Action of drugs on mucociliary clearance

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ABSTRACT

Two novel techniques have been developed for measurement of drug effects on mucociliary transport.

An optical method has been employed to measure transport rate of lead particles along the mucosal surface of frog oesophagus. Acetylcholine and parasympathomimetics have been shown to mimic the effect of vagal stimulation by accelerating transport. The reponse to vagal stimulation was inhibited by atropine or hexamethonium, but was not influenced by eserine. Adrenaline and sympathomimetics were without effect on particle transport rate. Mucolytics in clinical use, and a series of related compounds, have been compared; cysteine derivatives were ineffective, whereas bromhexine and ambroxol stimulated transport.

A beta-particle detector has been used to measure transport of phosphorus-32 labelled erythrocytes along the trachea of anaesthetised guinea-pigs, ferrets or rabbits. Vagal stimulation accelerated tracheal mucus velocity in guinea-pigs but not in ferrets. However, acetylcholine and pilocarpine accelerated tracheal mucus velocity in both species. Responses to vagal stimulation and to application of parasympathomimetic stimuli were inhibited by atropine. Isoprenaline was ineffective in both species but phenylephrine accelerated tracheal mucus velocity in guinea-pigs. Intravenous injection of ambroxol produced an acute acceleration of tracheal mucus velocity in guinea-pigs.

The pharmacology of mucociliary clearance has been reviewed and the present results have been discussed in the context of earlier clinical and experimental observations.

Abbreviations

Acetylcholine	ACh
Acetylcholine-esterase	AChE
Ammonia	NH3
Bovine serum albumin	BSA
Celsius	C
Chloride	Cl
Ciliary beat frequency	CBF
Citrate-phosphate buffer	Cit-ph
Forced expiratory volume	FEV1
Frog Ringer's solution	FRS
Gram	g
High pressure liquid chromatography	HPLC
Hour	hr
Hydrochloric acid	HCl
5-hydroxytryptamine	5-HT
Immunoglobulin A	IgA
Immunoglobulin G	IgG
Indium-III oxine	III-In
Isobutyl methylxanthine	IBMX
Mega Bequerel	MBq
Microgram	ug
Micro litre	ul
Micron	um
Millimetres	mm
Minute	min
Mucociliary transport	MCT
Phosphate buffered salt	PBS
Phosphorus-32	32-P
Prostaglandin E1	PGE1
Prostaglandin E2	PGE2
Prostaglandin F2 alpha	PGF2 alpha
Frequency	Hz

Slow reacting substance of anaphylaxis	SRS-A
Sodium	Na
Sulphur-35	35-S
Sulphur dioxide	SO2
Technetium-99	99-Tc
Tritiated glucose	3-H
Tracheal mucus velocity	TMV
Vasoactive intestinal peptide	VIP

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1.1) BACKGROUND

Cilia are hair-like projections which occur on the surface of both protozoan and metazoan cells. These organelles provide the propulsive force for locomotion of certain protozoa (ciliata), or for transport of fluids and particles over some epithelial linings in metazoan species. In only one animal phylum, the Nematoda, are cilia not found. Such a widespread distribution and the metachronal wave that characterises ciliary tracts, have attracted the attention of scientists since the early days of microscopy. Cilia were first reported by Anton van Leeuwenhoek in 1687 and were further described by Antonia de Heide a decade later. By the nineteenth century, when improved microscopes had become widely available, the first of many reviews on the occurrence and function of cilia was presented by Sharpey (1835). More recent reviews have been provided by Gray (1928), Lucas (1932) and Sleigh (1962).

In unicellular organisms, cilia provide a propulsive system to enable organisms to be freely motile (e.g. Paramecium) and to provide a system for food collection in both mobile (e.g. Paramecium) and sessile (e.g. Stentor) protozoa. In metazoan organisms, cilia subserve similar functions; for instance, cilia facilitate the collection of particulate material by filter-feeding species (e.g. Mytilus edulis); conversely, propulsion of fluid and mucus can serve to cleanse epithelial surfaces. It is in this latter context that the ciliary system of mammalian airways may be regarded, since ciliated bronchial epithelia propel plaques or sheets of mucus in an adoral direction, thereby providing a mechanism for removal of organisms and inorganic or organic debris entrapped during inhalation. This function

is termed "mucociliary transport". Effective mucociliary transport requires actively beating cilia and mucus of optimal physico-chemical properties, and, hence, understanding of function and regulation of ciliary activity and mucus secretion may provide a basis for understanding regulation of mucociliary transport.

In protozoan organisms, considerable technical ingenuity has been applied to the study of an assortment of environmental factors such as temperature, pH, ionic gradients and light on ciliary motion (Kinosito & Murikami, 1967). Invertebrates, such as the mussel (M. edulis), have been used extensively for study of ciliary motion, and in these preparations, 5-hydroxytryptamine (5-HT), dopamine, acetylcholine (ACh) and nerve stimulation have been shown to influence ciliary beat frequency (Bulbring et al., 1953; Aiello, 1960; Aiello and Guideri, 1964; Malanga, 1981). Measurement of ciliary beat frequency in intact vertebrates has been more difficult, since ciliated epithelia are in relatively inaccessible sites (e.g. airways, oesophagus, oviduct, Eustachian tubes). In addition, vertebrate ciliated epithelia contain large numbers of mucus secreting cells and glands interspersed amongst the ciliated cells. The sheet of mucus provided by these cells obscures the motion of underlying cilia. However, by shining incident light tangentially to a ciliated epithelium, it becomes possible to observe ciliary action as flickering in the mucus layer. Despite the invasive nature of the procedure for illumination and observation, this method has been used productively by many investigators (Lucas, 1935; Lierell & Moore, 1935; Dalham, 1956).

The epithelial surfaces of frog palate and oesophagus exhibit a very active mucociliary transport system, and have been used over the last

fifty years to study the effect of physiological and pharmacological agents on both ciliary activity and the rate at which particles are transported along these surfaces. (McDonald *et al.*, 1928; Pohle, 1931; Kordik *et al.*, 1952; Hill, 1957; Burn & Day, 1958; Milton, 1959; Sade *et al.*, 1970; King *et al.*, 1974; Braga, 1981; Morley and Sanjar, 1983). In earlier studies of the frog oesophagus, ciliary function was equated with the rate of transport of particles deposited on the mucus layer and "ciliary activity" and "particle transport rate" were interchangeable terms. However, changes in particle transport rate may arise from alterations in either the force or frequency of ciliary beat, in the amount and chemical composition of mucus or in the depth of periciliary fluid which lies between the epithelial surface and the mucus layer. Therefore, drug effects on the rate of particle transport need not conform to predictions based upon observations of a single parameter (e.g. ciliary beat frequency or mucus rheology). For this reason, *in vitro* preparations of ciliated cells, mucus secreting cells or mucus itself, may not be predictive of *in vivo* effects of drugs or other active materials on the overall process of mucociliary transport. For these reasons, the present study has focused upon use of *in vivo* preparations in intact or near-intact animals. As far as possible, discussion of cilia, mucus secreting apparatus and mucociliary transport will be confined to the mammalian respiratory epithelium and frog palate and oesophagus.

1.2) CILIA

1.2.1) Structure and function

Under the light microscope, cilia appear as hair-like structures with a length of between 5 and 100 um. These structures attracted considerable attention amongst light microscopists, but until the advent of electron-microscopy, very little was known about the fine structure of these organelles. The earliest electron-microscope studies were reported by Fawcett and Porter (1954). In cross section, cilia were observed to have a highly ordered internal structure which was surrounded by, and enclosed in, an extension of the cell membrane. It became apparent that both cilia and flagellae (longer than cilia found individually on cells), from a wide range of species and with different functions, had a constant internal structure which is now widely known as the "9 + 2" arrangement. This core structure is known as the axoneme and comprises nine doublet microtubules arranged circumferentially around a pair of central microtubules which are surrounded by a central sheath (Fig 1a). Each doublet consists of subfibres A and B. Subfibre A is a complete tubule, whilst subfibre B is incomplete, but shares a common wall with the subfibre A. Subfibre A of each doublet bears a radial spoke which links the doublet to the central sheath and a pair of dynein arms which contains the ATPase activity (Gibbons & Rowe, 1965). In addition to the radial spokes, the doublets are maintained in their geometrical lattice by a system of cross bridges that join adjacent doublets peripherally (Warner, 1970). This interdoublet link connects the terminal portion of the inner dynein arm to the adjacent subfibre B (Fig 1a). However, it is not clear whether there is a link to the arm or the subfibre A. The cilia

