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A PHYSIOLOGICAL, BIOCHEMICAL AND HISTOLOGICAL STUDY OF GOOSE TRACHEAL MUCIN AND ITS SECRETION

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Tracheal mucin secretion has been measured from a segment of trachea, isolated *in situ*, in anaesthetized geese by a method that involves radioactive labelling of tracheal mucus glycoproteins (Gallagher *et al.* 1975). Goose tracheal mucus comes entirely from goblet cells, since the goose trachea does not contain submucosal mucous or serous glands, and this method has been used to investigate the nervous and pharmacological control of the mucin secretion from these epithelial goblet cells. The mucins secreted have been collected, fractionated, and chemically analysed. Intracellular mucin has been examined histochemically, and the results of electron microscopic observations of epithelial cells and nerves are presented.

Acetylcholine increased tracheal mucin secretion, and this effect was completely blocked by atropine. Neither α- nor *β*-stimulant sympathomimetic amines affected tracheal mucin secretion. Stimulation of the peripheral cut ends of the descending oesophageal nerves increased tracheal mucin secretion and the majority of this response, approximately three-quarters, appeared to be cholinergic since this proportion was blocked by atropine. The mediator for the atropine-resistant part of the response is not known, but it appears not to be a *β*-adrenoreceptor stimulant since the response to nerve stimulation was unaffected by propranolol given at 34 μM intrasegmentally. Other possibilities are discussed. Atropine itself decreased the resting level of tracheal mucin secretion. The local anaesthetic, lignocaine, increased tracheal mucin secretion, while at the same time blocking the responses to acetylcholine and descending oesophageal nerve stimulation. The implications of this are discussed.

The electrophoretic, gel filtration and ion-exchange properties of goose tracheal mucins showed that they represented high molecular mass, negatively charged glycoproteins which could be labelled biosynthetically with [^{35}S]sulphate, [^{3}H]- and [^{14}C]glucose. These mucins could be stained with Alcian blue or periodic acid Schiff reagent. The carbohydrate composition was unusual for an epithelial glycoprotein in that fucose was absent and mannose was present in small quantities. The monosaccharides present in larger quantity were galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and sialic acid.

Histochemical analysis of tissue sections of gosling tracheas demonstrated that nearly all of the glycoprotein in epithelial goblet cells contained both sialic acid and sulphate residues. Sialated mucin was present also, but to a lesser extent, and many cells contained a mixture of sialated and sulphated mucins. The adult goose trachea had a high proportion of sialated glycoprotein.

Electron microscopy showed a range of epithelial cell types and intra-epithelial nerves also. Many of the nerves had neurosecretory vesicles suggestive of motor function and some were near to goblet cells.

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Introduction

The secretion and composition of tracheal mucus, in particular the tracheal mucins (tracheal mucus glycoproteins), have been studied previously in a number of species such as rabbit (Gallagher & Kent 1975), dog (Chakrin *et al.* 1972; Ellis & Stahl 1973) and cat (Gallagher *et al.* 1975). The mucins from all these sources contained fucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and sialic acid as the main monosaccharides, and could also be radioactively labelled by the administration of sodium [^{35}S]sulphate to the tracheal tissue (*in vitro* in the rabbit and *in vivo* in the cat). These secretions, however, invariably come from more than one source (epithelial goblet cells and submucosal mucous and serous glands), and only one investigation has as yet successfully separated goblet cell secretions from those of submucosal glands (see Stahl & Ellis 1973).

Most evidence to date suggests that although the submucosal glands have a functional efferent innervation, the epithelial goblet cells do not. Using histochemical techniques Florey, Carleton & Wells (1932) found that although electrical stimulation of the peripheral cut ends of the cervical vagus nerves of the cat emptied the tracheal mucous and serous glands of their secretions, it had no appreciable effect on the epithelial goblet cells. They concluded that these goblet cells were not innervated. Gallagher *et al.* (1975) found that electrical stimulation of the stellate ganglia in the cat increased tracheal mucin secretion. This effect also seemed to result solely from stimulation of mucous and serous glands since, histologically, the epithelial goblet cells appeared unaffected by the stimulus. By electron microscopy Walsh & McLelland (1974*b*) showed that the tracheal epithelium of the domestic fowl contained few, if any, motor fibres and none which were near the epithelial goblet cells. However, Jeffery & Reid (1973) showed that in the rat trachea there were motor fibres in the epithelium and some were close to the epithelial goblet cells.

Since the goose trachea appears to be similar to that of other birds studied, in that it only has epithelial goblet cells and no submucosal gland (see Results, §(*f*); Cover 1953; Purcell 1971; Walsh & McLelland 1974*a*), it provides an opportunity to study the control of secretion and composition of mucus from goblet cells alone.

Using a modification of a recently described method (Gallagher *et al.* 1975), which involves the collection of radioactively labelled tracheal mucin(s), the nervous and pharmacological control of tracheal mucin secretion in the goose has been investigated. The mucin, obtained from this precise cellular origin, has been chemically analysed. Histochemistry and electron microscopy have been used to study the tracheal epithelium.

Methods and materials

(*a*) *Anaesthesia*

Forty-seven adult geese, weighing 4–6 kg, and 7 goslings (unweighed) were used. They were from hybrid stock (*Anser anser* and *Cygnopis cygnoid*). Anaesthesia was induced either with a halothane (Fluothane, I.C.I.)/oxygen mixture, administered via a rubber hood (13 experiments), or with pentobarbitone sodium (Nembutal, Abbott), about 20 mg/kg injected intravenously (i.v.) through a wing vein (41 experiments). All the goslings had anaesthesia induced with halothane. The birds were placed supine and polyethylene catheters were tied into a

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brachial artery and vein. Anaesthesia was continued and maintained with supplementary doses of pentobarbitone sodium given through the venous catheter, and any halothane being given was then discontinued. Blood pressure was recorded from the arterial catheter by a strain gauge manometer (S.E. Laboratories). The animal's temperature was measured and kept constant, at approximately 41 °C, with a rectal thermistor probe and electric blanket (C. F. Palmer).

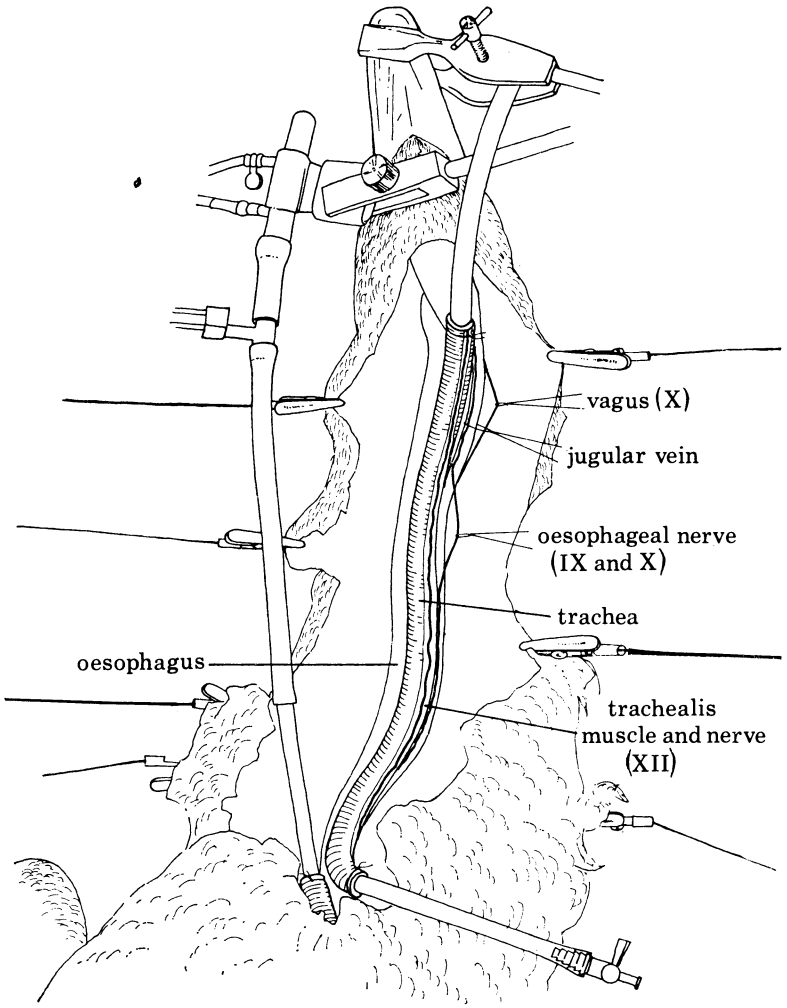

FIGURE 1. Diagram showing the insertions of the three tracheal cannulae. The animal breathed through the most caudal of the cannulae, and the isolated tracheal segment was washed out in a headwards direction with avian physiological solution via the rostral two cannulae. The trachealis nerves were left intact at the rostral end of the trachea but not normally at the caudal end except in experiments where the vagus nerves were to be stimulated. The courses of the vagus, trachealis and descending oesophageal nerves are also shown.

(*b*) *Anatomy*

A brief description of the anatomy and innervations of the cervical trachea is relevant to the experiments being described, since it appears not to have been studied systematically in the goose. However, much work has been done on the domestic fowl (*Gallus domesticus*) (see Hseih 1951; Watanabe 1960, 1964; Bubien-Waluszewska 1968) and the three geese that we studied appeared similar to the fowl in respect of the anatomy of the trachea and its innervation.

The cervical trachea lies on the ventrolateral surface of the oesophagus. There is a strip of

muscle running along either side of the trachea which consists of the trachealis lateralis, sterno-trachealis and sternolaryngeus muscles. Here these will be abbreviated to 'trachealis' muscle, since in the domestic fowl there is still much debate about the nomenclature of these muscles (McLelland 1965, 1968). The cervical trachea and surrounding structures appear to be innervated by the IX, X and XII cranial nerves (figures 1 and 2).

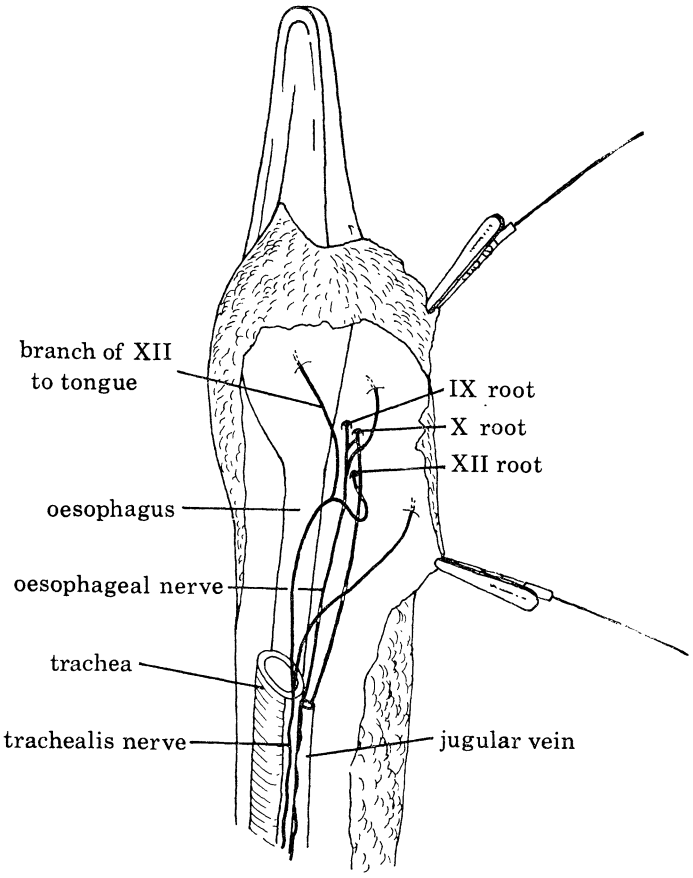

FIGURE 2. Diagram showing the courses and some of the anastomoses of the IX, X and XII cranial nerves high in the neck.

The glossopharyngeal (IX) nerve runs down the lateral aspect of the jugular vein, and divides into two main branches; the anterior laryngeal and the descending oesophageal nerves. Smaller branches supply the tongue, mouth and pharynx. The descending oesophageal branch runs along the ventral surface of the jugular vein, down the dorso-lateral aspect of the oesophagus. This branch innervates primarily the oesophagus and, in domestic fowl, may or may not innervate the trachea. In the goose fine branches were observed running from the descending oesophageal nerve to the trachea.

The vagus (X) nerve runs along the dorsal aspect of the jugular vein giving off many branches, only 3 of which are relevant to this description.

(1) The anastomotic branch to the glossopharyngeal nerve high in the neck. Through this branch the descending oesophageal branch of IX may derive some vagal fibres.

(2) The anastomotic branch to the hypoglossal nerve high in the neck from which the trachealis nerve may derive some fibres.

(3) The recurrent nerve. Both recurrent nerves arise from the vagi in the anterior part of the thoracic cavity, make acute turns, and run along the lateral aspects of the oesophagus. Above the crop they are continuous with the trunks of the descending oesophageal nerves. The recurrent nerves appear to supply the oesophagus, syrinx, trachea, and muscles attached to the trachea. In the domestic fowl they give off branches (i.e. the posterior laryngeal nerves) which run rostrally in the trachealis muscles, finally becoming continuous with the descending cervical branches of the hypoglossal nerves (i.e. the trachealis nerves). No branches were observed running from the vagus nerve directly to the trachea.

The hypoglossal (XII) nerve runs laterally to the vagus nerve, then crosses the vagus and glossopharyngeal nerves ventrally, just caudal to the vagus–glossopharyngeal anastomosis. It anastomoses with both the vagus nerve and the descending oesophageal branch of the glossopharyngeal nerve. It also gives off a branch (the descending branch of the hypoglossal nerve) that runs with the descending oesophageal nerve, but the main nerve courses in the trachealis muscle and is continuous with the posterior laryngeal branch of the recurrent laryngeal nerve.

There are also segmental nerves which leave the spinal cord at regular intervals in the neck, cross the jugular vein and vagus nerve dorsally, and innervate the skin. Some branches, however, appear to loop around to run across the ventrolateral surface of the oesophagus towards the trachea.

(*c*) *Experimental procedure and mucin collection*

The method of mucin collection and assay has been described previously in detail (Gallagher *et al.* 1975), and will only be outlined here.

A segment of cervical trachea (about 20–25 cm long) was isolated *in situ* with its nerve and blood supplies intact by inserting three polythene cannulae into the trachea. The trachea was exposed high in the neck and the trachealis nerves identified. The first cannula was inserted just distal to the larynx, pointing caudally, and was secured with a thread which passed around the trachea but did not enclose the trachealis nerves. The trachea was also exposed as low in the neck as possible. A wide slit was made in the trachea and into this the other two cannulae were inserted; one pointing caudally to the lungs (through which the animal breathed for the remainder of the experiment) and the other pointing rostrally. No special care was taken to spare the trachealis nerves at this level unless it was intended to stimulate the vagus nerves (see description of anatomy). Tidal volume and flow were measured from a Fleisch pneumotachograph head in series with the most caudal of the three tracheal cannulae. Airway CO_2 was monitored with an infrared absorption meter (Beckman LB2) which sampled gas from the tracheal tube at 500 ml/min.

Systemic arterial blood pressure, tidal volume, tracheal airflow, airway CO_2 and a signal trace were all recorded on u.v. sensitive paper (by a SE 6008 recorder) and also on magnetic tape (Ampex PR 500 tape recorder) together with a spoken commentary.

After the preliminary surgery the isolated tracheal segment was filled with an avian physiological saline solution (Cleugh, Gaddum, Holton & Leach 1961) and the tap on the tube at the caudal end of the segment closed so that the segment remained filled with solution. The ionic concentrations in the physiological saline solution were: Na^+, 149.3 mM; K^+, 2.68 mM; Ca^{2+}, 1.8 mM; Mg^{2+}, 0.105 mM; Cl^-, 143.5 mM; HCO_3^-, 11.9 mM; $H_2PO_4^-$, 0.417 mM. The secretions from this isolated tracheal segment were collected by flushing 25–26 ml physiological saline solution through the rostral two cannulae, usually every 15 min.

