mainly of a simple alveolar type (Fig. 5.18).

The mean total number of mucous cells in a pre-selected standard length of respiratory epithelium of the different regions of the respiratory tract of the hatched chicks, from day-old through to 13-day-old, was highest in the intrapulmonary primary bronchus and lowest in the middle nasal concha. However, the mean number of intraepithelial mucous glands was highest in the middle nasal concha and lowest in the secondary bronchus (Figs. 5.7 to 5.12).

Abbreviations for Figs. 5.1 to 5.12

MNC	Middle nasal concha
L	Larynx
CRT	Cranial trachea
CLT	Caudal trachea
IPB	Intrapulmonary primary bronchus
SB	Secondary bronchus

Fig. 5.1 Mean numbers of mucous cells and glands (±sd) in 15-day-old embryos

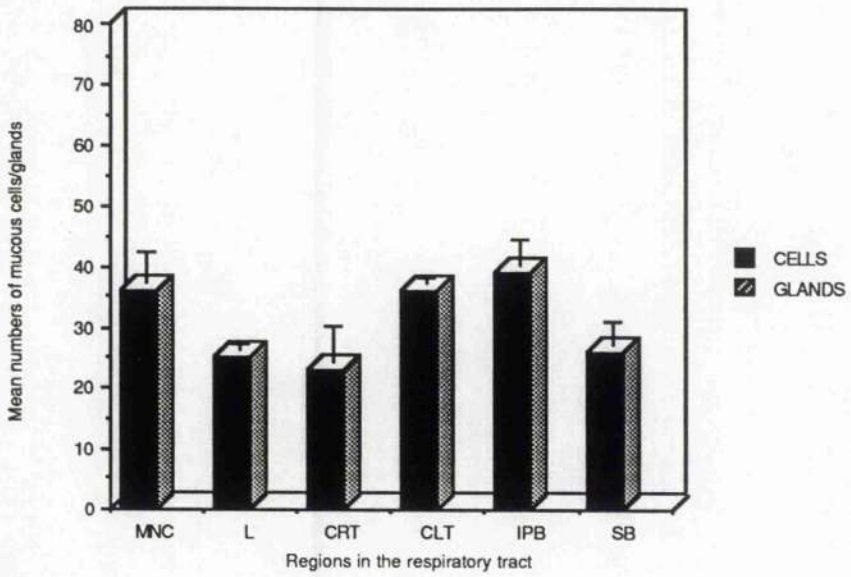


Fig. 5.2 Mean numbers of mucous cells and glands (± sd) in the 16-day-old embryos

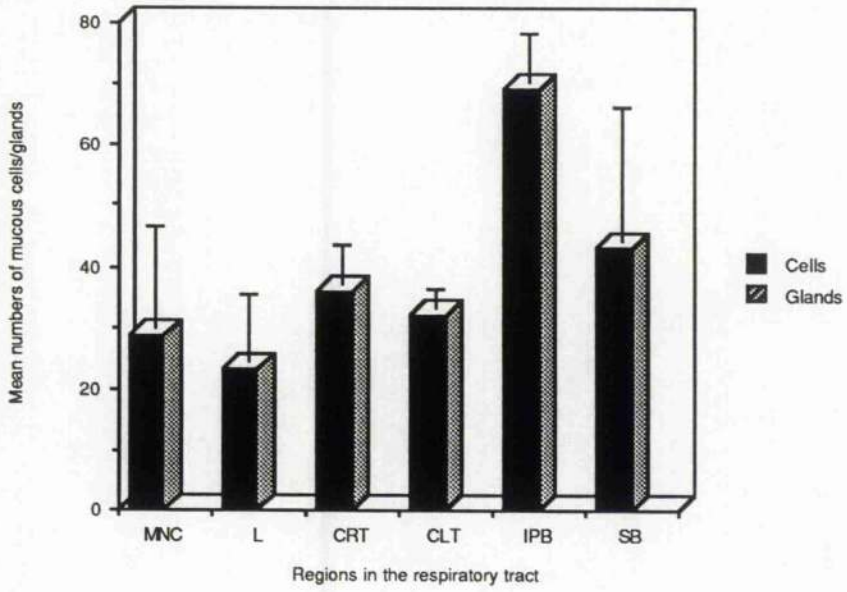


Fig. 5.3 Mean numbers of mucous cells and glands (±sd) in the 17-day-old embryos

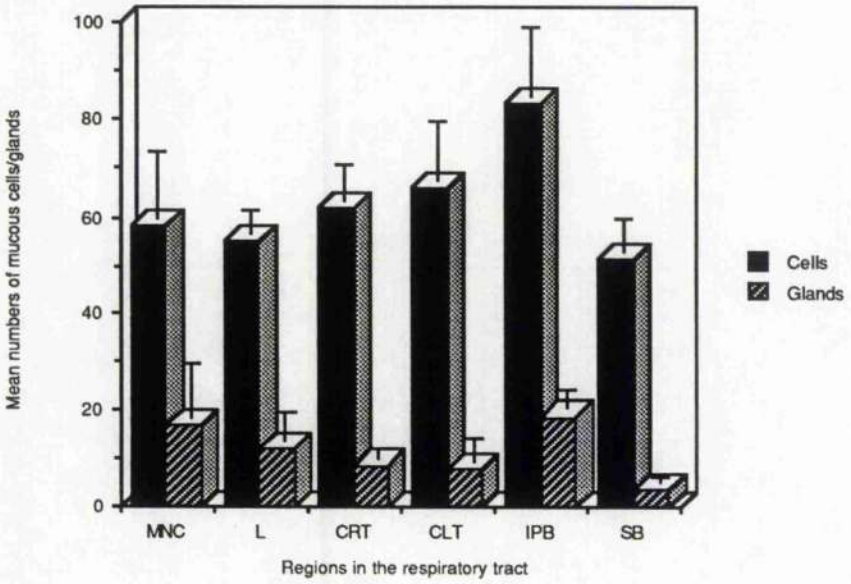


Fig. 5.4 Mean numbers of mucous cells and glands (± sd) in the 18-day-old embryos

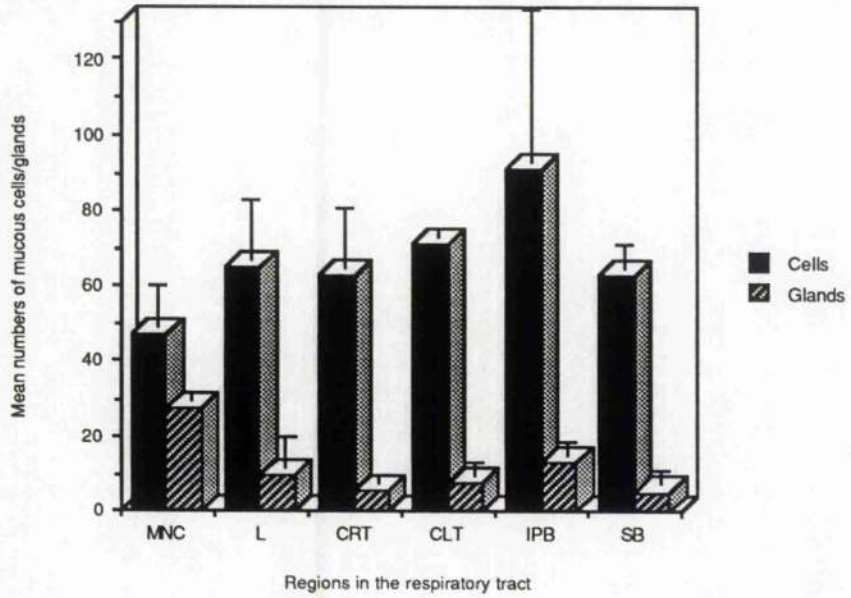


Fig. 5.5 Mean numbers of mucous cells and glands (± sd) in the 19-day-old embryos

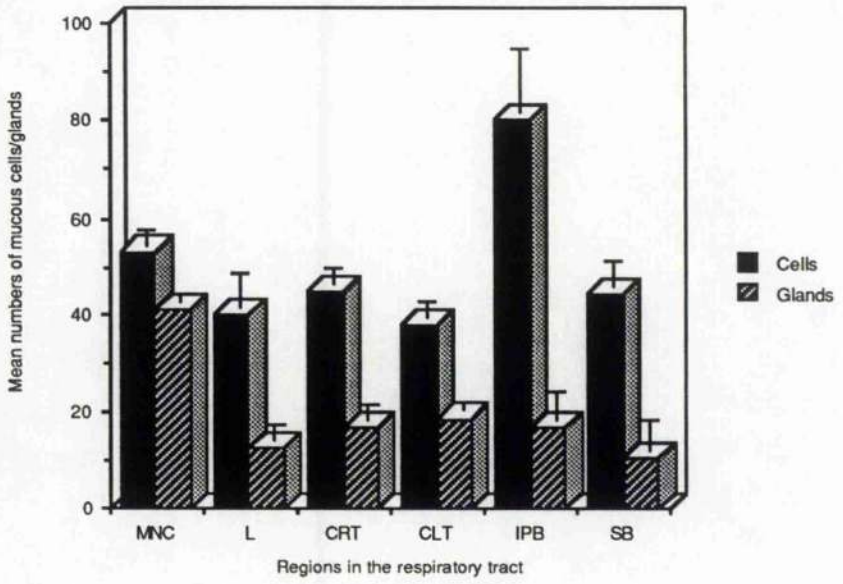


Fig. 5.6 Mean numbers of mucous cells and glands (± sd) in the 20-day-old embryos

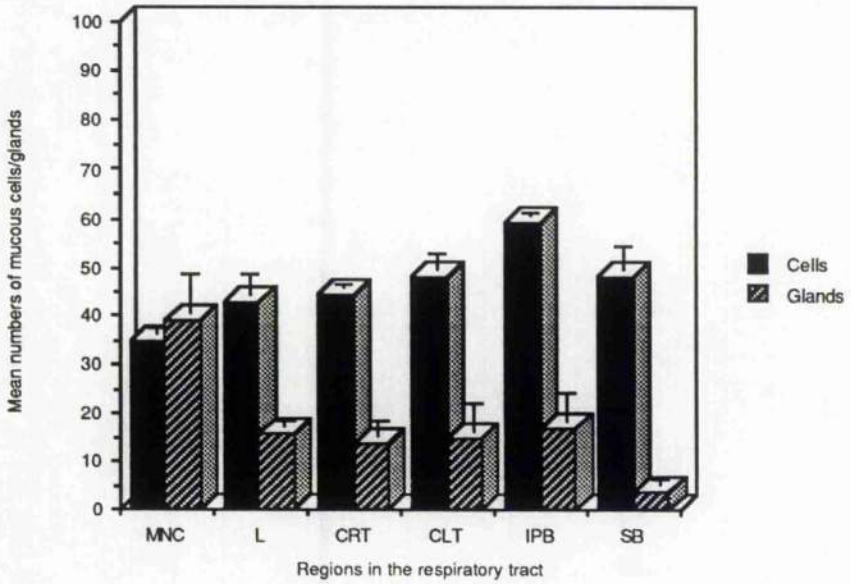


Fig. 5.7 Mean numbers of mucous cells and glands (± sd) in the 1-day-old chicks

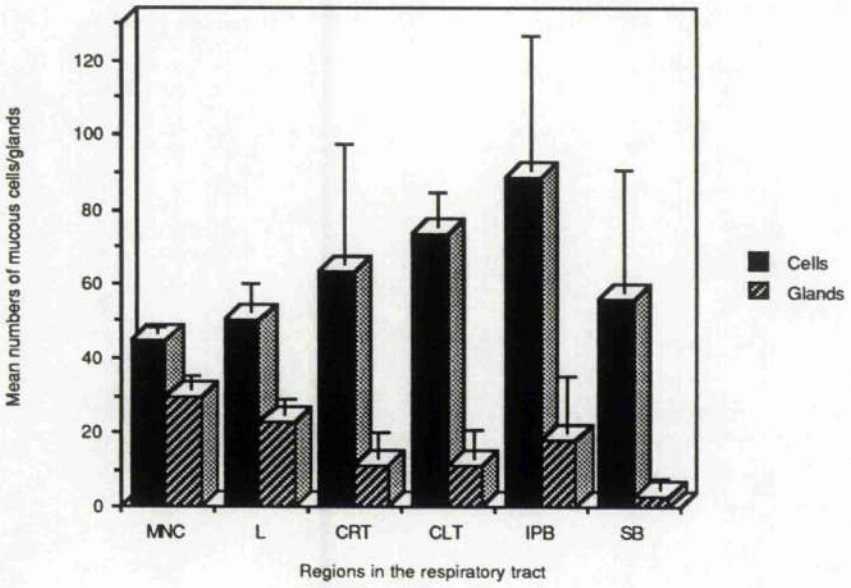


Fig. 5.8 Mean numbers of mucous cells and glands (± sd) in the 3-day-old chicks

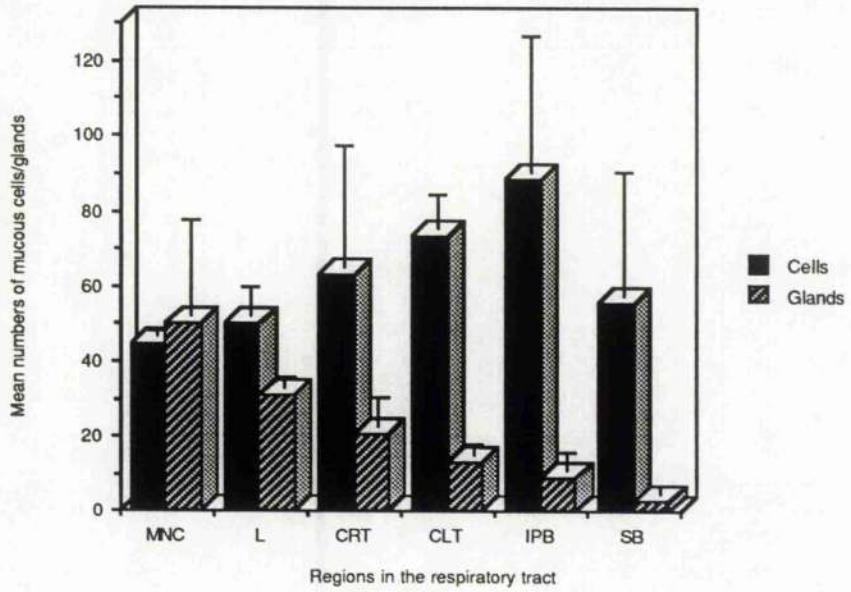


Fig. 5.9 Mean numbers of mucous cells and glands (± sd) in the 5-day-old chicks

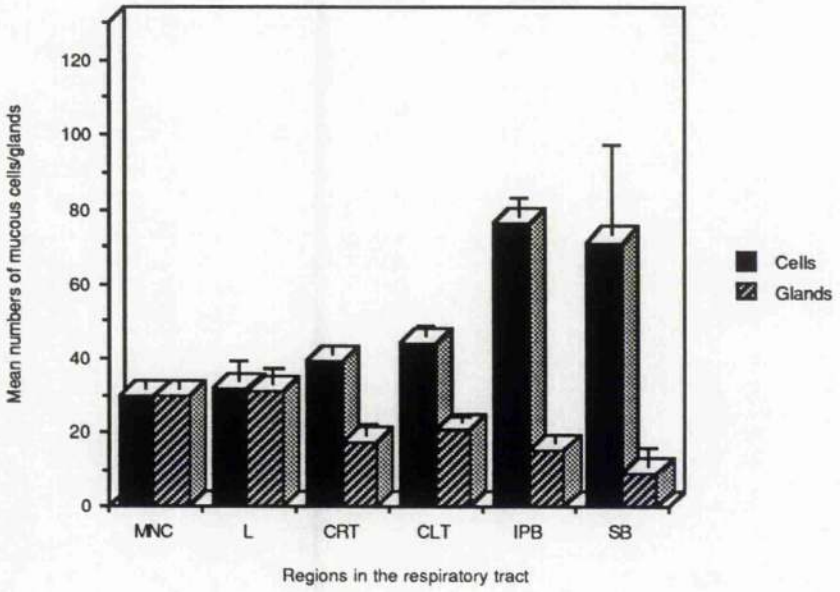


Fig. 5.10 Mean numbers of mucous cells and glands (± sd) in the 7-day-old chicks

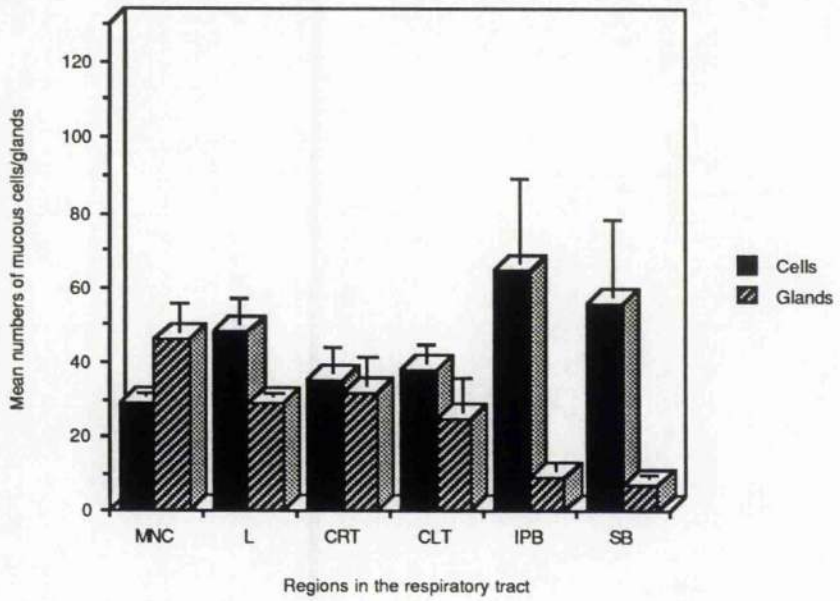


Fig. 5.11 Mean numbers of mucous cells and glands (± sd) in the 11-day-old chicks

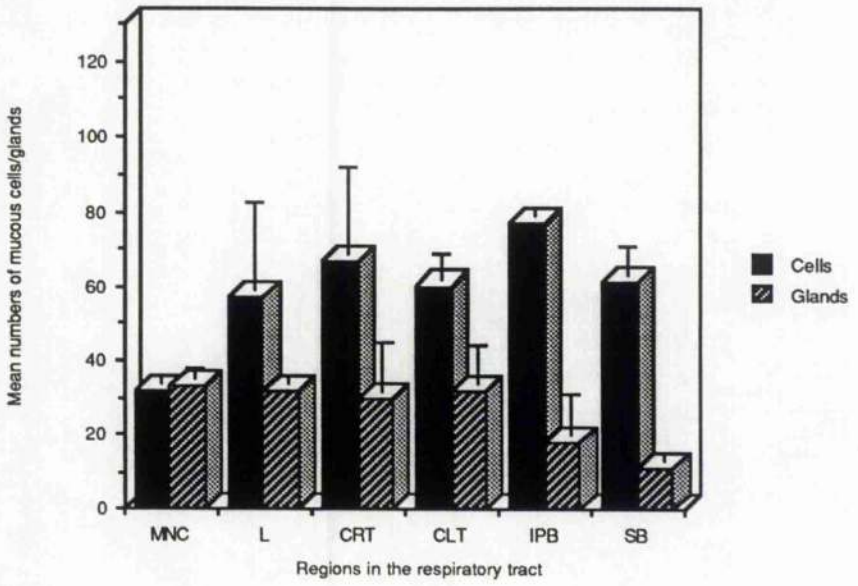


Fig. 5.12 Mean numbers of mucous cells and glands (± sd) in the 13-day-old embryos

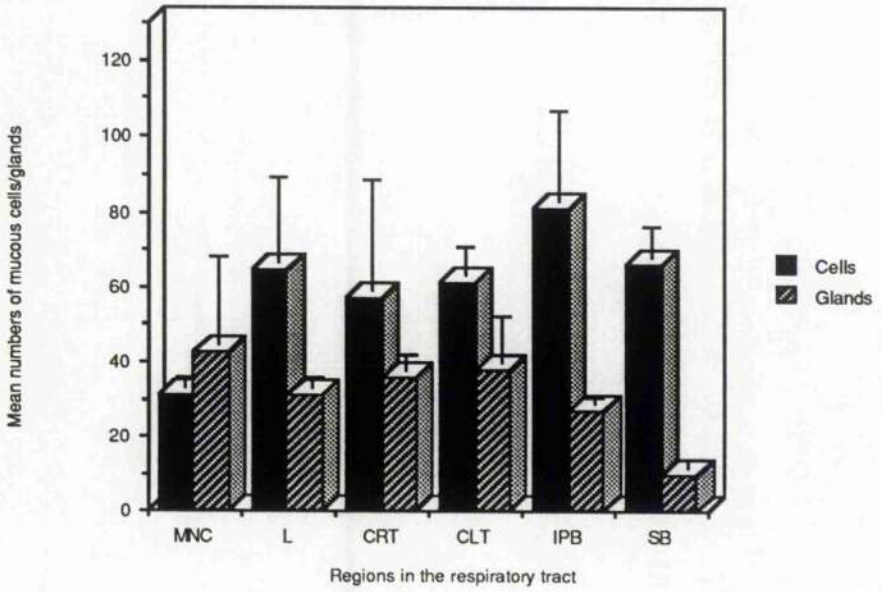


Fig. 5.13

Larynx. 15-day-old embryo.

Note presence of neutral mucus-producing cells (arrow).

X 120 AB/PAS

Fig. 5.14

Intrapulmonary primary bronchus. 15-day-old embryo.

Presence of acidic mucosubstance at the apical region of the cells (arrow).

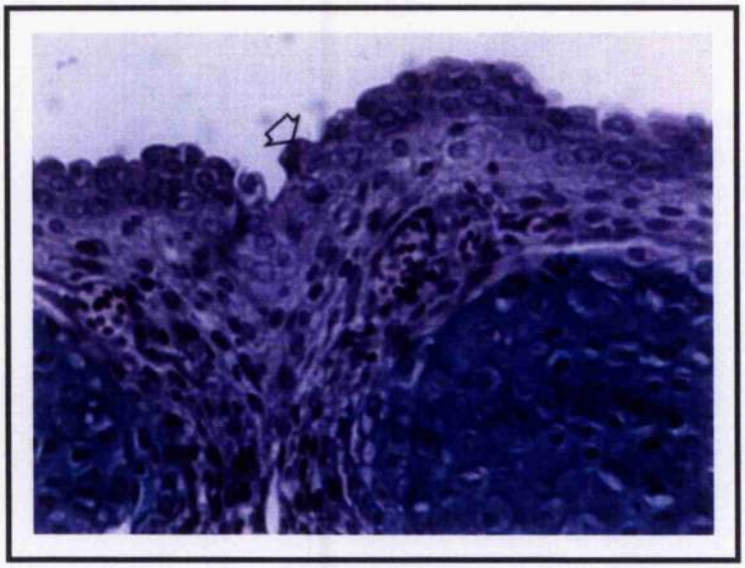
X 120 AB/PAS

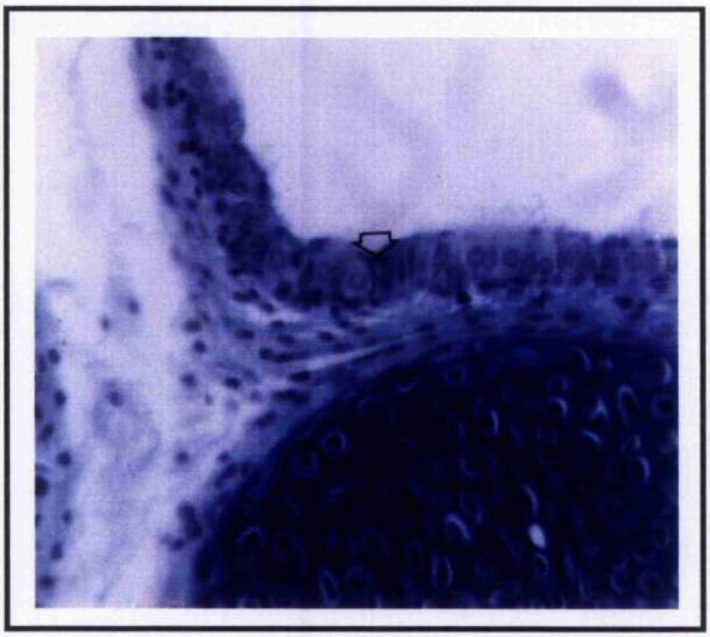
Fig. 5.15

Middle nasal concha. 18-day-old embryo.

Appearance of mixed mucosubstance containing blue and red staining secretory granules (arrow).

X300 AB/PAS





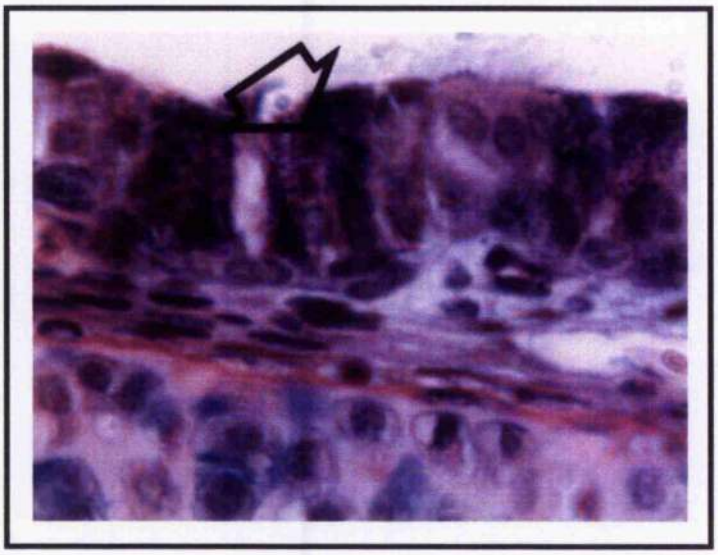


Fig. 5.16

Middle nasal concha. 17-day-old embryo.

Rudimentary intraepithelial mucous gland consisting of numerous acidic mucosubstance at the apical region of the cells (arrow).

X 300 AB/PAS

Fig. 5.17

Secondary bronchus. 19-day-old embryo.

Mucous cells distended with purple staining mucosubstance (arrow).

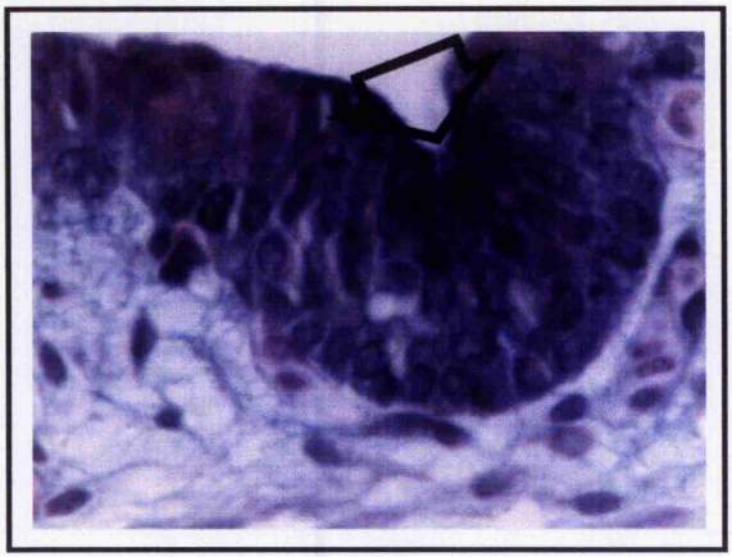
X300 AB/PAS

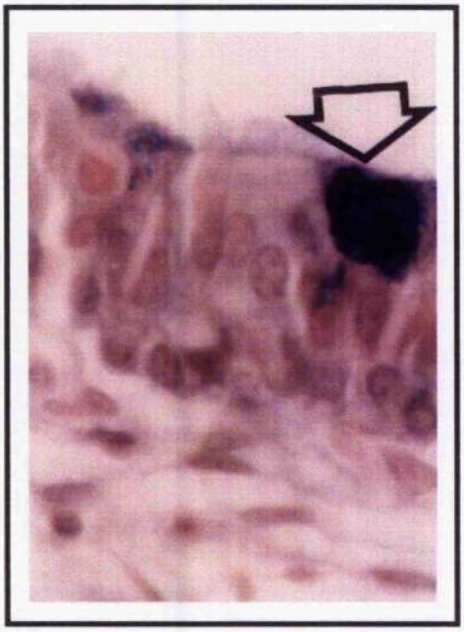
Fig. 5.18

Middle nasal concha. 3-day-old chick.

Numerous well-developed intraepithelial mucous glands containing acidic mucosubstance (arrow)

X 120 AB/PAS





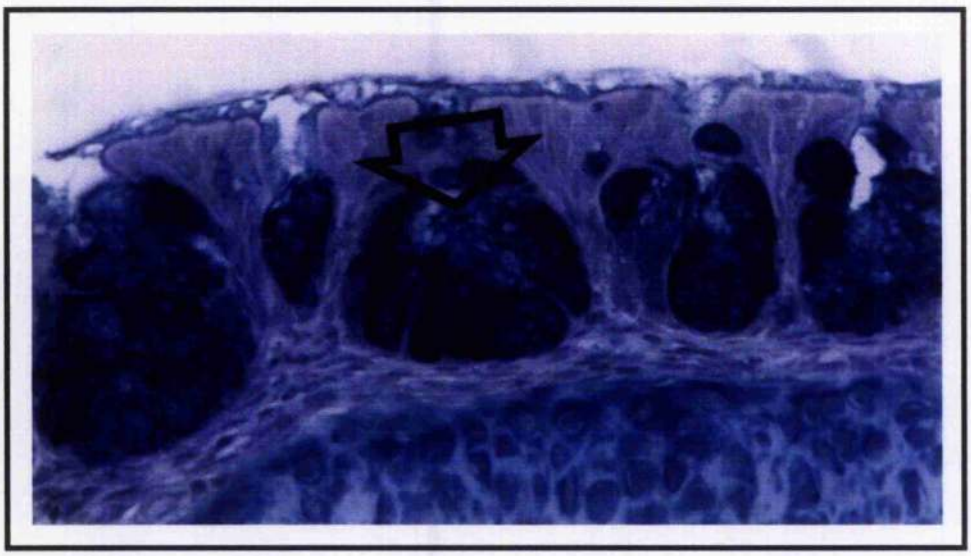


TABLE 10

QUALITATIVE ASSESSMENT OF THE MUCOSUBSTANCES IN THE RESPIRATORY TRACT OF DEVELOPING CHICKS

Age	Colour of muco-substance	Regions in the respiratory tract: Middle nasal concha		Larynx		Cranial trachea		Caudal trachea		Primary bronchus		Secondary bronchus	
		C	G	C	G	C	G	C	G	C	G	C	G
15-day-old embryo	Red	4+	-	4+	-	4+	-	4+	-	4+	-	4+	-
	Blue	1+	-	1+	-	1+	-	1+	-	1+	-	1+	-
	Purple	-	-	-	-	-	-	-	-	-	-	-	-
16-day-old embryo	Red	1+	-	1+	-	1+	-	1+	-	1+	-	1+	-
	Blue	4+	-	4+	-	4+	-	4+	-	4+	-	4+	-
	Purple	-	-	-	-	-	-	-	-	-	-	-	-
17-day-old embryo	Red	-	-	-	-	-	-	-	-	-	-	-	-
	Blue	4+	-	4+	-	4+	-	4+	-	4+	-	4+	-
	Purple	-	-	-	-	-	-	-	-	-	-	-	-
18-day-old embryo	Red	-	-	-	-	-	-	-	-	-	-	-	-
	Blue	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Purple	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+
19-day-old embryo	Red	-	-	-	-	-	-	-	-	-	-	-	-
	Blue	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Purple	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+
20-day-old embryo	Red	-	-	-	-	-	-	-	-	-	-	-	-
	Blue	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Purple	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+
1-day-old chick	Red	-	-	-	-	-	-	-	-	-	-	-	-
	Blue	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Purple	-	-	-	-	-	-	-	-	-	-	-	-
3-day-old chick	Red	-	-	-	-	-	-	-	-	-	-	-	-
	Blue	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Purple	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+
5-day-old chick	Red	-	-	-	-	-	-	-	-	-	-	-	-
	Blue	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Purple	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+
7-day-old chick	Red	-	-	-	-	-	-	-	-	-	-	-	-
	Blue	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Purple	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+
11-day-old chick	Red	-	-	-	-	-	-	-	-	-	-	-	-
	Blue	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Purple	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+
13-day-old chick	Red	-	-	-	-	-	-	-	-	-	-	-	-
	Blue	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Purple	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+

1+ - very few 2+ - few 3+ - many 4+ - very many
C - cells G - intraepithelial mucous glands

DISCUSSION

This section of the present study provides a basic quantitative and qualitative assessment of the mucus-producing apparatus in the developing embryos from 15-day-old through to 3-day-old chicks. The chick starts external respiration between the 19th to 21st day of incubation, as a result of internal pipping (Visschedijk, 1968a, 1968b; Burton and Tullett, 1985; Lasiewski, 1972), although it is probably not until hatching, that it is exposed to any great variety of potential pathogens when respiring. It is important to determine, therefore, how well developed the defensive mucociliary system of the respiratory epithelium at hatching, is in terms, not only of the degree of ciliation and of the lining epithelium, and its regional variations, but also of numbers and distribution of mucous cells and mucous glands, and the chemical nature of the mucoid secretion. Although Mohammed (1989) reported on the distribution of the mucous apparatus in the respiratory tract of adult chicken, the present study has demonstrated, for the first time in the bird, the presence of mucous cells and intraepithelial mucous glands throughout the respiratory system from the middle nasal concha through to the secondary bronchus in all age groups of chicks except 15 and 16-day-old embryos. Indeed Mohammed (1989) failed to demonstrate the presence of intraepithelial mucous glands in the secondary bronchus of the adult chicken at all. The present study also contradicts the findings of Chandra and Bharadwaj (1970), who documented that, though mucous cells were present in the larynx through to the secondary bronchus, mucous glands were present only in the proximal half of the trachea. The presence of developing intraepithelial mucous glands was first observed in the 17-day-old embryo in all the regions of the respiratory tract investigated in the present study. Such findings contradict those of Midtgard (1989), who reported that mucous glands were first seen in the middle nasal concha of the chicken, only in post-hatched chicks through to 5-month-old birds.

However, the present observations of such an early development of respiratory tract mucous glands find a similarity with those in man, where the first signs of mucous gland development appear as early as the 11th week of gestation in the rhinopharynx (Tos, 1977), at the 12th week of gestation in the trachea (Thurlbeck *et al.*, 1961), at the 13th week of gestation in the nose (Tos, 1975a) and at the 13th week of gestation in the bronchus (Tos, 1968; De-Haller, 1969). The appearance of neutral staining mucous cells only in 15 and 16-day-old embryos seen in the present study, suggests similar observations in man where neutral cells become less numerous with the increasing age of the foetus (De-Haller, 1969). The predominantly acidic nature of the mucosubstance produced throughout the respiratory tract of all the 17-day-old embryo onwards, as seen in this study, supports similar findings in the gosling (Jefferey, 1978) and man (De-Haller, 1969). The predominance of stored acidic mucosubstance has also been reported in the established respiratory epithelium of adult rabbits (Plopper *et al.*, 1984), cats (Jefferey, 1978), dogs (Spicer *et al.*, 1971; Wheeldon *et al.*, 1976; Majid, 1986), cows (Allan *et al.*, 1977), goats (Kahwa, 1992), horses (Pirie,1990), Rhesus and Bonnet monkeys (St. George *et al.*, 1984; Harkema *et al.*, 1987a) and men (Spicer *et al.*, 1971). It is worth noting, that although acidic mucosubstances predominate in the respiratory tract of most normal adult animals, neutral mucosubstances appear to predominate in the pig (Jones *et al.*, 1975). The present study also demonstrated the presence of mixed mucosubstances in the 18-day-old chick embryo onwards, alongside the more predominant acid mucosubstances. Such mixed mucosubstances presented either a purple or light purple staining reaction in the young chicks, a possibly significant difference from the findings in the adult chicken (Mohammed, 1989), where predominantly a dark purple mucosubstance was present. Such observations suggest that it may be interesting to undertake further investigations to determine the possibility of differences in

the histochemical nature of these mucoid secretions, on an age-related basis. In the present study, acidic mucin granules gradually accumulated in the mucous cells initially in the apical region and then spread down to the base, the first time such a feature has been noted in an avian species. Such findings support similar observations in man, where, in the development of the mucous cells, acid mucosubstances were first seen to occupy the apical region of the cells (De-Haller, 1969). In the present study, in general, there was a cranio-caudally increase in the number of mucous cells, with a peak in the intrapulmonary primary bronchus, whilst mucous gland numbers decreased cranio-caudally, being highest in the middle nasal concha and lowest in the secondary bronchus. This distribution pattern throughout the respiratory tract seen initially in the day-old chicks and persisting in all age groups of chicks has been reported here for the first time. Such findings are in agreement with those in the adult chicken by Mohammed (1989), who reported that this distribution pattern was similar from the middle nasal concha through to the intrapulmonary primary bronchus. A similar distribution pattern has been reported in the primates; the gland density is highest in the nose, low in the trachea, bronchi and nasopharynx in man (Tos, 1983) and there was a gradual anterioposterior increase in goblet cells density on both the septal and lateral walls in the nasal cavity monkeys (Harkema *et al.*, 1987a).