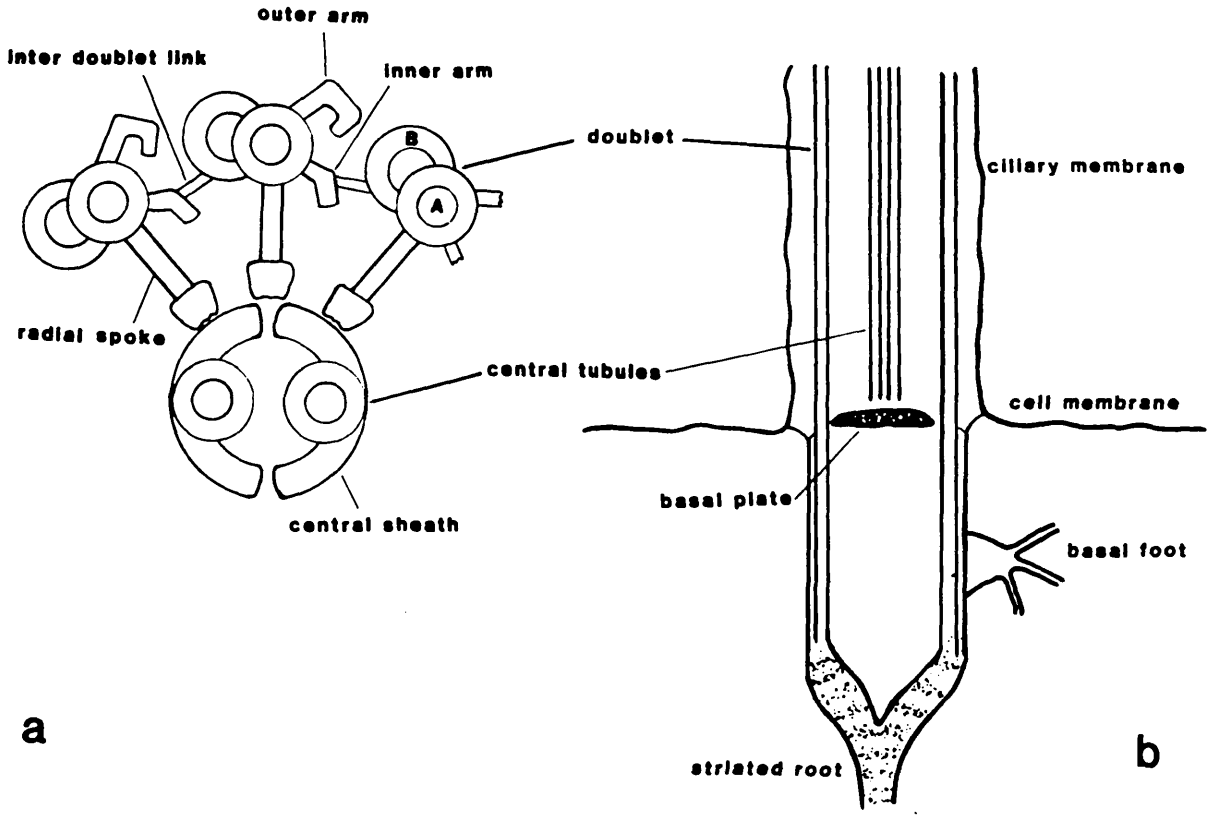


Figure 1. Diagramatic representation of electron microscopic appearance of a cilium in cross section (a) and longitudinal section.

are anchored to the cell by a basal body, or kinetosome, which is a cylindrical structure formed by 9 triplet microtubules. The 9 doublet microtubules of the axoneme of the cilia are continuous with the triplet structures. At the point where the shaft of the cilium enters the cell, there is a structure known as the base plate, within the cilium, sited at the level of the cell membrane. The central pair of microtubules stop at the base plate (Fig 1b).

Cilia perform their function by moving with a whip-like trajectory. There is an effective stroke which drives water or mucus over the epithelium followed by a whip-like recovery stroke in which the cilium swings out to one side as it regains its original position. The energy for this motion is provided by ATP (Hoffman-Berling, 1954; 1955). Abundant mitochondria, which provide the ATP required for ciliary motion, are found in the region of basal bodies.

1.2.2) Ciliated epithelium

Ciliated epithelia are found in many sites within vertebrate species. In the airways, ciliated cells are roughly columnar, approximately 20 um long and 7 um wide, tapering to 2 um at the base where they are in contact with the basement membrane. The baso-lateral surface of ciliated cells interdigitate with adjacent cells and form desmosomes (Rhodin, 1966). At the luminal surface, cells form tight junctions of the tripartite functional complex type (Inoue, 1974). The cell cytoplasm is electron lucent because it contains no secretory products or granules, nor are there many ribosomes. There is a well developed Golgi apparatus situated above the nucleus and abundant mitochondria are found in the upper cytoplasm, just below the apical row of basal

bodies. Each cell contains approximately 250 cilia which are 6 um long and 0.3 um wide; interspersed are approximately 120 microvilli. In the airways, the length of cilia and distribution of ciliated cells varies according to location and species. In man, tracheal cilia range from 5.4 to 6.6 um; comparable values have been reported for the dog (Serafini & Michaelson, 1977). However, length of cilia decreases in the lower airways, reaching a mean length of 3.7 um by the sixth airways generation in both man and dog (Serafini & Michaelson, 1977). The percentage of ciliated epithelial cells may also vary with location and species. Human trachea is reported to contain between 44 and 62% ciliated cells, which declines to between 2.8 and 19.5% in the fifth airways generation (Serafini & Michaelson, 1977). Tracheal epithelium in the dog has a smaller percentage of ciliated cells (22%), which declines to 2.7% by the third airways generation (Serafini et al., 1976). In addition to ciliated cells, abundant goblet cells are found in the tracheobronchial epithelium, where there are approximately 5 ciliated cells per goblet cell (Rhodin, 1966).

Ciliary beat frequency can be measured in exposed but intact mucosa by observing fluctuations in incident light due to ciliary action (Lucas, 1933; 1935; Dalham, 1960) or by using a photosensitive cell (Dalham & Rylander, 1962). High speed cinematography has been used to record ciliary activity (Proetz, 1932). The rate of ciliary beating can be measured in vitro from biopsy samples, or from brushings collected from the airways. This latter method was described by Yager et al. (1978) who showed that ciliary beat frequency of biopsy material collected from different levels of human airways was approximately 14 Hz.

1.2.3) Regulation of ciliary activity

Whether cilia or flagellae have intrinsic activity or are merely appendages powered by contractile elements from within the cell has long been subject to debate. The question was answered by the elegant experiments of Goldstein et al. (1970), who used a laser microbeam to sever a flagellum from Crithidia and observed that the detached flagellum was able to swim freely until energy reserves were expended. Although cilia and flagella can beat when detached from the cell, there exist intracellular mechanisms which co-ordinate beat frequency and the direction of beat as is clearly evident in protozoan species.

Burn (1950) suggested that the rhythmic activity observed in multi-cellular tissues, with no innervation, may be explained by the presence of local hormones. Acetylcholine (ACh) was proposed as the mediator most likely to perform this function, especially as such a role had been assigned to ACh in the rabbit auricle (Bulbring and Burn, 1949) and intestine (Feldberg and Lin, 1950). It was suggested that at low concentrations, ACh acted as a local hormone and was able to stimulate or maintain rhythmic contractions in the auricles, whereas at high concentrations, it acted as a true neurotransmitter by inhibiting the beating of auricles (Bulbring and Burn, 1949). This hypothesis was adopted for ciliated epithelia, since the presence of both ACh and acetylcholinesterase (AChE) had been demonstrated in Paramecium (Bayer and Wense, 1936) and Trypanosoma rhodesiense (Bulbring et al., 1949). In 1952, Kordik et al. found that the rate at which particles were transported (termed ciliary activity) along the frog oesophagus and rabbit trachea could be accelerated by low concentrations of ACh or eserine, but were inhibited by high concentrations of these drugs. These investigators were also able to extract AChE from the mucosa of

both preparations. The presence of AChE activity in the ciliated gill plates of M. edulis (Bullbring et al., 1953) and in the palatal mucosa of Bufo vulgaris formosus was reported subsequently (Satoh, 1959a,b). In accordance with these findings, Bulbring et al. (1953) showed that, in the ciliated gill plates of the mussel M. edulis, both ciliary beat frequency (measured stroboscopically) and particle transport rate were accelerated by low doses of ACh or eserine but were inhibited by high doses of these drugs or by atropine or d-tubocurarine. These observations were presented as proof for the "local hormone" theory, since nerves had not been demonstrated in the gill plates of mussels (Bulbering et al., 1953). However, Aiello (1960) reported that 5-HT or electrical stimulation of the branchial nerve (Aiello & Guideri, 1964) were potent stimulants of ciliary activity in this preparation; the presence of nerves was later confirmed by histological studies (Aiello & Guideri, 1965). Despite the demonstration that nervous control of ciliary activity is serotonergic (Aiello & Guideri, 1966) and dopaminergic (Paparo & Aiello, 1970; Malanga et al., 1981), ACh seems to be released from these nerves since hyoscyamine inhibits cilioexcitation due to nerve stimulation (Aiello & Guideri, 1964). It is apparent, therefore, that the concept of "local hormones" maintaining rhythmic activity cannot be applied to ciliated epithelium of molluscs. This concept is even less plausible in preparations such as the frog palate, since in the absence of stimulation, cilia stop beating (Lucas, 1935). Activity can be restored by mechanical stimulation of the epithelial surface or electrical stimulation of the 7th cranial nerve which is parasympathetic in nature (Lucas, 1935).