The radioactive precursors of tracheal mucins used were: sodium [^{35}S]sulphate, 1.0–8.0 mCi

i.v. (26 experiments) or 0.5–1.5 mCi directly into the tracheal segment (17 experiments); D-[U-^{14}C]glucose, 0.13–0.25 mCi directly into the tracheal segment (3 experiments) and D-[1-^{3}H]- or D-[2-^{3}H]glucose, 0.25 mCi directly into the tracheal segment (6 experiments). In 2 experiments no radioactivity was given. All radioactive materials were obtained from Radiochemical Centre, Amersham. When given directly into the tracheal segment the required amount of radioactive precursor was added to 10–15 ml physiological saline solution and this was flushed into the tracheal segment in the normal way.

Tracheal washings taken after the radioactive precurors administration contained both mucin-bound and unbound radioactivity. The samples were sonicated and dialysed to disperse the unbound radioactive ions or molecules and the mucin content was estimated by measuring the level of bound radioactivity by scintillation counting (as described previously, see Gallagher *et al.* 1975).

Blood samples were taken at the end of most experiments and sometimes at intervals during experiments, as a monitor of the level of plasma protein-bound radioactivity. Also at the end of 9 experiments, when the animal had been killed, ciliary motion within the tracheal segment was checked to see whether the epithelial layer of cells within the segment was still intact. This was done by opening the tracheal segment, raising the animal's head so that the trachea was tilted upwards and then placing graphite dust on the lower end of the tracheal segment epithelium. In only one animal was no particle movement detected. In the other eight the graphite particles were transported over the whole observed area of the epithelium.

(*d*) *Nerve dissections and stimulation*

Nerve dissections were carried out as required. (1) The descending oesophageal branches of the glossopharyngeal nerves were dissected clear of surrounding tissues below the anastomoses between the glossopharyngeal and vagus nerves. (2) The vagus nerves also were identified and cleared below the anastomoses between the glossopharyngeal and vagus nerves. In experiments where the vagus nerves were to be stimulated care was taken, when tying in the two caudal tracheal cannulae, to spare the branches of the recurrent nerves which ascend in the trachealis muscles (posterior laryngeal nerves). (3) The trachealis branches of the hypoglossal nerves were isolated just proximal to the point at which they joined the trachealis muscles (i.e. distal to the anastomoses of the hypoglossal and glossopharyngeal, the hypoglossal and vagus nerves, and also after the origin of the descending branch of the hypoglossal nerve). (4) Segmental nerves were dissected out just before their coursing across the oesophagus.

All nerves were sectioned and their peripheral cut ends electrically stimulated with pulses of 5–8 V, 1–2 ms duration at 10–12 Hz for 5–12 min of a 15 min sampling period.

(*e*) *Drugs and their administration*

Drugs were given directly into the tracheal segment in physiological saline solution. The following drugs were given at the final concentrations indicated: acetylcholine chloride (Sigma Chemical Co.) 0.28–55 μM; atropine sulphate (Antigen Ltd) 14 μM; adrenaline tartrate (Macarthy's Ltd) 55 μM; noradrenaline tartrate (Levophed, Winthrop) 30 μM; isoprenaline hydrochloride (Suscardia, Pharmax) 18 μM; propranolol hydrochloride (Inderal, I.C.I.) 34–340 μM and lignocaine hydrochloride monohydrate (Laporte Industries Ltd) 8.70 mM Atropine was also given i.v., 1 mg/kg, in a few experiments.

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(*f*) *Measurement of plasma contamination*

In experiments where the radioactive precursor [^{35}S]sulphate was given i.v. there was substantial radioactive labelling of plasma proteins, shown by scintillation counting of plasma samples, and so experiments were done to test the extent to which [^{35}S]sulphate labelled plasma proteins 'leaked' from the blood into the tracheal washings. In three experiments such plasma proteins were obtained by bleeding geese that had been given [^{35}S]sulphate label i.v. The blood from two of these experiments was heparinized and the cells were spun down. The plasma, dialysed for 5 days to disperse unbound radioactive sulphate ions, was then reinjected i.v. into another two geese. Blood samples and tracheal washings were taken over the next $2\frac{1}{2}$ h before a 1–2 mCi dose of sodium [^{35}S]sulphate was given i.v. to both geese. The blood obtained from the third goose was treated similarly except that it was not heparinized and was allowed to clot. The serum thus obtained was treated as was the plasma from the other two geese. In another goose a 12 ml blood sample was taken, mixed with 2 ml of Evans blue dye, which binds to plasma protein, and reinjected. Samples of plasma and tracheal washings were taken before and after dye injection and the concentration of dye in them was measured spectrophotometrically with a Beckman DB spectrophotometer at 470 nm. Estimations of non-dialysable [^{35}S]sulphate in plasma and in tracheal washings were done in parallel.

(*g*) *Analysis of results*

Any change in the output of bound radioactive count in response to a given stimulus is expressed as a percentage change compared with, where possible, the average output of radioactivity from the preceding and following control samples (bracketing controls). Some stimuli, however, had effects that lasted longer than the 15 min sample time (some due to the concentration used, e.g. acetylcholine, and some due to the experimental design, e.g. atropine, propranolol and lignocaine). In these cases the changes in output of bound radioactivity are expressed as percentage changes compared with the preceding control sample only (single control). Changes in mucin secretion are expressed as mean percentage changes (Δ) $\pm$ standard errors. All figures of probability were obtained using Student's *t*-test. In Results, n.s. signifies not statistically significant ($P > 0.05$).

(*h*) *Physical and chemical analysis of secretions*

Analytical techniques used for studying goose tracheal mucins were similar to those previously described for rabbit and cat mucins (Gallagher & Kent 1975; Gallagher *et al.* 1975). In brief, dialysed goose tracheal washings were concentrated by rotary evaporation and their glycoprotein and protein content was examined by electrophoresis on cellulose acetate (barbitone buffer 0.07 M, pH 8.6). These strips were then stained with Alcian blue (1 g/100 ml in 4 % (by vol.) acetic acid), periodic acid Schiff reagent (PAS) or nigrosine (0.02 g/100 ml in water). On some occasions mucins were digested with papain (0.1 M sodium acetate buffer, pH 5.5 containing 1 mM EDTA and 5 mM cysteine) at 60 °C for 4–6 h before further study. Incorporation of radioisotopes into these mucins was checked after electrophoresis by cutting the stained cellulose acetate strip into 0.5 cm segments and measuring the radioactivity in each.

The following gel filtration systems were used for fractionating goose mucins:

(i) Sepharose 2B, 83 cm $\times$ 1.25 cm, in sodium acetate buffer, pH 5.8, flow rate 5 ml/h.

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(ii) Sephadex G-200, 90 cm × 1.25 cm, in 0.05 M tris/HCl, pH 8.0, containing 0.1 M NaCl, flow rate 5 ml/h.

(iii) Sephadex G-200, 79 cm × 1.25 cm, in 50 mM phosphate, pH 7.2, containing 6 M urea, flow rate 5 ml/h.

(iv) Ultrogel AcA22 (from LKB), 69 cm × 1.7 cm, in 50 mM phosphate, pH 7.2, flow rate 10 ml/h.

Ion-exchange chromatography was carried out with DEAE cellulose DE-52 (Whatman) equilibrated with 20 mM phosphate, pH 7.2, containing 10 mM NaCl and 6 M urea, and packed into a column of bed size 5 cm × 1.25 cm. Goose tracheal mucins were dialysed against this buffer before application to the column, from which they were subsequently eluted by a linear gradient of NaCl from 10 mM → 0.7 M (in 20 mM phosphate and 6 M urea) in a total volume of 180 ml at a flow rate of 10 ml/h.

The monosaccharide composition of the mucins was determined by the gas–liquid chromatographic (g.l.c.) method of Clamp, Bhattie & Chambers (1971) after methonolysis of freeze dried samples at 90 °C under N_2.

(*i*) *Histology*

On the adult goose trachea histology and, in particular, histochemistry were made difficult by the presence of interlocking boney rings in the airway wall. Although decalcification is possible, it is not advised for histochemical studies (Charman & Reid 1972). For this reason histology was limited to mucosal wall, stripped from the bone of an adult goose and to conventional sections taken from tracheal and primary bronchi of 3 goslings 4–6 weeks of age: in the goslings the airway supporting plates are cartilaginous and amenable to section.

Tissue was fixed in 10 % neutral and buffered formal–saline, paraffin embedded and cut at 5–7 μm. From each animal one section was stained with haematoxylin and eosin and each of the three adjacent sections stained with one of the following combinations: (1) Alcian blue pH 2.6/periodic acid Schiff (AB2.6/PAS) (Mowry & Winkler 1956), (2) Alcian blue pH 1.0/periodic acid Schiff (AB1.0/PAS) (Spicer 1960), (3) Sialidase (Vibrio cholera–Koche) followed by AB2.6/PAS (Spicer & Warren 1960). Stained areas in the epithelium, indicating the presence of intracellular glycoprotein, were counted and expressed as numbers of goblet cells per high-power microscopic field. Each high power field represented a 0.44 mm length of epithelium: 40 fields were counted from each section. In all, over 6000 goblet cells were counted in about 150 mm of epithelium. The standard error of 3 repeat measurements for 396 goblet cells was ± 11 cells and this was considerably less than the variation between animals. Tracheal tissue from 1 gosling was taken for examination by electron microscopy. Small pieces were doubly fixed with 3 % glutaraldehyde and 1 % osmium tetroxide (both in 0.1 M cacodylate buffer at pH 7.2), embedded in Araldite, sectioned and stained with 1 % alcoholic uranyl acetate (Stempack & Ward 1964) and lead citrate (Reynolds 1963) and viewed in a Siemens 101 Elmskop electron microscope. 1 μm sections were stained with 1 % toluidine blue (Mercer 1963) for light microscopic examination and cell counts.

From one gosling given sodium [^{35}S]sulphate intrasegmentally autoradiographs were made of tissue sections, by using Kodak AR10 stripping film. Sections were prestained with PAS and stored in the dark at 4 °C for 1–2 weeks before development with Kodak D-19 developer for 5 min at 20 °C.

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Results

(a) *Characteristics of the method*

(i) *Intravenous labelling*

[^{35}S]sulphate was the only radioactive precursor given i.v. Bound radioactivity in the tracheal washings was scanty or absent in the first 30 min but subsequently rose to a plateau over 2 h (figures 4, 5 and 7). Blood samples collected during and at the end of some of the experiments showed that the blood was high in radioactivity, much of which was in a non-dialysable form. It is important for the interpretation of the results that little of the bound radioactivity in the tracheal washings resulted from leakage of [^{35}S]sulphate labelled plasma proteins across the tracheal epithelium. Three types of experiments have been performed in an attempt to measure this possible source of contamination. The results of these experiments are given below.

(1) Evans blue dye was injected i.v. into 1 goose. No dye was detectable in the tracheal washings. The concentration of blue marker was not increased by stimulation of the descending oesophageal nerves which did, however, increase the output of mucin-bound radioactivity in the tracheal washings. In this experiment no radioactivity in the tracheal washings appeared to be due to leakage of radioactively labelled plasma proteins.

(2) Radioactively labelled plasma proteins were injected i.v. in 3 geese. In two of these experiments the radioactivity of the tracheal washings taken over the next $2\frac{1}{2}$ h was around background level, and did not increase when acetylcholine was given directly into the tracheal segment (figure 3). When sodium [^{35}S]sulphate was given i.v. the level of radioactivity in the tracheal washings increased and under these circumstances acetylcholine given directly into the tracheal segment greatly increased the output of bound radioactivity. In these two experiments the calculated contributions to the 'plateau' of bound radioactivity from the leakage of radioactively labelled plasma proteins into the tracheal segment were 9 % and 0 % (see table 1). In the third experiment in this series the radioactivity of the tracheal washings after the injection of the radioactively labelled plasma proteins was significantly above the background level, though it was not increased when acetylcholine was given directly into the tracheal segment. The subsequent injection of sodium [^{35}S]sulphate i.v. increased the level of radioactivity in the tracheal washings, but to much less an extent than normally observed. Because of these two factors the calculated contribution to the 'plateau' of bound radioactivity from the leakage of radioactively labelled plasma proteins into the tracheal segment in this experiment was 60 % (see table 1).

(3) There is some chemical evidence of serum contamination in the tracheal washings, which will be discussed in Results, § (*e*).

(ii) *Intrasegmental labelling*

When the radioactive precursors were given directly into the tracheal segment the first sample (containing much of the radioactivity in unbound form) was discarded. Subsequent samples always contained mucin-bound radioactivity (figure 6). Generally the 'plateau' phase in these plots of bound radioactivity against time was not as constant as when [^{35}S]sulphate was given i.v. Often there was a maximum output of bound radioactivity within the first 2 h with a gradual fall in output after that (figure 6).

The concentration of protein-bound [^{35}S]sulphate in the blood was much lower when the radioactivity was given intrasegmentally (mean of 203 ± 243 count/min in a 0.5 ml sample of

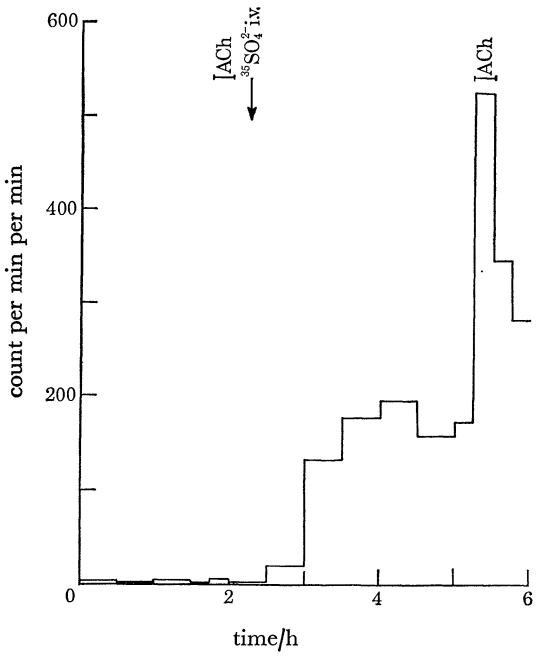

FIGURE 3. Plot showing the output of bound radioactivity in the tracheal washings in count per min per min of secretion against time (h). [^{35}S]sulphate labelled dialysed plasma was injected i.v. at time zero. Acetylcholine (ACh) given intrasegmentally at 5.5 μM produced no appreciable change in the output of bound radioactivity. 1.0 mCi sodium [^{35}S]sulphate was injected i.v. after $2\frac{1}{4}$ h ($^{35}SO_4$ i.v.) and the bound radioactivity of the tracheal washings increased as normal. Subsequent acetylcholine (ACh) intrasegmentally at 5.5 μM produced a large increase in tracheal mucin secretion (i.e. in the output of bound radioactivity).

TABLE 1. RESULTS FROM RADIOACTIVELY LABELLED PLASMA PROTEIN EXPERIMENTS

(Table 1 shows the calculated percentage contributions to the 'plateau' of bound radioactivity from the leakage of radioactively labelled plasma proteins into the tracheal segment.

All count per min (c.p.m.) values are compared with a water blank standard. Before [^{35}S]sulphate values refer to levels of radioactivity after the injection of the radioactively labelled plasma proteins, but before the injection of sodium [^{35}S]sulphate i.v. After sodium [^{35}S]sulphate values refer to levels of radioactivity after subsequent injection of the sodium [^{35}S]sulphate i.v. The tracheal washings c.p.m. values are the means of several unstimulated samples. The percentage contributions were calculated by multiplying the [^{35}S]sulphate level of radioactivity in the tracheal washings by the ratio of radioactivity in the blood after the [^{35}S]sulphate injection to that before the injection (ratio after: before, in table), and then expressing the value obtained as a percentage of the radioactivity in the tracheal washings after [^{35}S]sulphate administration.)

	blood radioactivity (c.p.m. in 0.5 ml aliquot)			tracheal washings radioactivity (c.p.m. in 0.5 ml aliquot)		calculated percentage contribution of blood radioactivity to tracheal washings radioactivity after [^{35}S]-sulphate i.v. (%)
expt. no.	before [^{35}S]sulphate i.v.	after [^{35}S]sulphate i.v.	ratio after: before [^{35}S]sulphate i.v.	before [^{35}S]sulphate i.v.	after [^{35}S]sulphate i.v.	
1	210.6	2031.9	9.7	1.8	197.4	9
2	417.6	2727.5	6.5	−2.1	169.5	0
3	264.8	2203.0	8.3	5.1	70.1	60

plasma, $n = 10$) than when given i.v. (mean of 3134 ± 1820 count/min in a 0.5 ml sample of plasma, $n = 18$). This means that when the label was given intrasegmentally the plasma radioactivity was only slightly greater than that in an equivalent volume of dialysed tracheal washings from unstimulated samples, and so leakage of radioactively labelled plasma into the tracheal segment was unlikely to vitiate the results.