CHAPTER 6

SCANNING ELECTRON MICROSCOPY OF THE RESPIRATORY EPITHELIUM OF CHICKS EXPOSED TO FORMALDEHYDE VAPOUR.

INTRODUCTION

One of the major problems in the commercial chick hatchery is the presence of potentially pathogenic microorganisms (Ide, 1979; Furuta and Maruyama, 1981; Maris, 1986; Gerrits, 1990; Cox and Bailey, 1996). In order to produce high hatchability and good healthy chicks, therefore, the use of disinfectants is a standard procedure designed to control and eliminate potential disease problems. Formaldehyde is one of the most widely used disinfectants in the commercial hatchery (Williams, 1970; Beesley, 1980; Hodgetts, 1987; Maris, 1986; Wilson and Mauldin, 1989; North and Bell, 1990; Deeming, 1992; Sainsbury, 1992). It is employed as a vaporous fumigant firstly at the setting of the incubating eggs and secondly immediately after transfer of the eggs to the hatcher (Anonymous, 1994; Buckle *et al.*, 1981; North & Bell, 1990; Gerrits, 1990; Gerrits & Dijk, 1991). However, formaldehyde vapour has been shown to cause intense irritation of the respiratory tract in many mammalian species including man (Schoenberg and Mitchell, 1975; Gamble *et al.*, 1976; Hendrick and Lane, 1977; Cockcroft *et al.*, 1982; Main and Horgan, 1983; Frigas *et al.*, 1984; Nordman *et al.*, 1985; Burge *et al.*, 1985; Malaka and Kodama, 1990), rodents (Swenberg *et al.*, 1980; Chang *et al.*, 1983; Kerns *et al.*, 1983; Monticello *et al.*, 1991) and rhesus monkey (Rusch *et al.*, 1983; Monticello *et al.*, 1989). The possibility therefore exists of a similar effect on the respiratory apparatus of chicks exposed to the vapour during the later stages of incubation. Indeed, it is surprising, given the widespread use of the fumigant that, with the exception of a few studies (Furuta *et al.*, 1989; Gerrits, 1990;

Gerrits & Dijk, 1991; Sander *et al.*, 1995), the effects of exposure to formaldehyde vapour in chicks has not been more extensively investigated.

The aim of the present study, therefore, was to investigate, by the use of scanning electron microscopy, the effect of formaldehyde vapour on the epithelial lining of the respiratory tract in commercially hatched chicks.

MATERIALS AND METHODS

Source of chicks

All chicks used in this study were exposed to 10.9 ppm formaldehyde vapour in a commercial hatchery, as detailed in Chapter 2. This study was organised into two sections: In the first section, chicks were exposed to formaldehyde vapour for varying periods of time. In the second section, 70 chicks were removed from the commercial hatchery at the end of the hatching period and were raised in chick pens and small groups were then sacrificed, and samples collected at pre-determined intervals, up to day 43. The number and age of chicks/chickens involved in this study is given in Table 11. All hatched chicks, were removed from the hatchery and distributed to broiler farms for rearing on.

Sample collection, processing of samples and photography for scanning electron microscopy

Samples were collected from the middle nasal concha, larynx, cranial trachea, caudal trachea, intrapulmonary primary bronchus and secondary bronchus of all birds, and then processed for scanning electron microscopic examination, as described in Chapter 2.

Lesion scoring

Scoring of any and all lesions observed was determined with reference to the severity of the lesion as graded below and the averaged

lesions from six pre-selected areas in each region of the respiratory tract were recorded in Table 12:

0 - No pathological change

1 - Clumping of cilia, blebs at the surface of the cilia, with or without focal deciliation.

2 - Multifocal deciliation.

3 - Large area of deciliation.

4 - Focal desquamation of the epithelium.

5 - Multifocal desquamation of the epithelium.

6 - Desquamation of a large area of the epithelium.

Analysis of variance using Newman-Keuls multiple range test was used to analyse the severity of all observed respiratory epithelial lesions against the different exposure times to the formaldehyde vapour.

TABLE 11

BIRDS USED IN THE SCANNING ELECTRON MICROSCOPIC INVESTIGATION OF THE EFFECT OF FORMALDEHYDE VAPOUR ON THE DEVELOPING RESPIRATORY EPITHELIUM OF CHICKS

Age (day-old)/ Exposure time (hrs)	No. of formaldehyde-exposed birds sampled
Section 1	Exposure effects
Embryo 18/ 0	6*
Embryo 19/6	6
Embryo 20/ 30	6
Chick 1/ 54	6
Section 2	Regeneration aspects
3-day-old chick	6
5-day-old chick	6
7-day-old chick	6
11-day-old chick	6
13-day-old chick	6
22-day-old chicken	6
29-day-old chicken	6
35-day-old chicken	6
43-day-old chicken	6

* Controls, these birds were removed before the formaldehyde vapour was liberated in the hatcher.

RESULTS

SECTION 1.

EXPOSURE EFFECTS OF FORMALDEHYDE VAPOUR ON THOSE BIRDS HATCHING WITHIN THE HATCHER.

Scanning electron microscopy revealed that the respiratory epithelium of hatching chicks fumigated with formaldehyde vapour at 10.9 ppm, resulted in severe pathological changes (Table 12). Obvious lesions were observed in the respiratory epithelium of all hatched chicks, and also chicks that had pierced the shell (external pipping) or chorioalantoic membrane (internal pipping); the latter case involved just a single individual. Those chicks exposed to formaldehyde vapour for 6 hours showed less severe lesions than those chicks exposed for 30 or 54 hours. The severest lesions were observed in the respiratory epithelium of chicks exposed for 54 hours ($P < 0.05$) (Table 12). The effects of the formaldehyde vapour were observed throughout the entire respiratory epithelium extending from the middle nasal concha to the lungs. With the exception of a few individuals, it was found that within each exposure group there was no significant difference ($P > 0.05$) in the nature and morphology of lesions observed between the various preselected areas. The results in this section have been based on the morphological changes observed in the respiratory epithelium of the preselected regions, at different times of exposure.

TABLE 12

AVERAGED LESION SCORE OF THE RESPIRATORY EPITHELIUM OF CHICKS EXPOSED TO THE FORMALDEHYDE VAPOUR

Exposure Time (Hours)		Middle nasal concha	Larynx	Cranial trachea	Caudal trachea	Primary bronchus	Secondary bronchus
0 *	Average	0.00	0.00	0.00	0.00	0.00	0.00
	SD	0.00	0.00	0.00	0.00	0.00	0.00
6	Average	1.00	1.00	1.00	1.00	1.33	1.00
	SD	0.00	0.00	0.00	0.00	0.75	0.00
30	Average	2.67	2.33	2.83	2.83	1.00	1.00
	SD	1.51	1.97	1.83	2.23	0.00	0.00
54	Average	4.33	3.67	3.67	3.67	3.00	2.67
	SD	1.15	1.86	1.86	1.86	1.86	1.37

* Control observation

Analysis of variance for data

	F-level	P-level		
Treatment	56.74	0.000	significant	($P < 0.05$)
Region	1.97	0.143	not-significant	($P > 0.05$)

18-day-old embryo not exposed to the formaldehyde vapour (the control group)

No pathological lesions were observed in the middle nasal concha, cranial or caudal trachea, the intrapulmonary primary bronchus or the secondary bronchus of any of the six embryos from the control group. The normal organisation of the respiratory epithelium was observed (Fig. 6.1), with patches of mature dense cilia occasionally interrupted by developing ciliated cells and mucous cells.

19-day-old embryo exposed to formaldehyde vapour for 6 hours.

The middle nasal concha of five 19-day-old embryos that had pipped the egg shell (external pipping), and one that had pierced the chorioallantoic membrane (internal pipping), when exposed to 10.9 ppm of formaldehyde

vapour demonstrated similar lesions in the respiratory epithelium. There was evidence of an increase in viscous mucus secretion, leading to clumping (Fig. 6.2) or disorientation of cilia: Frequent matting of cilia was also observed, such cilia lying flat on the mucosal surface instead of assuming their normal upright position (Fig. 6.3).

The laryngeal epithelium of all six embryos also demonstrated evidence of an increase in mucus secretion which resulted in a thick mucus covering on the laryngeal surface (Fig. 6.4). Clumping of cilia was also frequently seen at the mucosal surface of the larynx.

The cranial and caudal trachea demonstrated a thick layer of mucus covering the mucosal surface, with clumping of cilia again a common feature (Fig. 6.5).

The mucosal surface of the intrapulmonary primary bronchus demonstrated an increase in mucus production, leading to agglutination of the cilia. In one embryo, in addition to the observed increase in mucus secretion, a large area of deciliation was also observed.

The bronchial surface of all the secondary bronchi demonstrated similar lesions to those observed in the proximal regions of the respiratory tract; i.e. an obvious increase in mucus secretion, leading to matting of the cilia.

20-day-old embryos exposed to formaldehyde vapour for 30 hours

The pathological changes observed on the mucosal surface of the middle nasal concha of 20-day-old embryos exposed to formaldehyde vapour for 30 hours, produced an averaged lesion score of 2.67, indicating that the mucosal surface presented small isolated multifocal areas of deciliation or more extensive larger areas of deciliation. Within this averaged score, however, there was significant individual variation. Thick mucus secretion, the appearance of large areas of deciliation (Fig. 6.6) and the

observation of frequent blebs on the cilial walls (Fig. 6.7) were common features in these embryos, such individual variation was demonstrated by one particular embryo, which exhibited severe lesions associated with multifocal desquamation of the epithelium.

The laryngeal surface demonstrated a general matting and clumping of the cilia, but individual variation was again observed in response to formaldehyde vapour exposure. One individual showed large areas of non-ciliated microvilious cells another embryo showed multifocal deciliation, whilst a third embryo showed desquamation of a large area of the surface epithelium.

The changes seen in the cranial and caudal trachea were similar to those observed in the larynx. Changes included clumping of cilia (Fig. 6.8) and frequent epithelial sloughing. The latter, readily recognised by the damaged epithelial surface, was either localised or widespread (Fig. 6.9), at times exposing the sub-mucosal layer. Some individuals exhibited large areas of deciliation characterised by the presence of short stubby surface microvilli (Fig. 6.10). The common feature in the cranial or caudal trachea of all the 20-day-old embryos was the presence of thick mucus on the mucosal surface, frequently causing clumping of the surface cilia.

The mucosal surface of the intrapulmonary primary bronchus of all the 20-day-old embryos demonstrated clumping of cilia. Following 30 hours exposure to formaldehyde vapour, the epithelial lining of the secondary bronchus also persistently presented an image of clumped cilia (Fig. 6.11).

Day-old chicks exposed to formaldehyde vapour for 54 hours.

The day-old chicks that remained exposed to the formaldehyde vapour for 54 hours demonstrated the severest lesions ($P < 0.05$). As in the previous groups examined, there was still some degree of individual variation in the pathological response to the formaldehyde vapour, with

some individuals exhibiting large areas of exfoliated epithelium (Fig. 6.12), and others large areas of deciliation, on the middle nasal concha. Despite such individual variation, the most common lesions observed in the middle nasal concha of all embryos were extensive deciliation (Fig. 6.13) and clumping and disorientation of cilia (Fig. 6.14). In this group, mucus secretion was still very prominent, although in some individuals such mucus appeared organised into a lattice-like surface sheet (Fig. 6.15).

Clumping and disorientation of surface cilia was evident and a common feature in the larynx of all the day-old chicks in this group. Other pathological changes included large areas of exfoliation of the epithelium (Fig 6.16), large areas of non-ciliated microvillous cells were extensive, and in some birds the mucosal surface showed multifocal deciliation on the larynx.

Large areas of non-ciliated microvillous cells and clumping of cilia were the most common pathological features in the cranial and caudal trachea of these day-old chicks. Yet again, responses were individually variable, with some birds exhibiting large areas of exfoliation of the epithelium, and in some parts of the trachea large areas of non-ciliated microvillous cells were observed (Fig. 6.17), to excessive mucus secretion which encouraged clumping of cilia, and in some individuals demonstrated isolated multifocal deciliation.

The intrapulmonary primary bronchus of all day-old chicks demonstrated clumping and disorientation of the cilia with large areas of deciliation. One individual presented only clumping of the cilia in the primary bronchus, another demonstrated multifocal desquamation of the epithelium with obvious clumping of the cilia and blebs on the cilial wall.

The mucosal surface of the secondary bronchus of all day-old chicks demonstrated a common increase in mucus secretion, with clumping and disorientation of the cilia and blebs on the cilia wall. Two individuals

presented small isolated multifocal areas of deciliation on the mucosal surface, whilst two other individuals presented, large areas of surface deciliation. One chick demonstrated a more severe lesion, with multifocal desquamation of the epithelium on the mucosal surface of the secondary bronchus.

SECTION 2

REGENERATION ASPECTS.

The aim of this section was to investigate how long those lesions produced by exposure to formaldehyde vapour lasted in these birds, and when the epithelium began to show signs of regeneration. As detailed in section 1, clumping of cilia was recognised as the least severe pathological lesion presented after exposure to formaldehyde vapour. Further lesions observed in order of severity included varying degrees of deciliation through to focal exfoliation, the latter being the most severe pathological lesion observed.

The lesions observed in the middle nasal concha down to the secondary bronchus in 3-day-old to 13-day-old chicks are predominantly similar, as indicated by the levelling out of the mean lesion score as shown in Fig. 6.36 to Fig. 6.41, consisting of clumping of cilia, varying degrees of deciliation and relatively mild exfoliation of the epithelium. However, there was still, within this plateau, a significant degree of individual variation in response to the formaldehyde vapour exposure. From day 13 to day 29, however, in the middle nasal concha through to the secondary bronchus, there was a rapid fall in the mean lesion score (Fig. 6.36 to Fig. 6.41), indicating a rapid regeneration of the damaged epithelium.

Middle Nasal Concha

3 through to 7-day-old chicks

The middle nasal concha of 3-day-old to 7-day-old exposed chicks demonstrated various surface morphological changes. The most frequently observed pathological change was clumping or disorientation of cilia. In addition, severe sloughing of the epithelium was observed in one 7-day-old chick, and focal desquamation of the epithelium was seen in one 3-day-old chick. However, three of the 18 chicks examined (one 3-day-old, one 5-day-old and one 7-day-old) demonstrated normal ciliation of the middle nasal conchal respiratory epithelium.

11 through to 22-day-old chickens

The middle nasal concha of all chicks from 11 to 22-day-old again demonstrated clumping and disorientation of cilia, with the exception of one individual that exhibited normal ciliation of the concha. In addition to these various pathological changes, a few individuals exhibited additional features. A thick mucous blanket was occasionally observed covering the middle nasal concha of one 11- and one 13-day-old chick (Fig. 6.18). In addition large areas of stubby microvillous cells were encountered on the middle nasal concha of one 11-day-old chick and one 22-day-old chicken, with some of the microvillous cells exhibiting growing cilia, indicating that a progressive ciliogenesis was underway in these individuals (Fig. 6.19). Focal sloughing of the epithelium was also observed in two 22-day-old birds, associated with an apparent basal cell hyperplasia (Fig. 6.20).

29 through to 43-day-old chickens

A normal ciliated surface epithelium was observed covering the middle nasal concha of all 29, 35 and 43-day-old chickens (Fig. 3.6).

Larynx

3 through to 7-day-old chicks

The laryngeal surface of the 3, 5 and 7-day-old chicks demonstrated various pathological changes, from clumping of cilia to varying degrees of deciliation and exfoliation of the epithelium. Large areas of flattened microvillous cells were first seen in the larynx of one 5-day-old chick (Fig. 6.21). On the luminal surface of a number of the epithelial cells observed in these areas emerging short cilia were observed, whilst occasionally long cilia were seen growing from isolated cells.

11 through to 22-day-old chickens

This group presented similar features at the laryngeal surface. These included large areas of microvillous cells within which islands of narrow, elongated ciliated cells were frequently observed (Fig. 6.22). Short growing cilia were often seen emerging from these microvillous cells; presumably indicating the presence of regenerating ciliated cells. In one individual the microvillous cells presented a particularly flattened appearance (see Fig. 6.21). A thick mucous blanket was observed covering the laryngeal surface of one 11-day-old chick and one 22-day-old chicken. In areas where there was no thick mucous covering, active mucous cells were frequently seen (Fig. 6.23).

29 through to 43-day-old chickens

The larynx of all the 29, 35 and 43-day-old chickens exhibited the normal surface organisation of the respiratory epithelium (see Fig. 3.14), except for one 29-day-old chicken which demonstrated a relatively large patch of microvillous cells containing an isolated island of ciliated cells and occasional mucous cells, recognised by the presence of a sparse distribution of microvilli and sometimes the appearance of mucous granules

and pits or pores on the mucosal surface (Fig. 6.24).

Cranial trachea

3 through to 7-day-old chicks

Commonly observed pathological changes on the surface of the cranial trachea included deciliation and clumping or disorientation of cilia (Fig. 6.26). However, sloughing of the surface epithelium was also frequently seen in a number of chicks.

One individual exhibited numerous plate-like projections from the apical surface of some of the lining epithelial cells, these projections appearing to be packed with mucous granules (Fig. 6.26).

11 through to 22-day-old chicken.

Less severe morphological changes, varying from deciliation to clumping of cilia, were observed in these groups of birds. Stubby microvillous cells and short cilia were also occasionally seen on the epithelial surface of the cranial trachea (Fig. 6.27). However, more severe lesions, such as sloughing of the epithelium, were observed on the mucosal surface of the cranial trachea in three chickens. Large areas of flattened microvillous cells, containing isolated individual, or clumps of, ciliated cells, were also occasionally observed on the luminal surface of three chicks.

29, 35 and 43-day-old chickens

The cranial trachea of the 29, 35 and 43-day-old chickens exhibited a normal organisation of the epithelial surface (see Fig. 3.19)

Caudal trachea

3 through to 7-day-old chicks

Observed lesions were more severe in the trachea of 3-day-old chicks

than in the 5-day-old chicks, and least severe in the 7-day-old chicks. The most frequent lesions seen in the caudal trachea were a universal clumping or disorientation of cilia, along with, in some individuals, large or multifocal areas of deciliation. At low magnification such deciliated areas were seen to be composed primarily of microvillous cells, which themselves appeared in a variety of shapes and sizes. Many of these cells appeared to be regenerating ciliated cells, as they were characterised by the presence of long or short cilia, or just a single cilium. Exfoliation of the epithelium was observed in a number of chicks. The degree of sloughing of the epithelium varied, however, with cases of less severe sloughing leaving the basal cell layer intact, whilst cases of severe sloughing eroded both the superficial and basal cell layers.

The mucosal surface of two 7-day-old chicks presented a normal respiratory epithelial lining, with long mucous strands readily seen interspersed amongst the dense cilia.

11 through to 22-day-old chickens

Lesions in these groups of birds varied from the commonly observed clumping or disorientation of cilia, to areas of noticeable deciliation. These latter areas, composed primarily of flattened polygonal microvillous cells, contained many cells undergoing apparent ciliogenesis. Some of the microvillous cells had very sparsely distributed surface microvilli, and occasional pits were seen on the epithelial surface; such features were probably indicative of mucous cells. Although four chickens out of 18 demonstrated a normal respiratory epithelial lining on the surface of the caudal trachea, severe sloughing of the surface epithelium was observed in three other chickens (Fig. 6.28). In the latter cases, basal cell proliferation was also noted.

29 through to 43-day-old chickens

The mucosal surface of the caudal trachea of 29, 35 and 43-day-old chickens observed under SEM exhibited a normal ciliated respiratory epithelial lining (Fig. 3.18).

Intrapulmonary primary bronchus

3 through to 7-day-old chicks

Generally the intrapulmonary primary bronchus of the chicks showed the range of mild pathological changes varying from clumping and disorientation of cilia, with or without focal deciliation or multifocal deciliation. Changes seen at this level were similar to those seen in the upper levels of the respiratory tract, but were present in a much less severe form. However, the mucosal surface of the intrapulmonary primary bronchus of one 3-day-old chick demonstrated sloughing of a large area of the epithelial surface, thus exposing the underlying sub-mucosa (Fig. 6.29). Three 7-day-old chicks demonstrated a normal organisation of the respiratory epithelium of the intrapulmonary primary bronchus.

11 through to 22-day-old chickens

The most frequent feature seen on examination of the intrapulmonary primary bronchus in these groups of birds was clumping of cilia; occasional areas of deciliation were also seen. A thick mucous blanket was frequently observed on the epithelial surface. Where the mucosal surface was not covered by this mucous blanket, numerous active mucous cells were observed amongst the thick carpet of cilia (Fig. 6.30). Three chickens presented a normal respiratory epithelial lining of the intrapulmonary primary bronchus.

29 through to 43-day-old chickens

The mucosal surface of the intrapulmonary primary bronchus of 29, 35 and 43-day-old chickens presented a normal respiratory epithelial lining, *viz* a folded epithelium with long straight cilia interrupted by mucous cells and intraepithelial mucous glands on the mucosal surface (see Fig. 3.21).

Secondary bronchus

3 through to 7-day-old chicks

At this level, and in these groups of birds, epithelial changes varied from clumping of cilia to multifocal deciliation. Matting of the cilia was frequently observed, along with numerous multifocal areas of microvillous cells, often covered by mucus (Fig 6.31). However, two chicks demonstrated severe sloughing of the epithelial surface of the secondary bronchus (Fig. 6.32), with relatively few ciliated cells remaining intact (Fig. 6.33). Three 7-day-old chicks demonstrated a normal organisation of the respiratory epithelium of the secondary bronchus.

11 through to 22-day-old chickens

Clumping and disorientation of the cilia was the most common lesion observed (Fig. 6.34). A thick mucous covering was often seen on the mucosal surface. Numerous mucous cells were also frequently observed on the mucosal surface when this covering mucus blanket was absent (Fig. 6.35).

29, 35 and 43-day-old chickens

The epithelial surface of the secondary bronchus of these age groups demonstrated the normal organisation of the respiratory epithelium (see Fig. 3.25).

Fig. 6.1

Middle nasal concha. 18-day-old embryo.

Untreated. SEM of normal ciliated covering, note the dense carpet of long and straight cilia, and a mucous cell (arrow) emerging amongst the ciliary carpet.

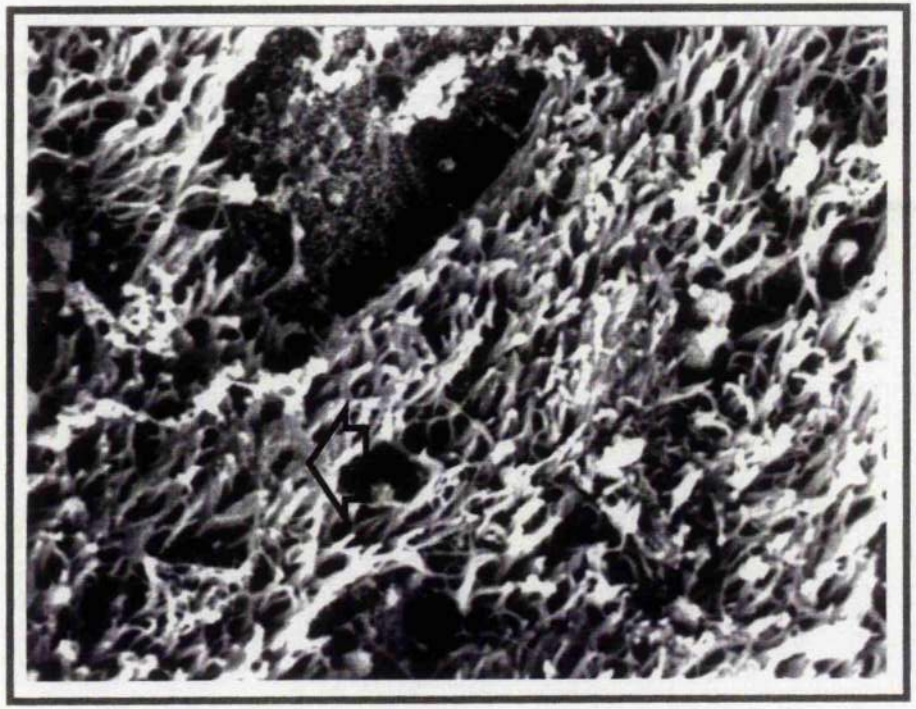
X 5, 500

Fig. 6.2

Middle nasal concha. 19-day-old embryo.

Treated. Embryos exposed to formaldehyde vapour for 6 hours. Increase in mucus secretion and clumping of cilia are apparent (arrow).

X 2,750



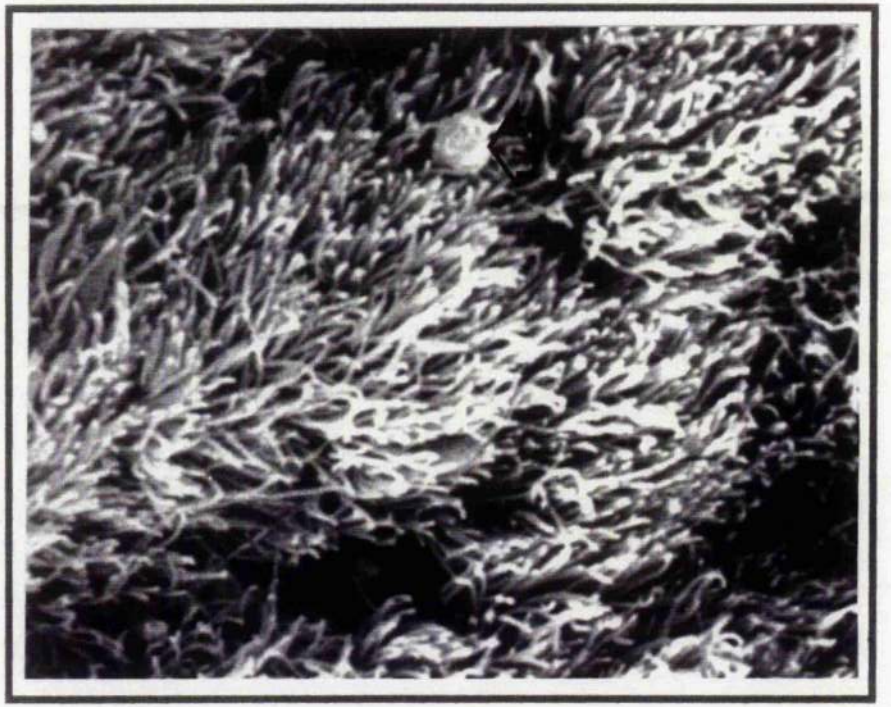


Fig. 6.3

Middle nasal concha. 19-day-old embryo.

Treated. Embryos exposed to formaldehyde vapour for 6 hours.

Cilia are matted and lie flattened on the surface.

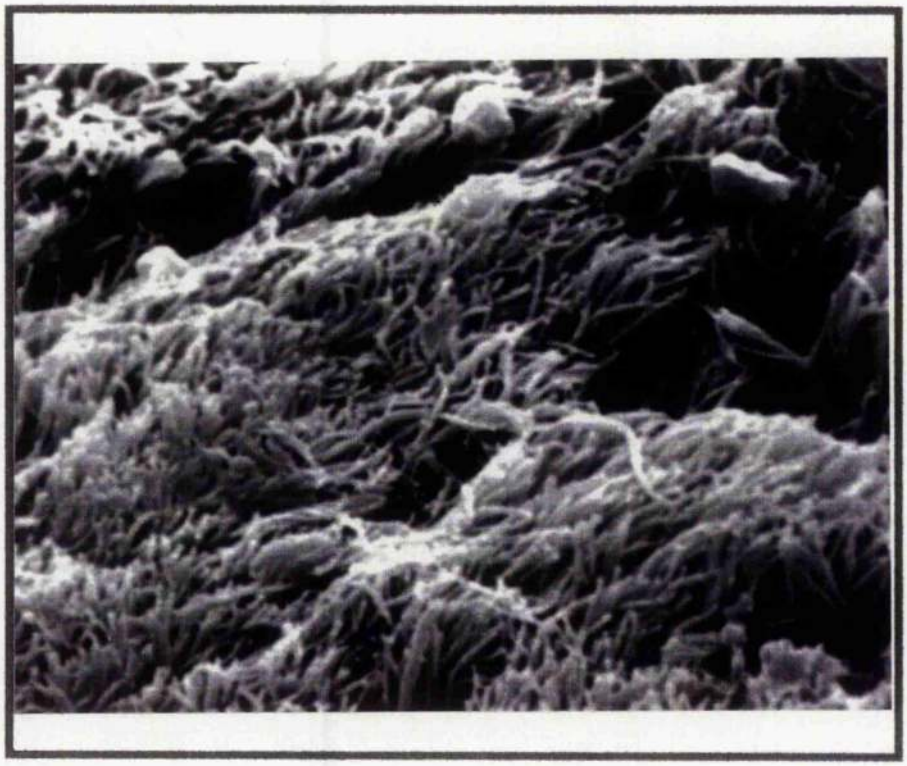
X5,500

Fig. 6.4

Larynx. 19-day-old embryo.

Embryo exposed for 6 hours to formaldehyde vapour. Note conspicuous accumulation of excessive mucus (M) secretion lying on the surface of the ciliary carpet. The non-ciliated microvillous cells (arrow) seen distributed amongst the ciliated cells are a normal feature of the lining epithelium of the larynx.

X5, 500



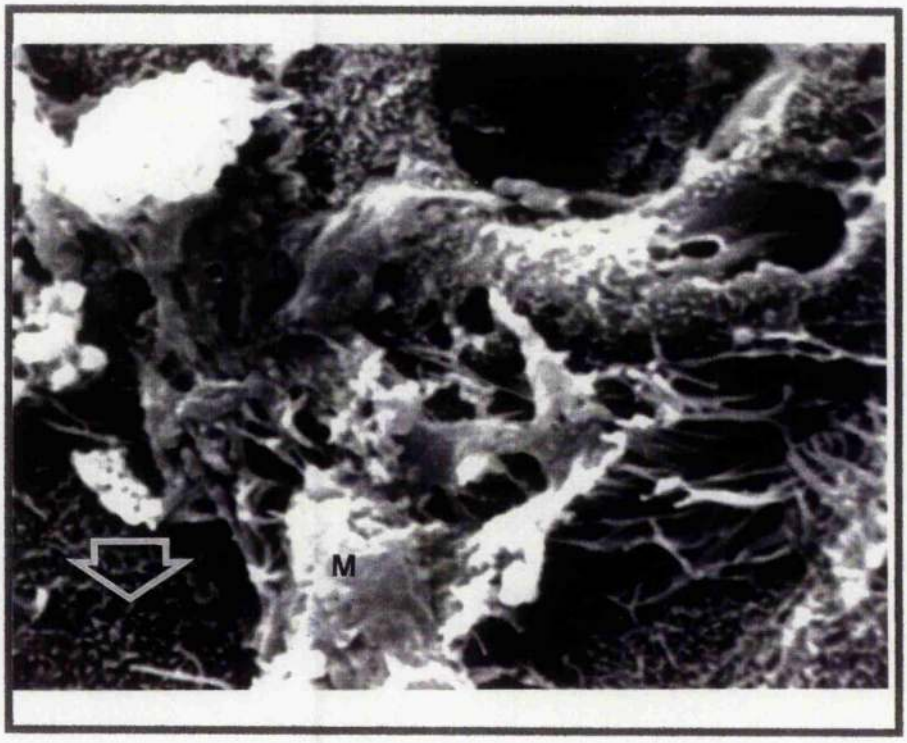

M

Fig. 6.5

Cranial trachea. 19-day-old embryo.

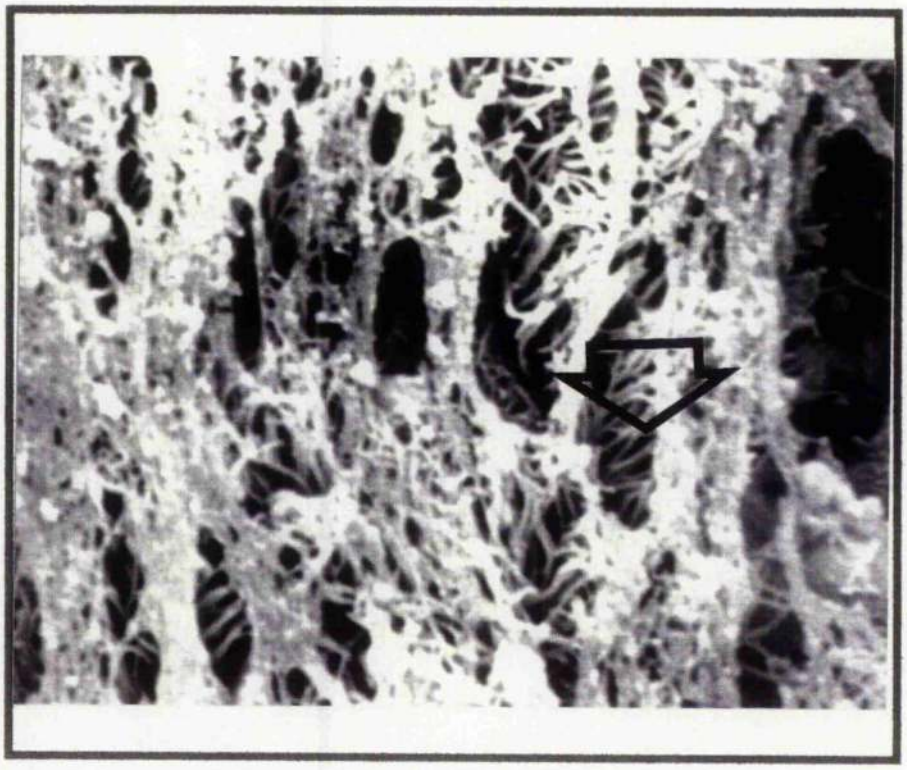
Clumping and disorientation of cilia (arrow) was observed.

X5, 500

Fig. 6.6

Middle nasal concha. 20-day-old embryo. Note large area of deciliation (arrow).

X1,270



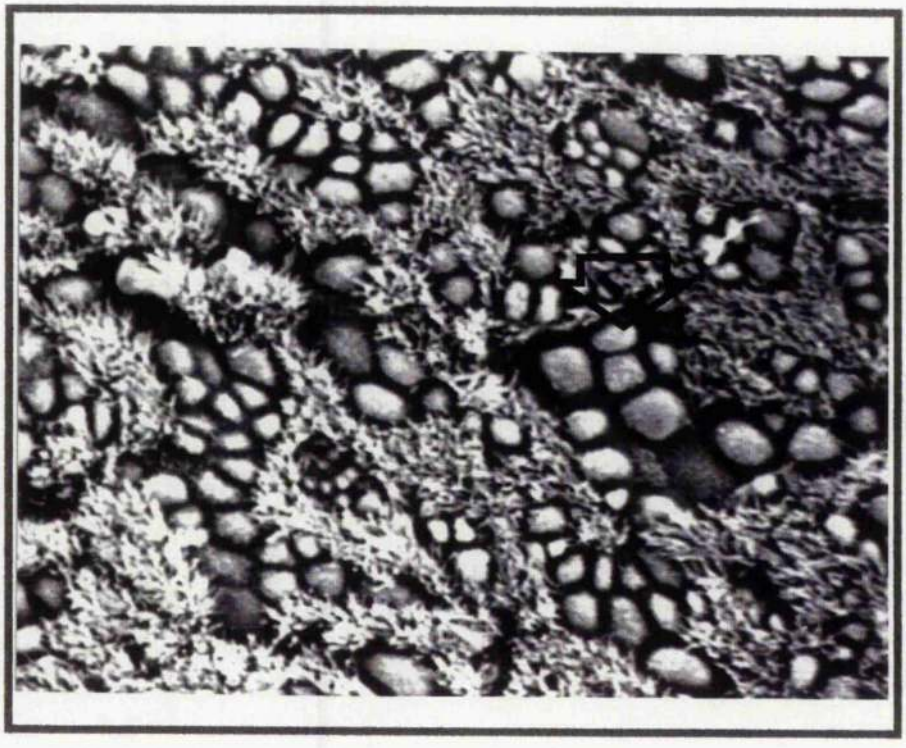


Fig. 6.7

Middle nasal concha. 20-day-old embryo.

Exposed to formaldehyde vapour for 30 hours. Blebs (arrow) are seen on the cilial wall.

X11,250

Fig. 6.8

Cranial trachea. 20-day-old embryo.

Exposed to formaldehyde vapour. Clumping of cilia and blebs on the cilial wall (arrow).