Control of ciliary beating in mammalian airways has been studied using a variety of methods (see above). There is circumstantial evidence of

nervous control, since fine unmyelinated nerves have been observed around the base of ciliated cells in human (Rhodin, 1966) and feline (Messerklinger, 1958) trachea. It is difficult to study the effect of pharmacological agents on cilia in intact airways due to the presence of mucus; however, an *in vitro* method has been described by Iravani (1967), which permits measurement of beat frequency in groups of cilia. Using this technique, isoprenaline, ACh, theophylline, 5-HT and histamine have been reported to increase ciliary beating, although the effects observed were modest (Iravani & Melville, 1975a). Measurement of ciliary beat frequency in biopsy samples from the airways is an attractive alternative, since ciliary activity is not obscured by mucus and preparations seem to last for several hours. Pharmacological agents have been tested on this system; but, only modest effects have been reported and large drug concentrations have been employed (Clarke, 1983). Similarly, observations that have been made using cultured tracheal epithelium, have required high concentrations (10-4 g/ml) of ACh to increase the rate of rotation of these clumps (Corsen and Allen, 1956). Brush biopsy samples have been used to determine the effect of antigen challenge upon ciliary activity of sensitive animals (Maurer *et al.* 1982). Samples obtained from the trachea of sheep with *Ascaris suum* hypersensitivity, showed significant increase in beat frequency, after challenge. This effect was abolished by di-sodium cromoglycate (DSCG) (Maurer *et al.* 1982). These results are at variance with the results obtained whilst measuring tracheal mucus velocity during antigen challenge *in vivo*, when mucociliary clearance is acutely reduced. It has been postulated that slow reacting substance of anaphylaxis (SRS-A) may be responsible for cilioexcitation, either directly, or by involvement of other mediators, such as prostaglandins E1, E2 or histamine (Wanner *et al.*, 1983). However, PGE1 has been

reported to have variable effects on ciliary activity on rats, hamsters and monkeys (Iravani & Melville, 1975b).

1.3) MUCUS

1.3.1) Source of airways secretions

In the respiratory epithelium, secretions originate from four cell types: mucus cells, serous cells, Clara cells and goblet cells. Serous cells are found either in the lining epithelium or organised into submucosal glands. The goblet cells are found in abundance in the normal mucosa (approximately 6,800 cells per square millimetre) and, in chronic bronchitis, goblet cell incidence can approximately double (Breeze & Wheeldon, 1977). Larger numbers of goblet cells are found towards the caudal end of the trachea and are associated with the cartilaginous, rather than the membranous, parts. Goblet cells are numerous in the trachea of most animals, except pathogen-free rats (Jeffery & Reid, 1975). Very few goblet cells are found in the bronchioles of normal subjects; however, at sites of disease, large numbers may be found (Thurlbeck et al., 1975). Goblet cells have relatively dense, electron opaque cytoplasm, with many mucus granules and ribosomes in the apical cytoplasm. The cell tapers towards the end that touches the basement membrane. Lower parts of the cell form desmosomes with neighbouring cells. The nucleus is small and found at the base of the cell and there is a well developed Golgi apparatus above the nucleus, associated with extensive rough endoplasmic reticulum, which is involved in protein synthesis. Carbohydrates are added to the protein and sulphur bridges are formed within the Golgi apparatus before release (Peterson and Leblond, 1964).

Serous cells are not found in all mammalian species. They have been distinguished as a specific cell type in the rat airways and have been

shown to contain neutral mucosubstance in the apical regions (Jeffery & Reid, 1975). Serous cells rest on the basement membrane with the cell apex exposed to the lumen. Their cytoplasm is electron dense and contains abundant rough endoplasmic reticulum. The function of these cells is not well defined; it has been suggested that they may be converted to goblet cells in the course of experimental bronchitis (Jeffery, 1978).

Clara cells are columnar cells which are found in the epithelium of terminal bronchioles. At this level, ciliated cells are shorter and non-ciliated cells are predominantly of the Clara type. The baso-lateral membranes of Clara cells inter-digitate with neighbouring cells. The nucleus is deeply invaginated; the apical cytoplasm contains profuse smooth endoplasmic reticulum and a prominent Golgi apparatus, which is indicative of a secretory function. However, secretions are not mucoid but possibly a lipoprotein (Azzopardi & Thurlbeck, 1969) that may form a surface-active layer in the bronchioles.

The bulk of mucus secretion in the airways arises from submucosal glands which are found in abundance in sheep, cows, cats, ferrets and humans where they extend from trachea to the small bronchi (Goco et al., 1963; Korhonen et al., 1969). The volume of mucus secretion from submucosal glands has been estimated as 40 times greater than that from goblet cells (Reid, 1960). Three regions are recognisable in submucosal glands: ciliated duct, collecting duct and secretory tubules. The ciliated duct is lined by tracheal epithelium which extends into the gland (approximately 350 um). The opening to the luminal surface is approximately 65 um, widening distally to 90 um.

The collecting duct is 800 um long and 250 um wide, and is lined with columnar cells (30-70 um tall). The lining cells are thought to determine the water and ion concentration of glandular secretions (Meyrick et al., 1969).

Secretory tubules arise from the collecting duct and contain mucus and serous cells. Mucus cells occupy the proximal section of the secretory tubules. These cells are large and can reach a height of 500 um and a width of 70 um. The nucleus is flattened against the base of the cell and the supra-nuclear Golgi apparatus is associated with many vacuoles and vesicles. Secretory ducts are lined by columnar cells 25 to 30 um high and containing secretory material. Serous secretory tubules arise at the end of mucus tubules. Serous cells are 20-30 um high and pyramidal in shape, containing a round basal nucleus. The Golgi apparatus is supranuclear and is associated with many vesicles (Meyrick et al., 1969; Meyrick and Reid, 1970).

1.3.2) Constituents of mucus

In normal individuals, collection of bronchial secretions is difficult, since there is no expectoration. Usually, bronchial secretions are mixed with upper respiratory tract fluid and saliva and swallowed. However, mucus production can be stimulated in healthy subjects by inhalation of ACh, histamine, prostaglandin F2 alpha (PGF2-alpha) or citric acid (Lopez-Vidriero et al. 1977). PGF2-alpha is the most effective of these substances. Usually sputum, which is a mixture of saliva, upper respiratory tract secretions and hyper-secreted bronchial mucus is used to study the properties and constituents of mucus. Sputum comprises the secretory products of goblet cells, serous and

mucus cells of submucosal glands, a transudate derived from plasma, surfactant derived from Type II pneumocytes, intact cells and cell debris (DNA), dust particles and bacteria (Masson and Hermans, 1973; Boat and Matthew, 1973).

Normal bronchial secretions consist of 95% or more water, 2-3% glycoprotein, 0.1-0.5% proteins and 0.1-0.5% lipids (Masson and Heremans, 1973; Boat and Matthew, 1973). Bronchial secretions are hyper-osmolar with respect to serum (Boat & Matthew, 1973). A large range of proteins which are derived from either plasma, by exudation, or secreted by the epithelial lining, are found in bronchial secretions. Exudated plasma proteins form a very small portion of the total protein content, of which albumin, immunoglobulins G and A (IgG & IgA) and alpha-1 anti-chymotrypsin are the most important components. The latter two are found at higher concentrations in sputum compared to plasma, indicating an active secretory process. Secreted plasma proteins include immunoglobulin A, lactoferrin, lysozyme and amylase which are considered to have a defensive role (Querinjean et al., 1971; Creeth, 1978).