Similar results were obtained with the other radioactive precursors given intrasegmentally. With [^{14}C]glucose the mean concentration of bound radioactivity in a 0.5 ml sample of plasma was 434 count/min while the mean concentration of bound radioactivity in a 0.5 ml sample of tracheal washings from unstimulated samples was 4089 ± 536 count/min ($n = 26$). With [^{3}H]-glucose the mean concentration of bound radioactivity in a 0.5 ml sample of plasma was 25 count/min while the mean concentration of bound radioactivity in a 0.5 ml sample of tracheal washings from unstimulated samples was 757 ± 86 count/min ($n = 82$). It therefore appears that with intrasegmental administration of the radioactive precursors the level of plasma radioactivity is not high enough for any plasma protein leakage to contribute significantly to the radioactivity in the tracheal washings.

(*iii*) *Repeatability of controls*

A change in mucin secretion caused by a stimulus was assessed by comparing the output of bound radioactivity during the stimulus with that in the bracketing controls (see Methods). Successive controls varied to some extent even when no stimulus intervened and we have measured this variation in controls where there was no intervention separating them. A total of 240 pairs of controls were examined, the mean difference between them being $15 \pm 1\%$. The results from early experiments, where sonication was not used to homogenize the samples, were significantly worse than those of later experiments where samples were sonicated. The mean difference between successive non-sonicated controls was $24 \pm 3\%$ ($n = 35$) while that for sonicated controls was $14 \pm 1\%$ ($n = 205$).

(*b*) *Drug induced effects*

(*i*) *Cholinergic influences*

Acetylcholine was given intrasegmentally at concentrations between 0.28 and 55.0 μM on thirteen occasions. At all doses it caused a large increase in tracheal mucin secretion. The overall mean increase was $+296 \pm 48\%$ ($n = 13$, $P < 0.001$) (figure 4).

Atropine given directly into the tracheal segment (14 μM) caused a significant reduction in mucin secretion ($\Delta = -30 \pm 5\%$, $n = 16$, $P < 0.001$) (figure 4). Atropine was invariably given to the animal at a time when the resting output of radioactively labelled mucin was slowly declining, and so it might be argued that the same decrease would have been seen even had no drug been given. However, when allowance was made for the slow decline in resting output (by subtracting from the drug-induced percentage change in resting output the percentage change between the two preceding control values), the atropine effect was still significant ($\Delta = -25 \pm 7\%$, $n = 16$, $P < 0.005$). Atropine was also given i.v. (1 mg/kg) on three occasions, with inconsistent effects on mucin secretion, the changes in these three experiments being $+50\%$, -4% and -40%.

When acetylcholine was given intrasegmentally to atropinized animals, mucin secretion was unchanged ($\Delta = +6 \pm 6\%$, $n = 5$, n.s.). A paired t-test showed that a significant ($P < 0.01$) block of the acetylcholine action had been produced by atropine (figure 4). This was not due

to tachyphylaxis or exhaustion of some of the mucin-secreting cells as was shown experimentally when the response to acetylcholine was reversibly blocked in 4 animals when the local anaesthetic, lignocaine, was given directly into the tracheal segment (8.70 mM) and subsequently washed out (figure 7) (see Results, § (*d*)).

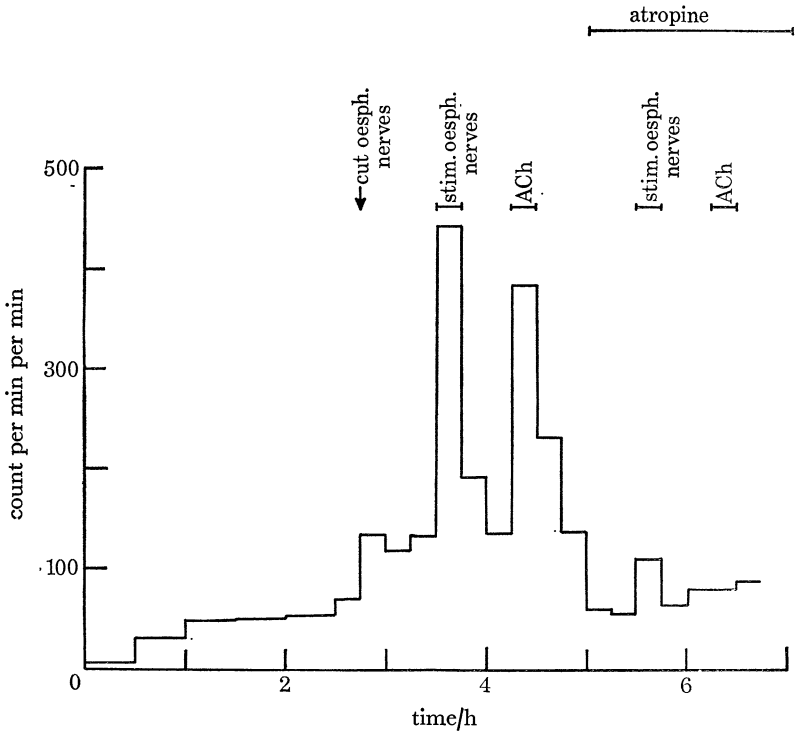

FIGURE 4. Plot of the output of tracheal mucin-bound radioactivity in count per min per min of secretion against time (h). 1.0 mCi sodium [^{35}S]sulphate was given i.v. at time zero. Section of the descending oesophageal nerves (cut oesph.ns) appeared to increase the resting mucin secretion. Both stimulation of the peripheral cut ends of the descending oesophageal nerves with pulses of 5 V, 2 ms duration at 10 Hz for 5 min (stim. oesph.ns) and acetylcholine given intrasegmentally at 5.5 μM (ACh) increased the mucin secretion. Atropine at 14 μM intrasegmentally (atropine) decreased the resting mucin secretion, and also produced a total inhibition of the response to acetylcholine (ACh) and an approximately 75% inhibition of the response to descending oesophageal nerve stimulation (stim. oesph.ns).

(*ii*) *Adrenergic influences*

The effects of three sympathomimetic amines given directly into the segment were tested. None had any significant effect on mucin secretion. Adrenaline (55 μM) caused a change of $-16 \pm 9\%$ ($n = 6$, n.s.); noradrenaline (30 μM) a change of $+5 \pm 10\%$ ($n = 5$, n.s.) while isoprenaline (18 μM) caused a change of $+28 \pm 20\%$ ($n = 7$, n.s.).

The adrenergic β-receptor blocker propranolol likewise had no significant effect on mucin secretion when given directly into the tracheal segment at either 34 μM ($\Delta = -11 \pm 12\%$, $n = 4$, n.s.) or 340 μM ($\Delta = +36 \pm 20\%$, $n = 6$, n.s.).

(*c*) *Nerve stimulation effects*

(*i*) *Vagus nerves*

The vagus nerves were sectioned in the neck, and the peripheral cut ends electrically stimulated in 5 geese. There was no significant effect on mucin secretion ($\Delta = -2 \pm 10\%$, $n = 5$ n.s.), but bilateral sectioning of the nerves was normally accompanied by irregular slow deep

breathing and stimulation of the peripheral cut ends typically produced hypotension and bradycardia.

(*ii*) *Trachealis nerves*

The trachealis nerves were sectioned and the peripheral cut ends were electrically stimulated in 6 geese. There was a small but insignificant increase in mucin secretion ($\Delta = +23 \pm 17\,\%$, $n = 8$, n.s.). Stimulation of the trachealis nerves caused the trachealis muscles to contract which in turn caused the tracheal segment to vibrate. This small and inconsistent increase in the mucin secretion may be explained by mechanical rubbing of the tracheal mucosa by the tracheal cannulae caused by this movement.

(*iii*) *Segmental nerves*

Two segmental nerves were sectioned and the peripheral cut ends were electrically stimulated at the same time in two experiments. There was no significant effect on mucin secretion; the changes being $+7\,\%$ and $-5\,\%$ (figure 6).

(*iv*) *Descending oesophageal nerves*

The descending oesophageal nerves were sectioned and the peripheral cut ends were electrically stimulated on thirty-six occasions. Sectioning the nerves produced a small increase in mucin secretion (figure 4) and also increased oesophageal peristalsis. Both effects can probably be explained by stimulation of axons while the nerves were being cut. Electrical stimulation of the peripheral cut ends greatly increased the mucin secretion ($\Delta = +196 \pm 15\,\%$, $n = 36$, $P < 0.001$) (figures 4, 6 and 7). This includes one occasion when the descending oesophageal nerves were stimulated in a 5 week old gosling. In this experiment the stimulation produced an increase in the mucin secretion of 203 % (figure 5) showing the tracheal innervation to be functional at this age. Oesophageal peristalsis was also seen during nerve stimulation.

When the descending oesophageal nerves were stimulated in atropinized animals there was still an increase in mucin secretion ($\Delta = +46 \pm 7\,\%$, $n = 14$, $P < 0.001$), but a paired t-test showed this increase was significantly less than that before atropine ($P < 0.001$) (figure 4). As with the atropine block of the acetylcholine effect this was not due to tachyphylaxis or exhaustion of some of the mucin-secreting cells as shown by six experiments.

In two of the experiments the descending oesophageal nerves were stimulated repeatedly in the absence of any atropine, and the effect was seen hardly to diminish in magnitude. The increases in mucin secretion in these two experiments were: $+216\,\%$, $+176\,\%$, $+193\,\%$, $+262\,\%$ and $+191\,\%$, $+181\,\%$, $+160\,\%$ respectively. In another experiment the descending oesophageal nerves were stimulated twice before and twice after the administration of atropine given directly into the tracheal segment at 14 μM. The increases in mucin secretion in this experiment were $+111\,\%$ and $+109\,\%$ before atropine, and $+29\,\%$ twice after atropine. In the other experiments the response to descending oesophageal nerve stimulation was reversibly blocked when the local anaesthetic lignocaine was given directly into the tracheal segment (8.70 mM) and subsequently washed out (figure 7) (see Results, §(*d*)).

To test whether the atropine-resistant effect was mediated through β-adrenoreceptors, the descending oesophageal nerves were stimulated before and after β-blockade. The β-receptor adrenergic blocking drug propranolol was given directly into the tracheal segment at 34 and 340 μM. Descending oesophageal nerve stimulation in the presence of propranolol, 34 μM, still

produced an increase in mucin secretion ($\Delta = +134 \pm 34\,\%$, $n = 4$, $P < 0.05$). Although this effect was slightly reduced when compared with the stimulation before propranolol, a paired t-test showed the decrease in effect not to be significant (figure 6). When the descending

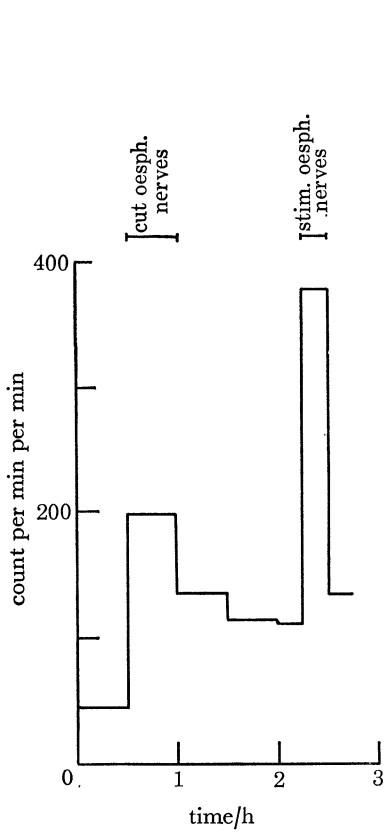

Fig. 5

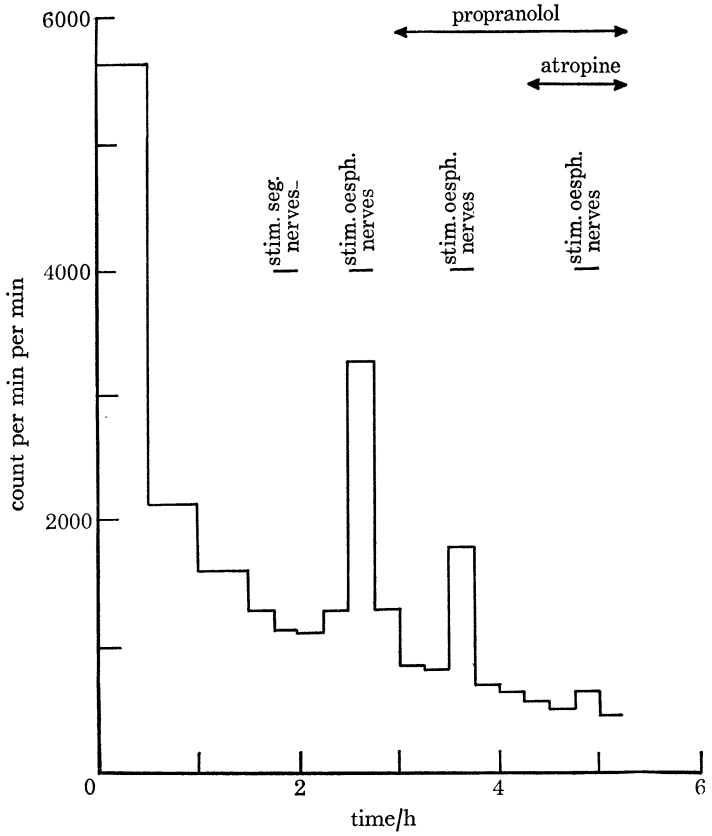

Fig. 6

Figure 5. Plot of the output of tracheal mucin-bound radioactivity in count per min per min of secretion against time (h), in a 5 week old gosling. 1.5 mCi sodium [^{35}S]sulphate were given i.v. at time zero. Section of the descending oesophageal nerves (cut oesph.ns) appeared to increase the resting mucin secretion. Stimulation of the peripheral cut ends of the descending oesophageal nerves with pulses of 6 V, 2 ms duration at 12 Hz for 12 min (stim. oesph.ns) produced a large increase in mucin secretion.

Figure 6. Plot of the output of tracheal mucin-bound radioactivity in count per min per min of secretion against time (h). 250 μCi [^{3}H]glucose were given intrasegmentally at time zero minus 1 h, and washed out of the segment at time zero. Stimulation of the peripheral cut ends of two segmental nerves with pulses of 7 V, 2 ms duration at 10 Hz for 10 min (stim. seg.ns) had no effect on mucin secretion. Stimulation of the peripheral cut ends of the descending oesophageal nerves with pulses of 6 V, 2 ms duration at 10 Hz for 5 min (stim. oesph.ns) increased the mucin secretion. This response was largely unaffected by propranolol given intrasegmentally at 34 μM (propranolol) but greatly decreased by a subsequent dose of atropine given intrasegmentally at 14 μM (atropine); (note, the propranolol remained in the tracheal segment when the atropine was given intrasegmentally).

oesophageal nerves were stimulated after a subsequent dose of atropine (14 μM intrasegmentally) while propranolol (34 μM) remained in the tracheal segment, there was still an increase in mucin secretion ($\Delta = +25 \pm 7\,\%$, $n = 3$, $P < 0.05$) (figure 6), but a paired t-test showed that the total decrease produced by propranolol and atropine was significant ($P < 0.05$). Although propranolol at 340 μM virtually abolished the atropine-resistant part of the response it had the same effect on the atropine-sensitive part of the response to descending oesophageal nerve

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stimulation. It therefore appears that at this high concentration the propranolol was having a local anaesthetic action (see Discussion).