X5,500

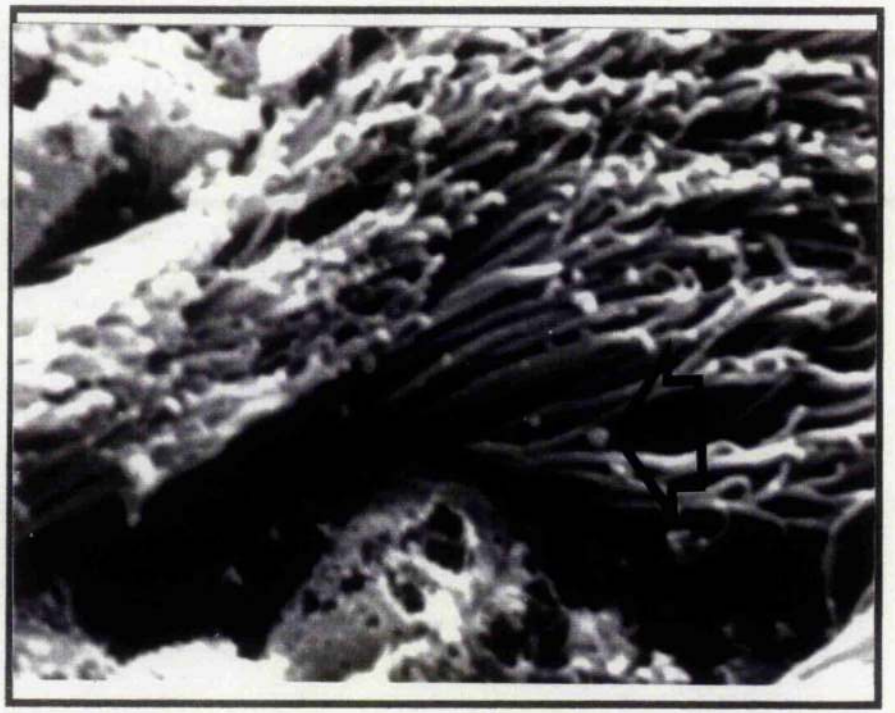


Fig. 6.9

Cranial trachea. 20-day-old embryo.

Note most of the epithelial cells have been sloughed off leaving only a few ciliated cells intact. Blebs (arrow) are seen on the cilial wall of the latter.

X5,500

Fig. 6.10

Cranial trachea. 20-day-old embryo.

Three different types of surface morphological changes

1. Deciliation; surface with short cilia
2. Sloughed epithelial surface; cell with rugged surface
3. Exfoliation of the epithelial cell; crater-like structure on the mucosal surface.

X1,270

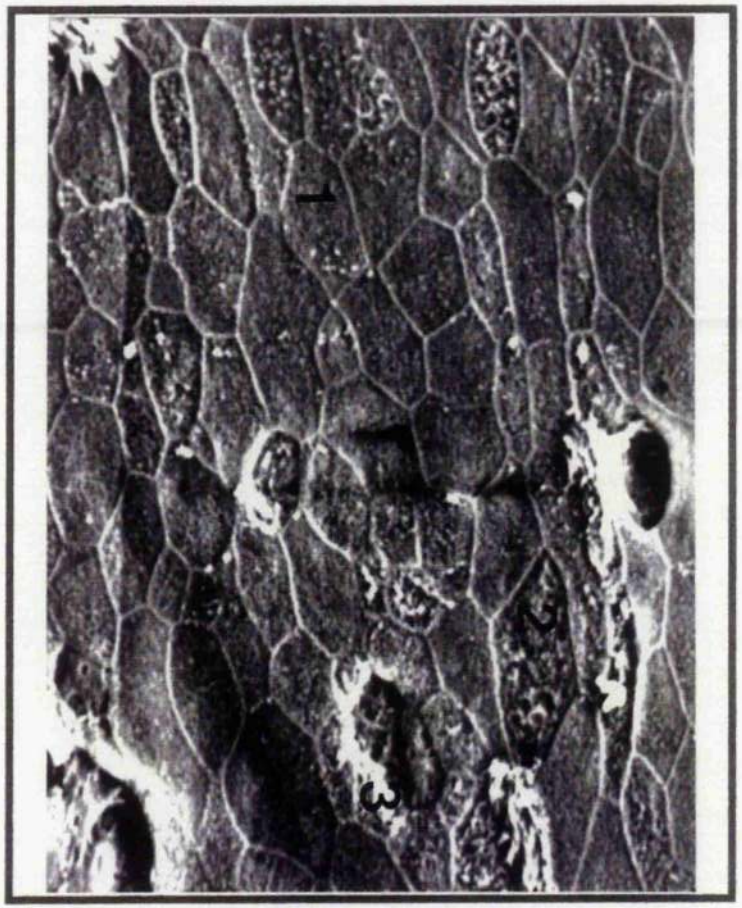


Fig 6.11

Secondary bronchus. 20-day-old embryo.

30 hours after exposure to the formaldehyde vapour.

Clumping and disorientation of the cilia. Note mucus covering the cilia.

X5, 500

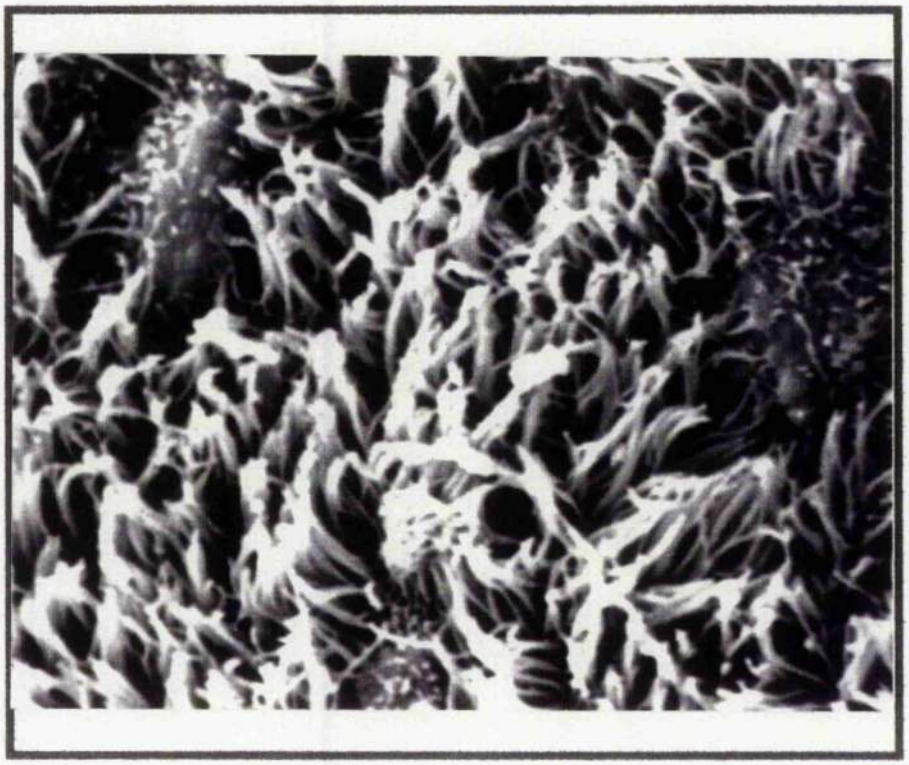
Fig. 6.12

Middle nasal concha. 1-day-old chick.

Exposed to formaldehyde vapour for 54 hours.

A large area of sloughed epithelium, exposing the basal cells (arrow).

X720



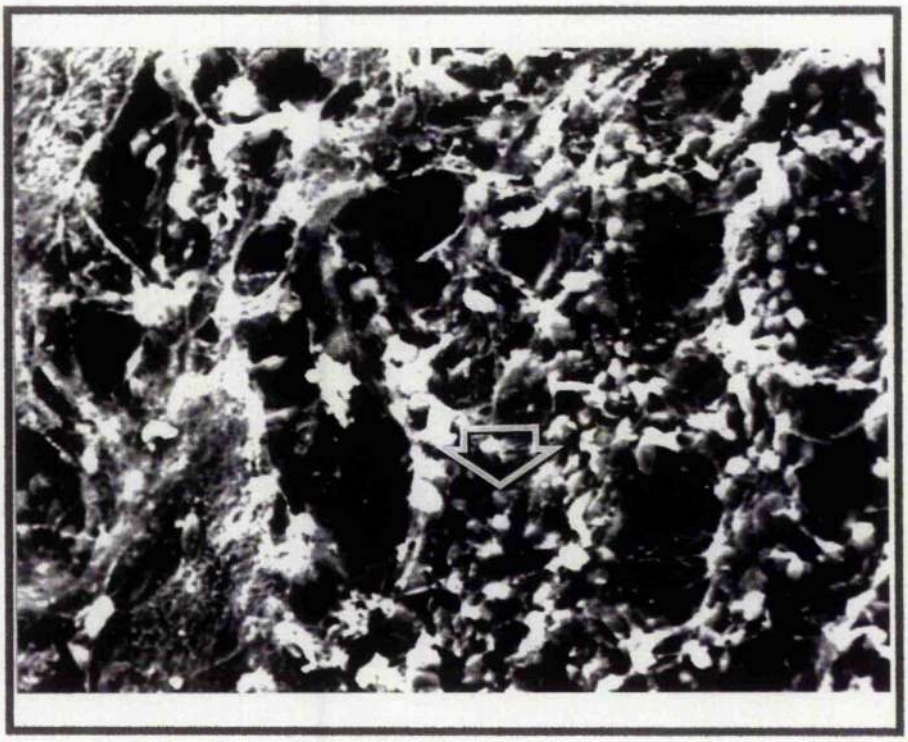


Fig. 6.13

Middle nasal concha. 1-day-old chick.

Chick exposed to formaldehyde vapour for 54 hours.

Deciliation is obvious on the mucosal surface (arrow).

Although normal microvillous cells (mv) are still apparent.

Deciliated cells are readily recognised by their smaller size and short stubby degenerated surface cilia.

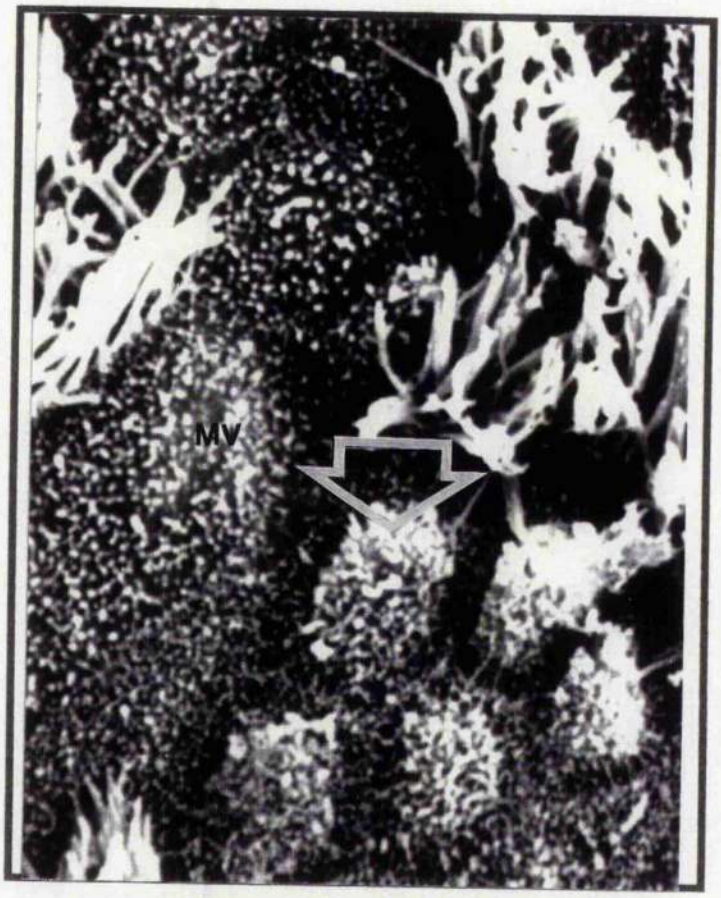
X5,500

Fig. 6.14

Middle nasal concha. 1-day-old chick.

Extensive clumping of the cilia

X2,750


MV

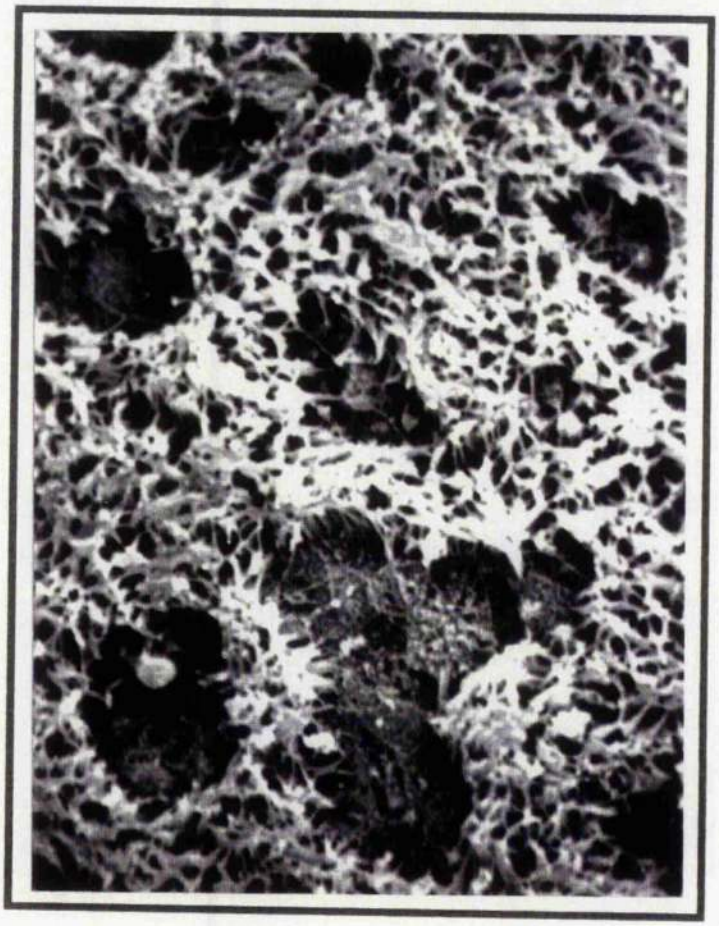


Fig. 6.15

Middle nasal concha. 1-day-old chick.

Mucous network covering the mucosal surface of the middle nasal concha.

X5,500

Fig. 6.16

Larynx. 1-day-old chick.

Extensive exfoliation of the mucosal surface of the after 54 hours exposure to the formaldehyde. Note the exposed basal lamina (arrow).

X1,270

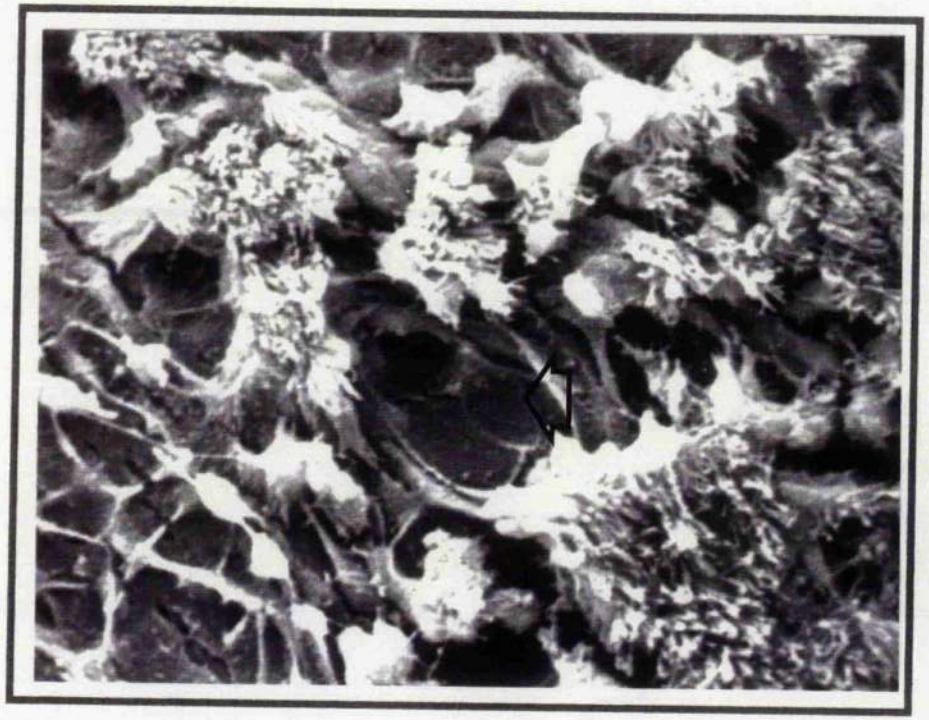


Fig. 6.17

Cranial trachea. 1-day-old chick.

Large area of non-ciliated microvillous cells on the mucosal surface after 54 hours exposure to the formaldehyde vapour.

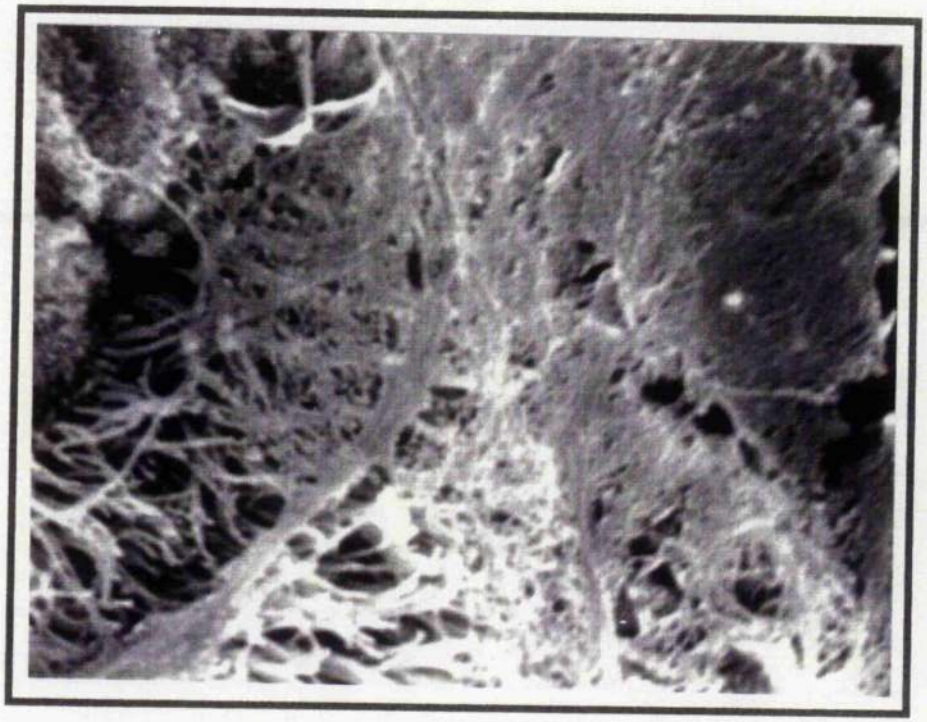
X1,270.

Fig. 6.18

Middle nasal concha. 11-day-old chick

Thick mucous blanket on the epithelial surface

X5, 500



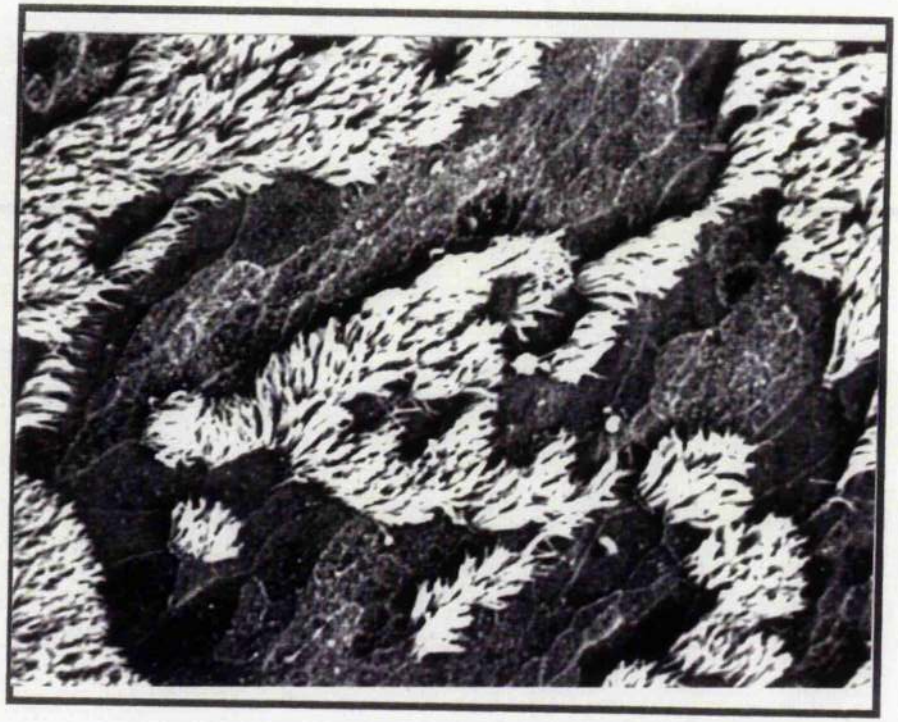


Fig. 6.19

Middle nasal concha. 11-day-old chick.

Large area of protruding microvillous cells with varying density of the microvilli. Note long cilia on a few of the epithelial cells and mucus (arrow) being extruded from the intraepithelial gland.

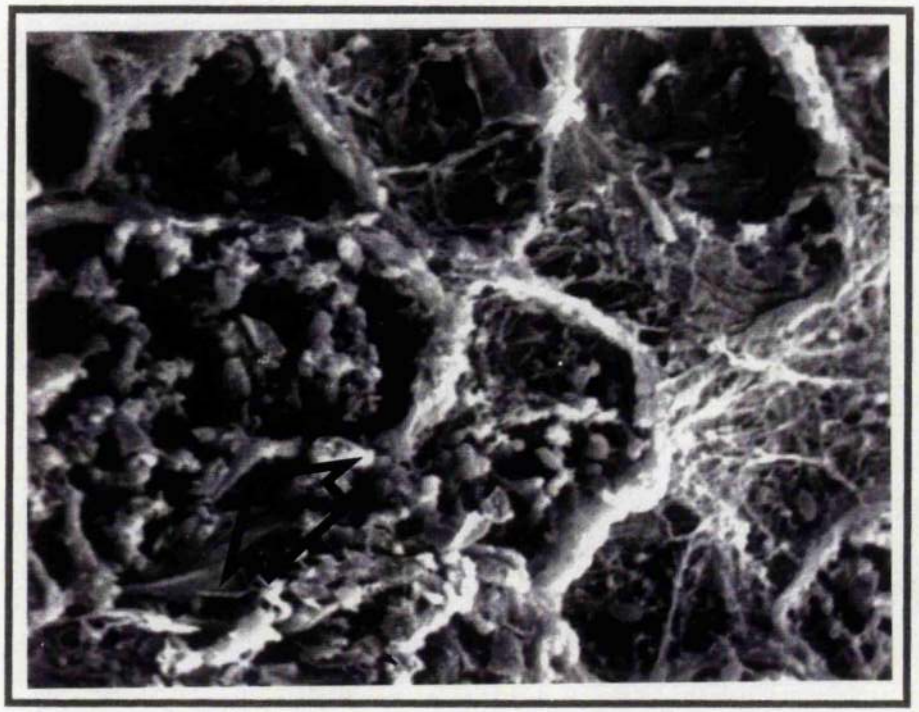
X 5,500

Fig. 6.20

Middle nasal concha. 22-day-old chicken

Sloughed epithelium. Note area of basal cells hyperplasia (arrow)

X 720



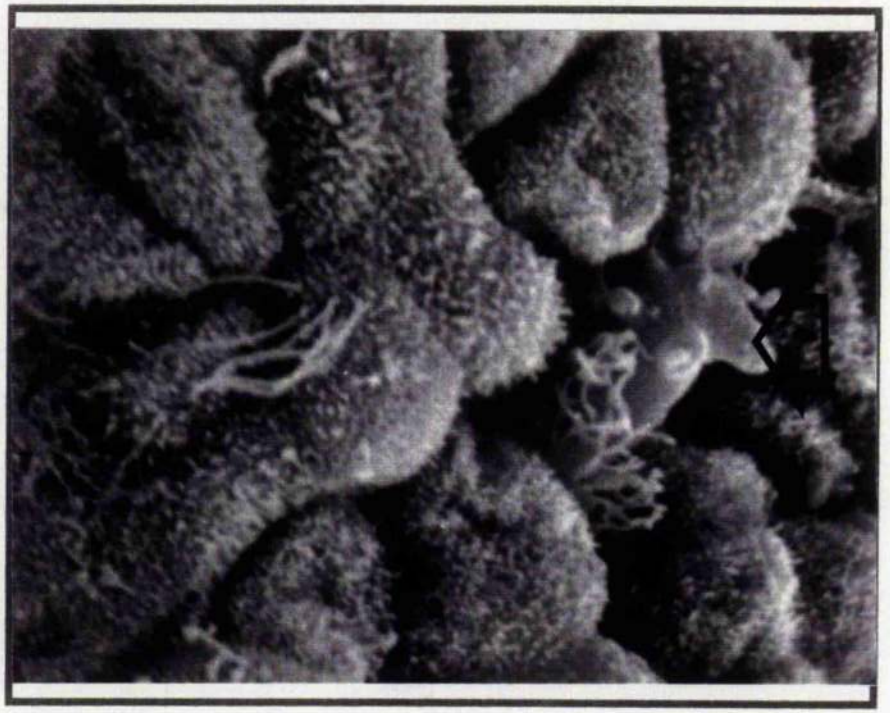


Fig. 6.21

Larynx. 5-day-old chick.

Short cilia emerging from the flattened mucosal surface

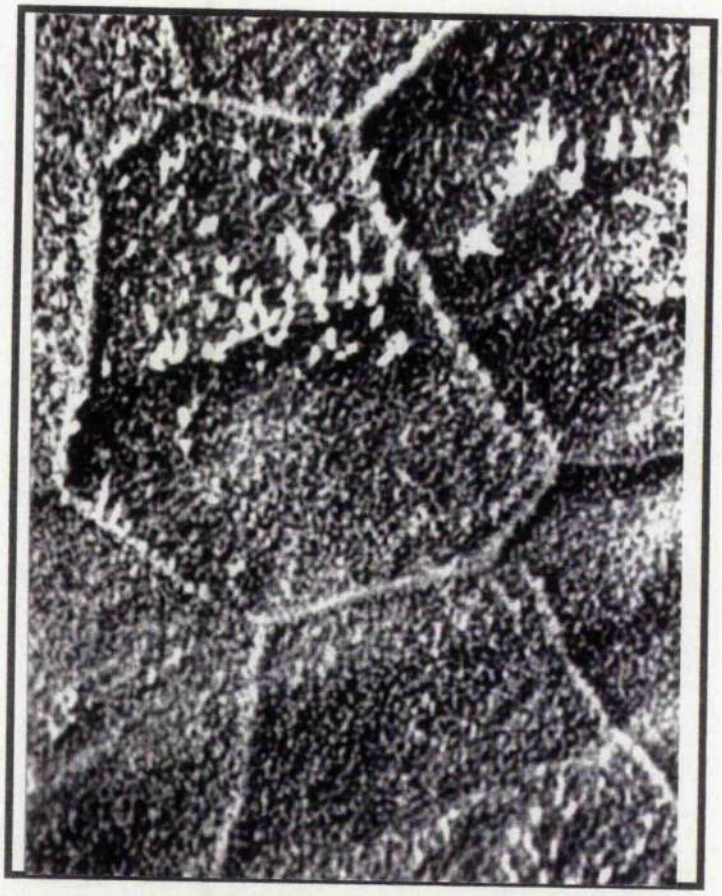
X 5, 500

Fig. 6.22

Larynx. 11-day-old chick

Long tapering cilia projecting from the cell margins (arrow), giving the impression of the ciliated cells being squeezed between adjacent microvillous cells (mv)

X 5, 500




mv

Fig. 6.23

Larynx. 11-day-old chick.

Numerous active mucous cells are seen (arrow). A coalescence of mucous granules can be seen on the less dense microvillous cells (open arrow)

X5,500

Fig. 6. 24

Larynx. 29-day-old chicken.

A patch of microvillous cells on the mucosal surface. Note isolated flattened ciliated cells (arrow) and a pore on one of the microvillous cells (open arrow) could possibly be mucous cell.

X 720

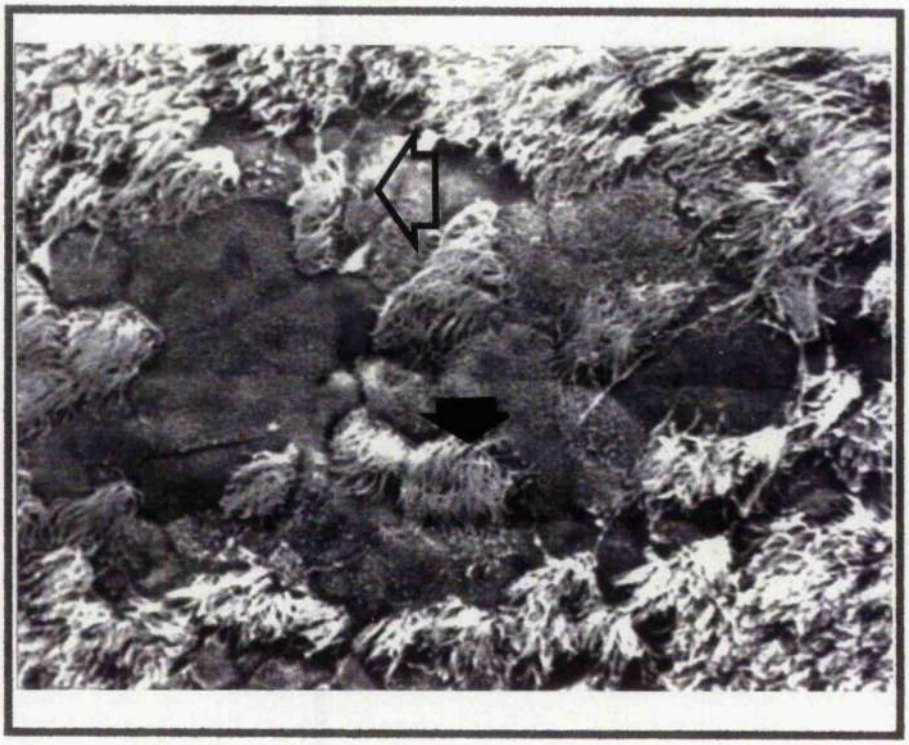


Fig. 6.25

Cranial trachea. 7-day-old chick.

Disorientation of cilia.

X 2, 750

Fig. 6. 26

Cranial trachea. 7-day-old chick.

Plate-like projections with numerous mucous granules (arrow) seen through the plasmalemma.

X 2, 750

Fig. 6.27

Cranial trachea. 11-day-old chick.

Stubby microvillous cells (arrow) and short cilia (open arrow) emerging from the microvillous area surrounded by long cilia.

X 5, 500

Fig. 6.28

Caudal trachea. 11-day-old chick.

Eroded epithelium (arrow), note basal cell proliferation (open arrow) from the intact epithelial cells.

x 720

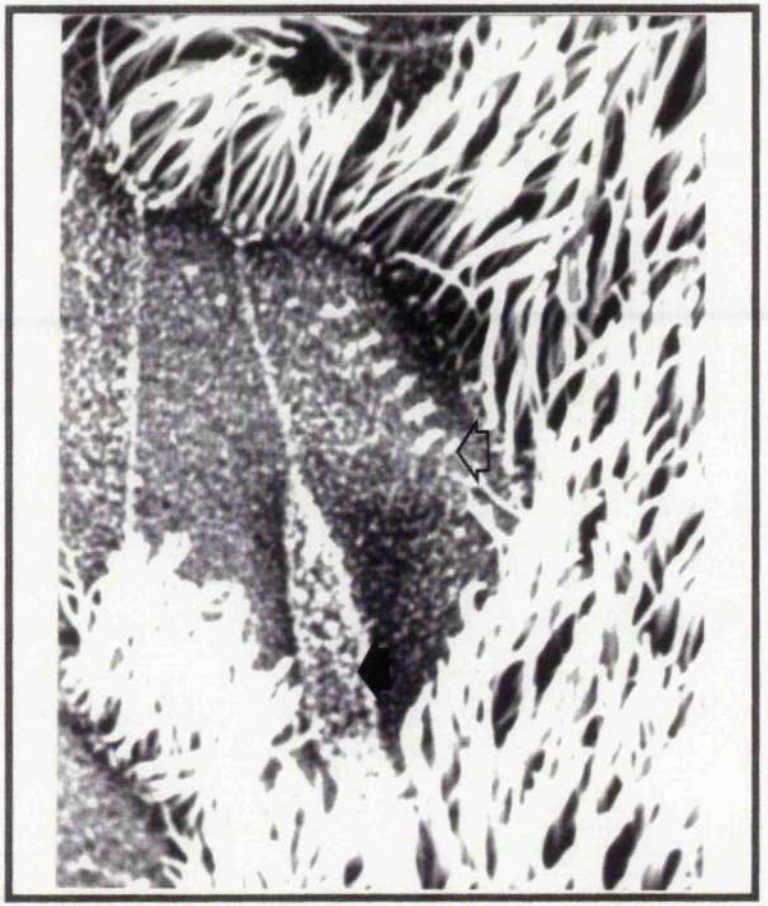


Fig. 6.29

Intrapulmonary primary bronchus. 3-day-old chick.

Exfoliation of a large area of the epithelial surface.

X 720

Fig. 6.30

Intrapulmonary primary bronchus. 13-day-old chick.

Note presence of numerous apical protuberances typical of mucous cells emerging amongst the thick carpet of cilia (arrow).

X 2, 750

Fig. 6.31

Secondary bronchus. 3-day-old chick.

Matted cilia (arrow) and stubby microvillous cells covered with mucus (open arrow).

X 5, 500

Fig. 6.32

Secondary bronchus. 5-day-old chick.

Exfoliation of the epithelial cells.

X 1, 270

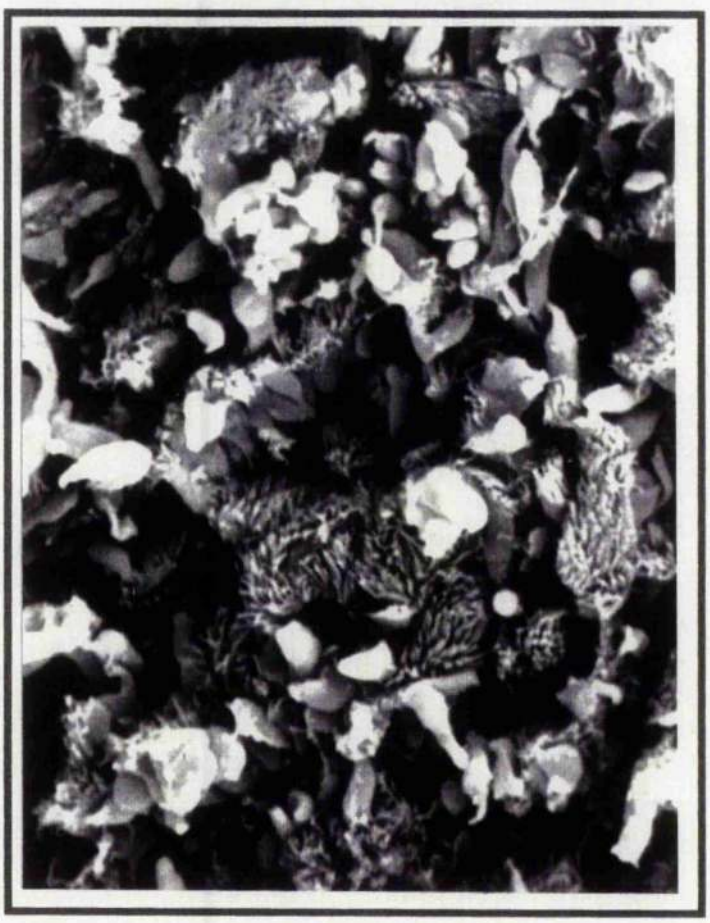


Fig. 6.33

Secondary bronchus. 5-day-old chick.

A few ciliated cells still anchored to the sub-mucosal layer.

X5, 500

Fig. 6.34

Secondary bronchus. 11-day-old chick.

Note clumping of cilia.

X5, 500

Fig. 6.35

Secondary bronchus. 7-day-old chick.

Numerous mucous cells seen emerging amongst the cilia.

X 2, 750

Fig. 6.36 Averaged lesion score (±sd) in the middle nasal concha.

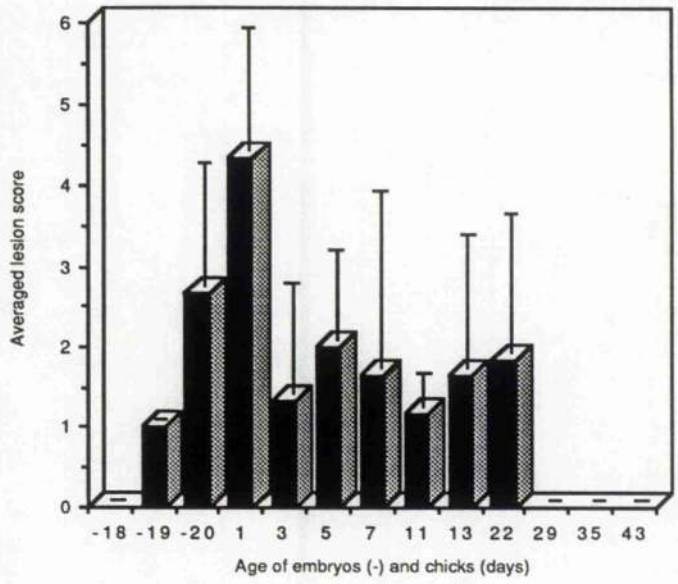


Fig. 6.37 Averaged lesion score (±sd) in the larynx.