Sputum can be divided into 2 fractions: a sol layer, in which the soluble components are found, and a gel layer, which gives sputum its characteristic physical properties. Mucus glycoproteins of high molecular weights comprise 50-80% of the macromolecules in sputum. These molecules are of variable size and contain a diversity of sugar side chains, so that it is difficult to isolate glycoproteins as an homogeneous entity (Roberts, 1978). Freshly produced mucus glycoprotein has a molecular weight of several million, of which well over half represents carbohydrate components which are oligosaccharide

units of 8-10 sugars studded along the polypeptide backbone, at about every third amino acid. The linkage between carbohydrate and protein is termed "O-glycosidic", because the linkage is through an oxygen atom. The oligosaccharide units project from the polypeptide chain, giving a structure that has been likened to a test-tube brush (Reid and Clamp, 1978). The glycoprotein can be solubilised by hydrogen bond cleavage using urea (Roberts, 1974), disulphide-bond cleavage agents (Havez et al., 1967) or by proteolytic enzymes (Brogan, 1959). Mucus glycoproteins (bronchial) have been reported to contain 14.1% fucose, 22.6% galactose, 19.1% glucosamine, 12.8% galactosamine and 5.8% sialic acid; there is no mannose or uronic acid (Roberts, 1978). By sub-fractionation of bronchial glycoproteins, Havez et al. (1968) have reported three distinct groups: neutral glycoproteins, sialylated glycoproteins and sulphated glycoproteins. These observations have been questioned by others (Roberts, 1974; Boat et al. 1976), who failed to separate sialylated glycoproteins and sulphated proteins but found that there was a continuous variation with respect to sialic acid and sulphate content. In experimental animals, bronchial mucus can be obtained by introduction of a tracheal pouch (Wardell et al., 1970) or by collection from excised trachea. Inter-animal variation has been reported (Kent and Widdicombe, 1977).

1.3.3) Physical properties and function

The physical properties of bronchial secretions or sputum arise from the glycoprotein fraction which has a gel-like structure. Mucus exhibits properties of a liquid and of a solid when responding to stress and behaves as some combination of the two; hence, mucus is said to have viscoelastic properties. Viscosity of sputum can be

measured by applying a shear stress to the sample and observing the resultant rate of strain. However, the measured or apparent viscosity is dependent on the applied stress. At high shear rates, changes in apparent viscosity may be permanent, which is consistent with rupture of the macromolecular chains of the gel structure (Palmer et al., 1970; Charman and Reid, 1972). This property may be important during cough (King, 1980). When stress is applied to mucus, it flows steadily until the stress is removed; there is a recoil action with partial recovery of the strain. This response has been used to measure elasticity of mucus (Barnett and Dulfano, 1970).

Effective mucociliary transport requires the presence of mucus (Sade et al., 1970; King et al., 1974) of optimal viscoelastic properties and concentration of glycoproteins. Using the frog palate, Shih et al. (1977) reported that transport was most effective when mucus samples contained 1.5-2.0% (w/v) glycoproteins, although higher concentrations (up to 5%) were also transported (30-50% less effectively). This observation has important implications, since drying of airways can be predicted to reduce effective mucociliary transport and subsequent humidification can be expected to restore transport to normal values; experimental observations have confirmed these points (Dalham, 1956; Asmundsson & Kilburn, 1970; Forbes, 1973; Hirsch et al., 1975). Inhalation of steam has been used for many years to improve expectoration and, more recently, in the treatment of lower respiratory disease (Gibson, 1974).

Changes in viscosity and elasticity of mucus affect mucociliary transport. Although increased viscosity reduces particle transport rate, alteration in elasticity has been reported to be the more

important determinant of transport rate (Dulfano and Adler, 1975), such that a slight change from an optimal value will produce large decreases in transport rate. It is therefore not surprising to find that mucociliary clearance and tracheal mucus velocity are severely impaired in patients with a variety of lung diseases (Santa Cruz et al., 1974; Wood et al., 1975; Foster et al., 1978). Certain drugs have been used in respiratory disease because of their capacity to modify the rheological properties of mucus, and are collectively referred to as "mucolytics". These compounds are mostly cysteine derivatives and exhibit a capacity to disrupt di-sulphide bridges which cross-link and form the glycoprotein polymer. Compounds, such as N-acetylcysteine, are administered as an aerosol and have been reported to increase expectoration dramatically (Webb and Jackson, 1962; Sheffner et al., 1964). More recently, compounds such as S-carboxymethylcysteine, which are administered orally, have been introduced. Havez et al. (1970) have reported that treatment with this compound changes the composition of sialylated glycoproteins and may exert its effect by removal of sialic acid or by impairing incorporation of sialic acid residues. Despite marked improvement of respiratory parameters (e.g. FEV1) following treatment with S-carboxymethylcysteine (Edwards et al., 1976), no improvement of mucociliary transport was observed (Thomson et al., 1975; Goodman et al., 1978). Other mucolytics, such as bromhexine and its analogue, ambroxol, have been reported to increase mucociliary transport in both experimental animals (Iravani & Melville, 1974; Melville et al., 1980) and in patients (Thomson et al., 1974; Aurnhammer et al., 1977; Weiss et al., 1981). The viscosity of sputum in vitro is decreased by saline and water mist (Palmer, 1960). Accordingly, inhalation of aerosolised hypertonic saline has been found to significantly increase mucociliary clearance and sputum

expectoration in patients with chronic bronchitis (Pavia et al., 1978).

In addition to entrap ment of inhaled particles and their subsequent removal, mucus may subserve a further protective function by providing a barrier between the external environment and epithelial cells. Direct contact between environmental and industrial pollutants, such as sulphur dioxide (SO2) and ammonia (NH3), etc. can be injurious to these cells. By analogy with the stomach, where mucus protects the gastric mucosa from lytic enzymes and hydrochloric acid (HCl) (Morson, 1955), it is not unreasonable to assign a similar protective role to bronchial mucus. More recently, it has been suggested that mucus may form a macromolecule-proof coating for cell surfaces (Edwards, 1978) and, hence, prevent direct contact between proteolytic enzymes and viruses with epithelial cells.

1.3.4) Regulation of mucus secretion

Regulation of airways secretion has been investigated for many decades. The earliest studies on the regulation of airways secretion were carried out by Kokin (1896, as cited by Widdicombe, 1978) and Calvert (1896). The former reported that electrical stimulation of the vagus nerve promoted secretion from submucosal glands. Many studies have since confirmed this observation (Florey et al., 1932; Perry and Boyd, 1942; Gallagher et al., 1975; Ueki et al., 1980). In addition to nerve stimulation, mucus production can be enhanced by pharmacological agents and irritants which may be physical (dust or tactile stimulation of the airways) or chemical (ammonia vapour). Since mucus is a mixture of secretions from epithelial cells, goblet cells and submucosal glands, attempts have been made to identify the

source of secretion. On the basis of histological studies, Florey et al. (1932) reported that vagal stimulation emptied mucus glands, leaving goblet cells unaffected. This effect of nerve stimulation was mimicked by pilocarpine. However, dilute ammonia emptied both goblet cells and glands; it was suggested, therefore, that mucus glands, and not goblet cells, were innervated. Using an isotopic method for detection of radiolabelled glycoproteins, Gallagher et al. (1975) confirmed the effects of vagal stimulation in the cat and, in addition, reported that atropine inhibited the stimulatory effect of vagal stimulation or pilocarpine. They also reported that electrical stimulation of the stellate ganglion and beta-adrenoceptor agonists stimulated mucus secretion, an effect that was inhibited by propranolol.

Autoradiographic studies have been used to localise the sites of accumulation of radiolabelled glycoprotein precursors. Gallagher et al. (1978) reported that radioactive sulphur (35-S) was incorporated into the submucosal glands and tritiated glucose (3-H) was taken up by epithelial cells, predominantly. Goblet cells contained very little of either marker. Most of the tritiated glucose was found along the luminal border of epithelial cells. Electrophoresis of mucus revealed two bands, a fast moving band containing mostly 35-S and staining with Alcian Blue and a slow moving band which stained with periodic acid-Schiff reagent. In response to vagal stimulation or pilocarpine, 35-S rich mucus was collected, whereas ammonia vapour caused the release of 3-H rich mucus, predominantly. These results confirm the earlier report from Florey et al. (1932), with the exception that a distinct glycoprotein was released from epithelial cell surface in response to ammonia rather than from goblet cells (Gallagher et al.,

1978). It remains uncertain whether goblet cells or secretory epithelial cells of mammalian airways are innervated. However, little doubt exists as to innervation of goblet cells in the trachea of the goose, where no submucosal glands exist (Phipps et al., 1977). Electrical stimulation of the descending oesophageal nerves increased mucus secretion (Phipps and Richardson, 1976; Phipps et al., 1977), an effect that was mimicked by ACh and inhibited fully by atropine. Adrenaline, noradrenaline or isoprenaline did not increase mucus secretion. There seems little doubt that cholinergic parasympathetic nerves and cholinergic agents are effective stimulants of mucus secretion in cats (Gallagher et al., 1975), geese (Phipps and Richardson, 1977) and ferrets (Basbaum et al., 1981; Borson et al., 1980). However, recently Peatfield and Richardson (1983a) failed to inhibit the effects of vagal stimulation with atropine in the cat.