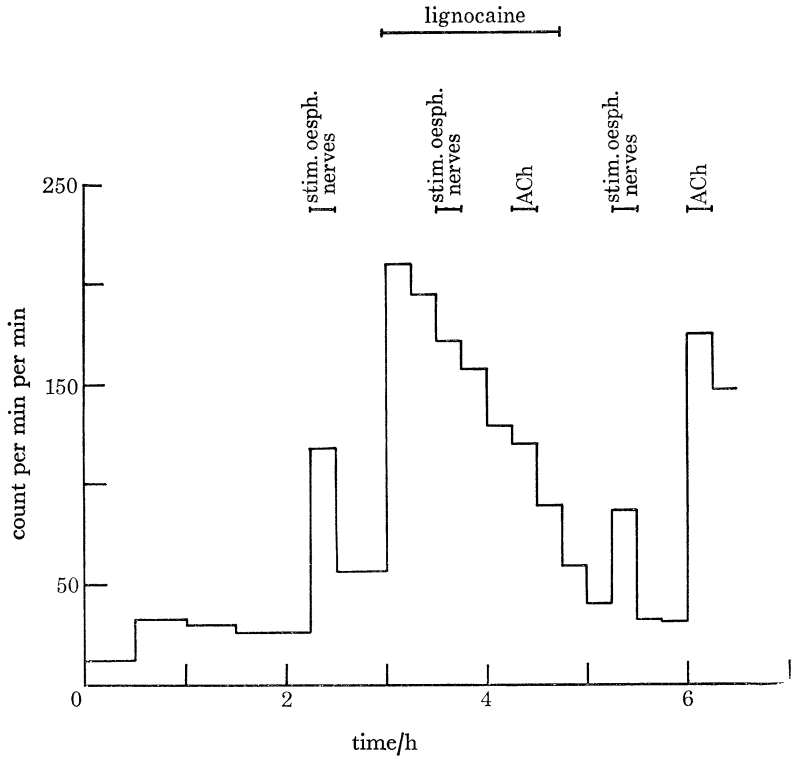

FIGURE 7. Plot of the output of tracheal mucin-bound radioactivity in count per min per min of secretion against time (h). 1.25 mCi sodium [^{35}S]sulphate were given i.v. at time zero. Stimulation of the peripheral cut ends of the descending oesophageal nerves with pulses of 6 V, 2 ms duration, at 10 Hz for 5 min increased the mucin secretion (stim. oesph.ns). Local anaesthetic, lignocaine, was given intrasegmentally at 870 μM (lignocaine), and produced a large increase in mucin secretion which persisted for the whole time the lignocaine remained in the segment, though the output did decline. When the lignocaine was subsequently washed out of the tracheal segment there was a decrease in mucin secretion. While the lignocaine was in the segment the tracheal mucin responses both to descending oesophageal nerve stimulation (stim. oesph.ns) and acetylcholine given intrasegmentally at 2.8 μM (ACh) were abolished. The tracheal mucin responses to these two stimuli were seen after the lignocaine had been washed out of the tracheal segment.

(*d*) *Lignocaine effects*

The local anaesthetic, lignocaine, was given directly into the tracheal segment at a concentration of 0.25 %, 8.70 mM, on seven occasions, and produced a large increase in mucin secretion ($\Delta = +244 \pm 60$ %, $n = 7$, $P < 0.005$) (figure 7). Some increase appeared to persist for the whole time that the lignocaine remained in the tracheal segment, although there was a gradual lessening in effect (figure 7). When the lignocaine was washed out of the tracheal segment there was a decrease in mucin secretion ($\Delta = -40 \pm 6$ %, $n = 7$, $P < 0.001$) which was still significant when allowance was made for the slow decline in resting output of radioactively labelled mucin ($\Delta = -32 \pm 7$ %, $n = 7$, $P < 0.005$).

As has already been mentioned (Results, §(*b*)) acetylcholine was given intrasegmentally before, during and after lignocaine. The changes in mucin secretion produced by acetylcholine under these circumstances are shown in table 2. The response to acetylcholine was abolished by lignocaine and returned when the lignocaine was washed out of the segment (figure 7). Paired

t-tests showed both the decrease in the acetylcholine response when lignocaine was given and the subsequent increase in the acetylcholine response when the lignocaine was washed out of the segment were significant ($P < 0.05$ and < 0.005 respectively). There was no significant difference between the response to acetylcholine before lignocaine was given and after lignocaine had been washed out of the segment.

TABLE 2. RESULTS FROM LIGNOCAINE BLOCKING EXPERIMENTS

Table 2 shows the effect of the local anaesthetic lignocaine given intrasegmentally (8.70 mM) on the responses of tracheal mucin secretion to intrasegmental acetylcholine and electrical stimulation of the peripheral cut ends of the descending oesophageal nerves.

pre-lignocaine	before lignocaine was added to the tracheal segment
plus lignocaine	while lignocaine was in the tracheal segment
post-lignocaine	after lignocaine had been washed out of the tracheal segment

(Values were calculated by comparing the count in samples during stimulation with means of count in bracketing controls. *n* indicates the number of experiments and *P* gives the probability of the effect being insignificantly different from zero.)

stimulation	Δ ± s.e. (%)		*n*	*P*
acetylcholine				
pre-lignocaine	+401	±87	3	< 0.05
plus lignocaine	+ 11	± 3	4	n.s.
post-lignocaine	+355	±42	4	< 0.005
descending oesophageal nerve stimulation				
pre-lignocaine	+210	±12	2	< 0.05
plus lignocaine	− 15	± 8	3	n.s.
post-lignocaine	+ 81	±29	3	n.s.

n.s., not significant.

Similar experiments were performed with descending oesophageal nerve stimulation. The changes in mucin secretion produced by descending oesophageal nerve stimulation before, during and after lignocaine are also shown in table 2. The response to descending oesophageal nerve stimulation was abolished by the lignocaine and partially returned after the lignocaine was washed out of the tracheal segment (figure 7). The reason that the recovery of response was incomplete was possibly that insufficient time was allowed after lignocaine washout before the nerves were stimulated again.

A similar abolition of the effect of nerve stimulation and acetylcholine was seen in all 6 experiments where lignocaine was given, even where the local anaesthetic's stimulation of mucin secretion was weak. For this reason we consider that lignocaine prevented these responses of the mucin secreting cells by some mechanism other than by making them secrete maximally (see Discussion).

(*e*) *Physical and chemical properties of goose tracheal mucins*

Native goose mucins, labelled biosynthetically with [^{35}S]sulphate or [^{14}C]glucose, eluted as a single radioactive peak in the void volume from a Sephadex G-200 column; the presence of 6 M urea in the eluting buffer did not affect the radioactivity elution profile (figure 8). [^{3}H]-Glucose labelled mucins also were eluted as a single peak in the void volume from an Ultrogel

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AcA22 column, although in this gel filtration system there was a small degree of 'tailing' not observed on Sephadex G-200 (figure 9). On Sepharose 2B, [^{35}S]sulphated labelled mucins yielded only a shallow radioactive plateau in the void volume region, with the major radioactive peak being eluted approximately mid-way between the void volume and the total volume of the column (figure 9). From these results it appears that the major radioactively labelled mucins secreted by the goose trachea are of high molecular mass (greater than 200 000) and molecular size heterogeneity is evident from the shoulder observed on the Sepharose 2B peak (figure 9). Papain digestion had little effect on the molecular size of these mucins.

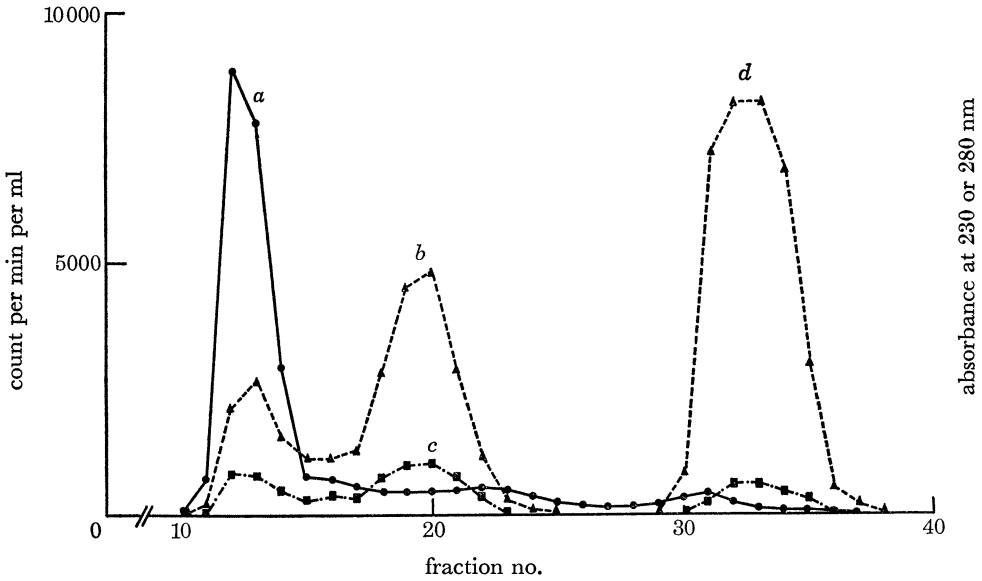

FIGURE 8. Fractionation of [^{35}S]sulphate labelled goose tracheal mucins by Sephadex G-200 gel filtration. A dialysed sample of the [^{35}S]sulphate labelled tracheal mucins was concentrated by rotary evaporation to approximately 1 ml before application to the column. Three ml fractions were collected at an elution flow rate of 5 ml/h. *a*, ●——● fraction radioactivity; *b*, ▲– – –▲ fraction absorbance at 230 nm; *c*, ■– · –■ fraction absorbance at 280 nm.

Electrophoresis of the unfractionated mucin and the main radioactive mucin peaks derived from the various gel filtration systems, showed a major Alcian blue positive band of diffuse, polyanionic character which on occasions, was partially resolved into two separate components (figure 10). This band contained the vast majority of the non-diffusible [^{35}S]sulphate radioactivity associated with the tracheal secretions and it also stained positively with PAS. These results show that the source of most of the non-diffusible radioactivity in the goose tracheal secretions is associated with a molecule(s) which, on molecular size, histochemical and electrophoretic criteria, has properties consistent with those of an epithelial mucin(s).

On comparison with a sample of goose serum, by electrophoresis followed by nigrosine or PAS staining, it was shown that the native goose tracheal secretions contained some components which corresponded to serum glycoproteins. However, these serum components, unlike the tracheal mucins, were not excluded from the Sephadex G-200 gel, and were thus readily separated from the tracheal mucins by this technique. In figure 8, peak *a* (the void volume peak) contained the tracheal mucins; peak *b*, characterized by minimal radioactivity but a relatively high u.v. absorption, was the principal source of the serum components; peak *d*

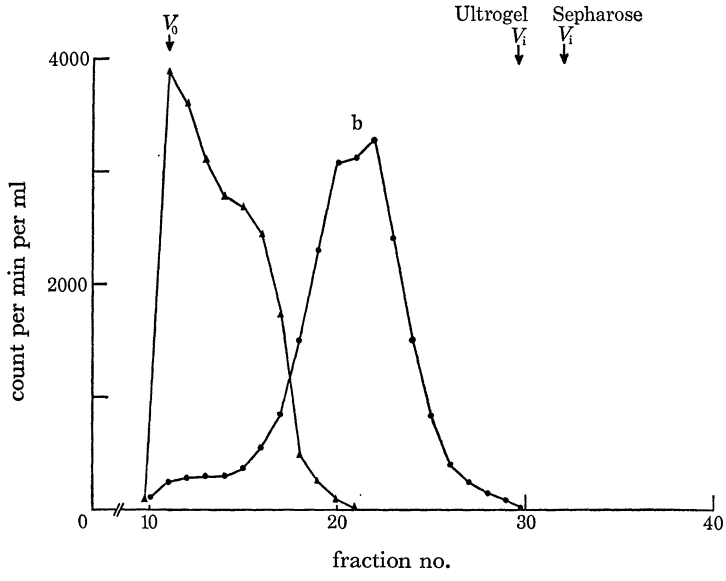

FIGURE 9. Comparison of the gel filtration profiles of radioactively labelled goose tracheal mucins on Ultrogel AcA22 (▲) and Sepharose 2B (●). Dialysed tracheal mucin samples, concentrated by rotary evaporation to about 1 ml, were applied to the two gel filtration systems and 3 ml fractions were collected from both columns. Mucins fractionated on Ultrogel AcA22 were labelled with [^{3}H]glucose and eluted at a flow rate of 10 ml/h; mucins fractionated on Sepharose 2B were labelled with [^{35}S]sulphate and eluted at 5 ml/h. Fractions 10–16 from the Ultrogel AcA22 column and fractions 16–26 from the Sepharose 2B column were pooled for analysis of their monosaccharides by g.l.c.

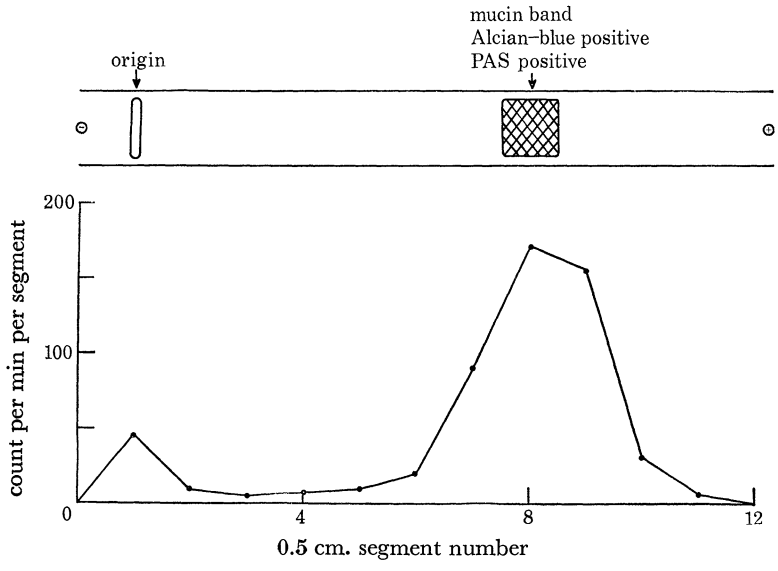

FIGURE 10. Cellulose acetate electrophoresis of [^{35}S]sulphate labelled goose tracheal mucins. [^{35}S]sulphate labelled tracheal mucins were electrophoresed (20 V/cm for 45 min) and located with either Alcian blue dye or PAS stain. The cellulose acetate strip was then cut into 0.5 cm segments and the radioactivity determined in each segment.

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arose mainly from the presence of antibiotics used in the dialysis medium to inhibit microbial growth.

Chromatography on DEAE cellulose DE-52 of the tracheal mucins, after separation from serum components on Sephadex G-200, gave a broad major radioactive peak which eluted between 0.1 M and 0.25 M NaCl and a second but minor peak at about 0.27 M NaCl (figure 11). When DEAE ion exchange was carried out in the absence of 6 M urea large losses of radioactivity occurred (above 50 %) and so it was essential first to dialyse the sample against 6 M urea before application to the column. Under these conditions about 90 % of the applied radioactivity was recovered from the column, eluted with a NaCl gradient (10 mM to 0.4 M) in buffered 6 M urea. Urea (6 M) has also been used to improve recoveries of mucins in sputum from DEAE-A25 Sephadex (Roberts 1974).