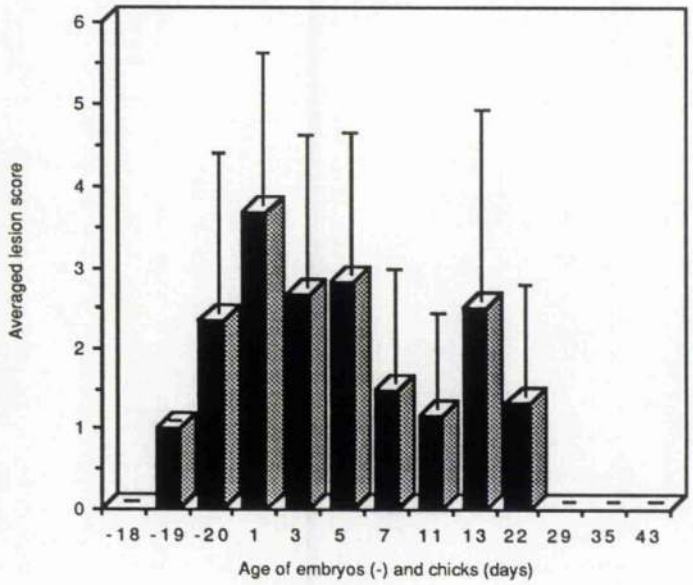


Fig. 6.38 Averaged lesion score (±sd) in the cranial trachea

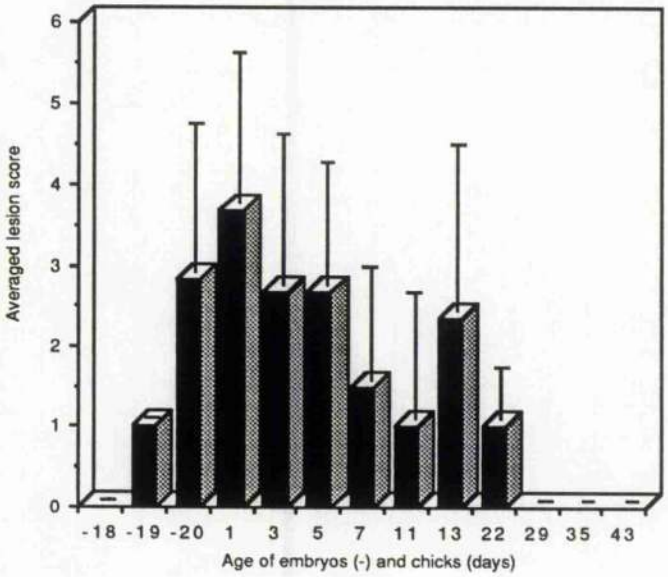


Fig. 6.39 Averaged lesion score (±sd) in the caudal trachea

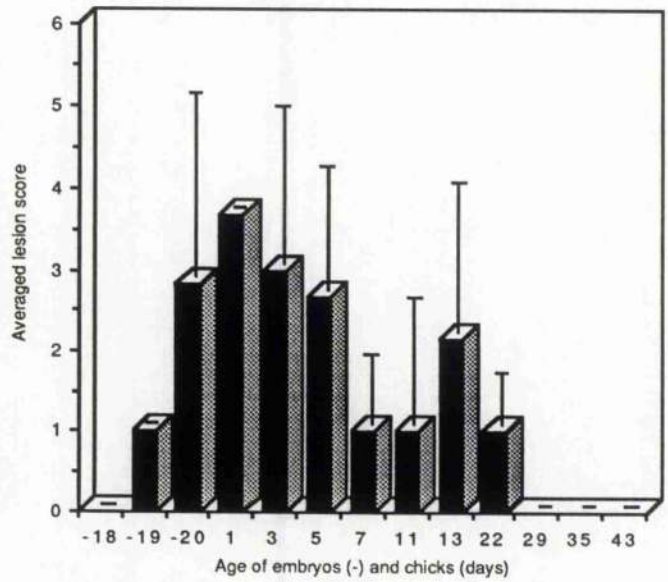


Fig. 6.40 Averaged lesion score (±sd) in the intrapulmonary primary bronchus

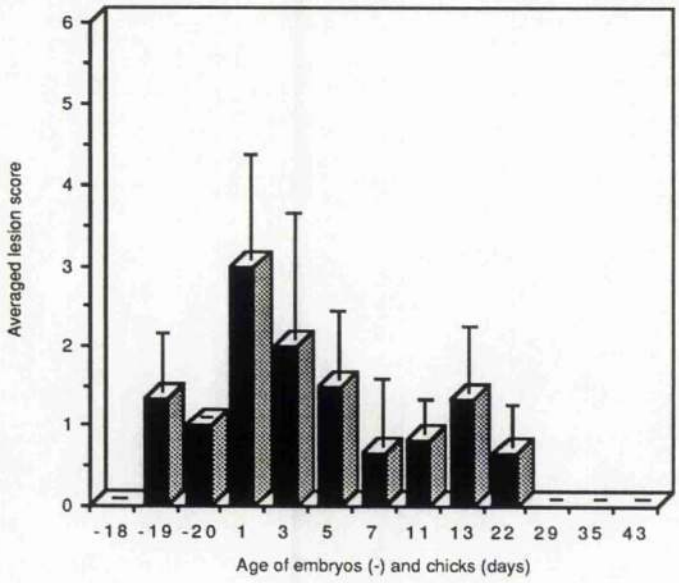
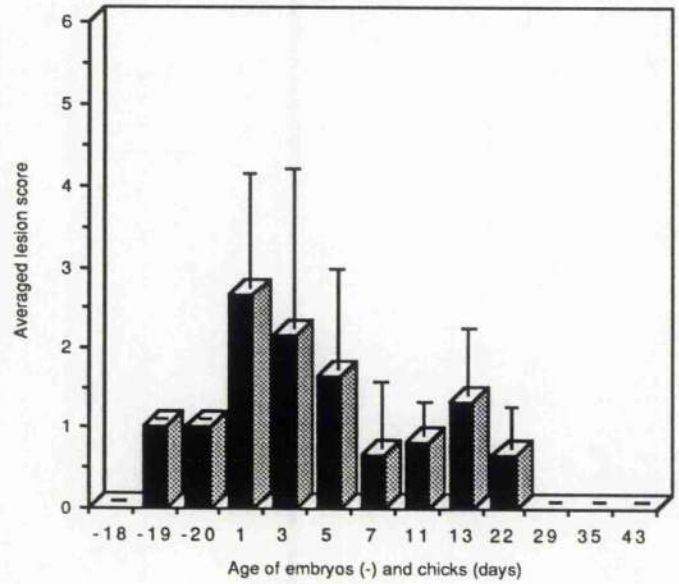


Fig. 6.41 Averaged lesion score (±sd) in the secondary bronchus



DISCUSSION

This study has demonstrated that fumigation of hatching chicks at a concentration as low as 10.9 ppm formaldehyde vapour causes pathological changes throughout the entire respiratory epithelium. Such observations contradict the findings of Furuta *et al.* (1989) who reported that low concentrations of formaldehyde vapour (8 ppm) did not appear to affect the respiratory epithelium of hatching chicks. Similar lesions to those observed in the present study in the epithelial lining of 19-day-old embryos through to 1-day-old chicks including clumping and disorientation of cilia, blebs on the cilial wall, and deciliation and sloughing of the epithelium were observed in SEM studies of the tracheal and bronchial epithelium by Furuta *et al.* (1989) and Sander *et al.* (1995) but only in response to much higher concentrations of formaldehyde.

The present SEM study has also attempted to compare, for the first time in the bird, the differing severity of the pathological lesions presented by hatching chicks as a result of the variations in exposure time to formaldehyde vapour fumigation. The observed results have demonstrated that, as might been anticipated, the longer the exposure time, the more severe the lesions within the respiratory epithelium. Light microscopic studies carried out to evaluate cell proliferation rates within the respiratory epithelium of the nasal cavity of rats and mice exposed to 0.5 to 15 ppm formaldehyde vapour for 3 to 12 hours per day for 1 to 10 days, demonstrate that the longer the exposure time, the higher the cell proliferation rate (Swenberg *et al.*, 1986). Similar transmission electron microscopic studies also demonstrated that longer exposure times increase cell turnover rates in the epithelial lining of the nasal cavity of rats subjected to formaldehyde exposure (Zwart *et al.*, 1988). Such findings support the present observations that epithelial responses to formaldehyde vapour are linked to

the duration of exposure. It may be, however, that not all air-borne chemically noxious substances produce similar effects, as Calderon-Garciduenas *et al.* (1992) has noted with respect to histopathological changes in the nasal mucosa of man following both short-term and long-term exposure to high levels of ozone. The degree of mucostasis and ciliostasis has also been shown to change with variations in exposure time to formaldehyde vapour in the nasal cavity of rats (Morgan *et al.*, 1986b).

Observations made during the course of this study suggest that regardless of age and duration of exposure, lesion severity is generally similar in all regions of the respiratory tract in any one individual. Such observations contrast markedly with earlier reports Gerrits (1990) and Gerrits & Dijk (1991) which indicated that the effects of formaldehyde vapour (at concentrations of 20-80 ppm) were restricted to the cranial part of the chick tracheal epithelium. Such findings contradict with Al-Mashhadani and Beck (1985), who noted that lesions seen in the respiratory tract of broilers exposed to 100 ppm of ammonia, were more severe in the lung than in the upper respiratory tract.

The observed increase in mucus secretion, and resultant matting of surface cilia, noted in the present study, confirms similar observations by Sander *et al.* (1995), who have suggested that it may be the irritant effect of formaldehyde vapour on the mucous cells, and/or an associated reduced motility of the cilial carpet, which leads to reduced clearance of the mucus blanket in exposed chicks. Similar increases in mucus secretion have also been reported as a result of exposure to other toxic gases, such as ammonia in chicks (Nagaraja *et al.*, 1983), sulphur dioxide in dogs (Greene *et al.*, 1984), and air pollution in rabbits (Gulisano *et al.*, 1995), passerine birds and small mammals (Llacuna *et al.*, 1993). Clinical cases such as acute bronchitis in rats have also been shown to produce similar effects (Iravani and As, 1972). Sulphur dioxide is also acknowledged to cause a

decrease in mucociliary activity in the trachea of the guinea pig (Knorst *et al.*, 1994). The present observations of blebs on the cilial wall, seen throughout the epithelial lining in the 19 and 20-day-old embryos and 1-day-old chicks, confirm the findings of Sander *et al.* (1995), who suggested that such blebs might weaken the cilia and eventually lead to cilial breakage and resultant deciliation. Such deciliation was a common feature of the epithelial lining of the respiratory tract in all chicks examined in this section of the study, again supporting similar observations by Sander *et al.* (1995). Deciliation has also been reported in the tracheal epithelium of chickens infected by *Mycoplasma gallisepticum* (Dykstra *et al.*, 1985). The reduced efficiency of the mucociliary clearance mechanisms within the respiratory tract, as a result of the interference of factors such as those just noted could be expected to result in the stagnation of large amounts of surface mucus. If such a situation continues over a long period, air is pressed out from the interciliary air spaces as the mucus sheet, often appearing in the form of lattice-like mucous strands (Tucker, 1974; present observations) sinks below the level of the cilial tips. As a result deep impressions are formed in the cilial carpet, and cilia become agglutinated along their whole length, leading to their entanglement and immobilisation (Tucker, 1974), a situation which causes yet further damage to the mucociliary clearance mechanisms.

In the present study, the respiratory epithelium of 3 to 7-day-old chicks demonstrated frequent clumping and occasional disorientation of cilia, deciliation and epithelial sloughing as a result of exposure to the formaldehyde vapour. Sloughing of the epithelium was seen either to be mild, involving only the epithelial cells, or severe where both the epithelial and basal cells were involved. All these features have also been reported in chicks exposed to higher concentrations of formaldehyde vapour (Furuta *et al.*, 1989; Gerrits, 1990; Gerrits and Dijk, 1991; Sander *et al.*, 1995), or in other species exposed to other toxic gases such as ammonia in man (Flury

et al., 1983), or ozone (acute ozone injury) in Bonnet monkeys (Wilson *et al.*, 1984) and rats (Pino *et al.*, 1992). Disorientation of cilia as seen in the epithelial lining of the chicks used in the present study, has also been reported in chronic sinusitis in man (Toskala *et al.*, 1995) and during ciliogenesis in cultured human respiratory epithelial cells (Yoshitsugu *et al.*, 1994). They suggest that prime factors involved in coordination of ciliary activity include ciliary elongation, beat amplitude and increased ciliated cell density. It is easy to see, therefore, how changes in ciliary morphology, such as ciliary damage, cilia disorientation and loss of ciliary cells, all factors noted in the present study, could affect the efficient functioning of the ciliary carpet. Yoshitsugu *et al.* (1994) have suggested that it is the loss of intercellular coordination between adjacent ciliated cells that causes the sort of surface disorientation of cilia observed in the present study.

The presence of large areas of flattened non-ciliated microvillous cells, as seen in the larynx of 3 to 7-day-old chicks in this study, has also been reported in the tracheobronchial epithelium of Bonnet monkeys in response to acute ozone injury (Wilson *et al.*, 1984) and in *in vivo* metaplastic restitution of airway epithelium due to mechanical injury in guinea-pig (Erjefalt *et al.*,1995). The latter study suggested that such areas were composed primarily of intermediate cells covering regions of injured epithelium. It was also suggested that these cells, in addition to serving as the precursor cells for the differentiated epithelium, might also serve a protective function by rapidly restoring epithelial integrity to an injured mucosa.

The finger-like projections, containing mucous granules emerging at the epithelial surface in the present study could be evidence of similar epithelial metaplasia as suggested in the SEM study of chronic sinusitis in man by Toskala *et al.* (1995). Thus in cases of severe or repeated epithelial damage, areas of squamous metaplasia may be a common feature. Such

areas would be inactive in terms of mucociliary clearance and thus more vulnerable to invasion by inflammatory agents, leading to further damage and chronic inflammatory changes.

In the 11 to 22-day-old birds studied, a thick mucus blanket was frequently seen on the epithelial surface throughout the respiratory tract, due to an apparent increase in the numbers of active mucous cells. Numbers of mature ciliated cells were also seen to increase in this age group, presumably as a result of the observed ongoing active ciliogenesis. Such ciliogenesis often characterised by the presence of numerous short cilia visible on the luminal surface of regenerating ciliated cells, was seen to begin at around 7 days of age in the present study. Similar observations of the appearance of short cilia have been reported in cases of chronic sinusitis in man (Toskala *et al.*, 1995), and in the rabbit bronchial epithelium as a result of exposure to polluted air (Gulisano *et al.*, 1995). Although the recognition of regenerating ciliated cells is aided by the appearance of short cilia at the luminal surface of these cells, it should be noted that many of the observed non-ciliated microvillous cells seen in the damaged respiratory epithelium will be, in fact, ciliated cells, unrecognisable due to their lack of cilia. Such a fact has also been noted by Gulisano *et al.* (1995) in their study of the alteration in the bronchial epithelium induced by air pollutants in rabbit. Groups of cilia aggregated into long narrow strips, giving the impression of ciliated cells being squeezed in amongst surrounding microvillous cells as in the present study has also been reported in the respiratory epithelium of adult chicken by Mohammed (1989). Such elongated ciliated cells show obvious similarities with a previous description in the dove (Wetzstein *et al.*, 1980). The latter authors reported the appearance of longer ciliated cytoplasmic extensions of the ciliated epithelial cells of the pulmonary bronchus lying between non-ciliated columnar epithelial cells. In general, the normal organisation of the

respiratory epithelium was seen to have re-established throughout the respiratory tract in 29 to 43-day-old chickens in the present study, although a few individuals presented a regenerated epithelium at an even earlier stage. Such re-epithelialisation of the airways as has been shown, by SEM and TEM studies, starts immediately, and occurs rapidly through migration of flattened secretory and ciliated cells (and presumably also basal cells) into the damaged areas (Erjefalt *et al.*, 1995). The duration of this regeneration process has also been shown to depend on the degree of damage to the underlying basal membrane (Hilding and Hilding, 1966; Ohashi *et al.*, 1991, Bootz and Reuter, 1992), as both mucous cells and ciliated cells regenerate from stem cells located at this membrane. The latter appear in large numbers 2 days after transplantation. The present observation of a complete reciliation of the lining epithelium in the 29-day-old chick can be favourably interpreted in the light of studies by Bootz and Reuter (1992), who reported that in studies on the free grafting of respiratory epithelium in the rat, ciliated cells will take 21 days to develop to maturity.

CHAPTER 7

TRANSMISSION ELECTRON MICROSCOPY OF THE RESPIRATORY EPITHELIUM OF CHICKS EXPOSED TO FORMALDEHYDE VAPOUR.

INTRODUCTION

The purpose of this study was to use the transmission electron microscope to further examine the effect of formaldehyde vapour on the respiratory epithelium of pre- and post-hatched chicks.

Although the effect of formaldehyde vapour on the epithelial lining of the respiratory tract has been well been documented by the use of light microscopy especially in the mammalian species (Cralley, 1942; Dalhamn, 1956; Swenberg *et al.*, 1980; Albert *et al.*, 1982; Swenberg *et al.*, 1983; Kerns *et al.*, 1983; Chang *et al.*, 1983; Rusch *et al.*, 1983; Jiang *et al.*, 1986; Maronport *et al.*, 1986; Morgan *et al.*, 1986a, 1986b, 1986c; Monticello *et al.*, 1989, 1991; Swiecichowski *et al.*, 1993; Bermudez *et al.*, 1994; Cassee *et al.*, 1996), relatively very little work has been carried out at transmission electron microscope level. The few studies that have been carried out at the ultrastructural level on the pathological changes induced by formaldehyde exposure have concentrated primarily on localised areas of the respiratory tract such as the nasal cavity of rats (Swenberg *et al.*, 1983; Rusch *et al.*, 1983 and Zwart *et al.*, 1988), hamsters (Rusch *et al.*, 1983) and monkeys (Rusch *et al.*, 1983), and the trachea of rats (Klein-Szanto *et al.*, 1981).

As for the irritant effect of the formaldehyde vapour on the respiratory tract of avian species, the only reported studies appear to be those in the domestic fowl using light microscopy (Furuta *et al.*,1989; Gerrits, 1990; Gerrits and Dijk, 1991; Sander *et al.*, 1995) and scanning electron microscopy (Furuta *et al.*, 1989; Sander *et al.*, 1995). This chapter appears, therefore, to be the first transmission electron microscopic study on the effect of formaldehyde vapour on the avian respiratory tract, specifically pre-

and post-hatched chicks.

MATERIALS AND METHODS

Source of chicks

Chicks used for this study were formaldehdye-exposed, and control chicks were obtained, as outlined in Chapter 2. The number and age of chicks involved in this study is shown in Table 13:

TABLE 13

BIRDS USED FOR TRANSMISSION ELECTRON MICROSCOPY (TEM) IN THE INVESTIGATION OF THE EFFECT OF FORMALDEHYDE VAPOUR ON THE TRACHEAL EPITHELIUM OF CHICKEN

Age of Chicks	No. of formaldehyde-exposed chicks
19-day-old embryo	3
20-day-old embryo	3
1-day-old chick	3
3-day-old chick	3
5-day-old chick	3
7-day-old chick	3
11-day-old chick	3
13-day-old chick	3
22-day-old chicken	3
29-day-old chicken	3
35-day-old chicken	3
43-day-old chicken	3

Sample collection and processing of samples for transmission electron microscopy

Chickens were euthanised, and the respiratory tract removed. Tissue samples were collected from the middle nasal concha, larynx and trachea. Processing of tissues for transmission electron microscopy was as detailed

in Chapter 2.

RESULTS

Middle nasal concha

19-day-old embryos through to 1-day-old chicks

Transmission electron microscopy demonstrated that clumping of the cilia was frequently seen in the middle nasal concha of the formaldehyde-exposed 19-day-old embryos through to 1-day-old chicks (Fig. 7.1). Where the luminal surface was devoid of cilia, clumping of microvilli was occasionally observed (Fig. 7.2). Deciliation (Fig. 7.3) was evident in all the chicks examined in this age group. Small balloon-like structures were frequently seen emerging from the cilial walls, as well as the microvillous walls (Fig. 7.4), and internal cilia were occasionally seen (Fig. 7.5). Ciliated cells undergoing degeneration (Fig. 7.3) were also observed, characterised by the presence of numerous vacuoles in the cytoplasm. In some of the necrotic areas, cells containing a large intracytoplasmic vacuole was frequently observed, and cell separation was usually obvious, due to the presence of large intercellular spaces (Fig. 7.6). Within the mucous cells, granules of varying electron density, either electron-opaque, electron-opaque with a darker core or electron-opaque with an electron-dense core, were well dispersed throughout the cytoplasm (Fig. 7.7). Numerous clearly recognisable mucous cells were observed in the basal regions of the respiratory epithelium (Fig. 7.8); these cells were thus located at a much deeper level in the epithelium than was normal. Developing ciliated cells with numerous centrioles, developing mucous cells containing numerous granules and lightly staining electron-lucent cells, containing numerous ribosomes and rough endoplasmic reticulum, a few mitochondria and occasionally few electron-dense granules, were frequently seen in the basal region (Fig. 7.9).

Various degrees of sloughing of the epithelium were observed. In less severe cases the mucous cells and ciliated cells were exfoliated, leaving the basal cells intact, whilst in the more severe pathological lesions, sloughing of the mucosal layer resulted in the exposure of the sub-mucosal layer (Fig. 7.10).

3-day-old chicks through to 22-day-old chicken

In this age group, clumping of cilia (Fig. 7.11) and deciliation (Fig. 7.12) were still observed in the epithelial lining of the middle nasal concha, although the balloon-like structures projecting from cilial and microvillous processes were less frequently observed. Necrotic cell separation from neighbouring cells was still frequently seen. In intact epithelial regions, an apparent increase in the number of active mucous cells, as determined by the presence of packed granules within the cell cytoplasm, was often being demonstrated (Fig. 7.13). Subjective observations suggested that there was a relative increase in the size of the intraepithelial mucous glands in the middle nasal concha in this age group of birds (Fig. 7.14). Secretory ciliated cells were also occasionally seen at the luminal surface of the epithelium in the middle nasal concha; such cells characteristically contained a large number of mitochondria, extensive rough endoplasmic reticulum, numerous centrioles and small numbers of either electron-dense (Fig. 7.15), electron-lucent, or electron-dense core (Fig. 7.16) mucous granules.

29-day-old through to 43-day-old chicken

A normal organisation of the epithelial lining cells was seen in the middle nasal concha of birds in this age group (Fig. 7.17), except for one region in the concha of one 29-day-old chicken, where cell proliferation at the edges of a desquamated area appeared to share a number of newly differentiated mucous cells invading this damaged area to provide cover for

the underlying sub-mucosa (Fig. 7.18).

Larynx

19-day-old embryos through to 1-day-old chicks

As in the middle nasal concha, deciliation (Fig. 7.21) and clumping of cilia associated with a thick covering of surface mucus were observed in all individuals examined in this age group (Fig. 7.19). Disorganisation of the microtubules within such clumped cilia was also observed occasionally (Fig.7.20), along with infrequently observed, dark-stained strips of luminal plasmalemma (Fig. 7.22). Balloon-like structures were frequently detected emerging from the cilial and microvillous walls of the cells lining the larynx. The early stages of cell sloughing detected in the larynx of this age group were associated with epithelial cell separation and plasmalemmal breakdown (Fig. 7.23). Frequently observed degenerating epithelial cells were frequently seen and were characterised either by the presence of numerous vacuoles at the apical region of the ciliated cells (Fig. 7.24) or numerous vacuoles and swollen mitochondria throughout the cytoplasm (Fig. 7.25). In the case of the mucous cells, such plasmalemmal destruction resulted in the release of large numbers of mucous granules into the laryngeal lumen (Fig. 7.26). More severe cases of epithelial sloughing were seen to result in the exposure of the basal cells (Fig. 7.27).

3-day-old chicks through to 22-day-old chicken

Deciliation and epithelial sloughing was still seen in the larynx of birds in this age group. However, some regions of the larynx of 5 of the birds in this age group demonstrated the presence of squamous metaplasia of the epithelial lining (Fig. 7.28). At the luminal surface the squamous cells carried scattered microvilli (Fig. 7.29) and were seen to contact neighbouring cells by means of numerous desmosomal attachments. Such squamous metaplastic cells, as typified by those in the mid-epithelial region,

were also seen to possess numerous intracytoplasmic tonofilaments and a centrally- located corrugated nucleus (Fig. 7.30). Occasionally dark staining cells are seen at the basal region of the epithelium (Fig. 7.31).

29-day-old through to 43-day-old chicken

Transmission electron microscopy revealed that all the chickens in this age group had acquired a normal epithelial organisation (Fig. 7.32).

Trachea

19-day-old embryos through to 1-day-old chicks

Transmission electron microscopy illustrated various pathological changes in the trachea of formaldehyde-exposed 19-day-old embryos through to 1-day-old chicks. Clumping of cilia was frequently observed (Fig.7.33), whilst the presence of balloon-like structures on the cilial wall, disorganisation of the microtubules within the cilia and cilial breakdown was observed in all birds in this age group (Fig. 7.34). Disintegration of cellular structure was also evident along with the development of large intercellular gaps (Fig. 7.35). In some mild cases of sloughing, isolated ciliated cells were seen to be left intact (Fig. 7.36) whereas in severe cases the entire mucosal lining was exfoliated, exposing the sub-mucosal layer (Fig. 7.37). In these birds, the mucous cells were seen to contain massive numbers of mucous granules (Fig. 7.38), causing the cells to appear more elongated than normal, due to the distribution of such granules into the more basal regions of the cells. The presence of such large numbers of mucous granules was related to the observed massive release of mucus onto the epithelial surface, often involving cell membrane destruction at the apical cell surface. A non-ciliated microvillous cell type, containing an abundance of rough endoplasmic reticulum at the apical region of the cytoplasm, a few mitochondria, and lacking any apparent secretory granules, was occasionally observed in the tracheal epithelial lining (Fig. 7.39).

3-day-old through to 22-day-old chicken

In this age group, clumping of cilia, and deciliation, together with ciliated cell breakdown were all observed (Fig. 7.40). A similar breakdown of mucous cells was also observed, often resulting in the release and dispersal of large quantities of mucous granules. The number of mucous cells was seen to increase markedly in the mid-region of some parts of the tracheal epithelium. Occasionally proliferation of mucous cells was noted just above the sub-mucosal layer (Fig. 7.41).

29-day-old through to 43-day-old chicken

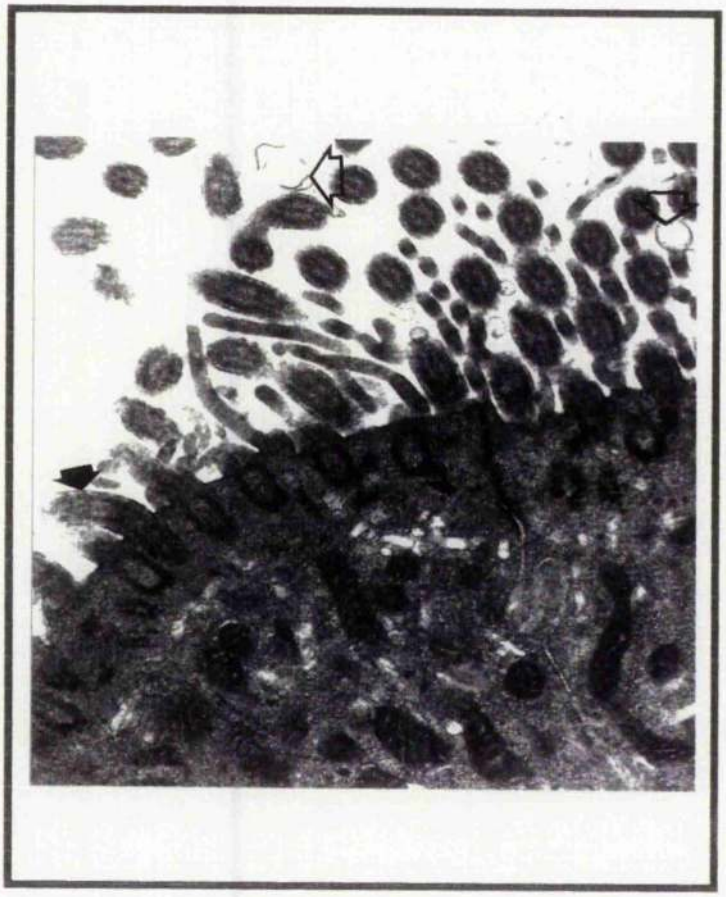
Transmission electron microscopy revealed that the epithelial lining of the trachea had acquired the normal muco-ciliary organisation in this group of birds (Fig.7.42).

Fig. 7.1
Middle nasal concha. 1-day-old chick.
Note the clumping of cilia (arrow) and a mucous globule on the epithelial surface (open arrow). Numerous vacuoles (*) seen in the cytoplasm of cells.
X 11,250

Fig. 7.2
Middle nasal concha. 1-day-old chick.
Clumping of microvilli (arrow) at the luminal surface of the mucous cells and the well distended rough endoplasmic reticulum (open arrows) in the cytoplasm of the mucous cells.
X 11,250

Fig. 7.3
Middle nasal concha. 1-day-old chick.
The cilia are short (arrow), note the balloon-like structures on the cilial and microvillous surface (open arrows) and numerous vacuoles in the cytoplasm of the cells.
X 22,500

Fig. 7.4
Middle nasal concha. 20-day-old embryo.
Balloon-like structures on the cilial walls (arrows) and microvillous walls (open arrow). X6700.
Insert: transverse section of the cilia with balloon-like structure emerging from the cilial wall (arrow).
X15,000



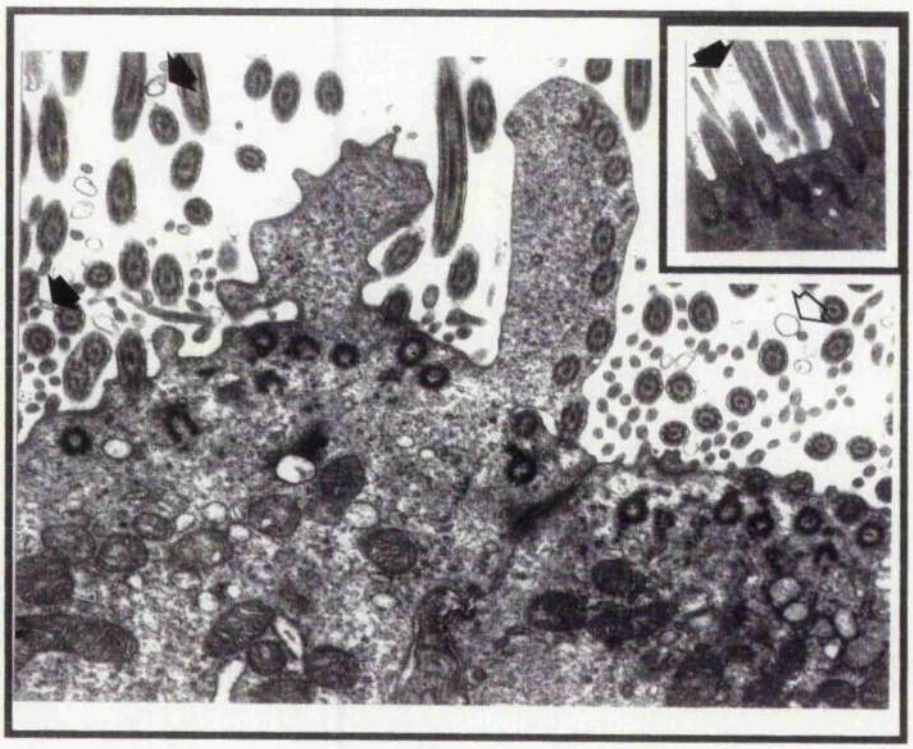
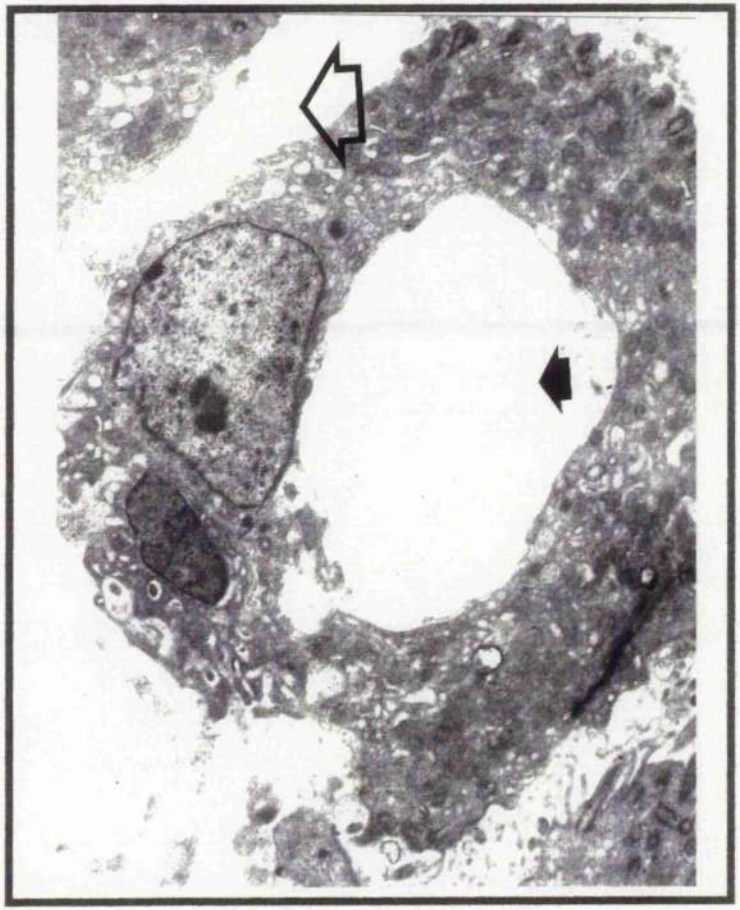


Fig. 7.5
Middle nasal concha. 1-day-old chick.
Note internal cilia (arrow) and deciliation (open arrow).
X 11,250

Fig. 7.6
Middle nasal concha. 1-day-old chick.
A necrotic ciliated cell, only a few basal bodies and a large vacuole (arrow) in the cytoplasm. Note the cell separation from left neighbouring ciliated cell and gaps (open arrow) with minimal cell contact.
X9,000



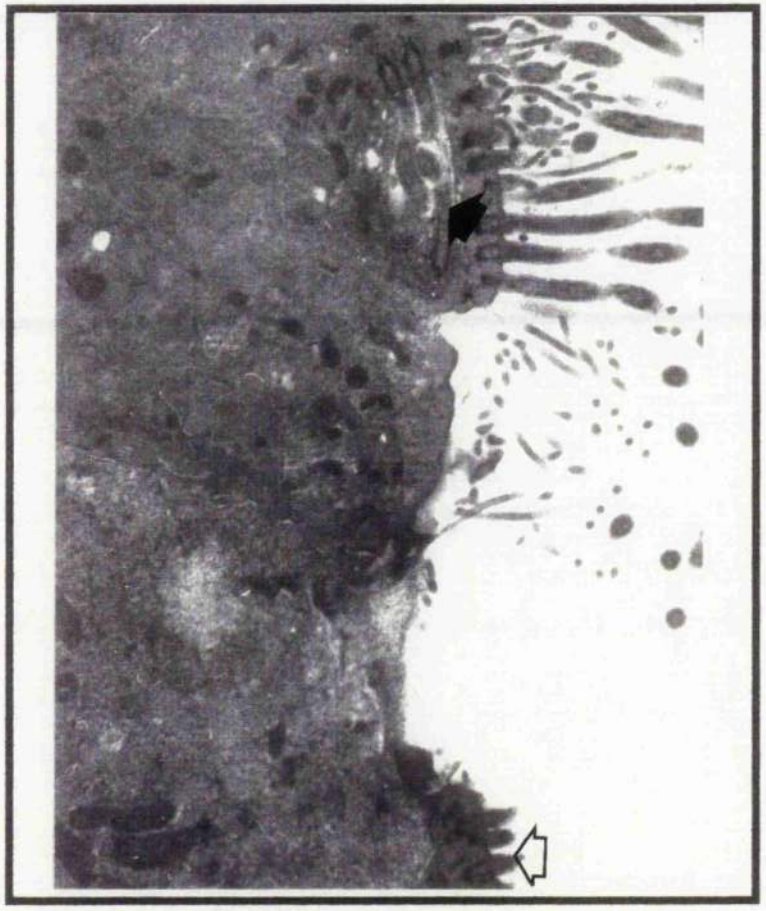


Fig. 7.7

Middle nasal concha. 20-day-old embryo.