Irritants may cause mucus secretion by direct action on the secretory cells, or by a reflex pathway. Widdicombe (1966) reported the presence of secretomotor fibres in both sympathetic and parasympathetic nerves. Electrical activity in these nerves was enhanced by mechanical stimulation of the larynx and by ammonia. Phipps and Richardson (1976) confirmed that both mechanical stimulation of the nasopharynx and inhalation of ammonia vapour increased mucus secretion; these effects were abolished by vagotomy. Mucus secretion is also enhanced by irritation of gastric mucosa (German et al., 1982); an effect that was reversibly abolished by cooling the abdominal vagi. In a more recent study, Peatfield and Richardson (1983b) reported that deposition of dust particles (charcoal and barium sulphate) into the trachea increased mucin secretion. Denervation inhibited 35-S glycoprotein, but had no effect on 3-H glycoprotein secretion. When dust was

introduced into the lungs, mucin production was enhanced and coughing was observed. On vagotomy, both effects were inhibited. A similar effect was observed in atropinised animals. In addition to sympathetic and parasympathetic pathways, non-adrenergic, non-cholinergic nerves may regulate mucus secretion. This may explain why atropine, in combination with propranolol and phentolamine, failed to inhibit mucus secretion in response to vagal stimulation (Peatfield and Richardson, 1983b). Vasoactive peptides, such as substance P or vasoactive intestinal peptide (VIP), have been reported to enhance mucus secretion from dog tracheal explants (Baker et al., 1977) and ferret trachea (Peatfield et al., 1983). Ferret trachea produces mucus in response to electrical field stimulation in the presence or absence of muscarinic, alpha- and beta-adrenoceptor antagonists (Borson et al., 1982).

With respect to adrenergic agents, there seems to be a diversity of opinion, which may reflect the animal species used for tests. Thus, in the cat, despite an earlier negative report (Gallagher et al., 1975), alpha-adrenoceptor agonists are reported to be more potent stimulants of mucus secretion than beta-adrenoceptor agonists (Phipps et al., 1980; Peatfield and Richardson, 1982) Similar results have been reported in ferrets (Borson et al., 1980; Basbaum et al., 1981) and dogs (Chakrin et al., 1973), although isoprenaline has been reported to increase glycoprotein secretion in ferrets in vitro (Peatfield et al., 1983). On the other hand, no increase of mucus secretion with beta-adrenoceptor agonists has been reported in rabbits, guinea-pigs (Boyd et al., 1943), dogs (Chakin et al., 1973), and human airways (Sturgess and Reid, 1972; Boat and Kleinerman, 1975).

The effect of inflammatory mediators on mucus secretion has been

studied. Histamine has been reported to have no effect on mucus secretion in the cat (Phipps and Richardson, 1976), dog (Chakin et al., 1973) or human airways (Sturgess and Reid, 1972). However, in a later paper Richardson et al. (1978) reported that, at a particular concentration, histamine increased mucin output. In the same study, 5-HT was without effect on mucin secretions. Prostaglandins F2-alpha and E1 are potent stimulants of mucus secretion in man (Lopez-Vidriero, 1978), cats, rats and monkeys (Iravani and Melville, 1975; Richardson et al., 1978). Leukotriene C4 has been reported to increase mucus secretion in a dose-related manner, but this effect was modest compared with other agonists (Peatfield et al., 1982).

Movement of water across the epithelium may play an important role in mucus clearance by increasing, or decreasing, fluidity of mucus. The existence of an active ion transport across tracheal epithelium has been reported (Oliver et al., 1975). A net movement of chloride ions (Cl^-) towards the lumen and a net movement of sodium ions (Na^+) towards the submucosa was demonstrated. These changes in ionic composition will be accompanied by a flux of water. Increased Cl^- secretion, or reduced Na^+ movement, might be expected to promote fluid production. Agents such as ACh, histamine, adrenaline and PGE1, increase active Cl^- secretion and decrease active Na^+ movement (Widdicombe and Walsh, 1980).

1.4) MUCOCILIARY TRANSPORT

Often, it may be convenient to investigate the pharmacology of cilia and of mucus secretion independently of one another, but *in vivo* these two processes are inter-dependent. Mucociliary transport has been measured in a wide range of animals using direct observation, isotopic or roentgenographic methods.

1.4.1) Measurement of mucociliary transport

Direct observation of the transport of marker particles along the mucociliary pathway has been used productively in mussels (Bulbring and Burn, 1953), frog palate and oesophagus (McDonald *et al.*, 1928; Kordik *et al.*, 1952; Hill, 1958; Sade *et al.*, 1970) and mammalian trachea (Asmundson and Kilburn, 1970). In these preparations, the epithelial surface exhibiting mucociliary transport is exposed and a marker particle is placed on the mucus layer; the passage of this particle, along a known path length, is timed and a rate of transport is calculated. With respect to mammalian preparations, this method is highly invasive and requires careful control of temperature and humidity; in contrast, molluscan and amphibian preparations are much more robust and are easier to use.

Other observational methods have been developed for use in mammals, which maintain the integrity of the airways to a large degree. Thus, Iravani (1967) described an *in vitro* method where the lungs were removed and the tracheobronchial tree was exposed by careful dissection. It was possible to observe ciliary activity and mucociliary transport by transillumination of the airways. Dalham

(1956) reported a less invasive method, in which a small window was excised from the trachea of an anaesthetised rat and transport of debris along the trachea was observed using a microscope.

More recently, fibre optic bronchoscopes have been used to deposit teflon discs into the trachea and, by filming the motion of these particles, it is possible to estimate the rate of transport (Sackner et al., 1973). This method is applicable to large mammals and has also been used in man (Santa Cruz et al., 1974). Two adaptations of this method exist. In the first method, the teflon discs are made radio-opaque with bismuth trioxide and insufflated into the airways through the inner channel of the bronchoscope. The adoral motion of these particles is recorded with a fluoroscopic unit coupled to an image intensifier, T.V. monitor and video recorder. Tracheal mucus velocity is calculated from the time taken for particles to traverse a given distance (Friedman et al., 1977). The second method, developed by Chopra et al. (1977) makes use of isotopically (technetium - 99) labelled albumin microspheres. A small suspension of these markers is placed on the mucosal surface of lower trachea via a catheter. Sequential pictures are taken with a gamma camera and the time taken for these markers to reach the larynx is used to calculate tracheal mucus velocity. Alternatively, tantalum powder may be insufflated into the airways via a catheter, which is advanced to just above the carina (Gamsu, 1973). Chest radiographs are taken immediately after deposition of tantalum and at regular intervals thereafter. The opacity immediately after deposition is taken to be 100%. The estimated amount of dust on each subsequent radiograph is expressed as a percentage of the initial radiograph. A less invasive method has been described by Yeates et al. (1975). Technetium - 99 (99-Tc)

labelled albumin microspheres are inhaled as an aqueous aerosol. Using a suitable inhalation manoeuvre, it is possible to deposit the aerosol at high concentrations in the large airways. The cephalad progress of these boli is monitored using a gamma camera.

All the methods described above have the advantage that mucociliary transport can be measured in anatomically defined areas and repeated measurement can be made (with the exception of the tantalum deposition method); hence, mucociliary transport can be measured before and after drug treatment. However, these methods are, to a greater or lesser degree, invasive. It is reasonable to expect excision of a tracheal window or insertion of a bronchoscope in the airways to produce a degree of irritation, which will stimulate mucus secretion (Phipps and Richardson, 1976). Mucus secretion and mucociliary clearance may be linked, since inhalation of dust enhances both mucus secretion (Peatfield and Richardson, 1983b) and mucociliary transport (Camner et al., 1973). Table 1 shows a comparison between tracheal mucus velocity values in man, using different methods. Faster transport rates are reported for bronchoscopic methods as compared with the radio-aerosol bolus technique which is non-invasive. The use of inhalation of radiolabelled aerosols as a non-invasive method of measurement of mucociliary transport was first described by Albert and Arnett (1955) and, since then, it has been used by many investigators. After inhalation and deposition of the radio-aerosol, the amount of radioactivity in the lungs is monitored externally using a gamma-scintillation detector. The percentage reduction in lung radioactivity, subsequent to deposition, gives an index of lung clearance. Recent improvements in the technique include using an isotope with a short half-life (e.g. 99-Tc; t1/2 = 6 h) and mapping

Table 1. Tracheal mucus velocities (mean ± SD) for healthy subjects.