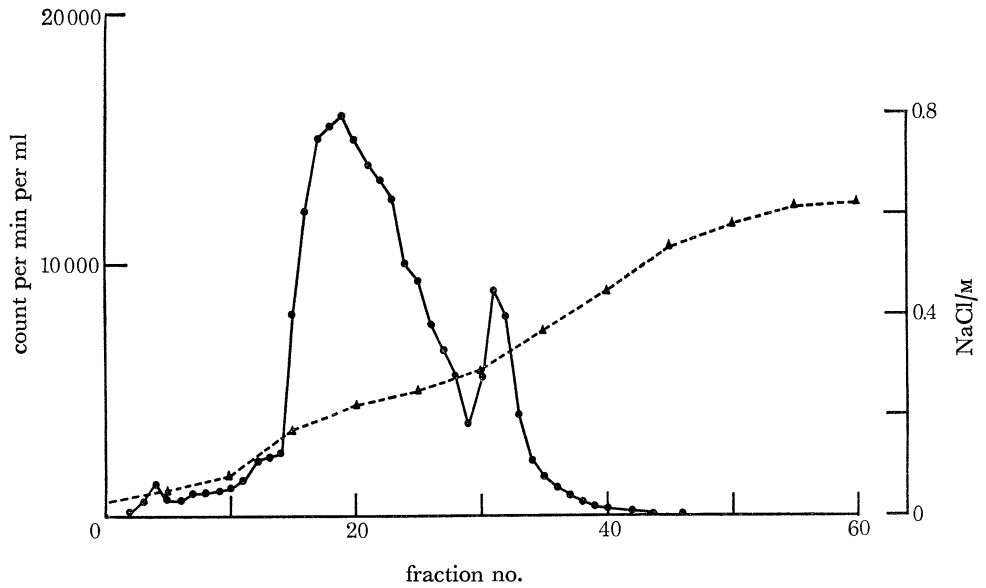

FIGURE 11. Fractionation of [^{14}C]glucose labelled goose tracheal mucins by ion-exchange chromatography on DEAE DE-52 cellulose. A sample of [^{14}C]glucose labelled tracheal mucins, which had first been isolated in the Vo from a Sephadex G-200 column, was dialysed against the starting buffer (20 mM phosphate, pH 7.2, containing 10 mM NaCl and 6 M urea) and pumped on to the column at a flow rate of 10 ml/h. Mucins were then eluted with a NaCl gradient from 0.01 M–0.7 M in a total volume of 180 ml. Three ml fractions were collected at an elution flow rate of 10 ml/h. *a*, ●——● fraction radioactivity; *b*, ▲– – –▲ NaCl/M gradient.

G.l.c. analyses of the monosaccharides found in various goose tracheal mucin fractions are given in table 3. Although there is considerable variation between the monosaccharide ratios of the various fractions, two features are most noteworthy:

(1) The absence of fucose, and the relatively high levels of sialic acid present compared with cat or dog tracheal mucins.

(2) The presence of mannose. Mannose was found in most mucin fractions analysed, although the concentration was usually low relative to that of other sugars present.

(*f*) *Histological observations*

(*i*) *Light microscopy*

The gosling and adult goose tracheal epithelium was pseudostratified, ciliated and columnar with goblet cells (figure 13, plate 1). No evidence of epithelial damage was found. The epithelium

was of a uniform thickness of about 20 μM and the depth of the ciliary layer was 6 μM. Goblet cells were distributed evenly along the entire length of the gosling trachea while in the adult they were often bunched together in shallow depressions as 'intraepithelial glands' (figure 14*a*, plate 1). Such 'intraepithelial glands' were not found in the gosling and in no animal was gland found in the submucosa.

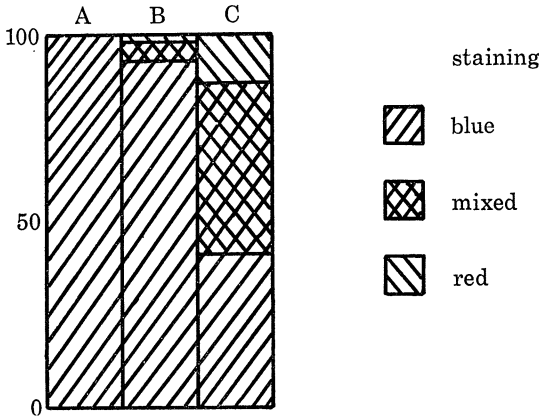

FIGURE 12. Percentage goblet cells staining either blue, red or showing a mixture of these colours after: (*a*) AB pH 2.6/PAS; (*b*) AB pH 1.0/PAS; (*c*) sialidase + AB pH 2.6/PAS.

Goblet cells were numerous in both the gosling and adult goose. In the gosling there were 20 goblet cells per high power (×40) field, each field representing a 0.44 mm length of epithelium. Sixty-five percent of goblet cells were full of secretion, in that stainable secretion was present from apex to base, while the remaining 35% had stainable secretion only at their apices (figure 14*b*, plate 1).

TABLE 3. CARBOHYDRATE COMPOSITION OF VARIOUS PREPARATIONS OF GOOSE TRACHEAL MUCINS

(Table 3 shows the carbohydrate composition of various preparations of goose tracheal mucins. Fractions A–E represent mucin samples obtained from different geese, which were fractionated by various methods. A, Vo peak from Sephadex G-200 (figure 8, peak *a*); B, peak *b* from Sepharose 2B (figure 9); C, Vo peak from Ultrogel AcA22 (figure 9); D1, DEAE-ion exchange chromatography fractions 13–29 (figure 10); D2, DEAE-ion exchange chromatography fractions 30–40 (figure 10); E, unfractionated sample analysed by Professor J. R. Clamp. * Control experiments showed that the high galactose value in this fraction arose from the agarose content of the Ultrogel AvA22.)

	molar rations of monosaccharides present (*N*-acetylglucosamine = 1.00) in various fractions					
monosaccharide	A	B	C	D1	D2	E
galactose	1.37	0.87	5.15*	2.14	2.05	1.39
N-acetylgalactosamine	1.60	1.18	1.19	1.01	0.53	0.67
N-acetylglucosamine	1.00	1.00	1.00	1.00	1.00	1.00
sialic acid	1.97	1.08	0.64	0.57	0.20	0.87
mannose	0.19	0.17	0.05	0.53	0.95	0.24

Histochemical methods were applied and the results are summarized in figure 12. These are preliminary findings and a fuller survey of both adult and gosling airway histochemistry is in preparation. While both acid and neutral glycoproteins were present, staining with AB 2.6/PAS showed that the acid predominated and was present in more than 95% of goblet cells. Both sulphated radicals and sialic acid contributed to the acidic properties. Staining with AB

1.0/PAS demonstrated that more than 90 % of goblet cells contained a glycoprotein which was sulphated. Treatment of this glycoprotein by sialidase showed that some 60 % of goblet cells contained a sialomucin which was susceptible to enzyme digestion, the remainder did not and retained their blue staining after Alcian blue at pH 2.6. Complete loss of AB staining was only found in about 10 % of goblet cells, the remainder retained some blue staining, this usually at the apex of the cell with magenta at its base (figure 15). Autoradiography showed a preferential uptake of radioactive sulphate by gosling epithelium with concentration in many of the goblet cells (figure 16). Some uptake was also seen over the cartilage.

The mean number of goblet cells counted, about 650 for each histochemical procedure, did not differ significantly from each other indicating that similar numbers of goblet cells were analysed with each histochemical method. Changes in the pattern of staining as a result of the three histochemical treatments were highly significant ($P < 0.001$) as judged by a χ^2-test.

Cell counts of three sections (1 μM thick, Araldite-embedded) of gosling tracheal epithelium gave the following results (mean number of cells per 5 oil immersion fields or 0.95 mm epithelium with ± standard error to give a measure of variation between animals): ciliated, 101.6 (± 5.4); epithelial serous, 0.3 (± 0.3); goblet, 24.0 (± 2.0); 'intermediate', 9.3 (± 0.7); basal, 67.7 (± 4.9); and total cell number, 203.0 (± 9.9). Therefore in the gosling goblet cells constituted some 12 % of all epithelial cells in the trachea.

(*ii*) *Electron microscopy*

Of the 8 epithelial and 2 migratory cell-types reported in airway epithelium (Jeffery & Reid 1975), 5 were identified in goose tracheal epithelium: ciliated, epithelial serous, goblet, intermediate and basal and also intraepithelial nerves (figures 17 and 18).

The ultrastructural features of epithelial cells were in general similar in the goose and the rat. However, goblet cell granules were larger, a maximum of 1.8 μM in diameter, and confluent with incomplete limiting membranes. The granules resembled those of the rat in their electron-lucency but, unlike the latter, were speckled with electron-dense areas (figure 18).

Intraepithelial nerves were found only infrequently: often several sections needed examination before a nerve profile was found. Most nerve profiles were at the base of the epithelium and only the occasional one was near the airway lumen. In each case they were recognized by their electron-lucent cytoplasm, neurotubules and/or neurosecretory vesicles (see figures 17 and

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Description of plate 1

Figure 13. Gosling tracheal epithelium showing goblet (arrows), ciliated, and basal cells. Three capillaries are present in the elastic lamina and cartilage is lowermost. 1 μm Araldite-embedded section; toluidine blue. (Magn. × 200.)

Figure 14. *a*, Adult goose tracheal epithelium illustrating goblet cells bunched together in shallow depressions to form 'intraepithelial glands'. AB pH 2.6/PAS. (Magn. × 200.) *b*, Intracellular secretion goblet cell in gosling epithelium. Some cells appear to be full of secretion while others have secretion only at their apices. Both magenta and blue-staining cells are represented. AB pH 1.0/PAS. (Magn. × 200.)

Figure 15. Intracellular goblet cell secretion following digestion of susceptible sialomucin by sialidase. Those cells showing mixed staining have Alcian blue staining at their apices with the remainder staining magenta. AB pH 2.6/PAS. (Magn. × 200.)

Figure 16. Autoradiograph of gosling epithelium demonstrating the uptake of [^{35}S]sulphate and its incorporation into secretion of many of the goblet cells. Some of the label remains as a thin layer adhering to the luminal surface of cells. Stained with PAS.

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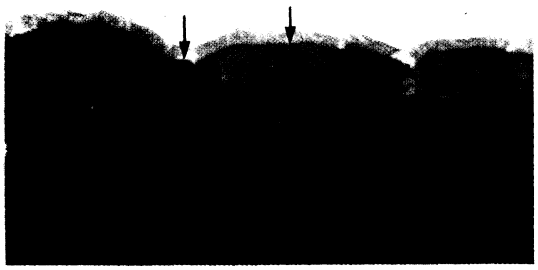

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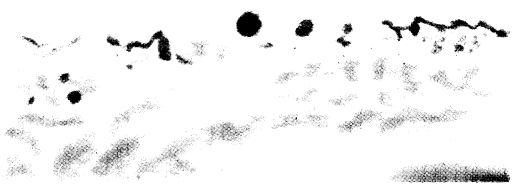

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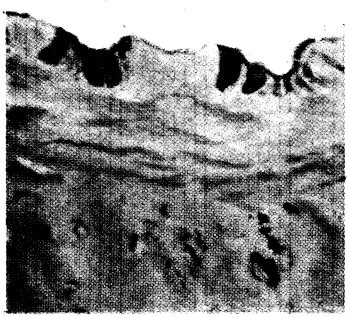

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FIGURES 13-16. For description see opposite.

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19). In a few nerve profiles small mitochondria were also included in section: the maximum mitochondrial diameter recorded was 0.2 μm. Of the nerve profiles with neurosecretory vesicles any one contained either clear vesicles only, dense-cored only or a predominance of clear with 1 or 2 associated dense-cored vesicles. From a sample of 40 the mean diameter of clear vesicles was 35.3 nm (±3.4) and for dense-cored 62.7 nm (±2.6).

At no time were membrane thickenings, characteristic of a synapse between nerve and cell, found but the intracellular gap between nerve and cell was always small; 16 nm or less.

Nerves were most frequently found associated with ciliated and basal cells and occasionally close to a non-ciliated, possibly secretory cell (figure 20). At one site subepithelial nerves appeared to pierce the epithelial basement membrane.

Discussion

The goblet cells of the goose tracheal epithelium, the only source of tracheal mucus in this species, have been shown to have a functional efferent innervation. Evidence for this comes from both physiological and histological results reported here. This innervation is primarily cholinergic in nature. Biochemical analysis has shown that the tracheal epithelial mucins are heterogeneous and of high molecular mass. Fucose is absent from the oligosaccharide side-chains while mannose is present. Histochemistry has shown the goblet cell intracellular secretion to be highly acidic.

(*a*) *Physiology*

The majority of these experiments were performed using [^{35}S]sulphate as the radioactive label and so in these only the secretion of sulphated mucins was being measured. However, results from our experiments in which radioactive precursors likely to label all the tracheal mucins ([^{14}C]glucose and [^{3}H]glucose) were used were qualitatively similar. The nerves supplying the goblet cells appear to run in the descending oesophageal branches of the IX cranial nerves since electrical stimulation of the peripheral cut ends of these nerves increased tracheal mucin secretion (though the fibres may have originated in either the IX, X or XII cranial nerves, due to the numerous anastomoses between these nerves high in the neck). The innervation appears to be primarily cholinergic (muscarinic) in nature since atropine significantly lessened the effect of nerve stimulation, though about 25% of the response to nerve stimulation, survived atropinization. This atropine-resistant portion of the response does not appear to be mediated through β-adrenergic receptors since it was not significantly affected by propranolol (34 μM). A higher concentration of propranolol (340 μM) did block this atropine-resistant part of the response. However, this concentration also blocked the atropine-sensitive part of the response and this action is best explained by the propranolol acting as a local anaesthetic (Morales-Aguilera & Vaughan Williams 1965; Goodman & Gilman 1975).

It is possible that the atropine-resistant part of the mucin secretion response to descending oesophageal nerve stimulation is cholinergic. Ambache (1955) reported several supposedly cholinergic effects in the gut and bladder of various species to be atropine-resistant (though many of these responses now appear to be non-cholinergic; Ambache & Freeman 1968; Ambache, Verney & Zar 1970; Ambache & Zar 1970). It seems more likely that this part of the response is mediated by a transmitter other than acetylcholine. Possible mediators of this part of the response include: dopamine, purines, histamine, 5-hydroxy tryptamine, prostaglandins,

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kinins, certain amino acids, though none of these have yet been tested. This part of the response remains to be investigated further.

Unlike the cat (Gallagher *et al.* 1975) there appears to be no sympathetic influence on tracheal mucin secretion in the goose. Evidence for this comes from several of the experiments reported here.

(1) Neither α- nor β-stimulant sympathomimetic amines had any effect on mucin secretion.

(2) Adrenergic β-receptor blockade with propranolol had no effect on the resting mucin secretion.

(3) Electrical stimulation of the peripheral cut ends of segmental nerves, which may carry sympathetic fibres, had no effect on the mucin secretion.

Section of the descending oesophageal nerves produced an increase in mucin secretion, though this is unlikely to represent an abolition of a resting inhibitory discharge as stimulation of the nerves themselves caused an increase in mucin secretion. This increase in secretion on sectioning these nerves is probably best explained by a transient stimulation of the nerves caused by the actual tying and sectioning. Because atropine lessened the resting secretion we feel that there is a resting stimulatory discharge in the descending oesophageal nerves.

The local anaesthetic lignocaine appeared to have two separate actions on the tracheal goblet cells; one a direct stimulatory action increasing mucin secretion, and the other an inhibitory action blocking the tracheal mucin responses to descending oesophageal nerve stimulation and locally applied acetylcholine. This direct action of lignocaine, increasing epithelial mucin secretion, does not seem to have been described before, though topical application of 1–2 % lignocaine is widely used to anaesthetize mucous membranes.

The stimulatory effect of lignocaine might be mediated by: (1) an action on the cholinergic receptors on the goblet cells (though lignocaine is not structurally similar to acetylcholine); (2) an action which involves the displacement of calcium ions from their binding sites (Ritchie & Greengard 1966), which in turn might stimulate secretion (Douglas & Poisner 1963; Ishida, Miki & Yoshida 1971), or (3) by some other undetermined mechanism. If lignocaine does act at cholinergic receptors to stimulate tracheal mucin secretion then administration of atropine before giving the lignocaine would probably block the stimulation. This experiment is planned.