Mucous cells with numerous granules of 4 different types are well dispersed throughout the cytoplasm.

1. Electron-lucent granules
2. Electron-opaque granules
3. Electron-opaque granules with an electron-dense core
4. Electron-opaque with darker electron-dense core

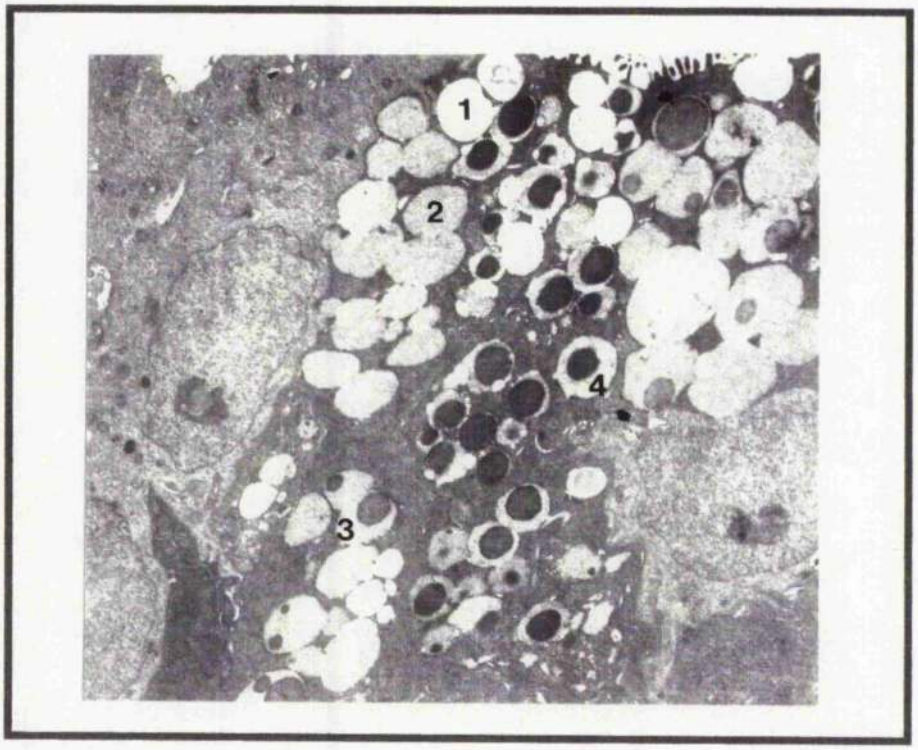
X6,000

Fig. 7.8

Middle nasal concha. 1-day-old chick.

Numerous mucous cells (arrow) at the basal region of the respiratory epithelium of the middle nasal concha. Note also the presence of a large cell (open arrow) with light staining cytoplasm containing numerous electron-dense granules, mitochondria and rough endoplasmic reticulum. Basal cells (BC) and basement membrane (BM).

X6,000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3
4

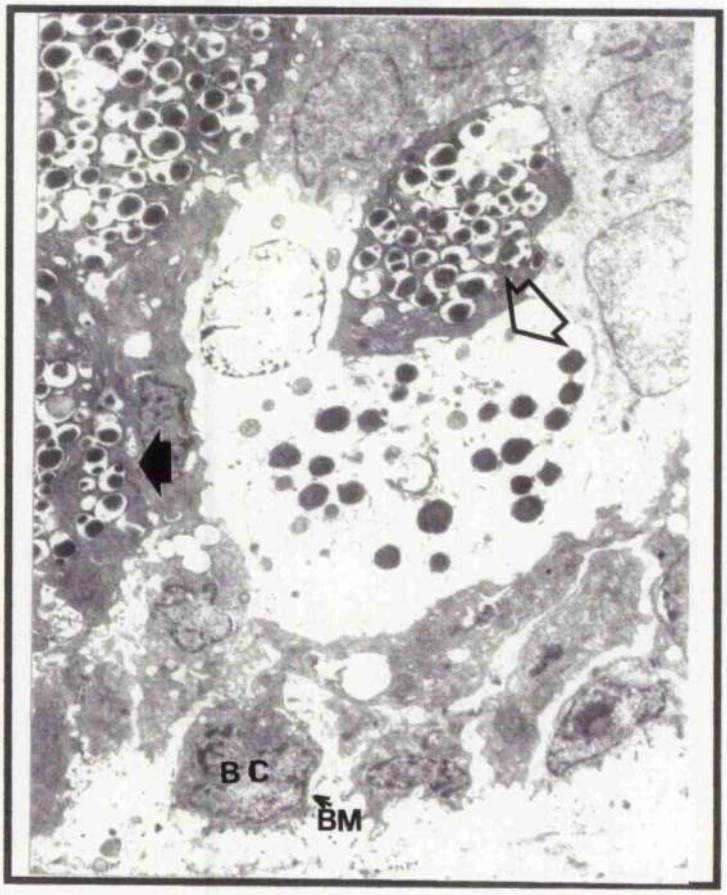

BC
BM

Fig. 7.9

Middle nasal concha. 19-day-old embryo.

Cells at the basal region.

1. Cells with numerous centrioles
2. Light staining cell with numerous ribosomes, rough endoplasmic reticulum and mitochondria and a few electron-dense granules.
3. Mucous cells with granules of various sizes and electron-density.

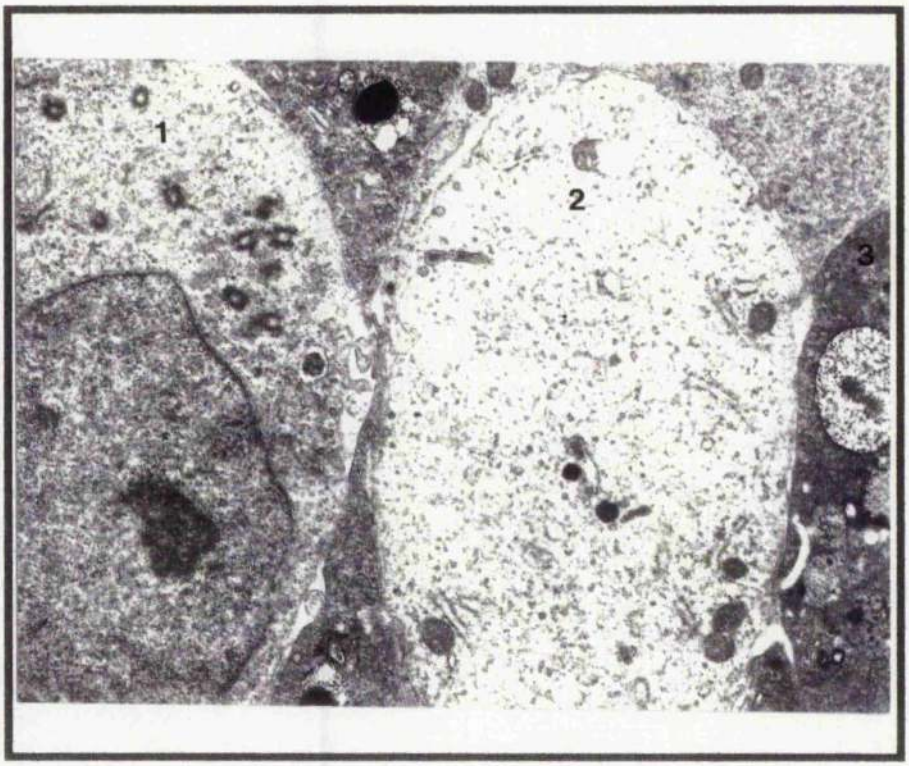
X9,000

Fig. 7.10

Middle nasal concha. 1-day-old chick.

Note severe damage to epithelial cells (arrow) exposing the sub-mucosal layer (open arrow). CT- connective tissue in the sub-mucosal layer.

X9,000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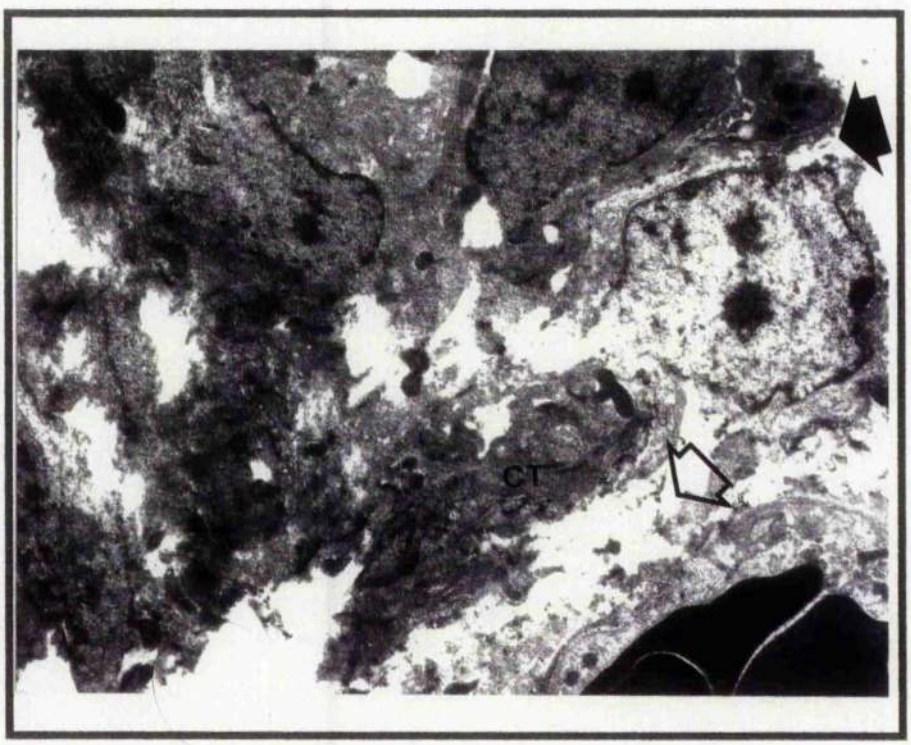

CT

Fig. 7.11
Middle nasal concha. 3-day-old chick.
Note clumping of cilia (arrow) and presence of numerous vacuoles (open arrow).
X15,000

Fig. 7.12
Middle nasal concha. 3-day-old chick.
Deciliated epithelial cell (arrow), basal bodies and rootlets are seen in the cytoplasm at the apical region of the ciliated cell. Note also large gaps between cilia (open arrow).
X9,000

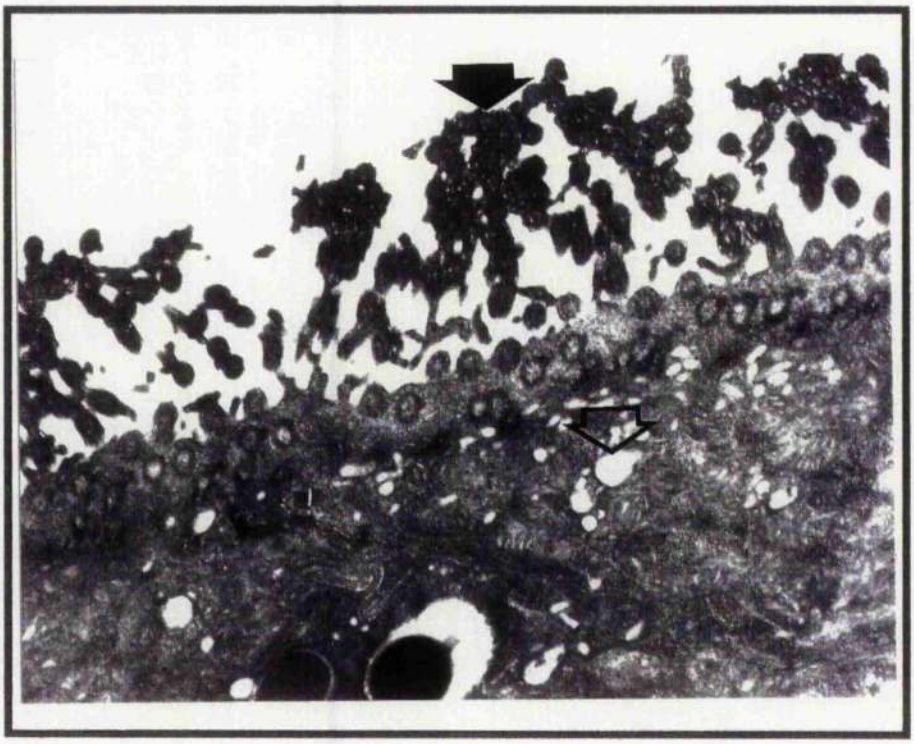
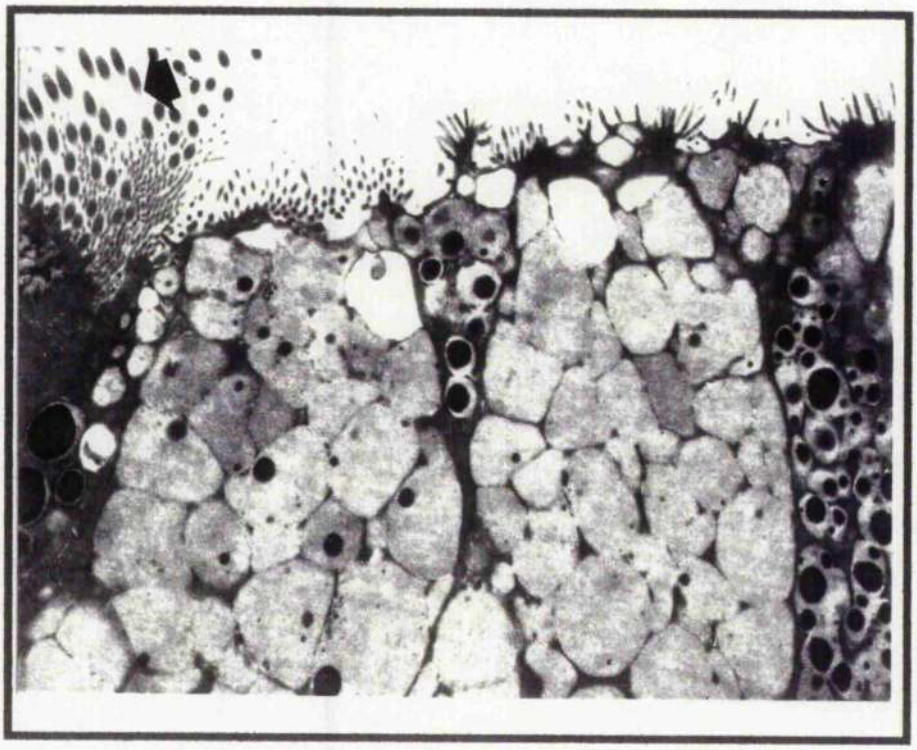


Fig. 7.13
Middle nasal concha. 7-day-old chick.
An apparent increase in the number of mucous cells and normal ciliation was seen (arrow).
X6,000

Fig. 7.14
Middle nasal concha. 5-day-old chick.
A cell with numerous centrioles and mucous granules (arrow) bordering an enlarged intraepithelial mucous gland.
X 6,000



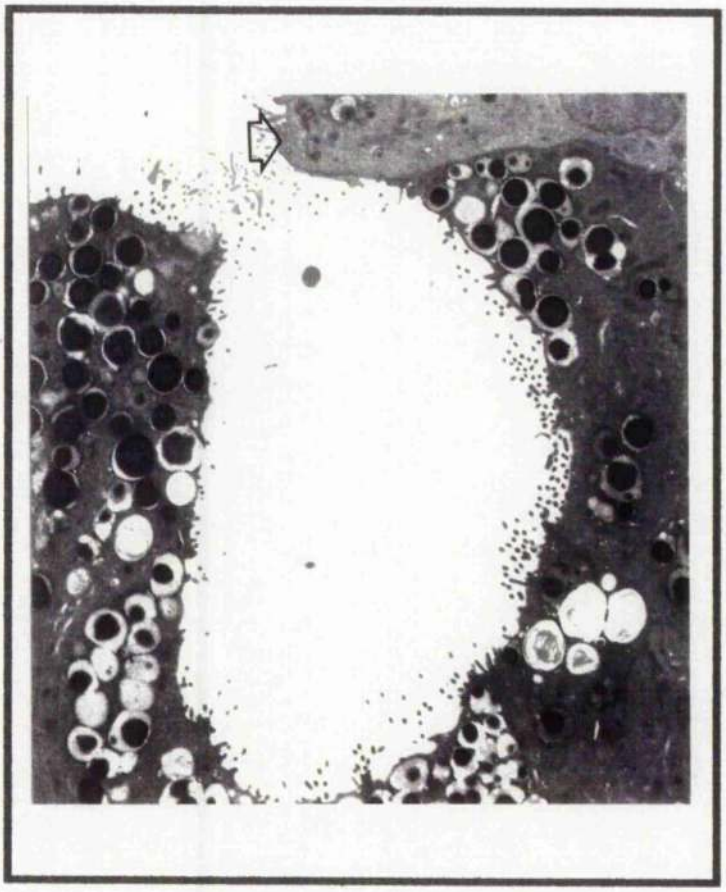
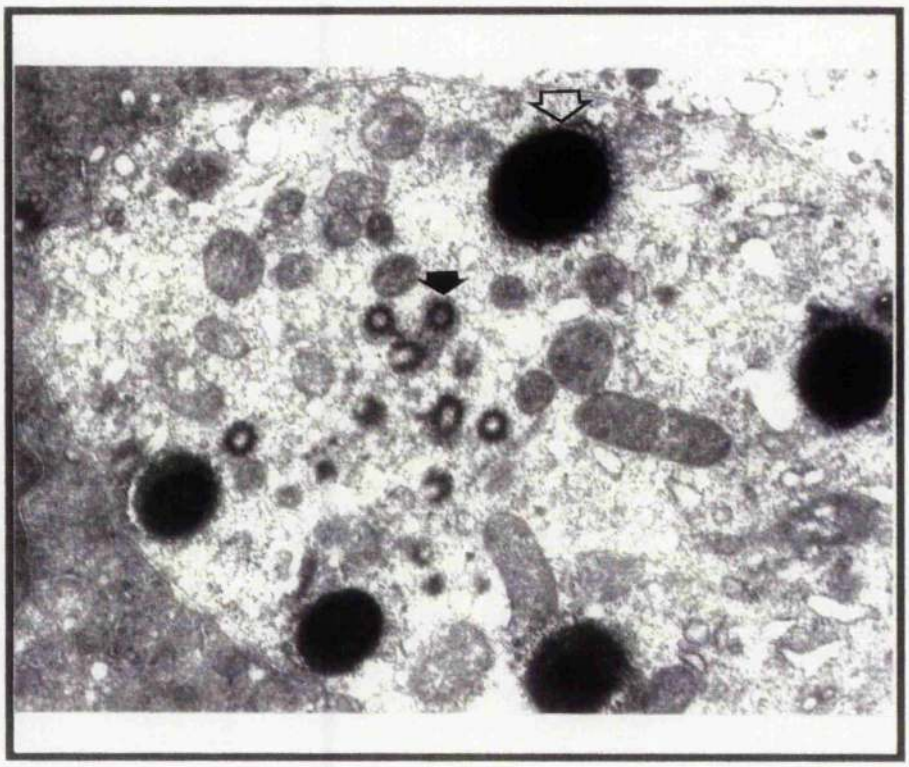


Fig. 7.15
Middle nasal concha. 5-day-old chick.
A cell with numerous centrioles (arrow), electron-dense granules (open arrow), mitochondria and rough endoplasmic reticulum.
X18,000

Fig. 7.16
Middle nasal concha. 5-day-old chick.
This cell has numerous centrioles (C), mitochondria (M) and rough endoplasmic reticulum (RER) and a few mucous granules of variable electron density (arrows).
X6,700



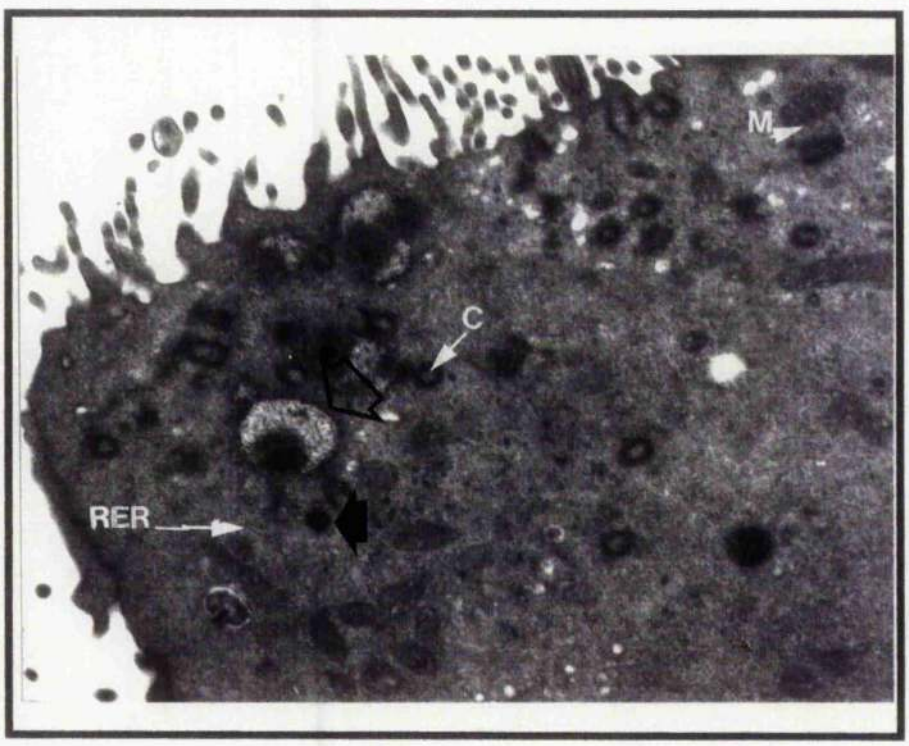

M
C
RER

Fig. 7.17

Middle nasal concha. 29-day-old chicken.

Normal organisation of ciliated and mucous cells lining the middle nasal concha.

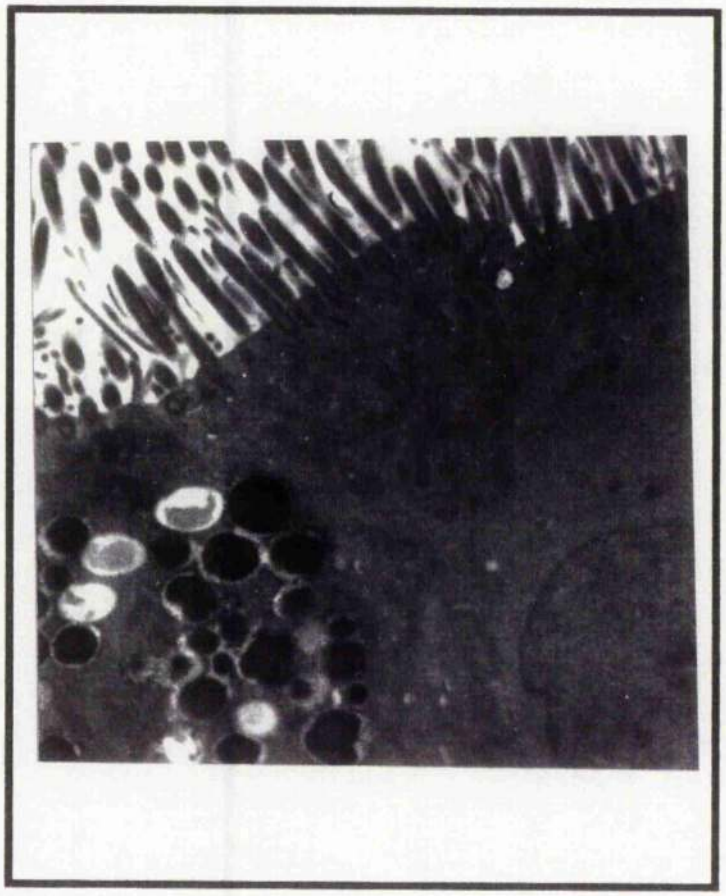
X9,000

Fig. 7.18

Middle nasal concha. 29-day-old chicken.

A single mucous cell is visible within the regenerating epithelium (arrow). Such mucous cells were frequently observed proliferating at the basal region of the epithelium. Ciliated cells (CC), basal cells (BC) and lumen (L) of damaged epithelial cell.

X6,000



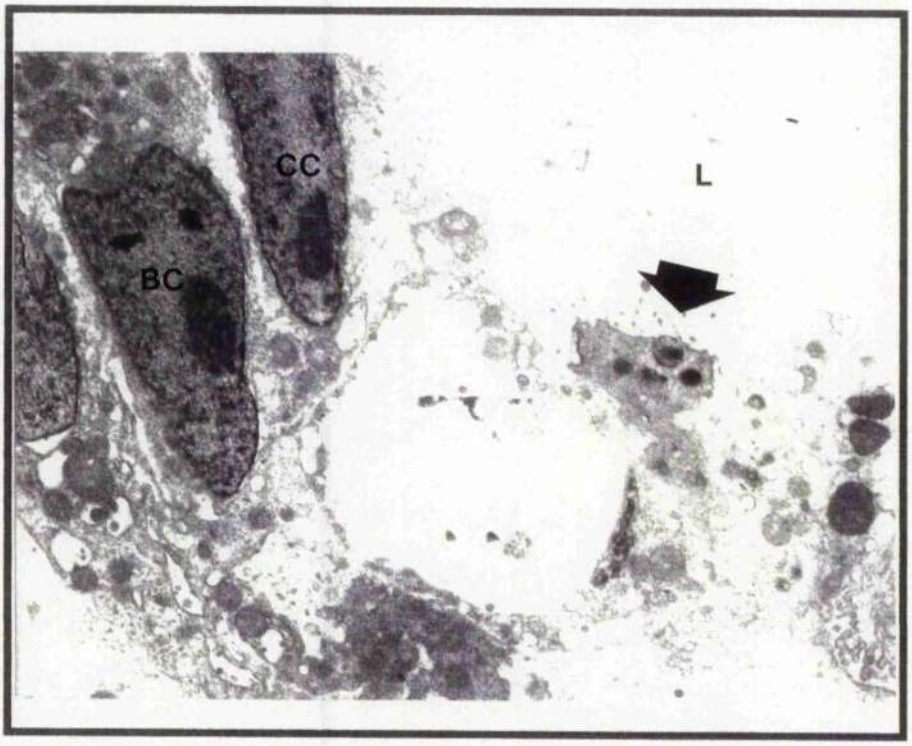
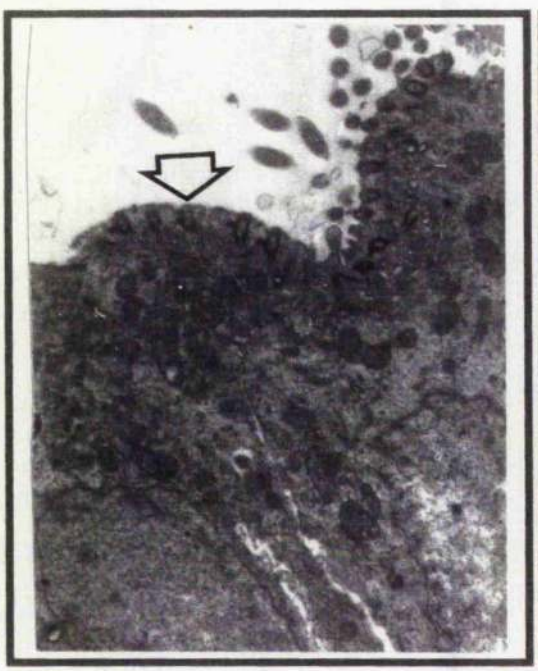

CC
L
BC

Fig. 7.19
Larynx. 1-day-old chick.
Thick mucus on the luminal surface covering the cilia. Arrow indicates clumping of the cilia and open arrow directs attention to balloon-like structures on the cilial walls.
X6,000

Fig. 7.20 (right)
Larynx. 19-day-old embryo.
Clumping of cilia. Note also the disorganisation of the microtubules (arrow).
X11,250

Fig. 7.21 (left)
Larynx. 20-day-old embryo.
Deciliation (arrow)
X9,000



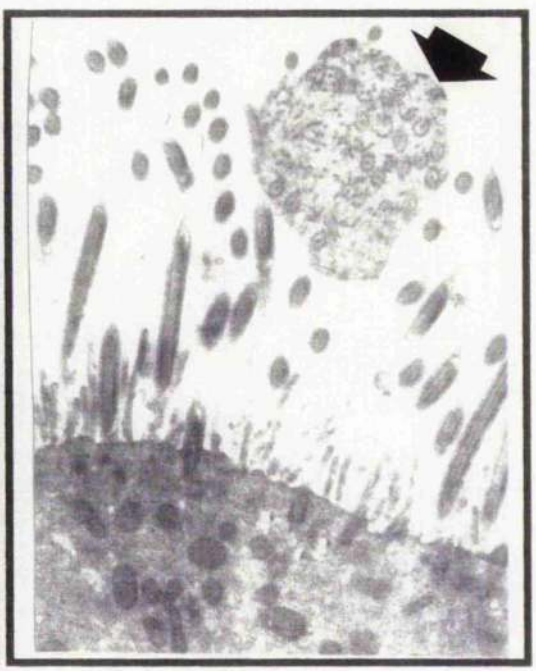


Fig. 7.22
Larynx. 20-day-old embryo.
Darkened plasmalemma at the luminal surface (arrow).
X9,000

Fig. 7.23
Larynx. 1-day-old chick.
Early stage of cell sloughing, ciliated cells are separated from the neighbouring cells. Plasmalemma breakdown (arrow). Note also balloon-like structures on the cilial walls (open arrow).
X11,2500

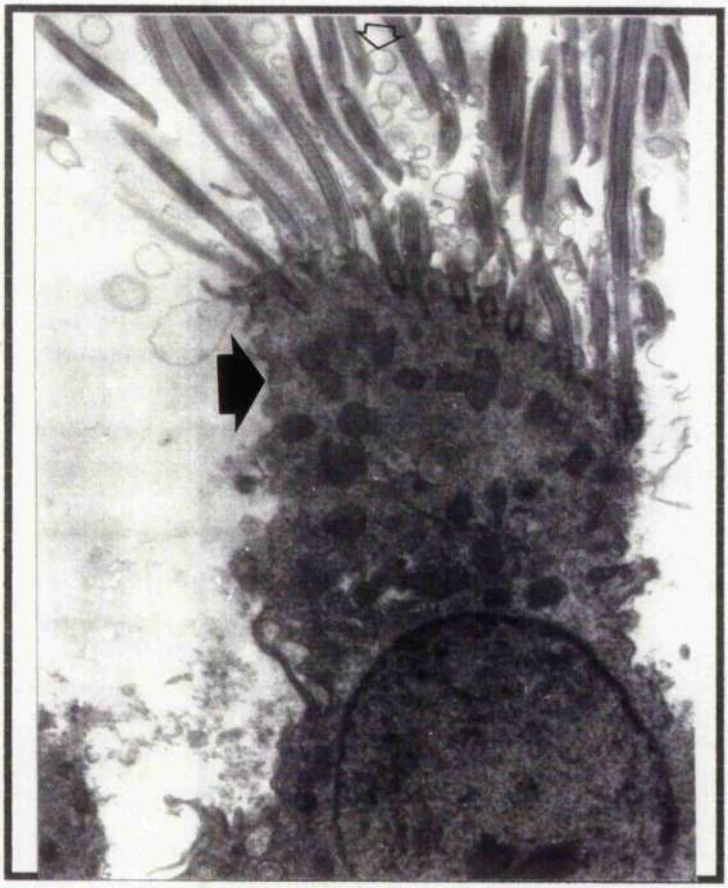
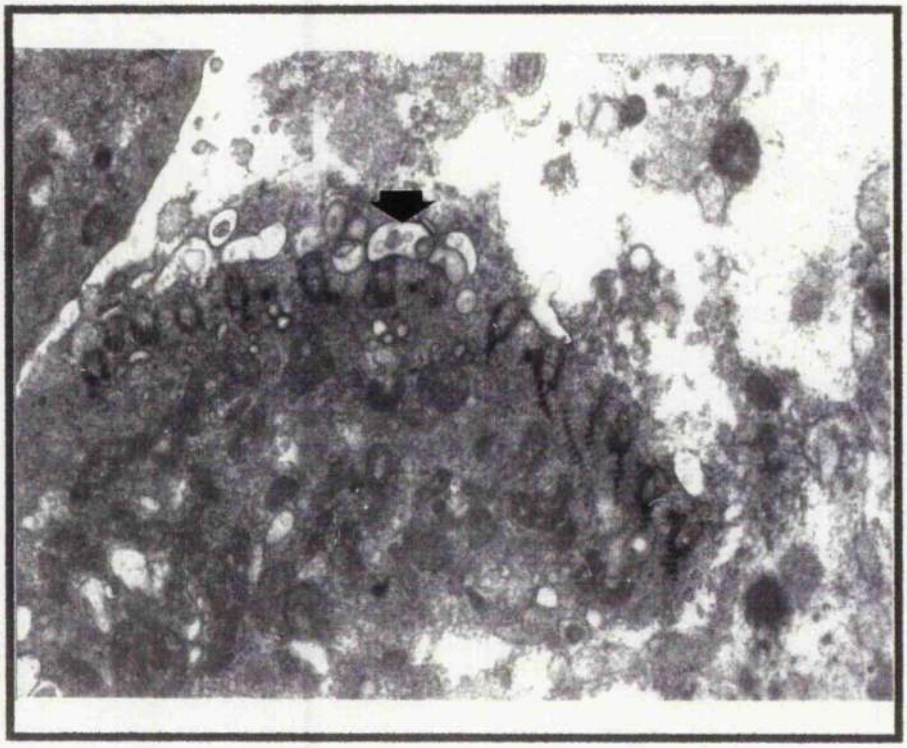


Fig. 7.24
Larynx. 20-day-old embryo.
Numerous vacuoles can be seen at the apical region of a degenerating ciliated cell (arrow).
X18,000

Fig. 7.25
Larynx. 20-day-old embryo.
Numerous vacuoles (arrow) are evident in an unidentified cell type undergoing degeneration.
X9,000



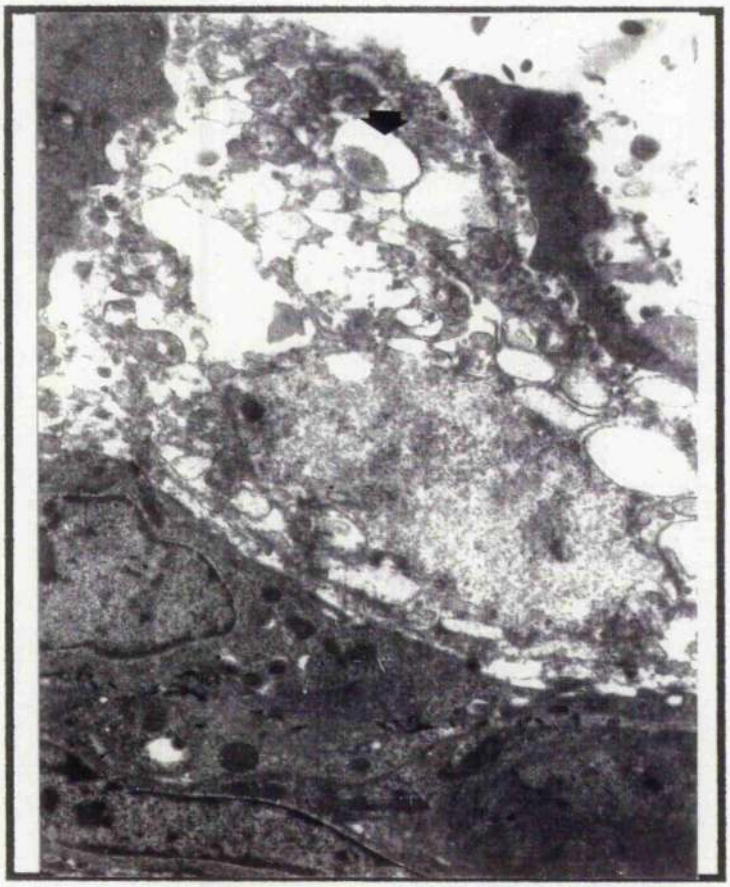
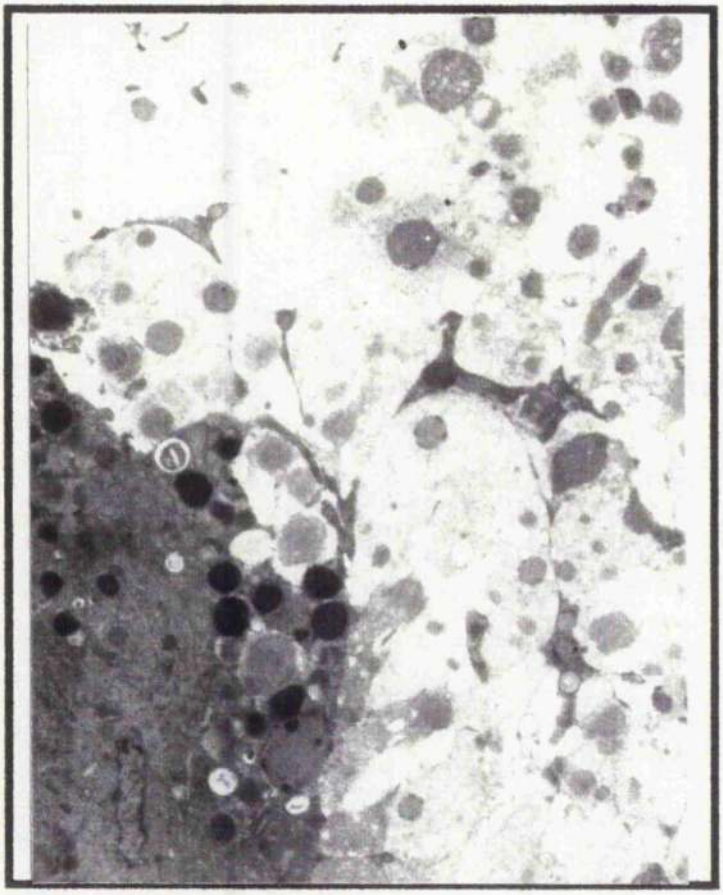