Technique	No. of subjects	TMV (mm/min)	Source	
Radioaerosol boli	42	4.7 *	Yeates et al.	(1975)
	7	4.4 ± 1.3	Wong et al.	(1977)
	8	6.3 ± 0.8	Foster et al.	(1978)
	4	4.9 *	Foster et al.	(1979)
	11	4.6 ± 2.4	Spektor et al.	(1979)
Cinebronchoscopy	16	21.5 ± 5.5	Santa Cruz et al.	(1974)
	20	20.1 ± 6.3	Wood et al.	(1975)
Radioisotopic using a bronchoscope	6	15.5 ± 0.7	Chopra et al.	(1979)
Roentgenographic	7	11.4 ± 3.8	Friedman et al.	(1977)
	10	10.1 ± 3.5	Goodman et al.	(1978)

* standard deviation not given.
Table reproduced from Pavia et al. (1980).

the initial distribution of particle deposition using a rectilinear gamma scanner (Dawson et al., 1971) or a gamma camera (Short et al., 1979). This is important, since central airways are reported to have a faster clearance rate than peripheral airways (Asmundson and Kilburn, 1970; Serafini et al., 1976), therefore, in conditions where airways obstruction is present, inha led particles are deposited centrally and appear to be removed more efficiently than normal lungs, where there is uniform deposition throughout the lungs (Pavia et al., 1980).

Although non-invasive, this method nevertheless suffers from certain disadvantages, including conversion of a three-dimensional event into a two-dimensional picture and the inability to estimate a rate of transport unless a gamma camera is used (Sanchis et al., 1972; Bateman et al., 1979). The use of isotopes for measurement of mucociliary transport in small mammals has not been fruitful, since gamma detectors are large in comparison to small laboratory animals and, to improve resolution, large amounts of lead shielding is required. Despite these disadvantages, several methods have been described for isotopic measurement of mucociliary transport in rats (Patrick and Stirling, 1977; Kenoyer et al., 1981), avian trachea (Baetjer, 1969) and guinea-pigs and rabbits (Felicetti et al., 1981). These methods are disadvantaged by being restricted to a single observation per animal.

1.4.2) Regulation of mucociliary transport

Since mucociliary transport is present in a wide range of species, it is not unreasonable to expect regulatory mechanisms to be different in each animal group. To avoid complication and confusion, each species or group of animals will be considered separately.

1.4.2.1) Molluscs

Filter feeding molluscs, such as the mussel (M. edulis) have been investigated widely. The earliest studies on this animal were reported by Bulbring and Burn (1953), who showed that ACh and eserine were stimulatory at low concentrations and inhibitory at high concentrations to both ciliary activity and mucociliary transport; atropine and d-tubocurarine were inhibitory. This preparation was subsequently reported to respond to 5-HT, dopamine and nerve stimulation (Aiello and Guideri, 1964; Malanga et al., 1981). Since ciliary beating and mucociliary transport responded to pharmacological agents in a similar way (Bulbring and Burn, 1953), agents affecting ciliary beating also influence mucociliary transport in a similar fashion (see 1.2.3).

1.4.2.2) Amphibians

Physiological regulation of mucociliary transport in amphibians has been studied intermittently throughout this century. Neuronal regulation of mucociliary transport had been suggested by McDonald et al. (1928), who reported that stimulation of the sympathetic chain accelerated particle transport rate, but that vagal stimulation was without effect. The concept of neurogenic control of palatal cilia was reinforced by the work of Lucas (1935), who reported that the accelerator fibres originated from the brain and were distributed within the seventh cranial nerve, but were cholinergic. This variation is reflected in the literature concerning the effects of humoral agents on ciliary transport in the frog oesophagus. Plattner and Hou (1931) reported that ACh (10-5 and 10-4 g/ml) increased ciliary activity, that

this effect was potentiated by eserine, but that eserine alone was without effect. These investigators observed that atropine was without effect on basal activity, but inhibited stimulation by ACh or adrenaline. On the other hand, Pohle (1931) reported that atropine inhibited ciliary movement. Blaich and Karl (1950) confirmed that ACh and adrenaline, in combination with penicillin, were stimulants to ciliary activity in the frog oesophagus; these effects were inhibited by atropine. In this study, eserine was reported to have no effect on basal ciliary activity. Kordik et al. (1952) and Burn and Day (1958) reported that acetylcholine (ACh), at 10 -6 g/ml, stimulated the rate of particle transport along the frog oesophagus and that atropine (10-6 g/ml) inhibited basal transport rate, whilst eserine at 10-5 and 10-4 g/ml stimulated basal transport rate. Both atropine and d-tubocurarine were reported to be inhibitory. On the other hand, Hill (1957) did not observe a stimulatory effect with ACh, nor did she observe the inhibitory effect of d-tubocurarine that had been reported earlier by Kordik et al. (1952). Milton (1959) repeated the studies of Kordik et al. (1952) and reported that ACh (10-6 g/ml) increased and tubocur arine decreased particle transport rate. However, the inhibitory effect of tubocurarine was observed only with bicarbonate Ringer's solution and not with phosphate Ringer's solution.

The effects of sympathomimetic agents have not been well characterised. Adrenaline has been reported to have some stimulatory effect (Plattner and Hou, 1931; Blaich and Klar, 1950). However, isoprenaline is without effect (Blair and Woods, 1969). More recently (Maruyama, 1983), noradrenaline has been reported to have a stimulatory effect at high concentrations, and an inhibitory effect at low concentrations. The action of biologically active peptides has not been reported.

1.4.2.3) Mammals

Minimal information exists as to the effect of nerve stimulation on mucociliary transport. In their introduction, Kordik et al. (1952) refer to the Russian literature and cite the work of Rozansky (1927), who reported an acceleration of mucociliary transport in the dog trachea, after vagal stimulation. A consensus of opinion exists with regard to the stimulatory action of ACh, which is supported by experimental data in rats (Iravani et al., 1975), dogs (Wanner et al., 1975) and man (Camner et al., 1974). The inhibitory effect of anti-cholinergic drugs, such as atropine, in dogs and man (Sackner et al., 1977; Yeates et al., 1975; Foster et al., 1976) and hyoscine in man (Pavia and Thomson, 1977) support cholinergic regulation of mucociliary transport. However, Chopra (1978) has reported a stimulatory effect of atropine on dog tracheal mucus velocity.

Sympathomimetic agents, and in particular beta-adrenoceptor agonists, have been subject to intense investigation, due to their widespread clinical useage. In rats (Iravani and Melville, 1974), cats (Blair and Woods, 1969), dogs (Sackner et al., 1976) and sheep (Sackner et al., 1979), isoprenaline and other beta-sympathomimetic agonists (terbutaline and carbuterol) have been reported to accelerate mucociliary transport. In man, conflicting data has been reported with respect to these agents. Thus, isoprenaline (Foster et al., 1976) and terbutaline (Konietzko et al., 1975; Camner et al., 1976) have been reported to stimulate mucociliary transport or to have no effect (Santa Cruz, 1974) in normal subjects. Similar discrepancy has been reported for patients with a variety of lung diseases (see Discussion).

Mucociliary transport in patients with chronic autonomic failure was no different compared with sex-matched controls (Jenkins et al., 1980). Results from the same laboratory indicated that selective and non-selective beta-blocking agents (atenolol and propranolol) did not alter mucociliary clearance (Bateman et al., 1980).

Steroids have been reported to improve mucociliary clearance in patients with stable asthma (Bateman et al., 1980), but had no effect in normal sheep (Sackner et al., 1977). The effect of vasoactive peptides on mucociliary transport is unknown.