The inhibitory effect of lignocaine on cholinergic stimulation might be mediated by: (1) an inhibition of acetylcholine at cholinergic receptors; (2) some intracellular block in the chain of mechanisms linking excitation of cholinergic receptors with secretion, or (3) lignocaine producing maximal stimulation of mucin secretion thus rendering other secretogogue agents ineffective. This last suggestion seems unlikely, however, since the changes in secretion produced by lignocaine were often smaller than the changes produced by the other stimuli (i.e. acetylcholine and descending oesophageal nerve stimulation) both before lignocaine and after lignocaine had been washed out of the segment.

The inhibitory effect of lignocaine on nerve stimulation-induced secretion could, in addition to the above, have been caused by its local anaesthetic action on pre- and/or post-ganglionic nerve fibres.

A full discussion of the advantages and disadvantages of the experimental method used here is given in Gallagher *et al.* (1975), however, further discussion of two points is relevant:

(1) Tracheal epithelium containing bound radioactivity might have sloughed off in the flow of the tracheal washing, and the radioactivity shown up as 'counterfeit' mucin. This is unlikely

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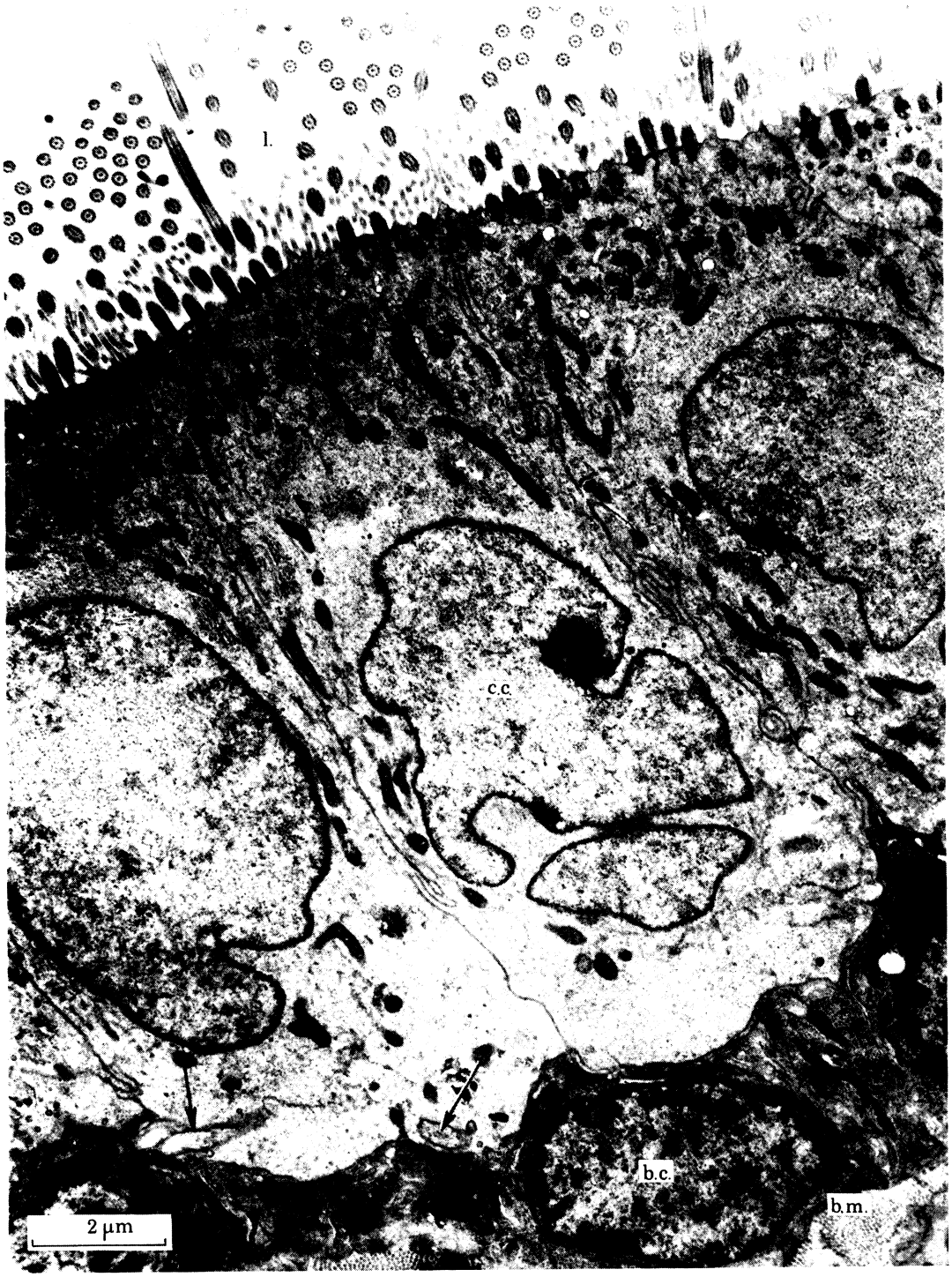

FIGURE 17. Electron micrograph of gosling tracheal epithelium showing ciliated (c.c.) and basal cells (b.c.) with intraepithelial nerves (arrows). Lumen and basement membrane (b.m.). Glutaraldehyde and osmium tetroxide: uranyl acetate + lead citrate. (Magn. × 10 000.)

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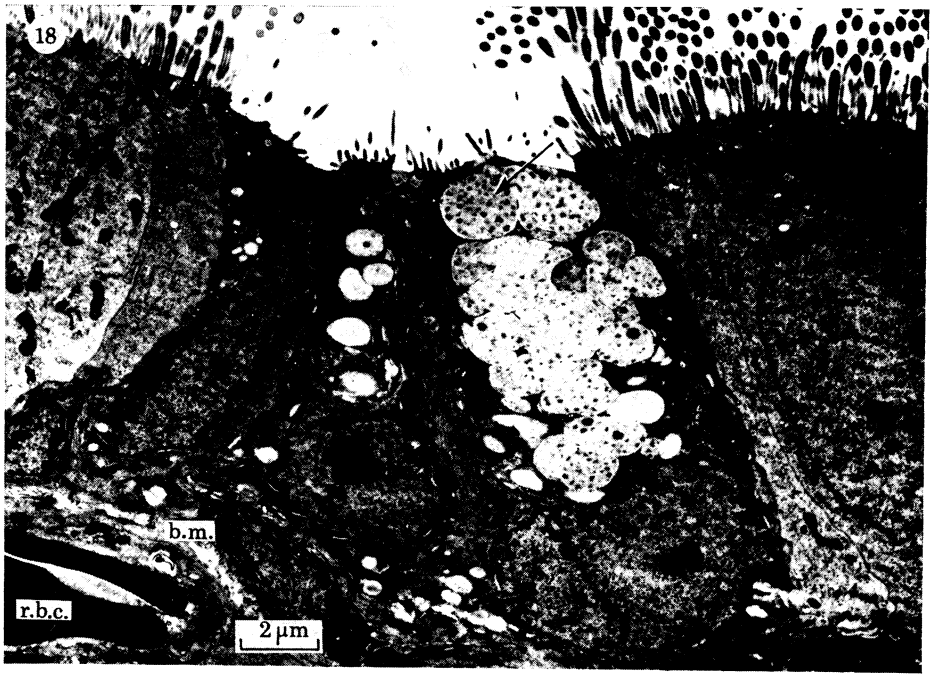

FIGURES 18 AND 19. For description see opposite.

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Description of plate 3

Figure 18. Gosling epithelium illustrating goblet cells with varying amounts of intracellular mucin: the secretory granules are confluent, electron-lucent but speckled with electron-dense areas (arrow). Basement membrane (b.m.) and capillary with nucleated red blood cells (r.b.c.). Glutaraldehyde and osmium tetroxide: uranyl-acetate + lead citrate. (Magn. × 5240.)

Figure 19. An intraepithelial nerve surrounded by a ciliated cell and close to the airway lumen. The nerve has electron-lucent cytoplasm and neurosecretory vesicles (arrow). Intercellular space (double arrows), lumen (l.) and cilia (c.). Glutaraldehyde plus osmium tetroxide: uranyl acetate plus lead citrate. (Magn. × 32 200.)

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FIGURE 20. Electron micrograph showing intraepithelial nerves (probably motor) associated with ciliated (c.c.), basal (b.c.) and nonciliated cells (n.c.c.). Lumen (l.). Glutaraldehyde plus osmium tetroxide: uranyl acetate plus lead citrate. (Magn. × 16 800.)

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as after a number of experiments ciliary transport was still present, and histology showed the epithelia to be intact (see Results, §(*f*)).

(2) In the cat very little leakage of radioactively labelled plasma proteins into the tracheal segment was found. This was also the case for the goose in three out of four experiments in which this was tested. However, in the fourth an appreciable leakage, and hence contribution to the radioactivity in the tracheal washings, was found. Whether this leakage resulted from an active transport process, a diseased tracheal epithelium or a damaged tracheal epithelium due to surgery is not known. Even in this fourth goose experiment the contribution of radioactivity to the tracheal washings from the plasma proteins is unlikely to have vitiated the results qualitatively, since the amount of leakage was unchanged by a dose of acetylcholine which did increase the radioactivity in the tracheal washings after the subsequent dose of [^{35}S]sulphate i.v. The leakage of radioactively labelled plasma proteins into the tracheal segment is only a problem when the radioactive label is given i.v. Plasma radioactivity levels were negligible when the radioactive precursors were given into the tracheal segment and results agreed with those from experiments in which the radioactive label was given i.v.

(*b*) *Biochemistry*

Monosaccharide analyses of all vertebrate mucins reported in the literature up to the present time have shown that fucose is one of the major neutral sugar components (Yosizawa 1972) and it is well established that fucose, along with sialic acids, can occupy terminal positions in the oligosaccharide chains of mucin-type glycoproteins. It seems, therefore, that fucose incorporation represents an important chain-termination step in glycoprotein biosynthesis. Fucose, however, is undetectable in goose tracheal mucins which means that the oligosaccharide chains have sialic acid at the non-reducing end or that another sugar replaces fucose. A study of the transferase enzymes involved in goose tracheal mucin biosynthesis would be an interesting exercise because the chain termination steps might possess unique features which would throw some light upon this 'dark' area of oligosaccharide assembly. In view of the rôle of fucose in determining certain blood group specificities (Watkins 1972) the blood group specificities of goose mucins would be of great interest.

It is noteworthy here that a sulphated glycoprotein has been isolated from another avian source, the allantoic fluid from chick embryos, and that this material was found to have little (Haukenes, Harboe & Mortensson-Egnund 1965, 1966) or no fucose (Meyer *et al.* 1967).

The mannose that was found in whole goose tracheal mucin preparations could have resulted, in part, from serum contamination. However, the detection of mannose in a sample of tracheal mucin which had been purified on both Sephadex G-200 and DEAE-cellulose (sample D, table 3) shows that mannose is a component sugar of goose tracheal mucins. Mannose has not been detected in the tracheal epithelial mucins of other species and in the goose it may substitute for fucose as a terminal neutral sugar.

As with cat tracheal perfusions administration of [^{35}S]sulphate either i.v. or intrasegmentally both appear to be valid methods for radioactively labelling the tracheal epithelial mucins (Gallagher *et al.* 1975); the non-diffusible isotope is associated with Alcian blue and PAS positive staining electrophoretic bands after fractionation by a variety of gel filtration system. In no experiment was there any significant radioactivity associated with serum glycoproteins, not even when [^{35}S]sulphate was administered i.v. rather than intrasegmentally. It is important to emphasize that Sephadex G-200 fractionation is an essential first step in the fractionation of

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goose tracheal mucins, since it separates most of the serum proteins which have passed into the tracheal segment from the radioactively labelled tracheal epithelial mucins. It is also apparent that [^{3}H]- and [^{14}C]glucose, administered intrasegmentally, are equally effective on the basis of the foregoing criteria as precursors in goose tracheal mucin biosynthesis.

(*c*) *Histology*

The glycoproteins of the gosling and adult goose trachea have been characterized histochemically and the ultrastructure of epithelial cells and their innervation has been described.

In the animals examined epithelial goblet cells were numerous while submucosal gland was never present, indicating that the source of all mucins collected was the epithelial goblet cell. This arrangement has been shown to be a feature of birds (Cover 1953; de Haller 1969; Purcell 1971; Walsh & McLelland 1974*a*).

The results for the gosling indicate that the simple alveolar 'glands' of the adult goose do not form until some time after the animal reaches 6 weeks of age.

The predominance of acid glycoprotein, and in particular sulphomucin, contrasts markedly with the predominance of neutral glycoprotein present in the trachea of the normal specific pathogen free rat (Jones, Bolduc & Reid 1973) but is similar to that described for the dog (Spicer, Chakrin, Wardell & Kendrick 1971). As almost all tracheal goblet cells of the goose contained sulphomucin it suggests that [^{35}S]sulphate label, used here as a marker for glycoprotein discharge, would have been incorporated readily by most of them. This is substantiated by autoradiographic evidence and also by the presence of an Alcian blue staining band on cellulose acetate electrophoresis which was associated with radioactive sulphate.

The presence of more than one type of acid glycoprotein within a single cell has been demonstrated previously in airway mucus-producing cells of other species (Lamb & Reid 1969; Jones *et al.* 1973). After treatment with sialidase, the loss of Alcian blue staining indicates the presence of 'susceptible' sialomucin. The special loss in the central zone of the cell, where the Golgi apparatus is located, has been found also for the rat goblet cell where it may indicate an early addition of sialic acid groups during mucous synthesis. From this histochemical study the presence of sialomucin resistant to digestion cannot be concluded: a further range of histochemical techniques will confirm or deny its presence. Some caution should be exercised in extrapolating the results from gosling to adult goose as histochemical differences in airway glycoproteins due to age have been reported, notably in man (Lamb & Reid 1972).

Electron microscopy has shown that in the gosling trachea the most common cell type is the ciliated columnar cell and this is in accord with the studies of Purcell (1971) and Walsh & McLelland (1974*a*) on the domestic fowl. In the goose the most common secretory cell is the goblet cell with large electron-lucent granules: the epithelial serous cell (see Jeffery & Reid 1975), though present, is rare. The heterogeneity of the goblet cell granules contrasts, however, with that seen in goblet cells of other species and may indicate heterogeneity of glycoprotein composition also.

Though nerve profiles were infrequent in gosling epithelium, the various types identified in the rat (Jeffery & Reid 1973) have been found in the gosling also. In a recent study, Walsh & McLelland (1974*b*) found intraepithelial nerves in the airways of the domestic fowl (*Gallus domesticus*) and like them we also found that most were close to the epithelial basement membrane. Walsh & McLelland (1974*b*) concluded that these nerves in the fowl were afferent and noted that no goblet cell was innervated. The physiological results of the present study strongly

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suggest that motor nerves do penetrate the airway epithelium and stimulate discharge of mucins from goblet cells. The presence of neurosecretory vesicles, observed by electron microscopy in large numbers within varicosities and terminals of these nerves, suggests a motor function (De Robertis & Bennett 1955; Wolfe, Axelrod, Potter & Richardson 1962; Palay 1956; Richardson 1966; De Robertis 1966). In neither goose nor chicken (Walsh & McLelland 1974*b*) was any synapse seen between nerve and adjacent cell and this indicates that transmitter release from any one nerve terminal probably affects more than one cell.