Fig. 7.26
Larynx. 1-day-old chick.
Massive release of mucous granules.
X6,000

Fig.7.27
Larynx. 1-day-old chick.
Sloughed epithelium exposing the basal cells (arrows)
Lumen (L).
-X15,000



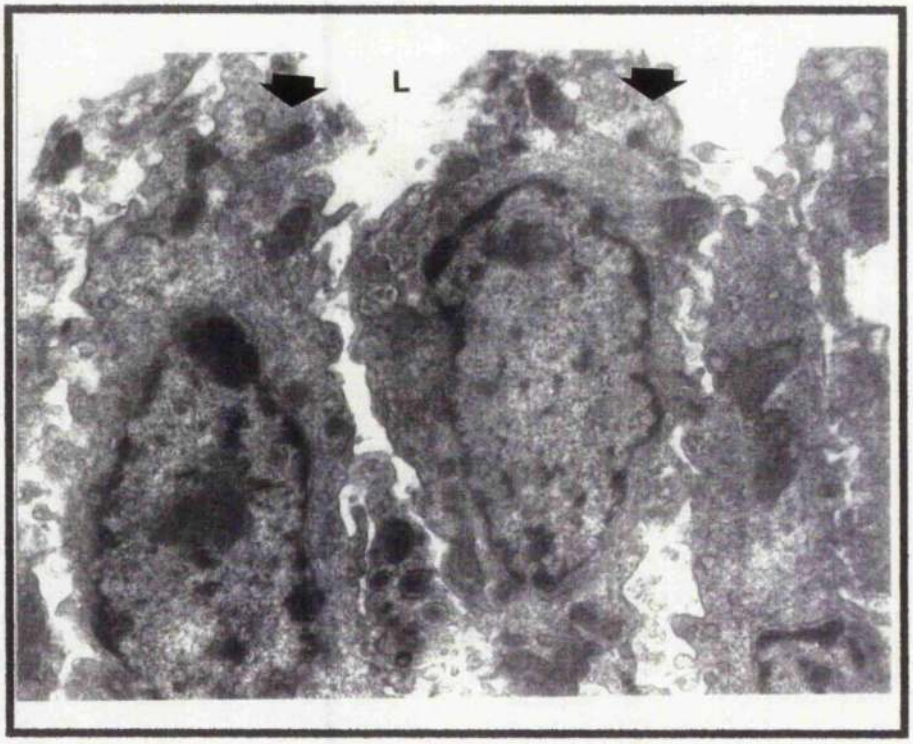
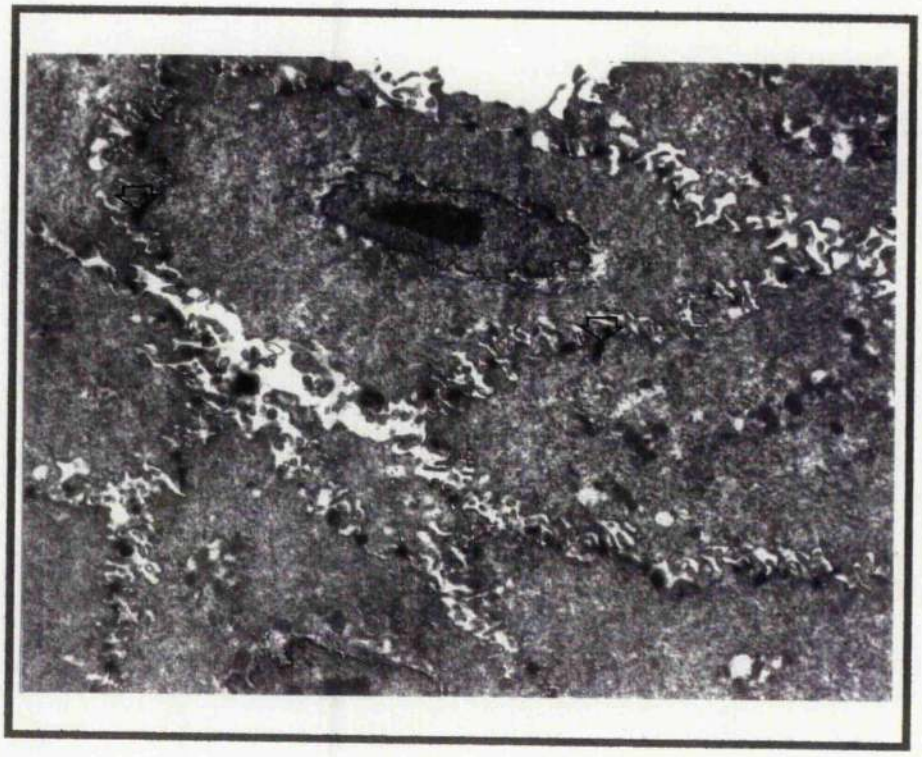

L

Fig. 7.28
Larynx. 11-day-old chick.
Squamous cell stratification in the epithelial lining of the larynx, note increase in the desmosomal attachments adjoining the cells (arrows).
X6,000

Fig. 7.29
Larynx. 7-day-old chick.
Squamous cells on the luminal surface of the larynx, note the few microvilli present at the apical surface (arrow) and the tonofilaments in the cytoplasm (open arrow). Attachment of cells is *via* numerous desmosomal attachments and interdigitation of cytoplasmic processes between neighbouring cells.
X15,000



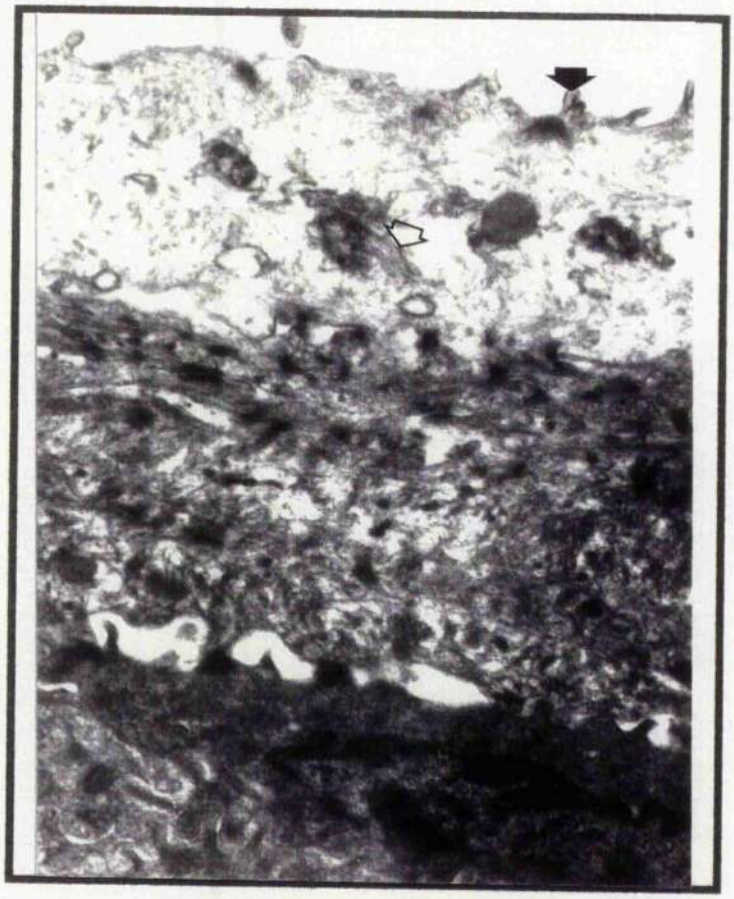


Fig. 7.30

Larynx. 11-day-old chick.

Tonofilaments (arrow) in the cytoplasm of the squamous cells in the mid-region of the epithelium. Note also the corrugated centrally located nucleus. The electron micrograph also illustrates the complex folding of the plasma membrane of adjacent cells and the large number of desmosomes which firmly anchor those cells to one another.

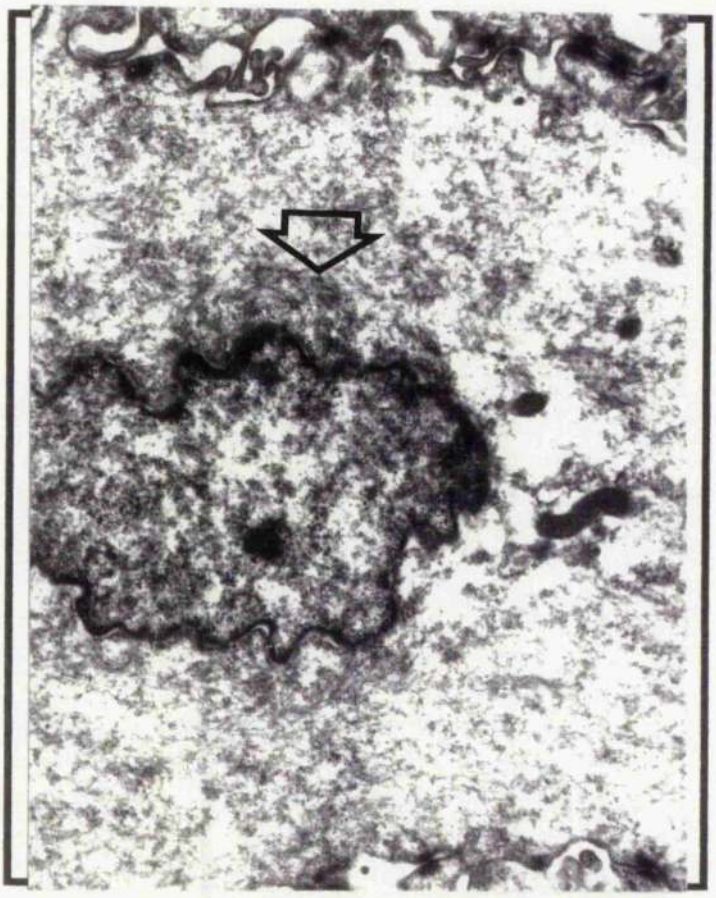
X6,700

Fig. 7.31

Larynx. 5-day-old chick.

Dark cell (arrow) at the basal region of the squamous metaplasia.

X9,000



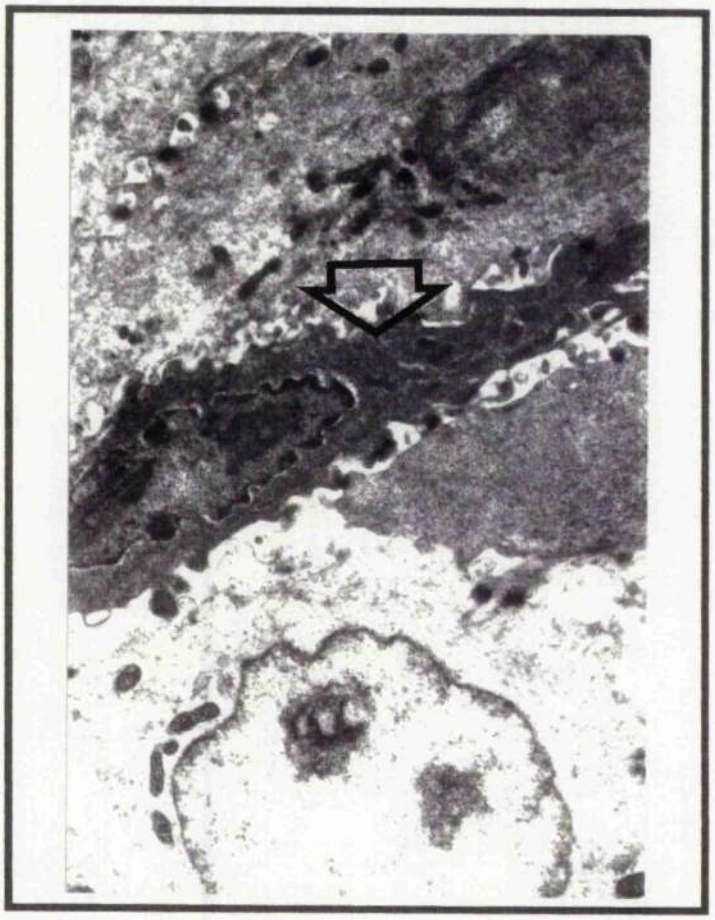


Fig. 7.32
Larynx. 13-day-old chick.
Normal organisation of the ciliated cells.
X6,000

Fig. 7.33
Trachea. 7-day-old chick.
Clumping of cilia in the epithelial lining of the trachea.
X18,000

Fig. 7.34

Trachea. 20-day-old embryo.

Pathological lesions within the cilia:

1. Breakage of the cilia
2. Balloon-like structures on the cilial walls
3. Disorganisation of the microtubules within the cilia.

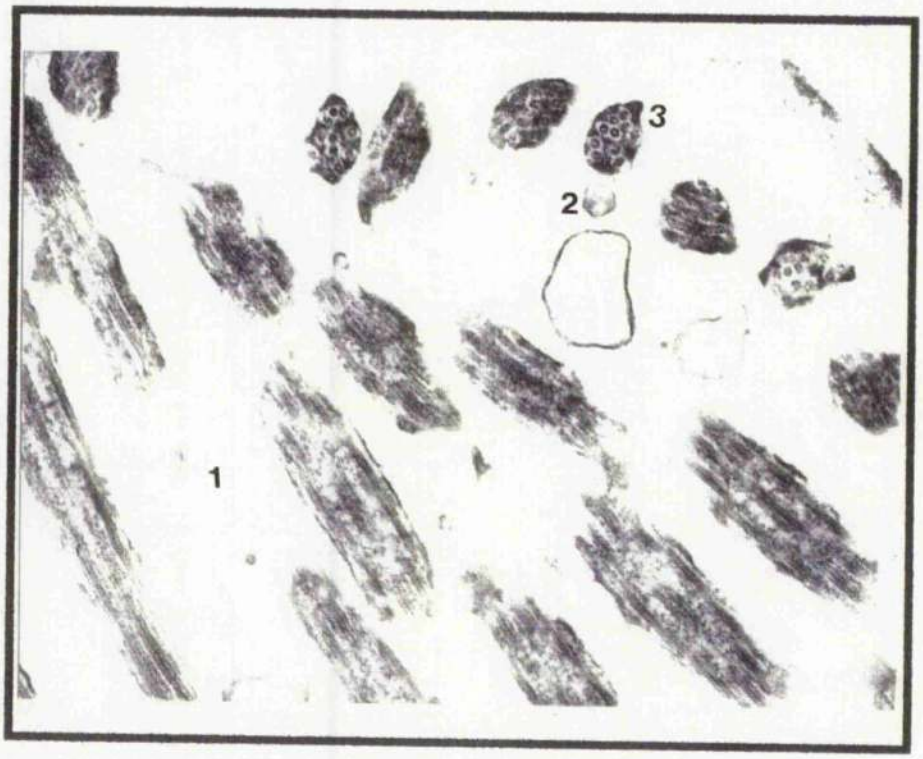
X31,500

Fig. 7.35

Trachea. 1-day-old chick.

Gaps (G) between cells and numerous vacuoles (arrow) and disintegration of intracellular structures in the epithelial cells lining the trachea.

X9,000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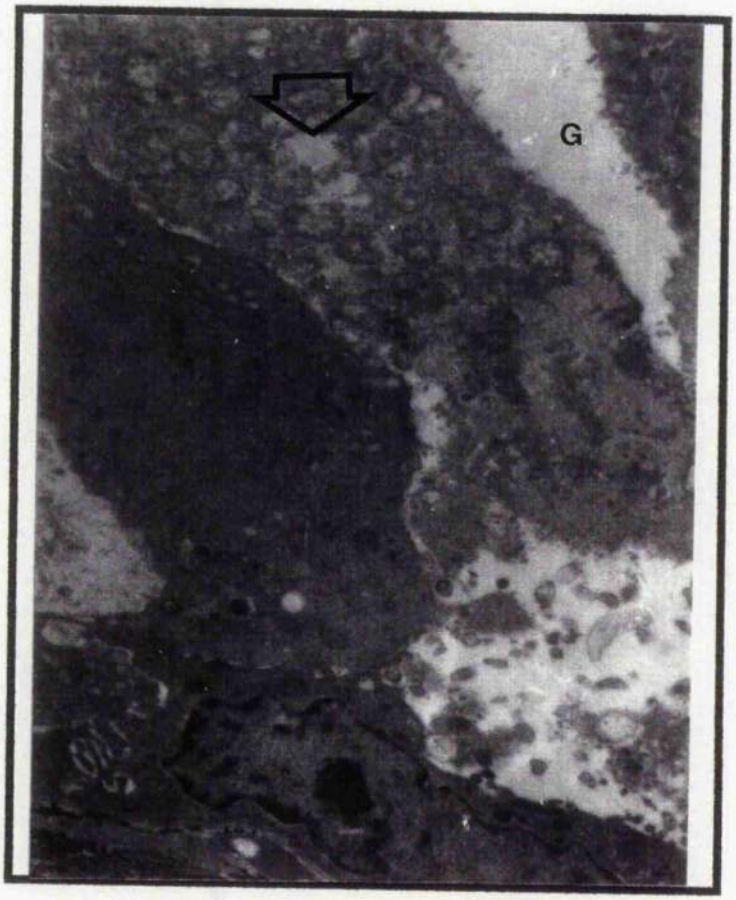
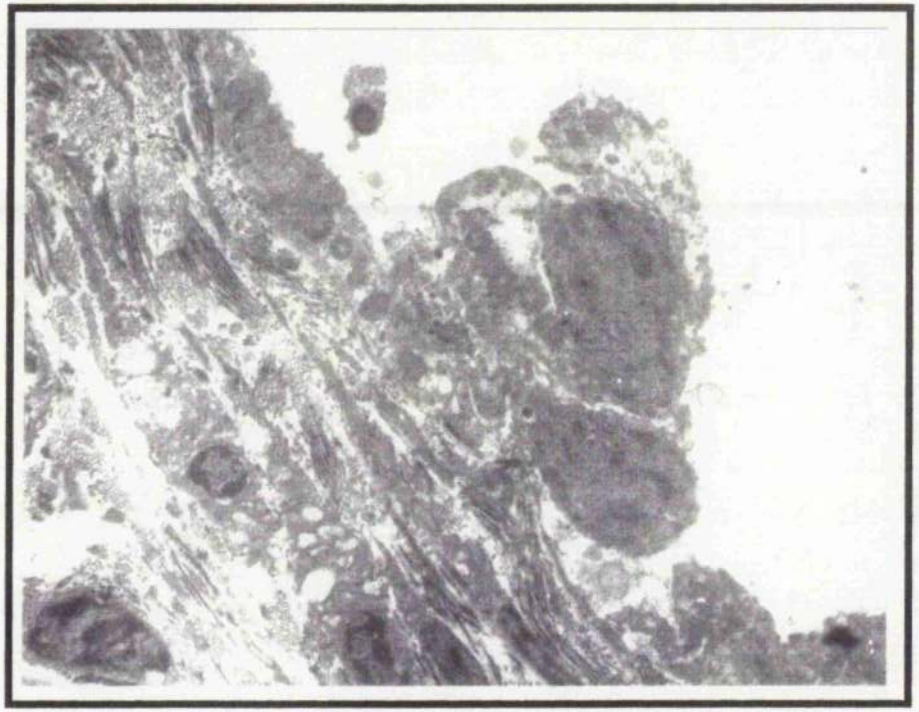

G

Fig. 7.36
Trachea. 1-day-old chick.
Two attached ciliated cells in the process of sloughing indicates a significant degree of epithelial damaged resulting from exposure to formaldehyde vapour.
X18,000

Fig. 7.37
Trachea. 1-day-old chick.
The degree of epithelial sloughing resulting from formaldehyde vapour exposure may be so severe as to expose the sub-mucosal layer.
X6,000



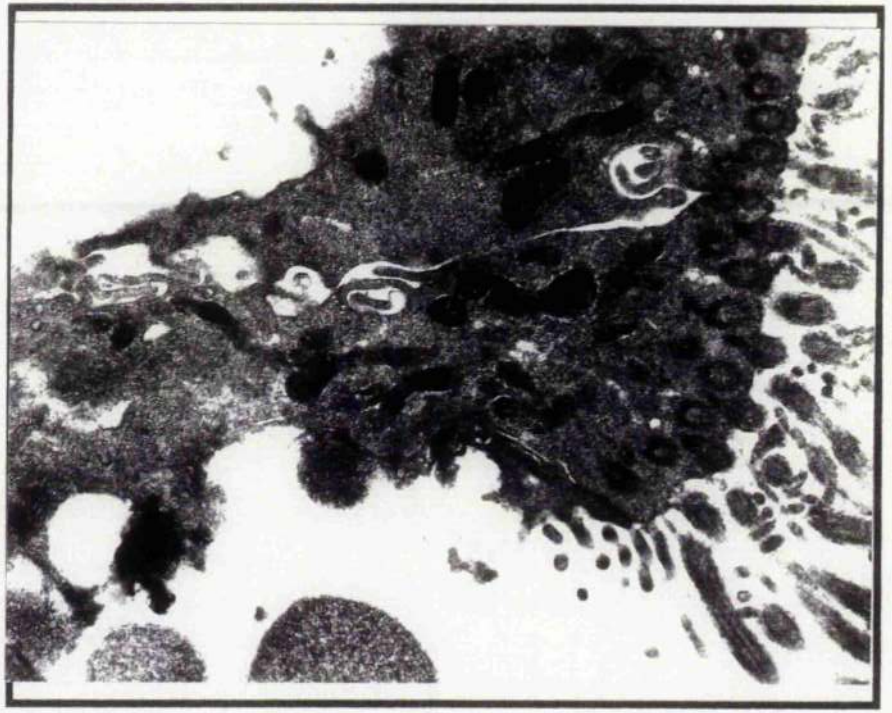


Fig. 7.38

Trachea. 20-day-old embryo.

Well dispersed mucous granules throughout the cytoplasm of apparently distended mucous cells.

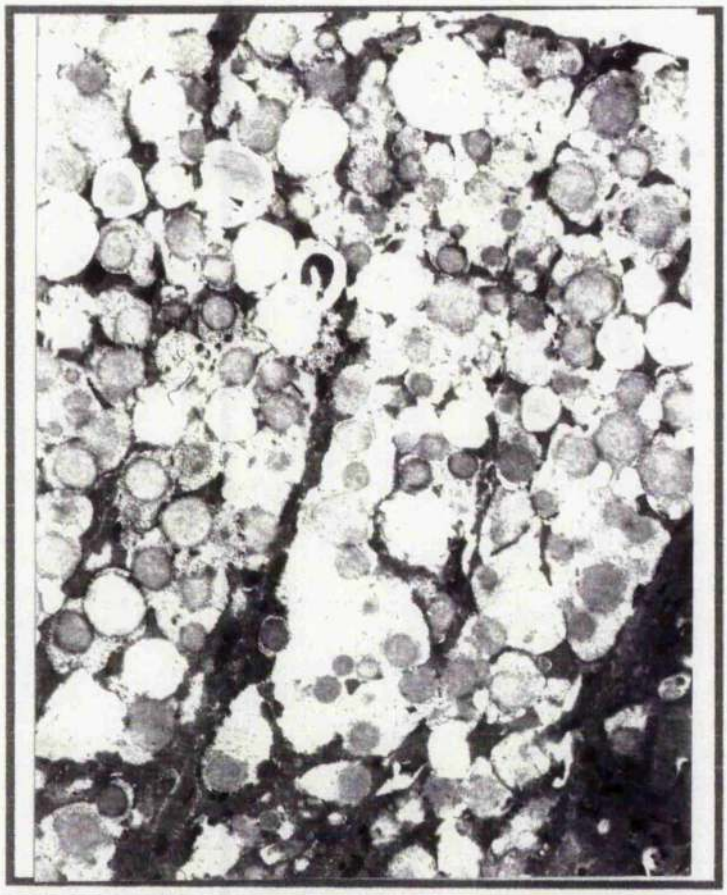
X6,000

Fig. 7.39

Trachea. 1-day-old chick.

A non-ciliated microvillous cell containing an abundance of rough endoplasmic reticulum (arrow).

X9,000



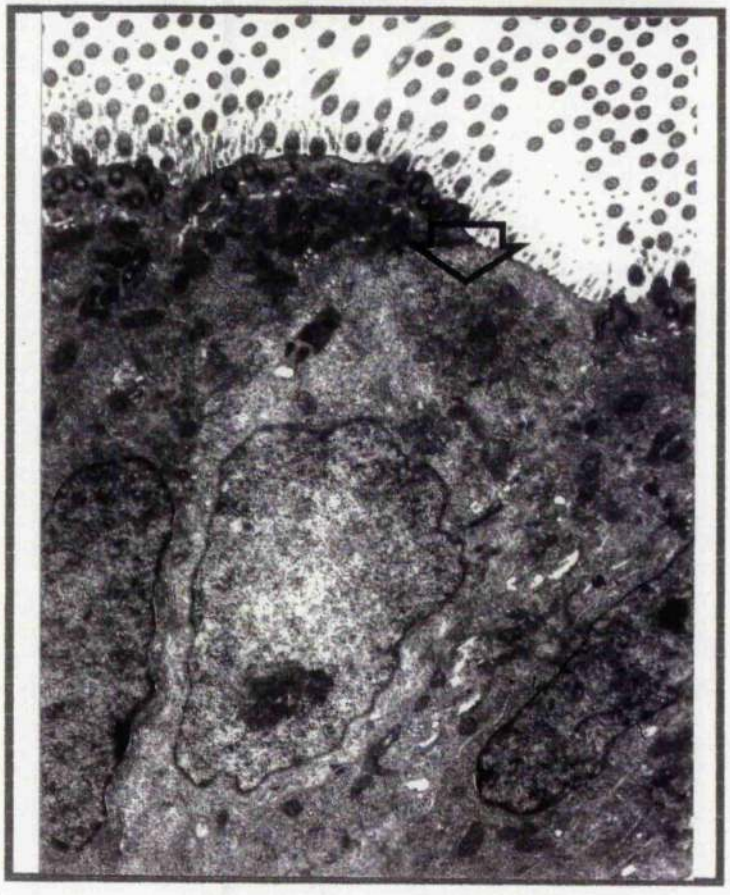


Fig.7.40

Trachea. 7-day-old chick.

A deciliated cell (arrow) illustrating its compliment of swollen mitochondria (open arrow).

X11,250

Fig. 7.41

Trachea. 11-day-old chick.

A mucous cell (arrow) with distended rough endoplasmic reticulum in the basal region of the epithelium.

X6,000

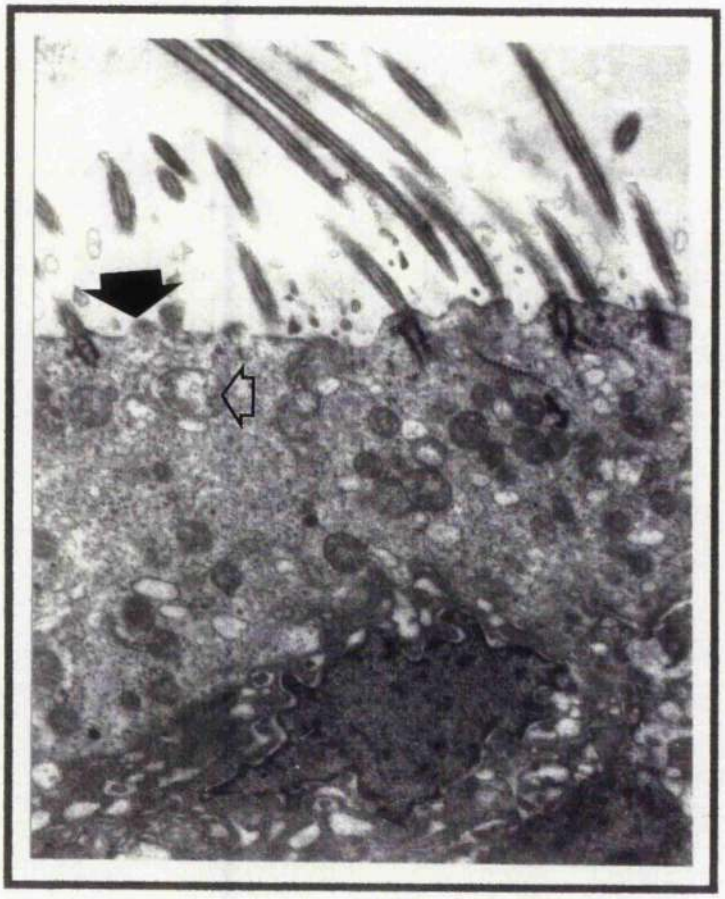
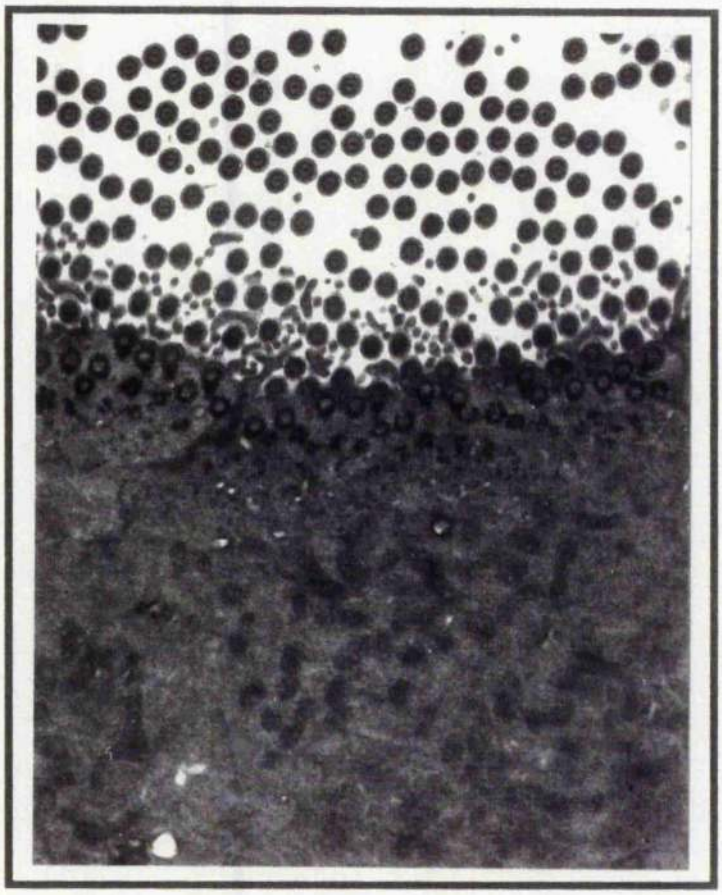


Fig. 7.42
Trachea. 29-day-old chicken.
Normal organisation of the ciliated cells.
X11,2500



DISCUSSION

In the present TEM investigation of the respiratory epithelium of chicks (from 19-day-old embryos to 43-day-old chickens) exposed to 10.9 ppm formaldehyde vapour during the last three days of incubation, the lesions and regenerative processes seen in the middle nasal concha, larynx and trachea were observed to be broadly similar, except for a few individual cases discussed separately. The most commonly observed pathological responses to such formaldehyde exposure, exhibited during the growth period (19-day-old embryo to the 22-day-old chicken) were clumping or loss of cilia and microvilli together with increased mucus production, degeneration and sloughing of the epithelium, such changes were followed by cell differentiation and regeneration, although these latter processes were not clear cut, as, areas of necrosis were often observed alongside regenerating cells. The 29, 35 and 43-day-old chickens, were seen to have a well-established normal respiratory epithelium lining the regions of the tract examined.

Effect on the mucociliary apparatus

The clumping of microvilli seen in the middle nasal concha in this study appears to be the first time such a feature has been noted in response to exposure to low concentrations of formaldehyde vapour. Together with the more commonly observed clumping of cilia, these features could be expected to produce in birds significant adverse effects on mucociliary and airway function, similar to those reported in mammals (Cralley, 1942; Dalhamn, 1956; Amdur, 1960; Swiecichowski *et al.*, 1993). Decreased ciliary movement within the respiratory epithelium exposed to formaldehyde vapour has been reported in the trachea of the rabbit (Cralley, 1942), rat (Dalhamn, 1956) and guinea pig (Amdur, 1960), in the latter case in response to concentrations of formaldehyde as low as 0.3 ppm , and for

exposure periods of one hour only. The relevance of exposure-time to observable formaldehyde-induced effects on mucociliary function has also been noted in studies on nasal mucociliary function in rats exposed to 15 ppm formaldehyde vapour, with increasing areas of mucostasis and ciliostasis being induced during longer exposure periods (6 hours) (Morgan *et al.*, 1986a, b). The increased production of mucus noted in the present study, due to increased secretory activity of the mucus producing cells, increased numbers of mucous cells, or both, could when considered alongside the observed clumping of surface cilia and microvilli, also be expected to lead to a degree of mucostasis and ciliostasis in the chick as well. Hypertrophy of goblet cells, similar to that seen in the chick in this present study, was also observed in rat respiratory epithelium in response to exposure to formaldehyde vapour (Monteiro-Riviere and Popp, 1986; Monticello *et al.*, 1991), and in cases of acute and chronic diseases of the respiratory tract in man (Ellefsen and Tos, 1972a).

Damage effects on cilia and microvilli

In the present study, direct damaging effects on surface cilia and microvilli, along with resultant loss of cilia and surface microvilli were the commonest observed as a result of exposure to the toxic effects of formaldehyde vapour. Such observations support earlier work, where deciliation of the respiratory epithelium due to the cytotoxic effect of formaldehyde vapour was seen in TEM studies in rats (Zwart *et al.*, 1988), and LM and SEM studies in the domestic chicken (Gerrits, 1990; Gerrits and Dijk, 1991; Sander *et al.*, 1995). Cilial loss was reported in light microscopic examinations of the cranial trachea of chicks exposed to 20-80 ppm formaldehyde vapour during the last three days of incubation (Gerrits, 1990; Gerrits and Dijk, 1991), whilst extensive loss of ciliation was similarly observed in scanning electron microscope studies in chicks exposed to 130

ppm formaldehyde vapour in the hatcher during the final three days of incubation (Sander *et al.*, 1995). Short cilia have been observed in a previous SEM study in the chicken (Sander *et al.*, 1995), and their presence has been reported at both LM and TEM levels in the nasal cavity of Wistar rats exposed to 3 ppm formaldehyde vapour for varying periods of time (Zwart *et al.*, 1988). Such deciliation effects appear to result from cilial breakdown due to the damaging effects of the formaldehyde vapour. The TEM observations in this study confirm such cilial breakdown, leading to the appearance of shortened cilia at the luminal surface. The balloon-like structures seen arising from the cilial and microvillous walls of all the 19 and 20-day-old embryos and 1-day-old chicks in the present study, a feature also noted in TEM observations in the nasal cavity of rats and mice (Jiang *et al.*, 1986; Monteiro-Riviere and Popp, 1986; Rautiainen *et al.*, 1992), are similar to the blebs observed in SEM studies in chicken trachea by Sander *et al.* (1995), who assumed that such structures indicated the sites of cilial weakness and thus of cilial breakage. Breakage at such sites could only result in the appearance of short cilia.

Degeneration and sloughing of epithelial cells

In the present study, epithelial cell degeneration and epithelial sloughing were also observed in responses to formaldehyde vapour. Such changes were characterised by cell separation leading eventually to vacuolar degeneration within the cell , as a prelude to epithelial cell sloughing. In cases of mild exfoliation, basal cells were usually left intact, but in cases of severe lesions, both epithelial and basal cells were exfoliated, to expose the sub-mucosal layer. Similar epithelial degeneration and sloughing have also been reported in LM and SEM studies of tracheal epithelium in newly hatched chicks exposed to 16 ppm formaldehyde vapour during the final three days of incubation (Furuta *et al.*, 1989), as well

as in the tracheal epithelium of hatching chicks to formaldehyde vapour at concentrations of 20-80 ppm (Gerrits, 1990; Gerrits and Dijk, 1991) and 130 ppm (Sander *et al.*, 1995). The degeneration and sloughing of epithelial cells due to the toxic effects of formaldehyde vapour is a common reaction and has been described in earlier reports in rats and mice (Chang *et al.*, 1983; Monticello *et al.*, 1991), and Rhesus monkeys (Monticello *et al.*, 1989). In addition, after noting that acute exposure (6 hours) to 15 ppm formaldehyde vapour caused early degeneration and sloughing of respiratory epithelial cells in the nasal cavity of rats, whilst the same treatment in mice, produced only a mild serous rhinitis and focal degeneration of the respiratory epithelium characterised by increased cytoplasmic granularity and isolated necrobiotic cells (Chang *et al.*, 1983), these authors suggested that such effects might be species related. Even exposure to relatively low concentrations of formaldehyde vapour, such as 3.2 ppm and 6 ppm, have also been shown to produce focal necrosis and sloughing of degenerated respiratory epithelium in the nasal cavity of rats (Cassee *et al.*, 1996) and the nasal cavity, trachea and bronchi of Rhesus monkeys (Monticello *et al.*, 1989) respectively.