2 MATERIALS AND METHODS

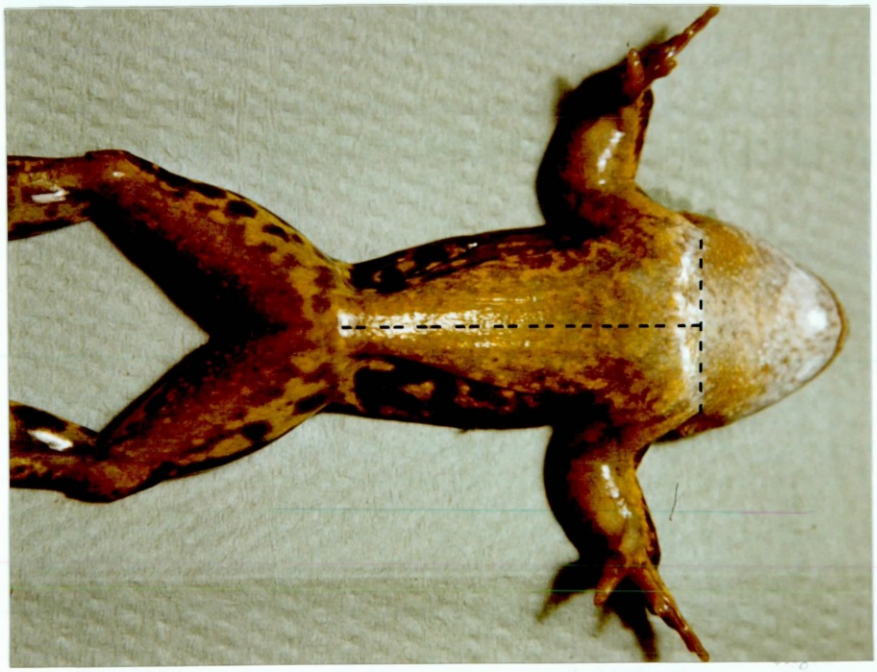
2.1) Amphibian studies

2.1.1) Animals

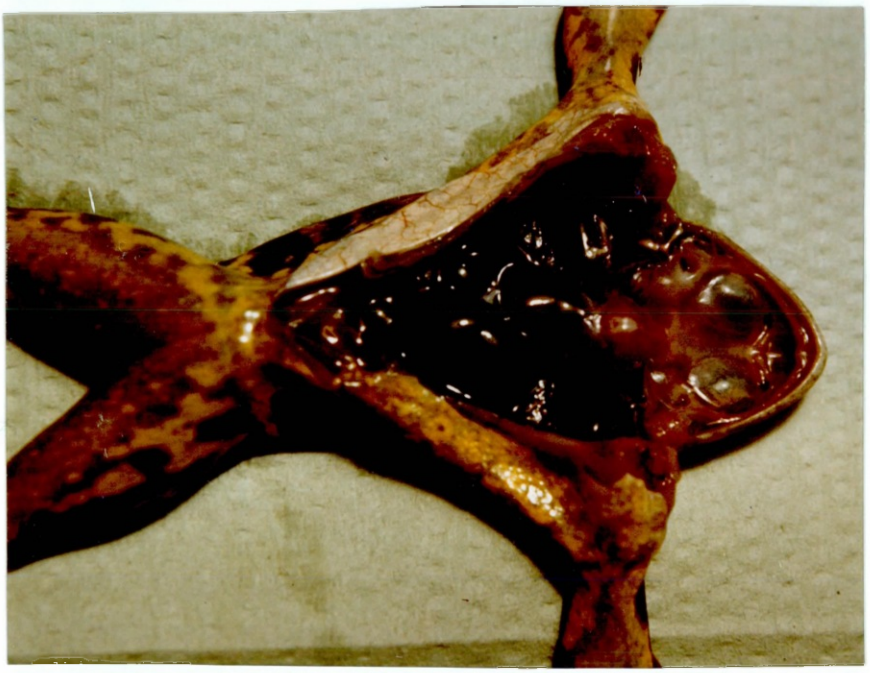
In order to select a species that would be suitable for this study, Rana temporaria, Rana pipiens and Xenopus laevis were examined in preliminary experiments, since all were readily available from a commercial source (Xenopus Ltd., Redhill). An increase of particle transport rate could be demonstrated in the oesophagus of all species following topical application of acetylcholine. R. temporaria was selected for further study, since the oesophagus was more robust and since this species was less expensive. Frogs were maintained at 4 degrees C, for, at this temperature, animals could be stored for long periods without feeding, and were easy to handle.

2.1.2) Dissection

Animals were pithed and the abdominal wall opened by an incision along the ventral midline, extending beyond the pectoral girdle which was transected. The lower jaw was removed by passing one blade of a pair of scissors into the mouth and cutting at right angles to the abdominal incision. Two halves of the abdominal wall were folded back; the heart, lungs and other viscera were removed, with the exception of the oesophagus and stomach. The ciliated lumen of the oesophagus was exposed by a longitudinal incision (Plate 1a, b, c). Three types of preparations were used.



a



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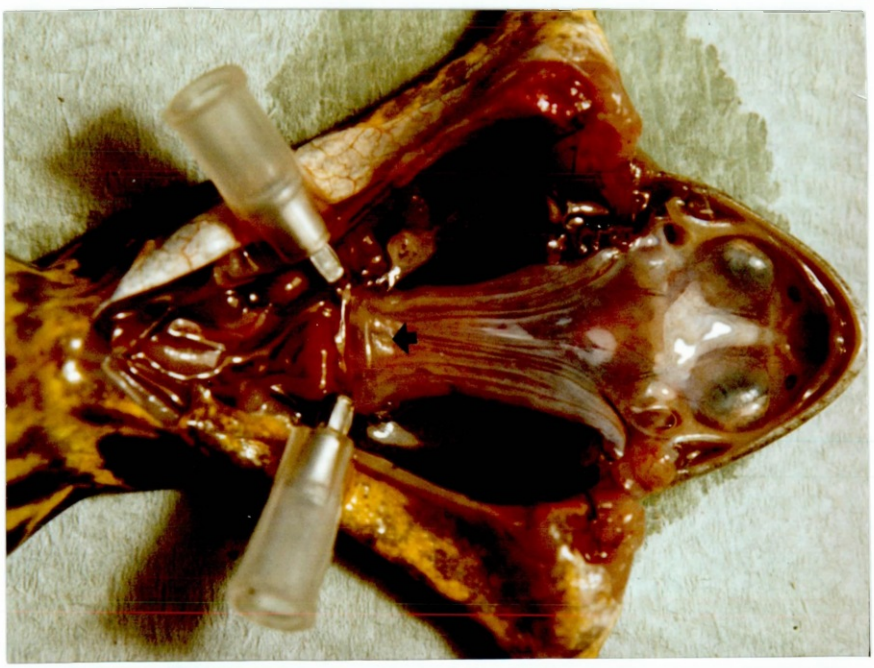


c

Plate 1. Stages in the dissection of the frog and preparation of an *in situ* model for measurement of oesophageal mucociliary transport. Incisions are made along dotted lines (a) and the abdominal walls are folded back, exposing the viscera (b). The viscera, including the heart and the lungs, are removed and the ciliated lumen of the oesophagus is exposed by a longitudinal incision. The proximal end of the oesophagus is pinned, with sufficient tension to flatten the luminal surface (c). Mucus accumulating at the distal end of oesophagus is clearly visible (arrow).

2.1.2.1) In situ preparation

The dissected frog was placed on a cork mat with the ventral surface uppermost. The head and proximal oesophagus were pinned to the cork mat with sufficient tension to flatten the luminal surface. The oesophagus was flooded in situ, at 5-10 minute intervals, with frog Ringer's solution (FRS) (Plate 1c). This preparation permitted study of the effects of topical application of pharmacological agents and of nerve stimulation (vagus and paravertebral sympathetic chain) on oesophageal mucociliary transport (MCT).

2.1.2.2) Chamber preparation

The palate and oesophagus were removed by transection of the body wall, at the level of second cervical vertebra, and by cutting across the cardiac portion of the stomach. The head and cut edge of the oesophagus were pinned onto a wax-lined chamber, under sufficient tension to flatten the luminal surface (Plate 2a) and the wax-lined chamber was promptly flooded with FRS (2 ml) to cover the preparation. In addition to measuring MCT, this preparation allowed collection of extruded mucus, which was analysed for protein content and sugar constituents (section 3.2).

2.1.2.3) In vitro preparation

The oesophagus was removed by cutting across the pharynx and stomach. After exposing the lumen by a longitudinal incision, the preparation was pinned onto a cork mat, with the luminal surface uppermost. The preparation was flooded at 5-10 minute intervals with FRS and was used

a

b

Plate 2. After dissecting the frog (2.1.2), the palate and oesophagus were removed by transection at the level of the second cervical vertebra and at the cardiac end of the stomach. The head and oesophagus were pinned into a wax lined cork chamber (a) and flooded with frog Ringer's solution (FRS). Mucociliary transport was measured *in vitro* by removing the oesophagus completely and pinning the cut ends under sufficient tension to flatten the luminal surface (b). A few drops of FRS are placed over the oesophagus to prevent drying. The arrow points to a group of lead particles which have been placed on the epithelial surface.

to study the effects of decentralisation on MCT (Plate 2b).