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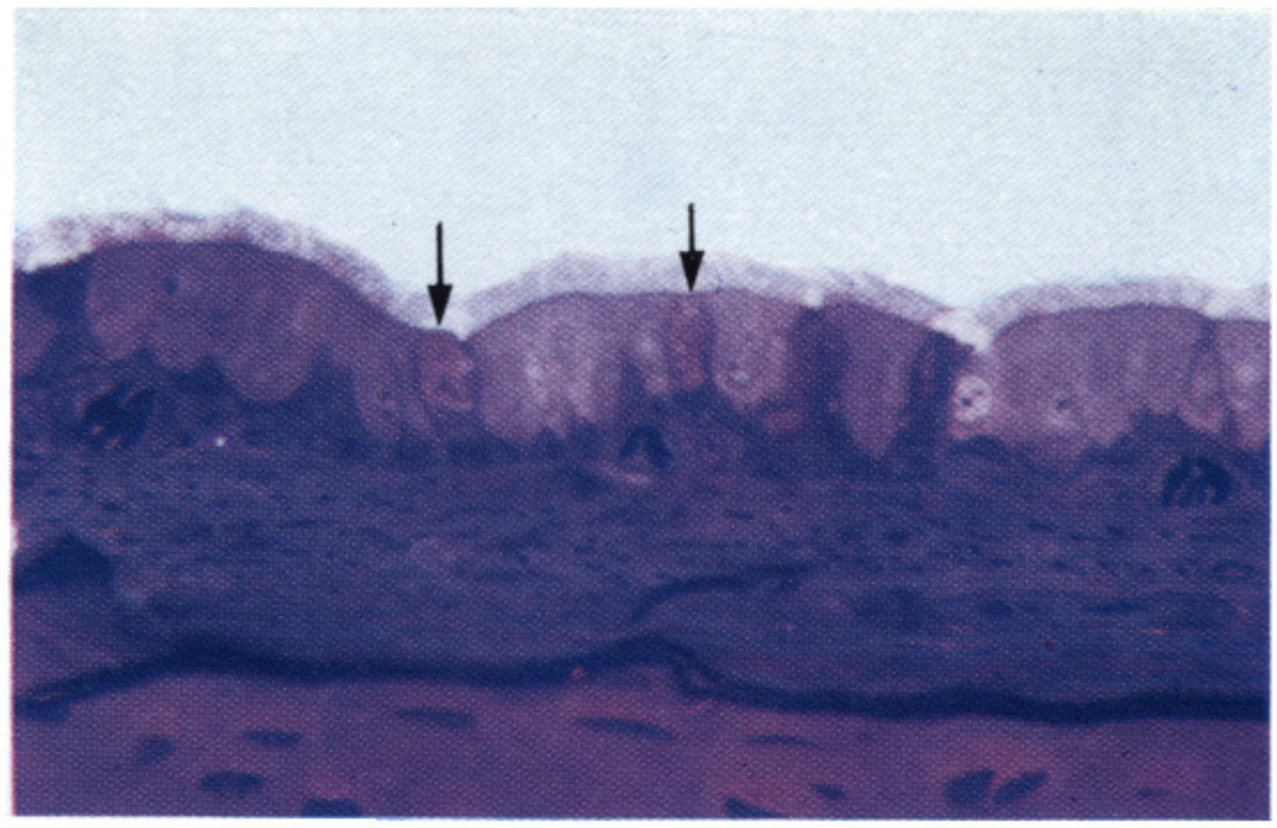
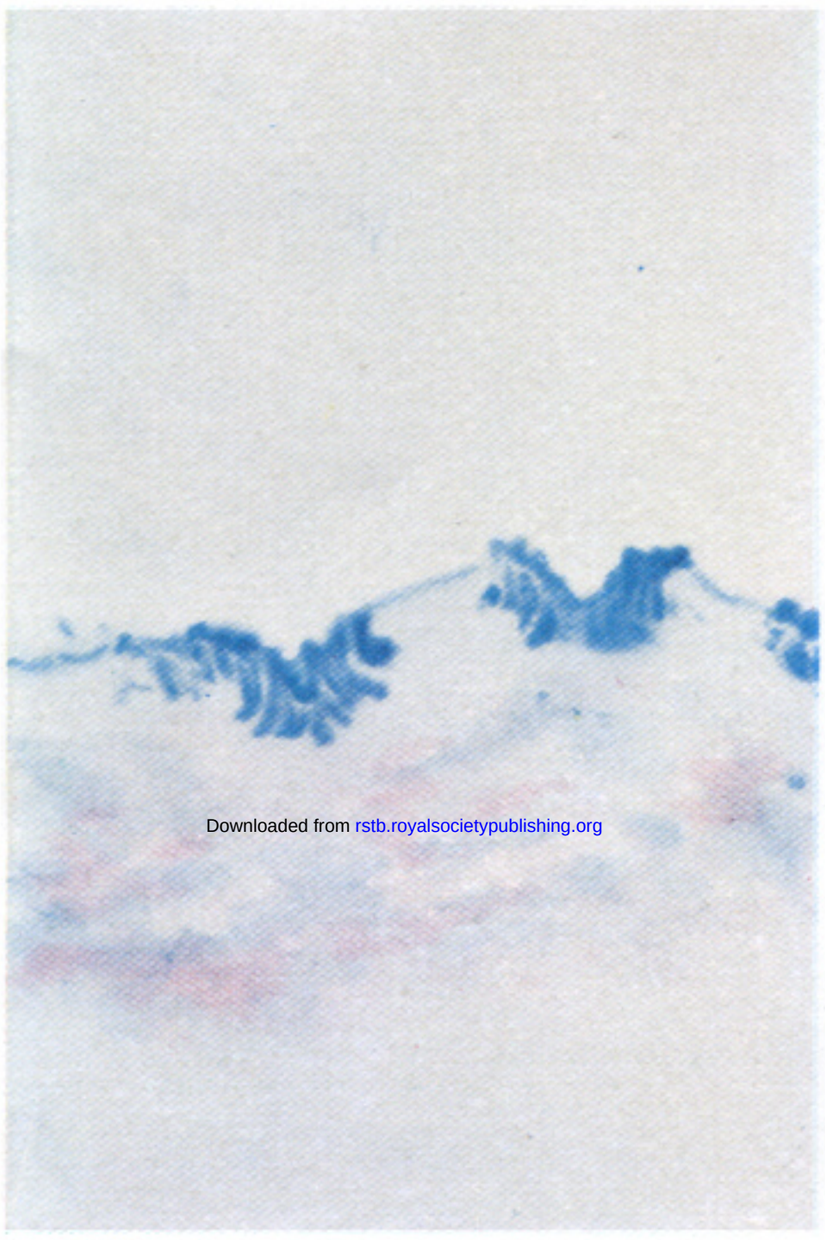


14*a*



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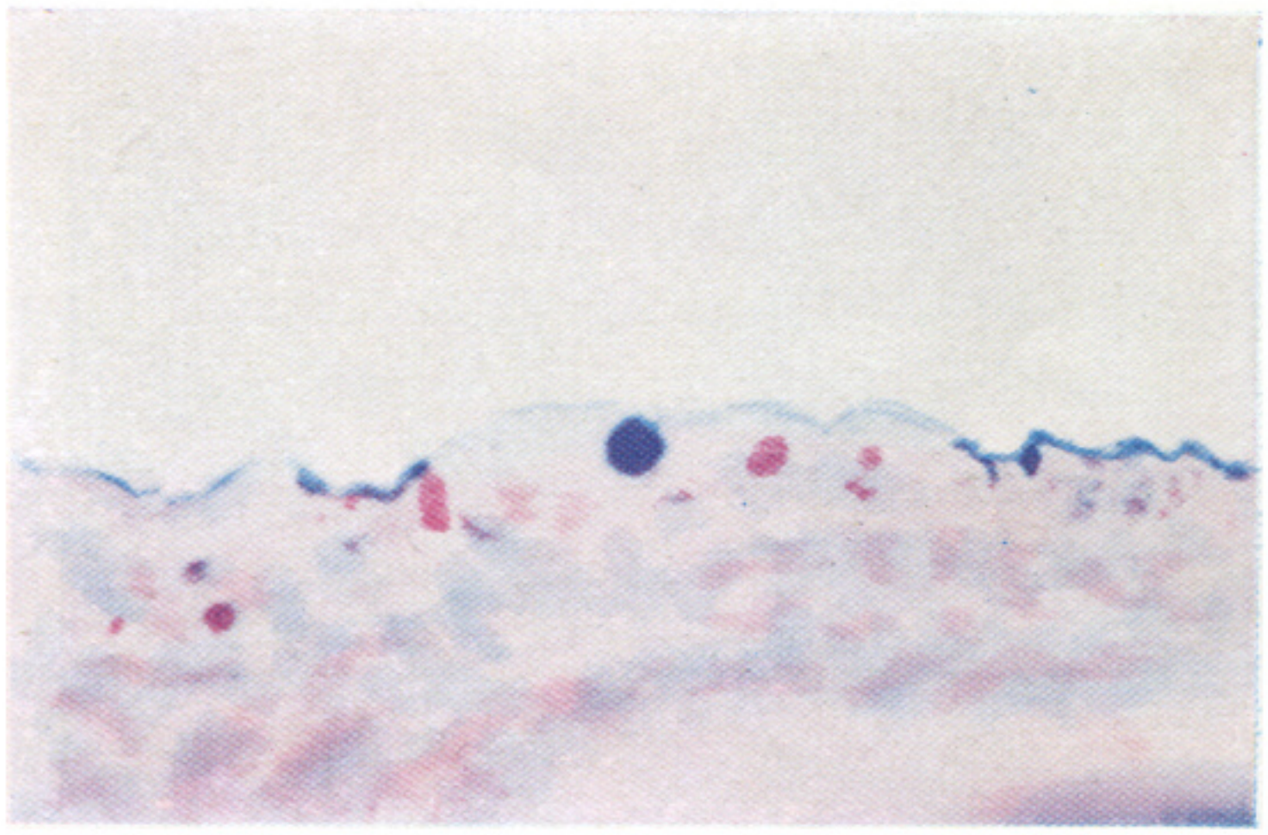

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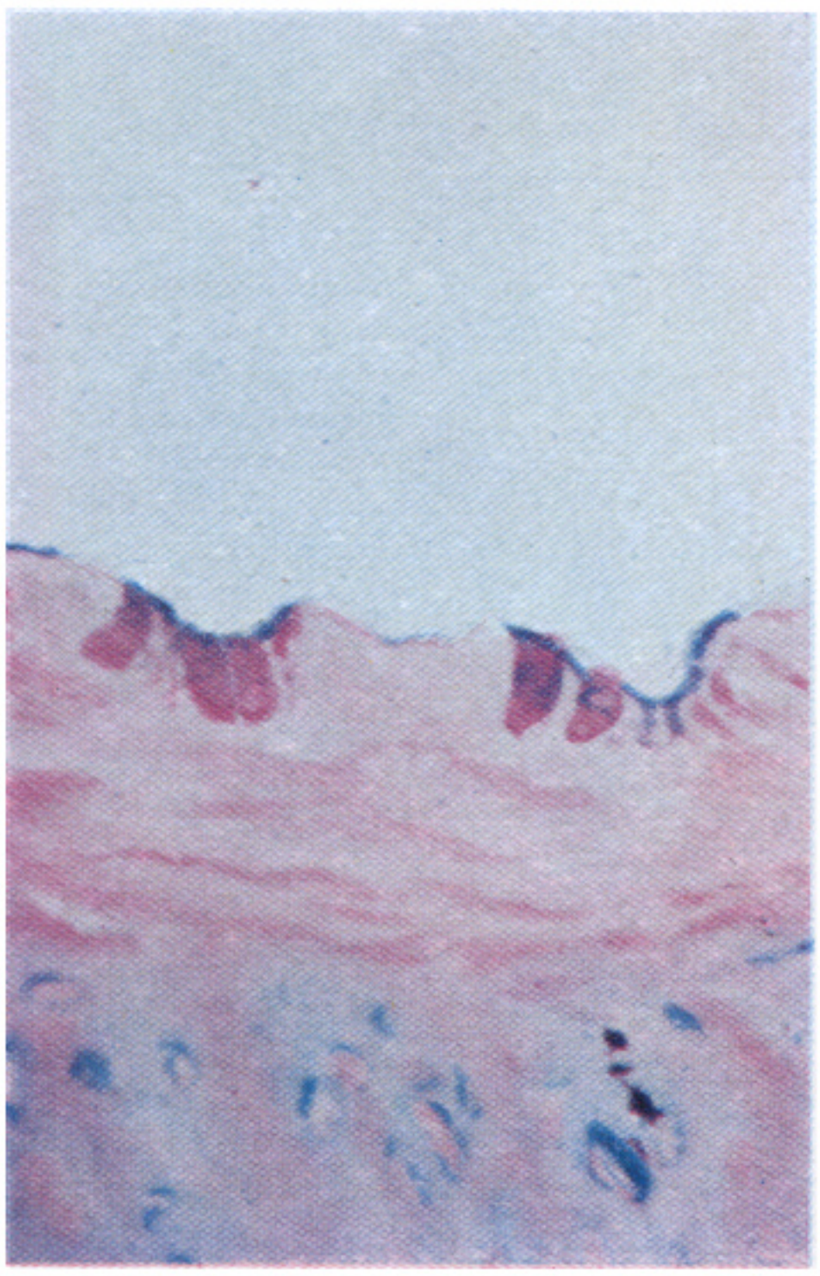
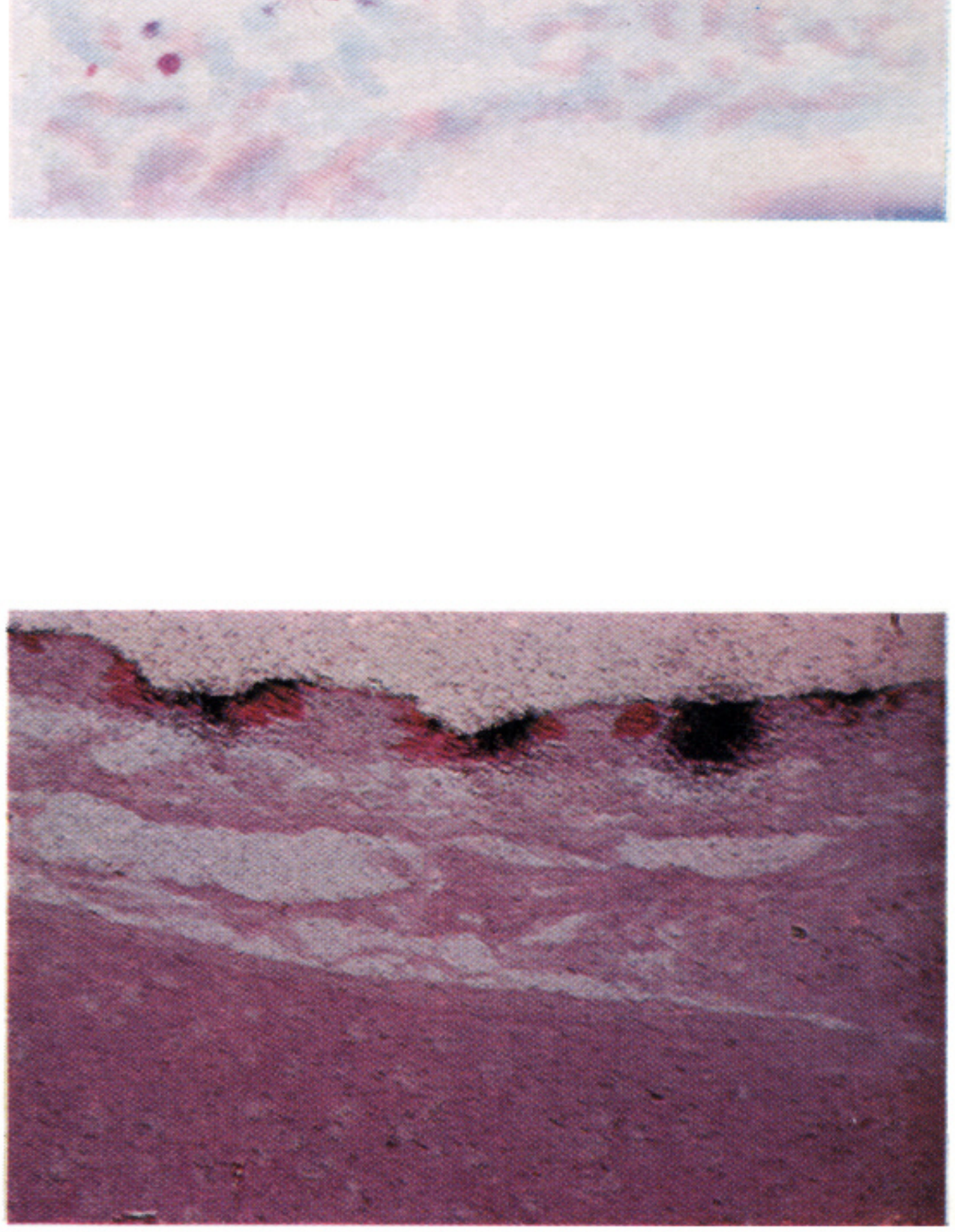