Cell proliferation

In the present study, the exact sequence of cell proliferation, occurring in response to the observed deciliation and epithelial sloughing resulting from formaldehyde exposure, could not be traced since lesions were sampled from different individual chickens. However, it is interesting to note that proliferation of mainly mucous cells originating from the basal region of the epithelial respiratory epithelium was seen in the middle nasal concha of one 29-day-old chicken, and in the trachea of one 11-day-old chick, whilst squamous metaplasia was frequently noted in the larynx of 5-day-old chicks to 22-day-old chickens. Such observations concur with the

findings of Erjefalt *et al.* (1995), who stated that airway reepithelialisation following mechanical injury in the trachea of guinea pig started immediately and occurred rapidly through the migration of flattened secretory and ciliated cells (and presumably also basal cells) into the damaged area from the surrounding normal boundaries. The time involved in such a regenerative process appears to depend on whether the basal cells and basement membrane remain intact or not (Ohashi *et al.*, 1991). Studies of the effects of mechanical injury on the nasal mucosa in rabbits showed that regeneration was completed within 5 days of injury in cases where the basal cells and basement membrane were unaffected, but that, where the entire nasal mucosa was injured, epithelial regeneration took up to 6 weeks to be completed. The participation of the mucous cell in the histiogenesis of regenerating tracheal epithelium, as noted in the chicken in this study, provides additional evidence for the hypothesis that mucous cells and basal cells always contribute to this process, irrespective of the nature of the injury (McDowell *et al.*, 1979).

Epithelial damage due to the cytotoxic effects of formaldehyde vapour has been shown to increase the rate of surface epithelial cell proliferation in numerous species including rat (Chang *et al.*, 1983; Zwart *et al.*, 1988), mouse (Maronpot *et al.*, 1986) and monkey (Monticello *et al.*, 1989), and in xenotransplanted human nasal respiratory epithelium (Klein-Szanto *et al.*, 1989). Swenberg *et al.* (1986) demonstrated that, following acute exposure (9 days) to formaldehyde vapour, the rat nasal epithelium exhibits a nonlinear concentration-dependent increase in cell proliferation rate which correlates with nasal epithelial cell cytotoxicity and cell death. This acute proliferative response was believed necessary to replace the damaged epithelium, and to protect the airway mucosa from further insult by repairing and adapting the epithelial barrier. This proliferative response may be attributed to a formaldehyde-induced cytotoxicity resulting from the

overloading of protective mechanisms such as mucociliary clearance, metabolic detoxication, and DNA repair (Swenberg *et al.*, 1983).

Such sensitivity in the cell proliferation response has also been shown to be species specific (Rusch *et al.*, 1983); the monkey and rat were more sensitive to formaldehyde exposure than the hamster. Sensitivity of cell proliferation also appears to be related to the concentration of formaldehyde vapour, the higher the concentration the more extensive the lesions in the respiratory epithelium of mouse (Maronpot *et al.*, 1986) and rat (Monticello *et al.*, 1991). More recently, the mean cell proliferative indices of the respiratory nasal epithelium of rats exposed to the formaldehyde vapour were reported to be dependent on its concentration, the higher the concentration the more significant the mean cell proliferative indices (Cassee *et al.*, 1996).

In the present study it was interesting to note the presence of an obvious squamous metaplasia in the larynx of five birds, a feature which, it has been suggested, may save to stabilise damaged areas of respiratory epithelium and thus reduce the possibility of further continuing formaldehyde-induced necrosis (Jiang *et al.*, 1986; Contran *et al.*, 1994). Such a feature supports earlier findings in the nasal cavity (Swenberg *et al.*, 1980; Rusch *et al.*, 1983) and trachea (Klein-Szanto *et al.*, 1981) of rat, the entire respiratory tract of the mouse (Maronpot *et al.*, 1986) and nasal cavity of the monkey (Rusch *et al*, 1983). The presence of occasional dark cells in the basal region of such squamous stratification in the larynx has never been reported in Aves, but concurs with the findings of Klein-Szanto *et al.* (1981) who worked on rat trachea.

In the present study, a non-ciliated microvillous cell was observed in the trachea of 1-day-old chick, the appearance of such a cell has been reported in the normal incubating chick (Walsh and McLelland, 1978) and adult chicken (Walsh and McLelland, 1974a). However it was interesting to

also note the appearance of large amount of rough endoplasmic reticulum in the apical cytoplasm of this cell. Such an ultrastructural feature, noted as a result of formaldehyde toxicity, agrees with earlier reports on the nasal respiratory epithelium of rats (Swenberg *et al.*, 1986; Monteiro-Riviere and Popp, 1986), and may be representative of a more metabolically active cell (Swenberg *et al.*, 1983).

Cells containing both centrioles and mucous granules occasionally were seen in the respiratory epithelium in the present study. These cells have been observed in the developing tracheal epithelial lining of hamster (McDowell *et al.*, 1985), chicks (Kalnins and Porter, 1969) and African clawed toad (Steinman, 1968) and have been suggested to represent primitive, undifferentiated cells (McDowell *et al.*, 1985).

Intracytoplasmic cilia were seen at times in the ciliated cells of the middle nasal conchal epithelium in the present study. Such structures were also noted in the respiratory epithelium of rats exposed to formaldehyde vapour (Monteiro-Riviere and Popp, 1986) and Rhesus monkeys exposed to ozone (Harkema *et al.*, 1987b), as well as in the regenerating respiratory epithelium of hamsters following mechanical injury (McDowell *et al.*, 1979). In addition, the disorganisation of microtubules in the damaged cilia as observed here, has been reported in human respiratory epithelial cells during ciliogenesis (Yoshitsugu *et al.*, 1994), where it resulted in interference of the normal intracellular coordination of the ciliary beat.

Whatever the damage suffered by the lining respiratory epithelium as a result of exposure to formaldehyde vapour in the present study, regeneration of the affected epithelium appeared to take place relatively quickly. By 29 days post hatching, most birds examined demonstrated a return to the normal organisation of the epithelial lining. An increase in number of cilia was noted, with individual cilia showing a normal organisation of the 9+2 microtubular configuration. Such observations

correspond to those of Bootz and Reuter (1992), who reported complete regeneration of the respiratory epithelium in the rat within 21 days following free grafting of the nasal septum.

CHAPTER 8

RESPONSE OF THE MUCUS PRODUCING APPARATUS OF THE RESPIRATORY TRACT OF CHICKS TO EXPOSURE TO THE FORMALDEHYDE VAPOUR.

INTRODUCTION

Hyperplasia of mucous cells and hypersecretion of mucus are important characteristics of human respiratory disorders, especially chronic bronchitis and cystic fibrosis (De-Haller and Reid, 1965; Lev and Spicer, 1965; Lamb and Reid, 1972). Similar responses by the mucous-producing apparatus also occur in the respiratory epithelium of animals experimentally exposed to chemical irritants such as sulphur dioxide and tobacco smoke (Lamb and Reid, 1968; Spicer *et al.*, 1974; Jones *et al.*, 1978a). Histochemical studies of the mucous cell components have also indicated that the degree of sulphation of glycoconjugates in human airway mucous cells is increased both in chronic bronchitis and in cystic fibrosis (Lev and Spicer, 1965; Jones and Reid, 1978). In addition it has been shown that chronic bronchitis in dogs results in an increase in sialomucins of glycoconjugates in the mucous apparatus of the tracheobronchial tree (Wheeldon *et al.*, 1976), mirroring the shift in the nature of secreted mucosubstances from neutral to acidic seen in laboratory animals exposed to chemical irritants (Lamb and Reid, 1968; Spicer *et al.*, 1974; Jones *et al.*, 1978). Although there is thus a significant body of work available on the histochemistry of the mucous-producing apparatus in the respiratory tract of a wide range of mammalian species exposed to toxic gases or subject to cases of chronic bronchitis, cystic fibrosis and pneumonia, there appear to be no quantitative studies available on the mucous-producing apparatus of the respiratory epithelium of any animal species exposed to formaldehyde vapour. The objective of this study was to investigate the basic quantitative and qualitative histochemical responses of the mucous-producing

apparatus of the respiratory epithelium of chicks to exposure to formaldehyde vapour in a commercial situation.

MATERIALS AND METHODS

Source of chicks

All control (non-exposed) chicks and chicks exposed to formaldehyde vapour were obtained as detailed in Chapter 2. The number and age of chicks involved in this study are shown in Table 14:

TABLE 14

CHICKS USED FOR LIGHT MICROSCOPY IN THE INVESTIGATION OF THE EFFECT OF FORMALDEHYDE VAPOUR ON THE RESPIRATORY EPITHELIUM OF CHICKS

Age of chicks	No. of formaldehyde-exposed chicks involved in light microscopy
19-day-old embryo	3
20-day-old embryo	3
1-day-old chick	3
3-day-old chick	3
5-day-old chick	3
7-day-old chick	3
11-day-old chick	3
13-day-old chick	3

Sample collection, processing of samples for light microscopy, counting of mucous cells and intraepithelial mucous glands and photography for light microscopy.

As detailed in Chapter 5, samples from the middle nasal concha, larynx, cranial trachea, caudal trachea, intrapulmonary primary bronchus and secondary bronchus were collected, processed and stained with AB/PAS (as detailed in Chapter 2) in order to identify the individual mucous cells and intraepithelial mucous glands contributing to the mucous-producing apparatus of the respiratory tract. The results of the study of these

parameters, in the normal developing embryo and chick, as detailed in Chapter 5, were used as control (non-exposed) values in determining the comparative effects of formaldehyde vapour on the mucous-producing apparatus in this section. The qualitative assessment of mucous cells and intraepithelial mucous glands numbers was carried out as also detailed in Chapter 2.

RESULTS

Middle Nasal Concha

Figure 8.1 shows that in the middle nasal concha of the 19 and 20-day-old embryos that had been exposed to 10.9 ppm formaldehyde vapour, there was a decrease in the mean numbers of mucous cells populating the respiratory epithelial lining. However, in the middle nasal concha of chicks from 3-day-old through to 13-day-old there was a slight increase in the mean numbers of such mucous cells. A concomitant decrease in the mean numbers of intraepithelial mucous glands in the epithelial lining of the middle nasal concha of most of the chicks exposed to the formaldehyde vapour was also noted (Fig. 8.7). Histological examination also demonstrated the more frequent sloughing of the surface epithelial lining as seen in the younger chicks (19-day-old embryos to 3-day-old) (Fig. 8.13) and also albeit by subjective assessment a probable increase in the size of the intraepithelial mucous glands in the older chicks (5 to 13-day-old chicks) (Fig. 8.14).

Histochemically, there was also an apparent change in the nature of the mucosubstances stored in the mucous cells and intraepithelial mucous glands as a result of exposure to the formaldehyde vapour. The mucous cells and intraepithelial mucous glands of the middle nasal concha of all 19 and 20-day-old embryos and 1-day-old chicks stained blue (indicative of the presence of acidic (AB+) intracellular mucous granules) (Table 15). However, from 3 to 13-day-old of age, both blue (acidic) and purple (mixed)

cells and glands were distributed in the respiratory lining of the middle nasal concha. Within this group (3 and 13-day-old chicks) the cells and glands were predominantly acidic whereas the 5, 7 and 11-day-old chicks showed contrasting mixed cells and glands. A very small number of cells containing (PAS+) mucosubstance were also observed in 7-day-old chicks.

Larynx

Figure 8.2 illustrates that there was a decrease in the mean numbers of mucous cells in the larynx of 19 and 20-day-old embryos as well as day-old chicks, followed by an increase in the mean numbers of mucous cells in the 3, 5 and 7-day-old chicks. However, there seemed to be a decrease in the mean numbers of mucous cells in the 11 and 13-day-old chicks. Figure 8.8 demonstrates a consistent decrease in the distribution of intraepithelial mucous glands in the larynx of the various age group of chicks exposed to the formaldehyde vapour as compared to the larynx of control chicks. The initial decrease in the mean numbers of mucous glands appeared to be due to the sloughing of the epithelium but in the later stages it may be due to the hypertrophy of the intraepithelial mucous glands or due to the squamous metaplasia in the epithelial lining of the larynx (Fig. 8.15)

AB/PAS staining of the mucous cells and intraepithelial mucous glands in the larynx demonstrated the presence of acidic mucosubstances in the 19 and 20-day-old embryos and also in day-old chicks. The 3-day-old chicks demonstrated cells containing predominantly mixed mucosubstances (purple staining), together with a few cells containing acidic secretory (blue staining) products. Approximately equal numbers of mucous cells containing either mixed or acidic mucosubstances were observed in the 5, 7, 11 and 13-day-old chicks along with very small numbers of cells containing neutral (red staining) mucosubstances. The intraepithelial mucous glands in the 3-day-old through to the 13-day-old chicks were populated by predominantly mixed (purple staining) mucous cells together with significantly smaller

number of acidic cells.

Cranial trachea

Figure 8.3 demonstrates that there was a slight decrease in the mean numbers of mucous cells in the cranial trachea from 19-day-old embryos and day-old chicks, followed by an increase in the mean numbers of mucous cells in the 20-day-old embryos, 3-day-old through to 13-day-old chicks. However, Figure 8.9 also shows that there was a slight decrease in the mean numbers of intraepithelial mucous glands in most age groups of the embryos and chicks.

AB/PAS staining of the mucous apparatus in the lining epithelium of the cranial trachea demonstrated the presence of purely acidic mucosubstance in the mucous cells and intraepithelial mucous glands of day 19 and 20 embryos as well as the day-old chicks. The mucous cells demonstrated an approximately equal distribution of mixed secretory products in the 3-day-old to 13-day-old chicks, whilst in the 5 , 7 and 13-day-old chicks, a few mucous cells producing neutral mucosubstance. The intraepithelial mucous glands produced purely acid mucosubstance from the 19-day-old embryos to 1-day-old chicks, whilst a mixture of acidic and neutral mucosubstance was seen in the 3 to 13-day-old chicks. In the 5, 7, 11 and 13-day-old chicks the secretions were predominantly mixed (purple staining) whilst in the 3-day-old chicks, acidic (blue staining).

Caudal trachea

Figure 8.4 illustrates that the mean numbers of mucous cells in the caudal trachea was lower in the 19-day-old embryos and day-old formaldehyde-exposed chicks compared to the unexposed chicks of the same age group. However, the mean number of mucous cells was comparatively higher in the 20-day-old embryo, 3, 5, 7, 11 and 13-day-old

chicks. Figure 8.10 demonstrates the relative decrease in the number of mucous glands in all the age groups of the formaldehyde-exposed chicks.

Qualitative assessment of the secretory nature of the mucous cells showed that in the caudal trachea of 19 and 20-day-old embryos and day-old chicks all cells stained positive for the presence of acidic mucosubstance. In the 3, 5, 7, 11 and 13-day-old formaldehyde-exposed chicks, the number of such mixed cells increased, to represent about half the total number of mucous cells present, the rest of the cells still being acidic in nature. A few red cells containing neutral mucosubstances were demonstrable in the 5, 11 and 13-day-old chicks. The mucous glands demonstrated that the mucosubstance in the 19 and 20-day-old embryos and day-old chicks was purely acidic, whilst in the 3-day-old chicks, a mixture of predominantly acidic and less frequently of mixed mucosubstances were seen. However, AB/PAS staining of all the mucous glands in the 5, 7, 11 and 13-day-old chicks indicated the presence of a predominantly mixed secretory product, with only occasional acidic glands being noted.

Intrapulmonary primary bronchus

Figure 8.5 illustrates that the mean numbers of mucous cells are relatively low in the 19 and 20-day-old embryos and day-old formaldehyde-exposed chicks compared to the control chicks. However, the mean numbers of cells are relatively high in the 3, 5, 7, 11 and 13-day-old chicks. Figure 8.11 shows that there is a comparatively low mean numbers of mucous glands in the intrapulmonary primary bronchus of all age groups of formaldehyde-exposed chicks compared to the control chicks.

The mucous cells of 19 and 20-day-old embryos and day-old chicks were predominantly acidic in nature, except for the infrequent purple staining mixed mucous cells seen in the day-old chicks. In the 3 to 13-day-old chicks there was a mixture in the nature of the mucosubstance, an equal

distribution of acidic and mixed cells in the 3, 5 and 7-day-old chicks, an almost total predominance of mixed cells in the 11-day-old chicks, and a predominance of acid cells in the 13-day-old chicks. The intraepithelial mucous glands contained only acid mucosubstance in the 19 and 20-day-old embryos and day-old chicks, whilst glands in the 3-day-old chicks were predominantly acidic in nature, with only a few glands containing mixed secretory products. However, in the 5 to 11-day-old chicks, glands were predominantly mixed in nature, and in the 13-day-old embryos were predominantly acidic again.

Secondary bronchus

Figure 8.6 shows a decrease in the mean numbers of mucous cells in the 19 and 20-day-old embryos, 1, 5 and 7-day-old chicks, and a slight increase in the mean numbers of mucous cells in the 3, 11 and 13-day-old chicks. Figure 8.12 demonstrates generally there is a lower mean numbers of intraepithelial mucous glands in the secondary bronchus of all ages of formaldehyde-exposed chicks compared to the control chicks with the exception of the 20-day-old embryos and 3-day-old chicks

Histochemical studies show that the mucous cells in the secondary bronchus are acidic in nature in the 19 and 20-day-old formaldehyde-exposed embryos whereas in the formaldehyde-exposed day-old chicks, only very small numbers of mixed and neutral secretory cells begin to appear. The secondary bronchus of the 3, 5, 7, and 11-day-old chicks demonstrates the presence of approximately equal numbers of acidic and mixed secretory cells, but the cells appeared predominantly acidic in nature again in the 13-day-old chicks. The mucous glands contained mainly acidic mucosubstances in the 19 and 20-day-old embryos and day-old chicks, and 3, 11 and 13-day-old chicks, but mixed mucosubstances was evident in the secondary bronchus of 5 and 7-day-old chicks.

TABLE 15

QUALITATIVE ASSESSMENT OF MUCOSUBSTANCES IN THE RESPIRATORY TRACT OF DEVELOPING CHICKS THAT HAVE BEEN EXPOSED TO THE FORMALDEHYDE VAPOUR DURING THE THREE LAST DAYS OF INCUBATION.

Age	Colour of muco-substance	Regions in the respiratory tract											
		Middle nasal concha		Larynx		Cranial trachea		Caudal trachea		Primary bronchus		Secondary bronchus	
		C	G	C	G	C	G	C	G	C	G	C	G
19-day-old	Red	-	-	-	-	-	-	-	-	-	-	-	-
embryo	Blue	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Purple	-	-	-	-	-	-	-	-	-	-	-	-
20-day-old	Red	-	-	-	-	-	-	-	-	-	-	-	-
embryo	Blue	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Purple	-	-	-	-	-	-	-	-	-	-	-	-
1-day-old	Red	-	-	-	-	-	-	-	-	-	-	2+	-
chick	Blue	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Purple	-	-	-	-	-	-	-	-	1+	-	1+	-
3-day-old	Red	-	-	-	-	-	-	-	-	-	-	-	-
chick	Blue	3+	4+	2+	1+	3+	3+	3+	3+	3+	3+	3+	3+
	Purple	1+	-	4+	4+	3+	1+	3+	1+	3+	1+	3+	1+
5-day-old	Red	-	-	1+	-	1+	-	1+	-	-	-	-	-
chick	Blue	3+	1+	3+	1+	3+	1+	3+	1+	3+	1+	3+	1+
	Purple	4+	3+	3+	4+	3+	4+	3+	4+	3+	4+	3+	4+
7-day-old	Red	1+	-	1+	-	1+	-	-	-	-	-	-	-
chick	Blue	3+	1+	3+	1+	3+	1+	3+	1+	3+	1+	3+	1+
	Purple	3+	4+	3+	4+	3+	4+	3+	4+	3+	4+	3+	4+
11-day-old	Red	-	-	-	-	-	-	1+	-	-	-	-	-
chick	Blue	3+	1+	3+	1+	3+	1+	2+	1+	-	-	3+	4+
	Purple	3+	4+	3+	4+	3+	4+	4+	4+	4+	4+	3+	1+
13-day-old	Red	-	-	1+	-	1+	-	1+	-	1+	-	-	-
chick	Blue	4+	4+	3+	1+	3+	1+	3+	1+	4+	4+	4+	4+
	Purple	1+	1+	3+	4+	3+	4+	3+	4+	2+	1+	2+	1+

C - Cells
G - Glands

1+ - very few
2+ - few
3+ - many
4+ - very many

Fig. 8.1 Mean numbers of mucous cells (± sd) in the middle nasal concha.

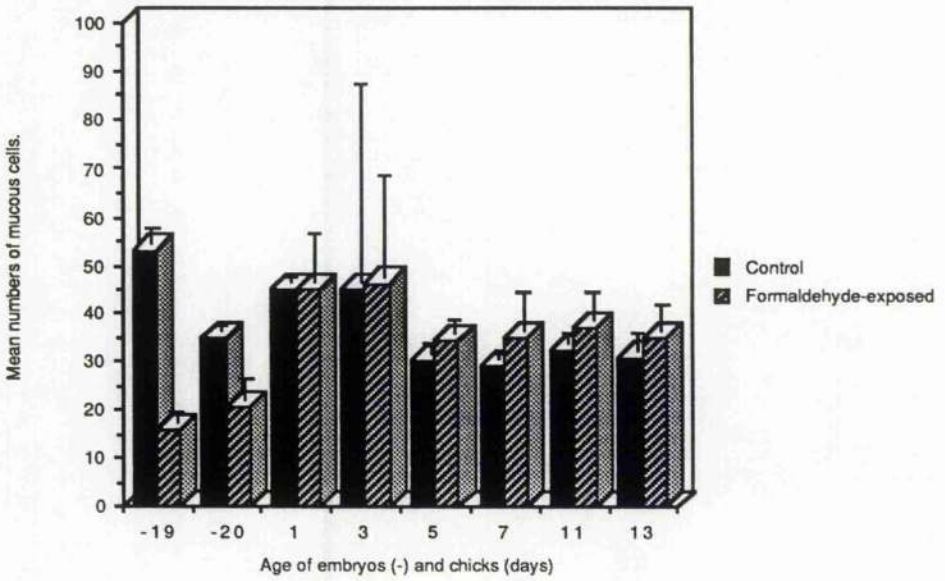


Fig. 8.2. Mean numbers of mucous cells (±sd) in the larynx.

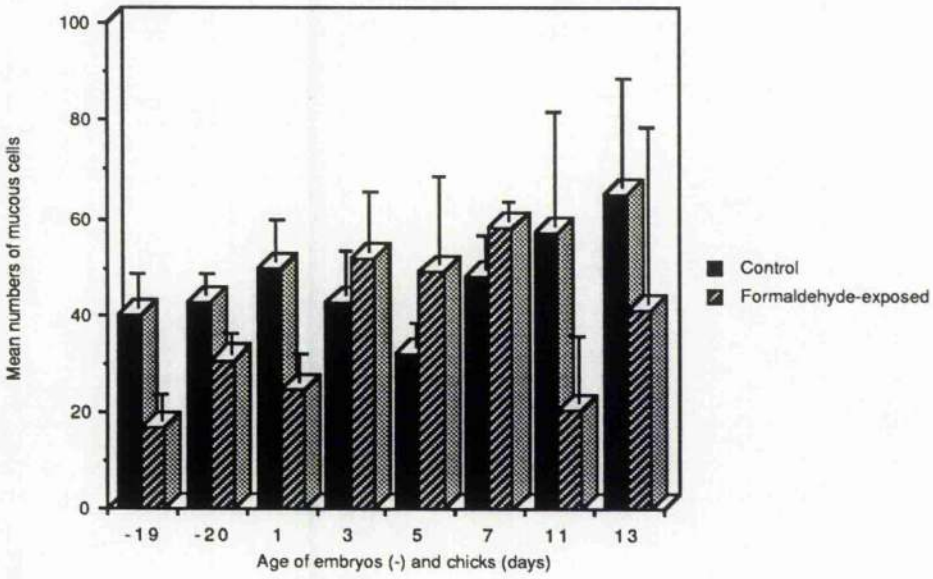


Fig. 8.3 Mean numbers of mucous cells (± sd) in the cranial trachea

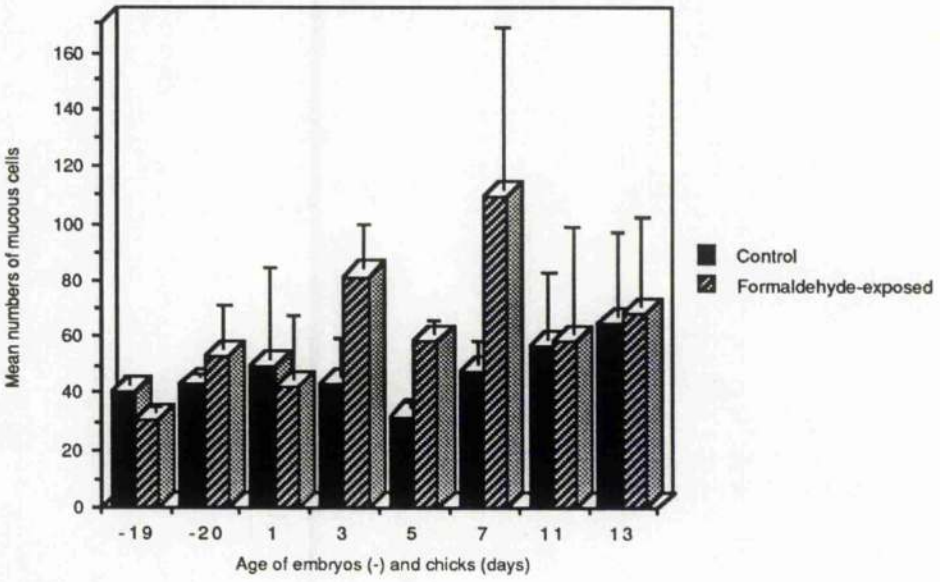


Fig. 8.4 Mean numbers of mucous cells (± sd) in the caudal trachea.

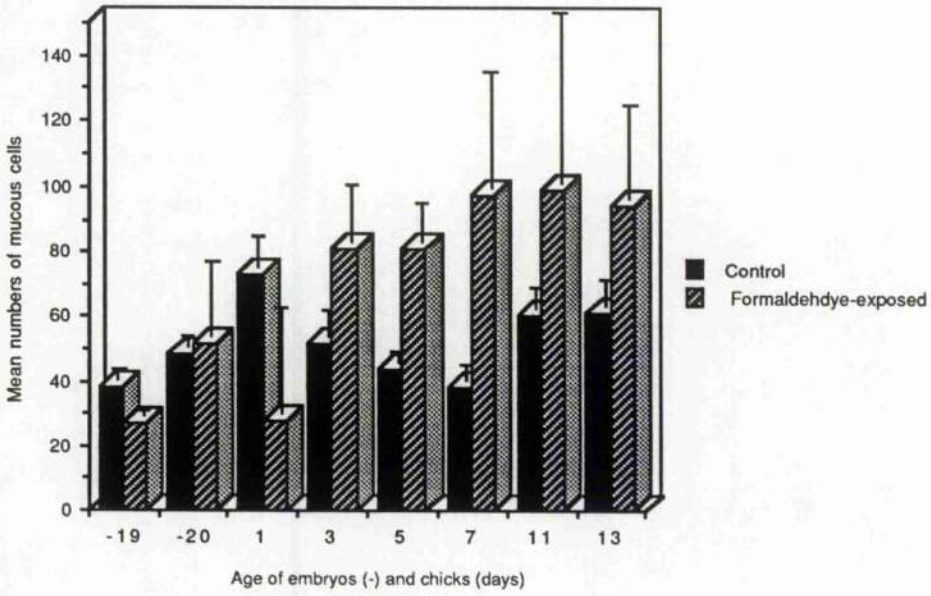


Fig.8.5 Mean numbers of mucous cells (±sd) in the primary bronchus

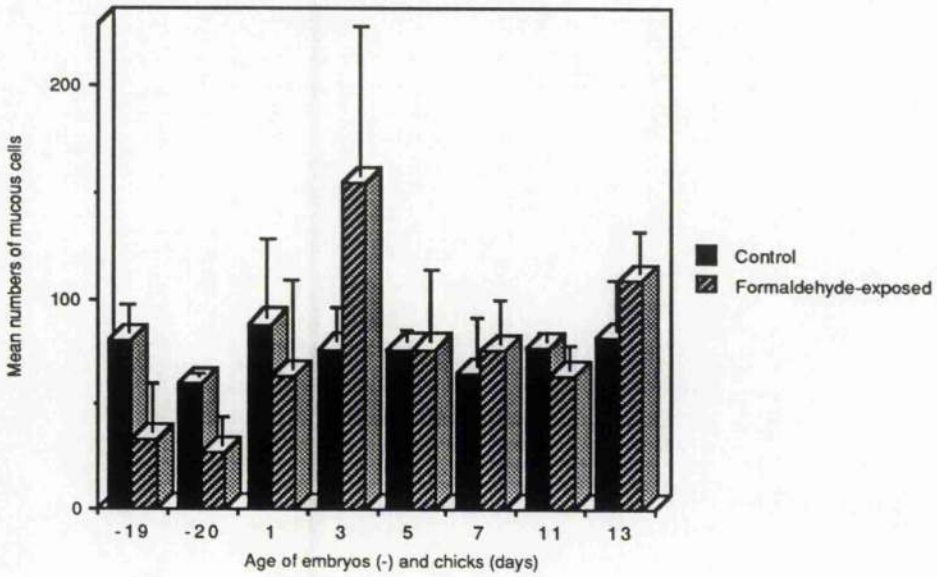


Fig. 8.6 Mean numbers of mucous cells (±sd) in the secondary bronchus

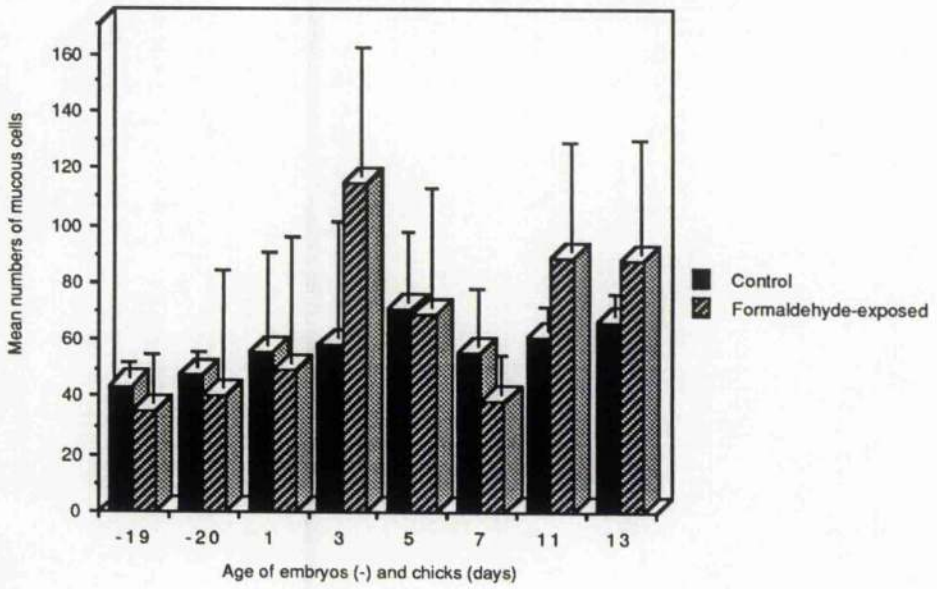


Fig. 8.7 Mean numbers of mucous glands (±sd) in the middle nasal concha

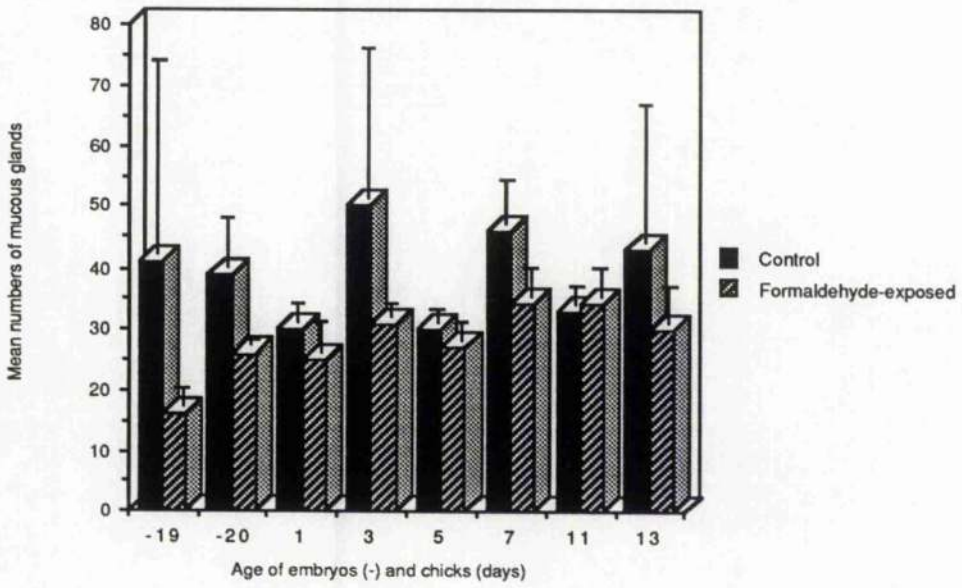


Fig. 8.8 Mean numbers of mucous glands (±sd) in the larynx

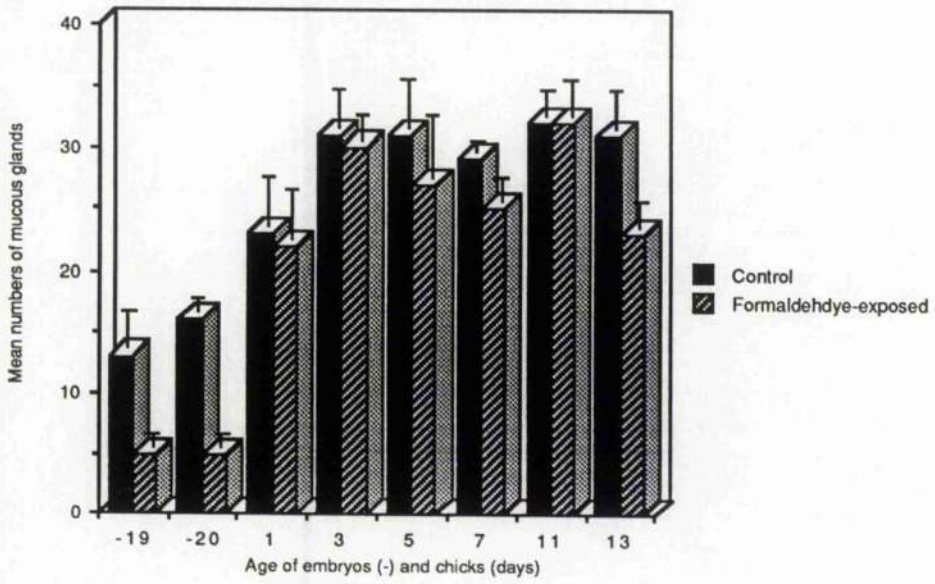


Fig. 8.9 Mean numbers of mucous glands (± sd) in the cranial trachea

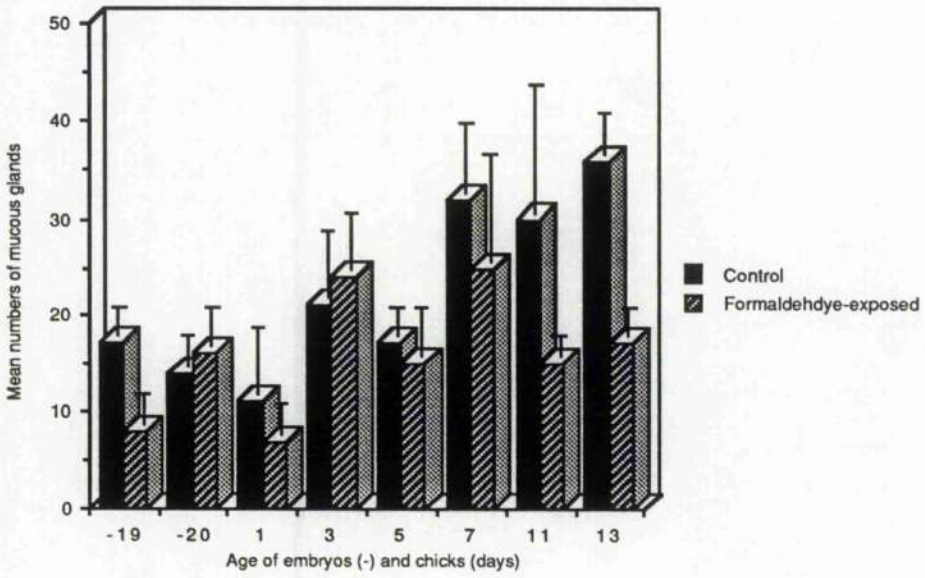


Fig. 8.10 Mean numbers of mucous glands (±sd) in the caudal trachea

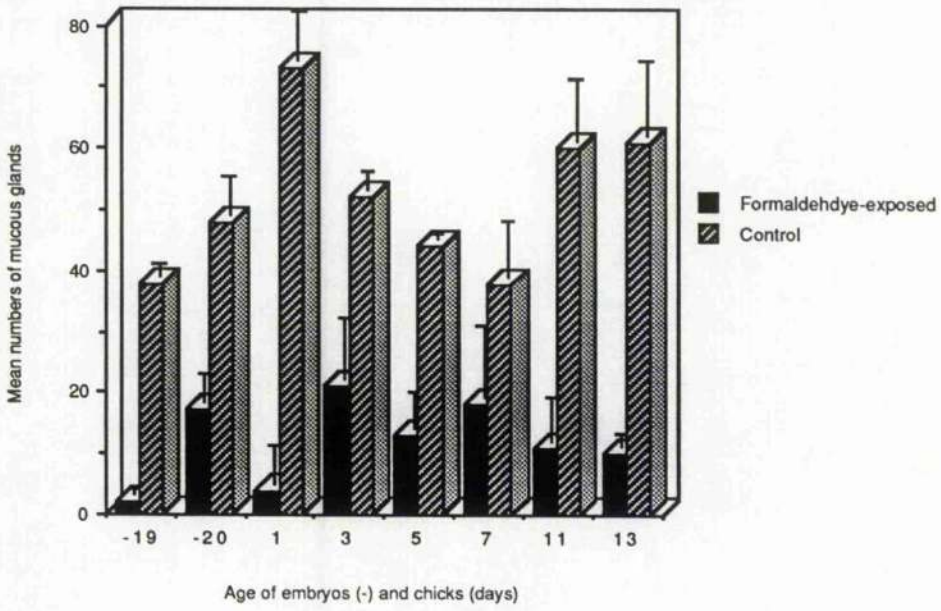
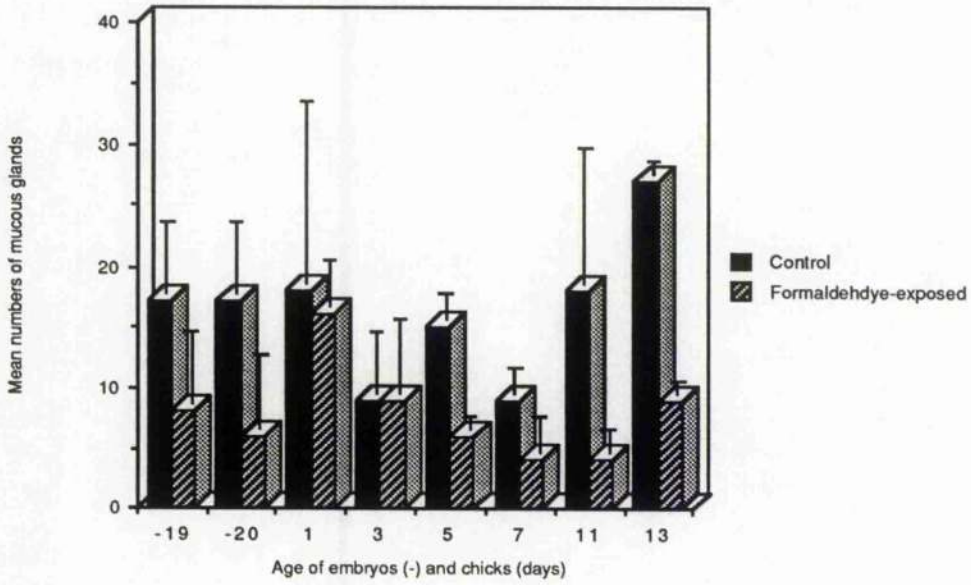