16



FIGURES 13-16. For description see opposite.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

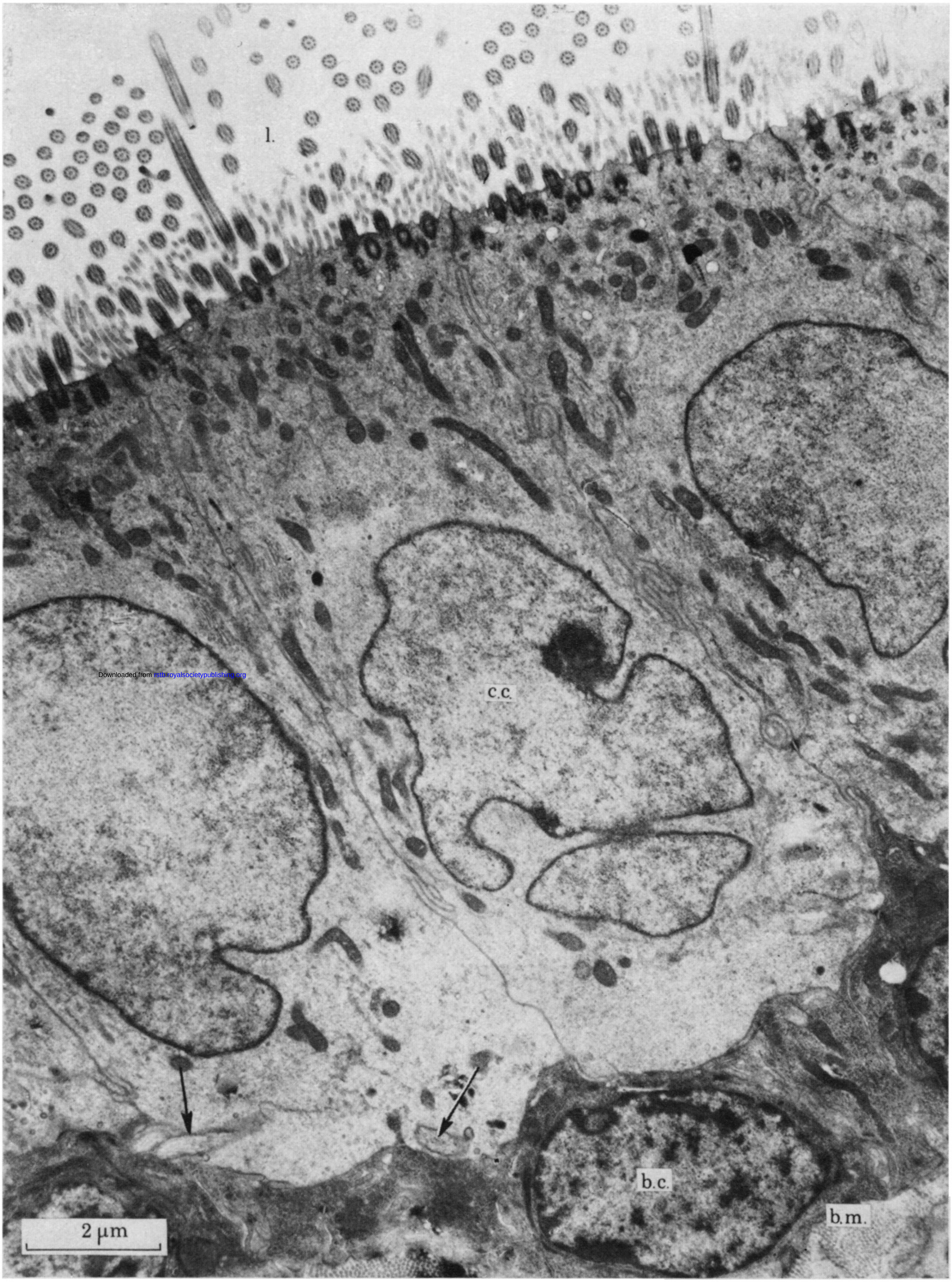
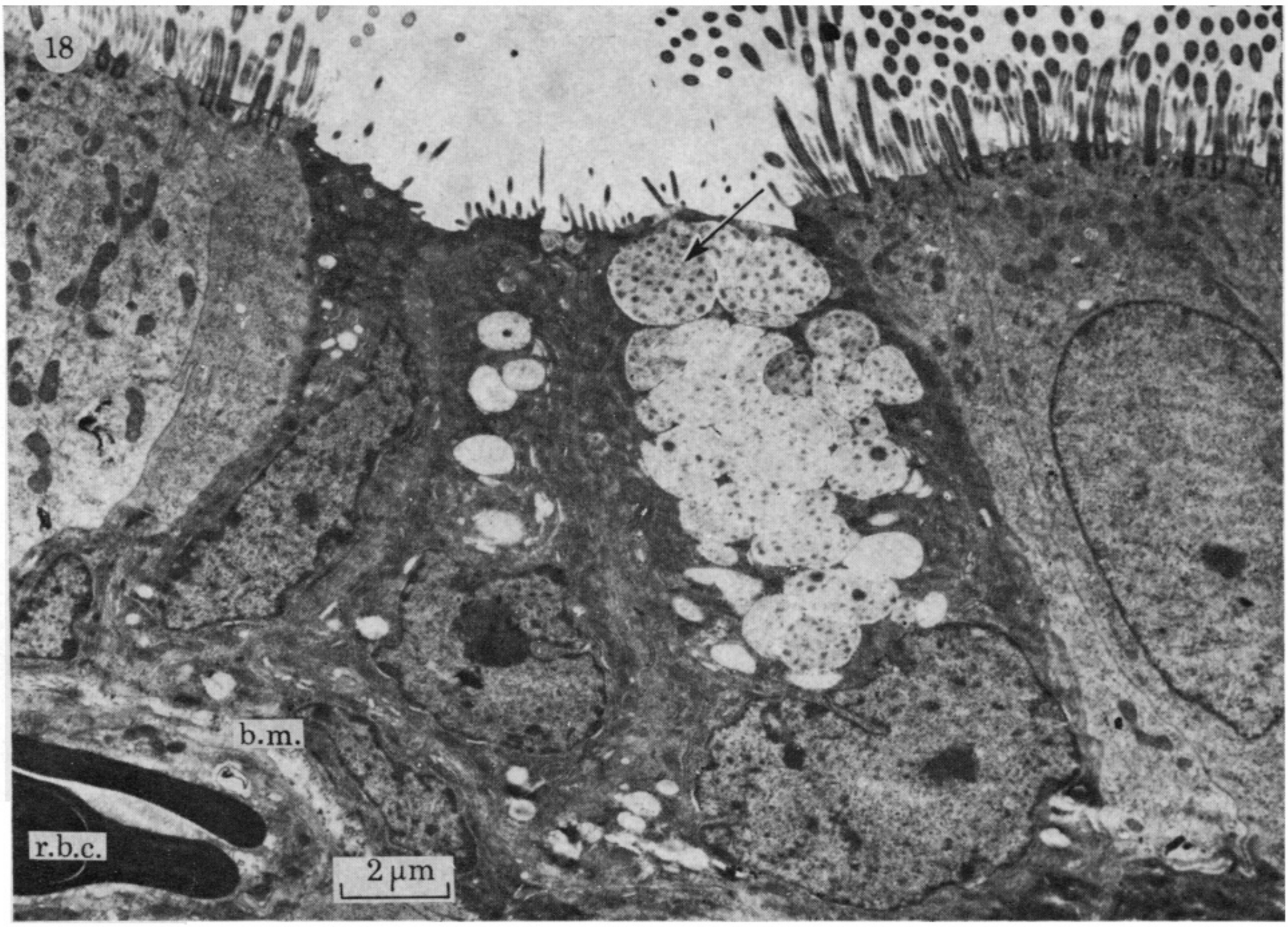
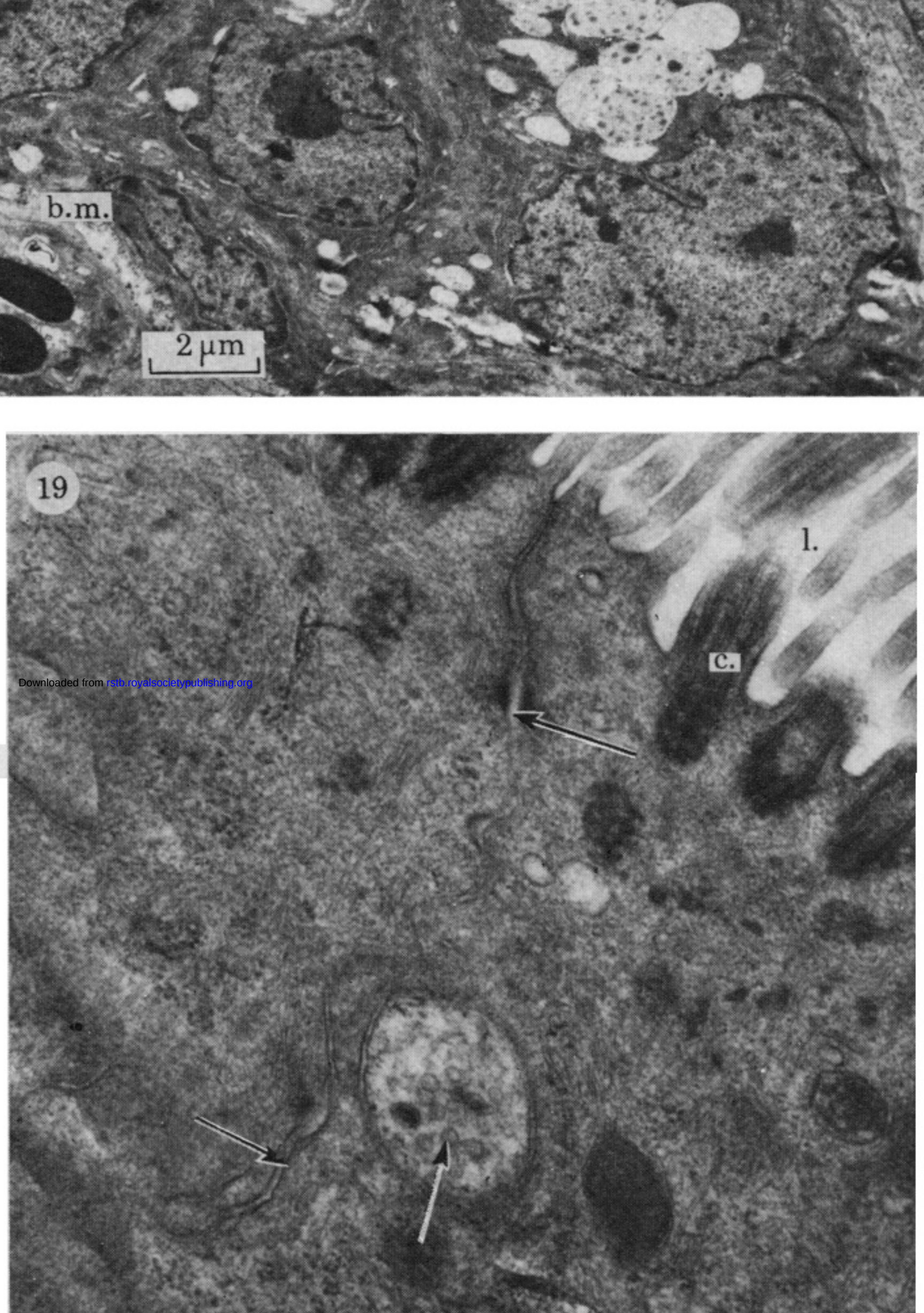


FIGURE 17. Electron micrograph of gosling tracheal epithelium showing ciliated (c.c.) and basal cells (b.c.) with intraepithelial nerves (arrows). Lumen and basement membrane (b.m.). Glutaraldehyde and osmium tetroxide: uranyl acetate + lead citrate. (Magn. × 10 000.)





FIGURES 18 AND 19. For description see opposite.

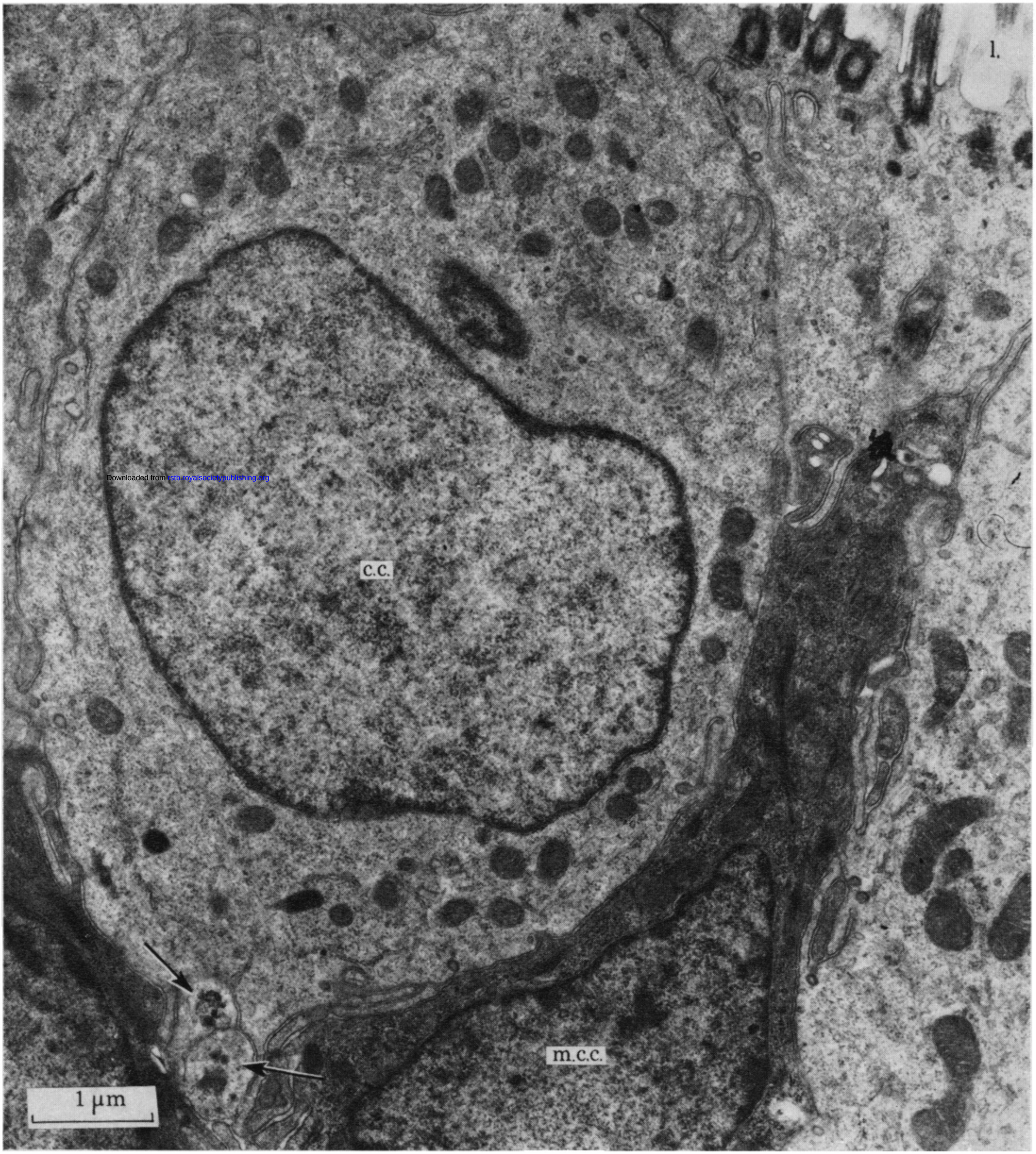


FIGURE 20. Electron micrograph showing intraepithelial nerves (probably motor) associated with ciliated (c.c.), basal (b.c.) and nonciliated cells (n.c.c.). Lumen (l.). Glutaraldehyde plus osmium tetroxide: uranyl acetate plus lead citrate. (Magn. × 16 800.)