Fig. 8. 11 Mean numbers of mucous glands (± sd) in the primary bronchus



Flg. 8.12 Mean numbers of mucous glands (±sd) in the secondary bronchus

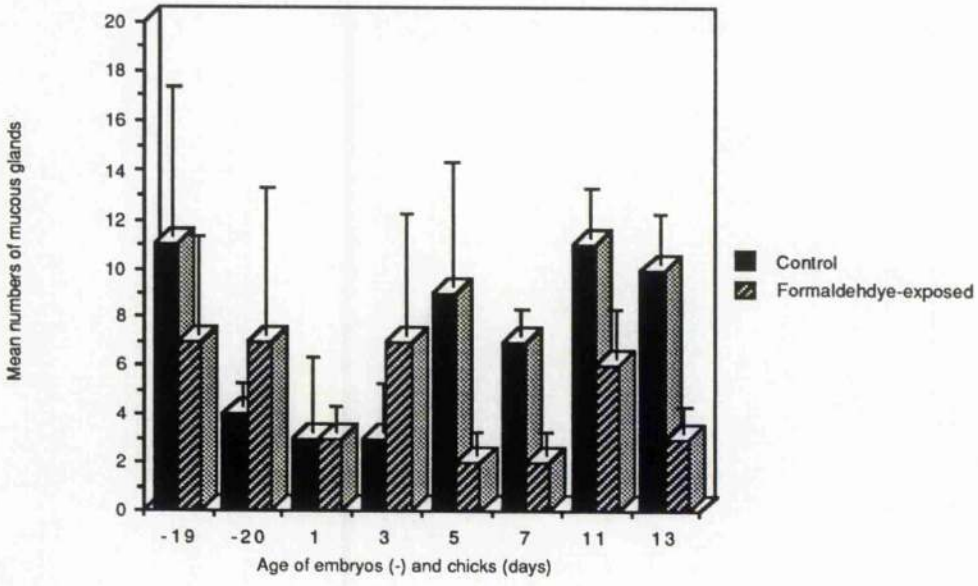


Fig. 8.13

Middle nasal concha. 1-day-old chick.

Sloughing of the epithelial lining involving the mucous cells (arrow).

X 120 AB/PAS

Fig. 8.14

Middle nasal concha. 7-day-old chick.

Appearance of enlarged intraepithelial mucous glands containing mainly mixed (light purple) mucosubstances.

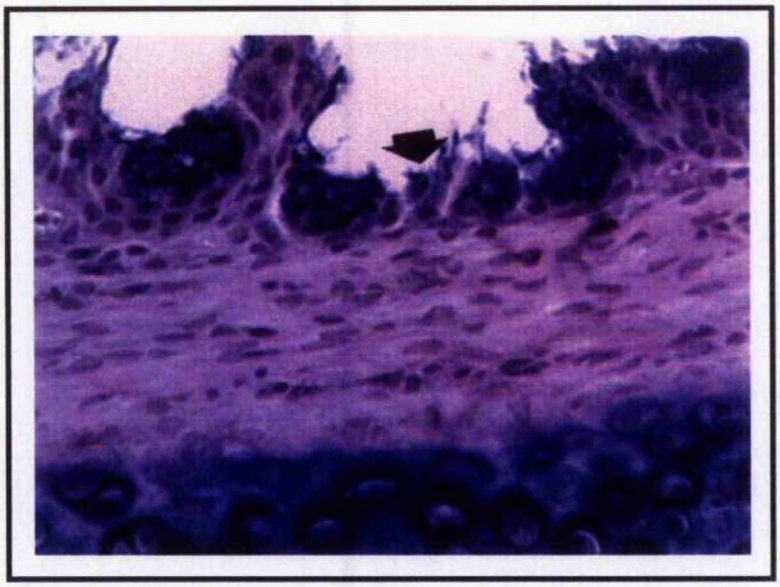
X120 AB/PAS

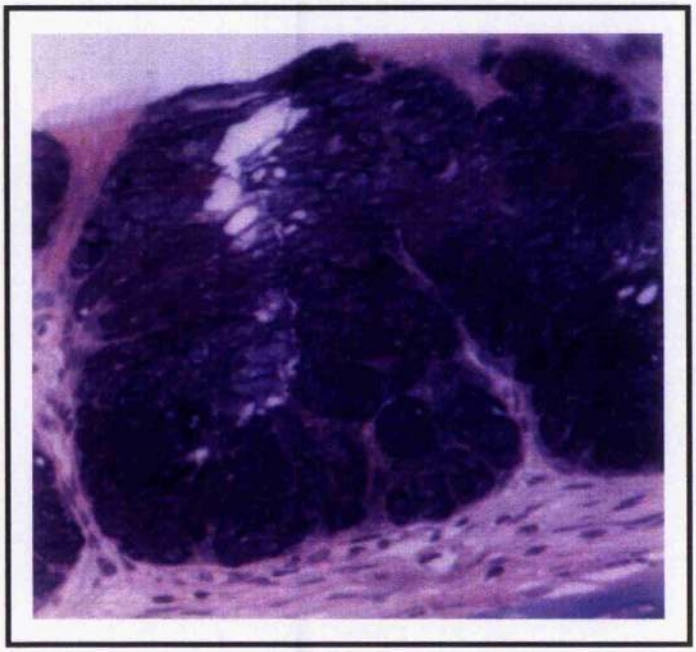
Fig. 8.15

Larynx. 5-day-old chick.

Stratification of squamous metaplasia in the epithelial lining.

X 120 AB/PAS





DISCUSSION

The purpose in undertaking this study was to provide a preliminary quantitative assessment of the distribution of mucous cells and intraepithelial mucous glands within the lining epithelium of the respiratory tract of formaldehyde-exposed chicks from 19-day-old embryos through to 13-day-old chicks, and to investigate the basic histochemical nature of the secretory products of such structures.

There appears to be a common pattern to the quantitative assessment of the mucous cells in the respiratory tract of the formaldehyde-exposed chicks, with an initial slight decrease in the mean numbers of mucous cells throughout the tract in the 19-day-old embryos through to the 1-day-old chicks. This appears to be the first time such an apparent initial decrease in the mean numbers of mucous cells has been associated with the irritant effect of formaldehyde vapour in either bird or mammal. Such observations are in agreement with those observed in the initial stages of cases of acute pulmonary diseases such as pneumonia, bronchopneumonia and pleuropneumonia and lung cancer in man (Ellefsen and Tos, 1972a) and an initial exposure to tobacco smoke in man (Jones *et al.*, 1978), in which it has been suggested that the apparent decrease in the number of demonstrable mucous cells is due to the overwhelming discharge of glycoprotein leading to fewer cells left containing secretory products which can be stained and thus identified. The possibility of such an explanation applying to the present observations is discussed in Chapter 9.

This initial decrease in mucous cell numbers appeared to be followed by an increase in the mean numbers of mucous cells throughout the respiratory tract in the 3-day-old through to 13-day-old chicks. It is interesting to note that these mucous cells are particularly sensitive to exposure to the formaldehyde vapour and that they increase in number under such

conditions. Such findings are in agreement with previous studies on the response of the mammalian respiratory epithelium to exposure to irritant gases such as cigarette smoke in the nasal cavity of rats (Lamb and Reid, 1969; Walker, 1983) and sulphur dioxide and cigarette smoke in the lower airways of rats and dogs (Jones *et al.*, 1978; Mawdesley-Thomas and Healey, 1973; Lamb and Reid, 1968). Mucous cell numbers have also been noted to increase in clinical cases such as enzootic pneumonia produced experimentally by infection with *Mycoplasma hyorhinis* (Jones *et al.*, 1975), cuffing pneumonia in calves due to *Mycoplasma dispar* (Allan *et al.*, 1977) and chronic bronchitis or cystic fibrosis in man (Reid, 1960; Sturgess and Imrie, 1982). Although the present investigation appears to provide the first available study detailing an increase in mucous cell numbers in the avian respiratory tract presumably in response to stress induced by exposure to formaldehyde vapour, an increase in mucous cell numbers has been reported in the oviduct epithelium of the domestic fowl under other stress conditions (Watt, 1989). The present study also noted a consistent decrease in the mean number of intraepithelial mucous glands throughout the respiratory tract in all age groups of chicks examined. The initial decrease in the number of mucous glands could be due to the sloughing of the mucous glands along with the epithelial lining or excessive secretion of the mucus. However, in the older chicks it may be the result of hypertrophy of the glands resulting in fewer numbers in the pre-selected length of the respiratory lining epithelium examined. Such observations are similar to the quantitative study of respiratory epithelial lining in man which indicated a decrease in the number of glands due to the presence of more hypertrophied glands in smokers (Ryder *et al.*, 1971; Kollerstrom *et al.*, 1977).

The present study demonstrates an identifiable shift in the nature of the secretory products produced by the mucus secreting apparatus of the

respiratory lining in the chick. In control chicks of the 19 and 20-day-old embryos and 1-day-old chick groups, the apparatus produces a mixture of predominantly acidic and less frequently mixed mucosubstances (documented in Chapter 5); in the formaldehyde-exposed birds in these age groups, however, the secretory products are purely acidic in nature. The presence of purely acidic mucosubstances as a result of exposure to formaldehyde, as described here for the first time in the bird supports similar observations in the respiratory tract of rats and dogs exposed to sulphur dioxide or cigarette smoke (Lamb and Reid, 1968; Spicer *et al.*, 1974; Jones *et al.*,1978) and monkeys subjected to long-term exposure to ozone (Harkema *et al.*, 1978b). In these cases a significant shift was observed in the acidic glycoconjugates from sialomucins to sulphomucins. Similar shifts in the nature of the mucoid secretions of the respiratory tract have also been reported in cases of chronic bronchitis in dogs where sialomucin was predominantly detected (Wheeldon *et al.*, 1976) and in calves with lesions of cuffing pneumonia due to infection of *Mycoplasma dispar*, where a switch from neutral to acidic mucoid secretory products in the form of both increased sulphomucins and sialomucins was reported (Allan *et al.*, 1977). The only reported case of an increase in acidic mucosubstance in bird as a result of increased stress appears to deal with the reproductive tract of domestic fowl (Watt, 1989).

In the present study, generally a consistent alteration in the nature of the mucosubstance was seen throughout the respiratory tract; 3-day-old chicks demonstrated predominantly acid rather than mixed mucosubstance, however, between 5 and 13-day-old chicks the situation was reversed, to a mainly mixed mucosubstance. When compared to the mucosubstance of normal chicks not exposed to the formaldehyde vapour, although a mixture of the mucosubstance was seen in the older chicks, acid mucosubstance predominated throughout the respiratory tract. The comparative results on

the histochemistry indicate that the nature of the mucosubstance does not revert to the normal condition until approximately 2 weeks after exposure to formaldehyde vapour. The present results confirm previous studies in that the reversibility of the type of mucosubstance was not immediate, the change in the type of intracellular mucus persisted at least 5 weeks after exposure to sulphur dioxide (Lamb and Reid, 1968).

It is not clear from this study whether changes in the chemical nature of the secretion from the non-ciliated microvillous cells lining the surface epithelium or cell breakdown due to the formaldehyde vapour (surface epithelium and mucous apparatus) are cause and effect, but it is conceivable that the release of a more acidic secretion onto the surface of the respiratory tract would cause tissue damage in the same way as hyper-activity in man causes ulceration of the gut (Dadoo *et al.*, 1995).

CHAPTER 9

SUMMARY AND CONCLUSIONS

The use of formaldehyde fumigation in the commercial poultry industry to effectively reduce the numbers of potential pathogens in the hatchery environment is common practice. And yet, despite reports that high concentrations of formaldehyde vapour in simulated experiments can induce significant damage to the tracheal epithelium in incubating chicks, the effects of relatively low concentrations of formaldehyde vapour employed in commercial situation on the respiratory epithelium of the developing chick have not been studied. Any attempt to understand and assess the nature and progression of the possible potential pathological effects of formaldehyde vapour on the respiratory epithelium of hatching chicks can only be successfully undertaken if a basic knowledge of the normal morphological features of the developing respiratory tract of chicks is acquired.

A review of the available established literature showed that studies on the adult respiratory epithelium, have focused mainly on a relatively wide range of mammalian species, such as rat, mouse, hamster, guinea pig, rabbit, coyote, ferret, monkey, dog, cat, ox, sheep, goat, pig, horse and man, whilst relatively few birds have been examined, such as chicken, goose, penguin, budgerigar, Ringed turtle dove, quail, duck, pigeon, Indian dove and turkey. Likewise, literature on the developing respiratory epithelium was more available in a wide range of mammalian species, such as rat, rabbit, dog, man and hamster, as compared to only the domestic chicken in the avian species. Most of these studies have employed the use of SEM to define the surface morphology of localised areas of the developing respiratory epithelium, or TEM to further characterise the cell types within the developing epithelial lining. The apparent lack of available information

concerning epithelial morphology throughout the developing respiratory tract of chicks determined that the primary objective of the first section of this study was to investigate the surface morphological structure of this epithelium and further characterise the cell types present in the latter. In addition, since the action of secreted epithelial mucus and cilia together constitute an important defense mechanism in the respiratory tract, light microscopy combined with AB/PAS staining was used to further investigate the distribution and nature of the mucosubstances present in the surface mucous cells and intraepithelial mucous glands of the developing respiratory epithelium. Having established the morphology of the normal developing respiratory epithelium, and also the distribution and nature of the mucosubstances involved with the mucociliary defense mechanism, the second section of this study dealt with both the effects of formaldehyde vapour on the epithelial lining of the entire developing respiratory tract of broiler chicks and the regeneration of a normal respiratory epithelium after such formaldehyde damage. This appears to be the first time such an investigation on the entire respiratory epithelium has been undertaken.

The surface morphology of the developing respiratory epithelial lining from 15-day-old embryos to 3-day-old chicks was studied as detailed in Chapter 3. Eighteen embryos and six post-hatched chicks were used and euthanised, and samples removed and processed as detailed in the procedures described in Chapter 2. It was established that in 15 to 16-day-old embryos, a non-ciliated microvillous cell type populated the entire respiratory epithelium from the middle nasal concha to the tertiary bronchus, with the exception of the intrapulmonary primary bronchus which presented a well developed mature, heavily ciliated epithelial lining. The secondary bronchus was seen to develop such a mature ciliated epithelial lining by the 17th day of incubation, whilst the middle nasal concha, larynx and trachea also presented a similar lining epithelium between the 19th and 20th day of

incubation. The tertiary bronchus was compacted by non-ciliated microvillous cells in the 15 to 16-day-old embryos; such non-ciliated microvillous cells flattened by the 17th day of incubation, and the presence of atria, infundibula and surfactant was noted.

The SEM studies detailed in Chapter 3 provided a basis for the characterisation of the cell types lining the developing respiratory epithelium by means of TEM, the results of this study being detailed in Chapter 4. Twenty-four chicks, 18 embryos and 6 post-hatched chicks, were used in this particular study, with samples from the middle nasal concha down to the tertiary bronchus being examined. In the 15 to 16-day-old embryos, the middle nasal concha, larynx, trachea and tertiary bronchus were seen to be lined by a two cell thick layer of undifferentiated cells, whilst the intrapulmonary primary bronchus had a well differentiated ciliated epithelial lining containing occasional granular endocrine cells. In the middle nasal concha, larynx and trachea of these embryos the upper cell layers were lined by differentiating ciliated and mucous cells whilst the lower cell layers were composed of undifferentiated cells in the 15 to 16-day-old embryos. At this stage contact between adjacent cells in the upper layer was by means of tight junctions, a few desmosomal contacts and short interdigitating of cytoplasmic processes; intercellular spaces were particularly noticeable at this stage. In the 17-day-old embryos to 3-day-old hatching chicks, ciliated, mucous and basal cells, and intraepithelial mucous glands, were differentiated and recognised in the middle nasal concha, larynx and trachea along with the rare appearance of an intermediate cell type in the larynx. Intercellular contacts were more established, with tight junctions at the luminal surfaces, more desmosomal contacts and relatively longer cytoplasmic interdigitations. Tertiary bronchi were represented by cord-like structures lined by columnar cells and surrounded by undifferentiated mesenchymal cells in the 15-day-old embryo, with lumina appearing in the

tertiary bronchus of the 17-day-old embryos where developing atrial cells were recognised by the presence of multivesicular bodies, Golgi apparatus, numerous vesicles, a few mitochondria and scattered rough endoplasmic reticulum. By the 18th day of incubation outpouchings of the tertiary bronchus were observed, these leading to the formation of atria and infundibula along with developing blood and air capillaries. Two types of inclusion body were seen in the atrial cells of 18-day-old embryos to 3-day-old post-hatched chicks; *viz* immature electron dense bodies and mature lamellated osmiophilic inclusion bodies. A trilaminar surfactant, released from the mature osmiophilic inclusion bodies, was seen in the atria and air capillaries of 18-day-old embryos onwards, the amount increasing in the post-hatched chicks.

In Chapter 5, AB/PAS staining was employed to investigate the presence and distribution of mucous cells and intraepithelial mucous glands as well as the nature of the mucosubstance in the developing respiratory epithelium from 15-day-old embryos through to 13-day-old chicks. 18 embryos and 18 post-hatched chicks were used in this study. Mucous cells were present in the middle nasal concha down to the secondary bronchus as early as in the 15-day-old embryo, mucous granules being first seen in the apical region of these cells and then increasing in numbers to fill up the mucous cells until eventually distending them. Developing mucous glands were first observed in the 17-day-old embryo, being distributed throughout the respiratory epithelium from the middle nasal concha through to the secondary bronchus. Both the developed mucous cells and intraepithelial mucous glands were well-developed in 19-day-old embryos. Neutral mucous cells were seen only in the 15 to 16-day-old embryos. Both the mucous cells and intraepithelial mucous glands from 18-day-old embryos to 13-day-old chicks demonstrated the presence of mixed mucosubstances, although acid mucosubstances were predominant. A cranio-caudal

increase in the numbers of mucous cells, which showed a peak in the intrapulmonary primary bronchus, together with a cranio-caudal decrease in intraepithelial mucous gland numbers, these being highest in the middle nasal concha and lowest in the secondary bronchus, was first seen in the 1-day-old chicks and persisted in all age groups of chicks. It was established that the mucociliary system of the respiratory epithelium of the chick was fully developed and established throughout the tract by the time the chick hatches.

The SEM study in Chapter 6 provides, a study of the effects of low concentrations (10.9 ppm) of formaldehyde vapour on the surface morphology of the entire respiratory epithelium of hatching chicks. Seventy eight broilers, consisting of eighteen embryos and sixty post-hatched birds, were used in this study, the results reported in two sections. Lesion scoring resulting from such formaldehyde exposure was determined with reference to the severity of lesions graded from 0 to 6 based on the degree of observed pathological change. Such changes included clumping of cilia, blebs at the surface of the cilia with or without focal deciliation, multifocal deciliation, large areas of deciliation, focal desquamation of the epithelium, multifocal desquamation of the epithelium and desquamation of large areas of the epithelium. In section 1, chicks exposed to the formaldehyde vapour for three different exposure times, i.e. 6 hours (19-day-old embryos), 30 hours (20-day-old embryos) and 54 hours (1-day-old chicks), showed that resultant lesions in the respiratory epithelium from the middle nasal concha through to the secondary bronchus were most severe in the longer exposure group (54 hours), and that there were no regional differences in the severity or nature of lesions irrespective of the different exposure times. In section 2, which involved an investigation of the longevity of the lesions produced by exposure to formaldehyde vapour together with regeneration of the epithelium; the mean lesion scores in 3-day-old to 13-day-old chicks

were seen to be similar, and predominantly involved clumping of cilia, varying degrees of deciliation and relatively mild exfoliation of the epithelium. From day 13 to day 29 post-hatching, the mean lesion scores decreased, indicating a regeneration of the damaged epithelium. In the 35 and 43-day-old chickens, a normal surface organisation of the respiratory epithelium was observed, revealing a complete regeneration of the epithelium.

Chapter 7 was undertaken to investigate the TEM effects of formaldehyde vapour on the respiratory epithelium of developing chicks, to augment the SEM observations detailed in Chapter 6. Thirty broilers, aged from 19-day-old embryos to 43-day-old chickens, were used in the study. The lesions and the regenerative processes seen in the middle nasal concha, larynx and trachea were observed to be similar. The most common pathological features seen were clumping of cilia and microvilli, increase in mucus production, the presence of balloon-like structures on the cilial and microvillous surfaces, loss of cilia and microvilli, degeneration and sloughing of the epithelium. Cell differentiation and regeneration, when observed, was not clear cut, with many cases exhibiting the co-existence of necrotic cells and regenerating cells. The ultrastructural features seen under TEM confirmed and extended the findings using the SEM. Some features such as clumping of microvilli, the presence of balloon-like structures on the microvillous walls and sloughing of microvilli not noticeable by SEM were well presented under TEM. The appearance of apparent short cilia seen at the luminal surface of the ciliated cells under TEM, was supported and reinforced by the three dimensional short cilia seen under SEM. The severity of sloughing of the epithelium also confirmed findings presented in Chapter 6, with TEM also clearly demonstrating whether such sloughing involved only the epithelium or the entire mucosal surface. Ultrastructural features of the cytoplasmic organelles of both degenerative and regenerative epithelial

cells were characterised by the use of TEM, with pathological ultrastructural features due to formaldehyde vapour exposure being seen in 19-day-old embryos through to 22-day-old chickens, and normal epithelial cells seen in the respiratory tract of 29, 35 and 43-day-old chickens.

In Chapter 8, AB/PAS staining was used to demonstrate and differentiate the distribution and nature of the mucosubstance in the mucous apparatus of respiratory tract after being exposed to 10.9 ppm formaldehyde vapour before hatching. Six embryos and eighteen post-hatched chicks were used in this study. Generally, there appeared to be an initial slight decrease in the mean numbers of mucous cells from the middle nasal concha, larynx, cranial trachea, caudal trachea, intrapulmonary primary bronchus and the secondary bronchus in the 19-day-old embryos through to the 1-day-old chicks. It was thought that this could have been due either to the destructive effect of the formaldehyde vapour or to excessive mucus secretion as confirmed by the SEM and TEM observations detailed in Chapters 6 and 7 respectively. In contrast, there was a general increase in the mean numbers of mucous cells throughout the respiratory tract in the 3-day-old through to 13-day-old post-hatched chicks. Such an increase in the density of the mucous cells would appear to increase the secretory capacity of the mucous membrane, and thus contribute to the excessive accumulation of mucus on the luminal surface of the respiratory lining. A consistent decrease in the mean numbers of intraepithelial mucous glands throughout the respiratory tract in all the pre- and post-hatched chicks could be due to the sloughing of the mucous glands, to excessive mucus secretion resulting in empty mucous cells, or to the hypertrophy of the glands resulting in lower numbers present in the pre-selected length of the respiratory lining epithelium examined. The result of the study also suggested that there was a shift in the nature of the mucosubstance in the mucus-secreting apparatus, the pattern of such alteration being similar throughout the respiratory tract

from the middle nasal concha down to the secondary bronchus. All the mucous cells and intraepithelial mucous glands appeared to contain purely acidic mucosubstances in the 19 and 20-day-old embryos and 1-day-old chicks. In the 3-day-old chick to the 13-day-old chick, the nature of the mucosubstance was seen to become mixed in nature. Future work is needed to determine when the mucosubstance reverts to its normal nature.

The combined SEM, TEM and LM studies described in Chapters 6, 7 and 8 respectively have provided, for the first time, a detailed morphological account of the effects of formaldehyde vapour exposure on the entire respiratory epithelium of the pre- and post-hatched chick in a commercial studies, such results being interpreted with reference to the comparative baseline studies of normal respiratory epithelial morphology detailed in Chapters 3, 4 and 5. The former exhibited a range of pathological effects to such exposure varying from relatively mild disorganisation of the surface cilial pattern to major degrees of epithelial sloughing. In addition, mucus production was also seen to be affected, with increased mucus output and changes in the nature of such secreted mucosubstances being observed. All these changes could reasonably be assumed to affect the mucociliary clearance mechanisms functioning within the lining epithelium of the entire respiratory tract of the pre- and post-hatched chick. Such effects would appear to last until about the fourth week post-hatching, when regeneration of the lining epithelium appeared to be completed. The severity of such lesions being related to time of exposure.

As seen in this study, day-old chicks that hatched in the commercial hatchery practising formaldehyde fumigation, presented an impaired respiratory epithelial lining. Given the damage to the respiratory epithelium of the post-hatched chick noted in this study, therefore, it is interesting to speculate whether or not vaccination of such chicks on 1 day of age, against such diseases as Infectious bronchitis and Newcastle disease, would

exacerbate respiratory tract damage and thus increase the likelihood of chronic ascitic development, as suggested by Lister (1996).

APPENDIX A

MEAN TOTAL NUMBERS (± SD) OF MUCOUS CELLS IN A PRE-SELECTED LENGTH (2.76 mm) AT DIFFERENT LEVELS OF THE RESPIRATORY TRACT IN THE DEVELOPING CHICK

	Mean no. of mucous cells in the respiratory tract					
Age	Middle nasal concha	Larynx	Cranial trachea	Caudal trachea	Primary bronchus	Secondary bronchus
15-day-old embryo	36 ± 5	25 ± 1	23 ± 6	36 ± 1	39 ± 4	26 ± 4
16-day-old embryo	29 ±16	23 ± 11	36 ± 6	32 ± 3	69 ± 8	43 ± 22
17-day-old embryo	58 ± 14	55 ± 5	62 ± 7	66 ± 12	83 ± 14	51 ± 7
18-day-old embryo	47 ± 11	65 ± 16	63 ± 16	71 ± 2	91 ± 40	63 ± 6
19-day-old embryo	53 ± 3	40 ± 7	45 ± 3	38 ± 3	80 ± 13	44 ± 6
20-day-old embryo	35 ± 1	43 ± 4	44 ± 1	48 ± 3	59 ± 1	48 ± 5
1-day-old chick	45 ± 1	50 ± 8	63±32	73 ± 9	88 ± 37	56 ± 32
3-day-old chick	31±3	43 ± 9	51 ± 14	52 ±7	76 ± 16	59 ± 39
5-day-old chick	30 ± 2	32 ± 5	39 ± 2	44 ± 3	76 ± 5	71 ± 24
7-day-old chick	29 ± 1	48 ± 7	35 ± 7	38 ± 5	65 ± 22	56 ± 20
11-day-old chick	32 ± 2	57 ± 23	67 ± 23	60 ± 7	77 ± 2	61 ± 8
13-day-old chick	31 ± 3	65 ± 22	57 ± 29	61 ± 8	81 ± 24	66 ± 8

APPENDIX B

MEAN TOTAL NUMBER (± SD) OF INTRAEPITHELIAL MUCOUS GLANDS IN A PRE-SELECTED LENGTH (2.76 mm) AT DIFFERENT LEVELS OF RESPIRATORY TRACT OF DEVELOPING CHICKS

	Mean no. of mucous glands in the respiratory tract					
Age	Middle nasal concha	Larynx	Cranial trachea	Caudal trachea	Primary bronchus	Secondary bronchus
15-day-old embryo	0	0	0	0	0	0
16-day-old embryo	0	0	0	0	0	0
17-day-old embryo	17 ± 11	12 ± 6	9±2	8 ± 5	19 ± 4	4 ± 1
18-day-old embryo	27 ± 3	10± 8	6 ± 2	8 ± 3	13 ± 3	5 ± 4
19-day-old embryo	41 ± 2	13± 3	17 ± 3	19 ± 2	17 ± 6	11 ± 6
20-day-old embryo	39 ± 8	16 ± 1	14 ± 3	15 ± 6	17 ± 6	4 ± 1
1-day-old chick	30 ± 3	23 ± 4	11 ± 7	11 ± 8	18 ± 15	3 ± 3
3-day-old chick	50 ± 25	31 ± 3	21 ± 7	13 ± 3	9 ± 5	3 ± 2
5-day-old chick	30 ± 2	31 ± 4	17 ± 3	21 ± 1	15 ± 2	9 ± 5
7-day-old chick	46 ± 7	29 ± 1	32 ± 7	25 ± 9	9 ± 2	7 ± 1
11-day-old chick	33 ± 3	32 ± 2	30 ± 13	32 ± 10	18 ± 11	11 ± 2
13-day-old chick	43 ± 23	31 ± 3	36 ± 4	38 ± 12	27 ± 1	10 ± 2

APPENDIX C

AVERAGED LESION SCORE (± SD) OF THE RESPIRATORY EPITHELIUM OF CHICKS EXPOSED TO THE FORMALDEHYDE VAPOUR

Age	Regions in the respiratory tract					
	Middle nasal concha	Larynx	Cranial trachea	Caudal trachea	Primary bronchus	Secondary bronchus
18-day-old embryo	0	0	0	0	0	0
19-day-old embryo	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1.3 ± 0.8	1 ± 0
20-day-old embryo	2.7 ± 1.5	2.3 ± 2.0	2.8 ± 1.8	2.8 ± 2.2	1 ± 0	1 ± 0
1-day-old chick	4.3 ± 1.5	3.7 ± 1.9	3.7 ± 1.9	3.7 ± 0.0	3 ± 1.3	2.7 ± 1.4
3-day-old chick	1.3 ± 1.4	2.7 ± 1.9	2.7 ± 1.9	3.0 ± 1.9	2 ± 1.6	2.2 ± 1.9
5-day-old chick	2.0 ± 1.1	2.8 ± 1.7	2.7 ± 1.5	2.7 ± 1.5	1.5 ± 0.9	1.7 ± 1.2
7-day-old chick	1.7 ± 2.2	1.5 ± 1.4	1.5 ± 1.4	1.0 ± 0.9	0.7 ± 0.8	0.7 ± 0.8
11-day-old chick	1.2 ± 0.4	1.2 ± 1.2	1.0 ± 1.6	1.0 ± 1.6	0.8 ± 0.4	0.8 ± 0.4
13-day-old chick	1.7 ± 1.6	2.5 ± 2.4	2.3 ± 2.0	2.2 ± 1.8	1.3 ± 0.8	1.3 ± 0.8
22-day-old chicken	1.8 ± 1.7	1.3 ± 1.4	1.0 ± 0.6	1.0 ± 0.6	0.7 ± .5	0.7 ± 0.5
29-day-old chicken	0	0	0	0	0	0
35-day-old chicken	0	0	0	0	0	0
45-day-old chicken	0	0	0	0	0	0

APPENDIX D

MEAN NOS. OF MUCOUS CELLS (± SD) IN THE RESPIRATORY TRACT OF FORMALDEHYDE-EXPOSED CHICKS

Age	Regions in the respiratory tract					
	Middle nasal concha	Larynx	Cranial trachea	Caudal trachea	Primary bronchus	Secondary bronchus
19-day-old embryo	16 ± 2	17 ± 5	31 ± 1	27 ± 2	32 ± 24	35 ± 17
20-day-old embryo	21 ± 4	31 ± 4	53 ± 15	52 ± 23	27 ± 13	40 ± 42
1-day-old chick	45 ± 10	25 ± 6	42 ± 23	28 ± 32	63 ± 42	50 ± 43
3-day-old chick	46 ± 21	52 ± 12	81 ± 16	81 ± 17	155 ± 69	115 ± 44
5-day-old chick	34 ± 3	49 ± 18	59 ± 4	81 ± 11	76 ± 34	69 ± 41
7-day-old chick	35 ± 8	58 ± 4	109 ± 57	97 ± 36	75 ± 21	39 ± 13
11-day-old chick	37 ± 6	21 ± 13	59 ± 37	99 ± 52	63 ± 11	89 ± 37
13-day-old chick	35 ± 5	41 ± 36	68 ±32	94 ± 28	109 ± 19	88 ± 40

APPENDIX E

MEAN NOS. OF INTRAEPITHELIAL MUCOUS GLANDS (± SD) IN THE RESPIRATORY TRACT OF FORMALDEHYDE-EXPOSED CHICKS

Age	Regions in the respiratory tract					
	Middle nasal concha	Larynx	Cranial trachea	Caudal trachea	Primary bronchus	Secondary bronchus
19-day-old embryo	16 ±3	5 ± 1	8 ± 3	2 ± 1	8 ± 6	7 ± 4
20-day-old embryo	26 ± 1	5 ± 1	16 ± 4	17 ± 5	6 ± 6	7 ± 6
1-day-old chick	25 ± 5	22 ± 4	7 ± 3	4 ± 6	16 ± 4	3 ± 1
3-day-old chick	31 ± 2	30 ± 2	24 ± 6	21 ± 10	9 ± 6	7 ± 5
5-day-old chick	27 ± 3	27 ± 5	15 ± 5	13 ± 6	6 ± 1	2 ± 1
7-day-old chick	34 ±5	25 ± 2	25 ± 11	18 ± 12	4 ± 3	2 ± 1
11-day-old chick	34 ± 5	32 ± 3	15 ± 2	11 ± 7	4 ± 2	6 ± 2
13-day-old chick	30 ± 6	23 ± 2	17 ± 3	10 ± 2	9 ±1	3 ± 1

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