2.1.3) Equipment

Preparations were observed with the aid of a stereomicroscope (Wild M8). A cold source of light (Volpi 2500), fitted with twin fibre optic light guides provided illumination. The stereomicroscope housed a television camera (Hitachi Denshi HV.17 TV), which was connected to a video tape recorder (Sony AV 3620 CE) and to a television monitor (Hitachi Denshi VM-173 U) (Fig 2). The microscope included a beam splitting prism, so that particle transport could be observed directly through the microscope, or on the T.V. monitor, and recorded simultaneously. The motion of particles along the oesophagus was aboral; the television camera was aligned so that the path of particles was parallel to the horizontal axis of the monitor screen.

2.1.4) Measurement of mucociliary transport.

The monitor screen was calibrated by placing a ruler under the microscope (magnification set at x 18) so that an image of the ruler was displayed, with its edge parallel to the pathway of particle transport. A 5 mm path was defined on the monitor screen as two vertical lines. The screen was sub-divided by two parallel horizontal lines to give three equal segments. Such sub-division of the screen allowed estimation of mucociliary transport in central and peripheral regions of the oesophagus (Fig 2). Lead filings (0.02-0.08 mm range of maximum diameters) were used as marker particles. Prior to recording an observation, fine forceps were used to place a group of particles (20-50) at the oral end of the oesophagus. Physical contact between

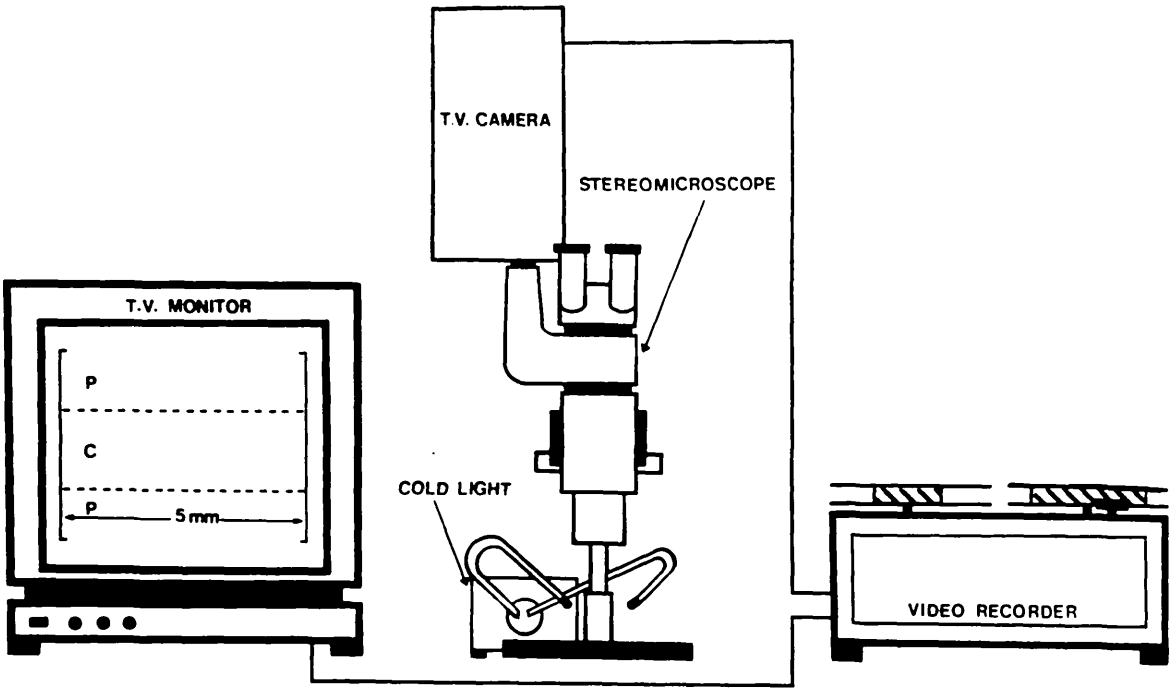


Figure 2. Equipment used for measurement of mucociliary transport in the frog oesophagus.

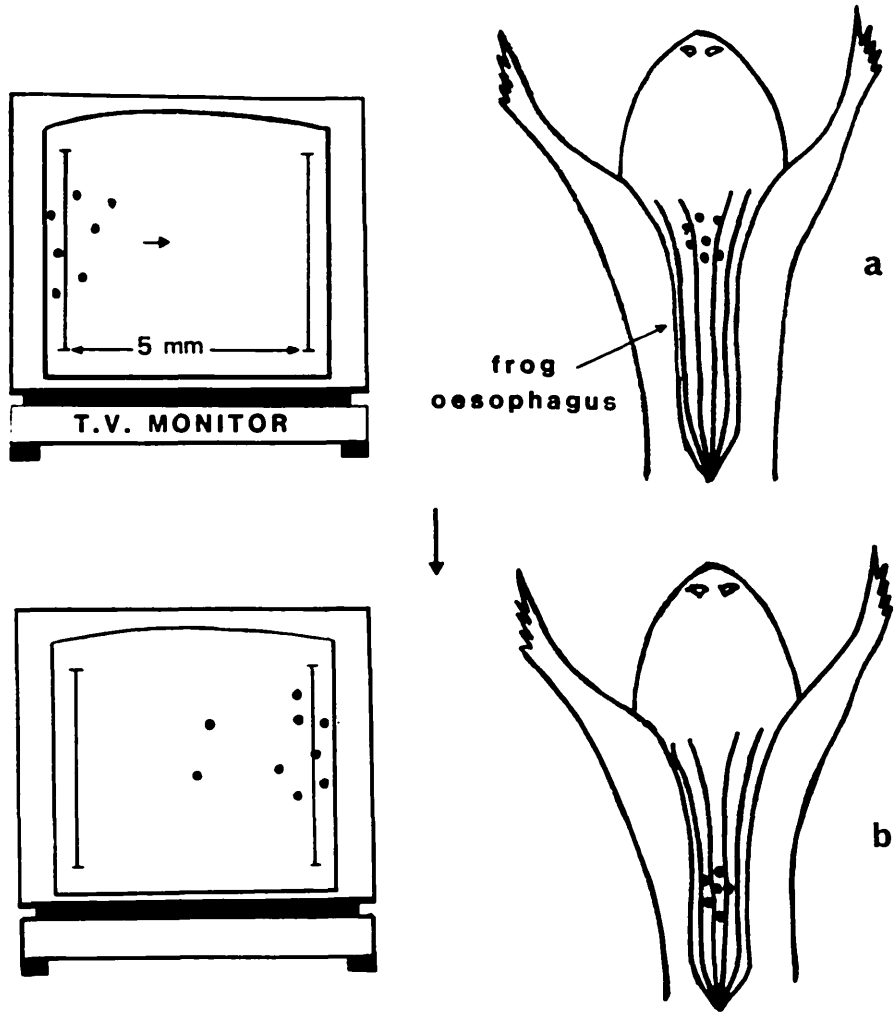


Figure 3. Diagrammatic representation of measurement of mucociliary transport. Lead filings are placed at the oral end of oesophagus using fine forceps (a) and the passage of particles, between the calibration lines, timed with a digital stop-watch (b). Rate of transport is calculated in mm/sec.

forceps and the epithelium stimulated transport and hence was avoided. For any treatment, mucociliary transport was measured by timing the motion of at least 10 particles across the 5 mm path, using a digital stop watch (Fig 3). From this data, a mean rate of transport was calculated in mm/sec. MCT and "particle transport rate" are used interchangeably.

2.1.5) Site of measurement of mucociliary transport

To find the most suitable area for measurement of MCT along the oropharynx, particle transport rate was measured at 1) fore-palate; 2) mid-palate; 3) pharynx; 4) upper oesophagus; 5) lower oesophagus, using 2 mm path lengths.

2.1.6) Drug administration

Drugs were dissolved in frog Ringer's solution (FRS) of the following composition (mM): NaCl 112; NaHCO3 4.7; NaH2PO4 2H2O 1.0; CaCl2 1.08; pH 7.3 $\pm$ 0.1 and applied topically in a final volume of 2 ml. A contact time of 2 minutes was allowed, during which transport rates were determined. Prior to each treatment, MCT was measured immediately after application of frog Ringer's solution (saline control). Application and aspiration of drug solutions of increasing concentrations provided data for dose-response curves.

2.1.7) Nerve stimulation

2.1.7.1) Vagal stimulation

The vagus nerve on the right side was positioned over nickel coated copper electrodes of an electrical stimulator (Fenton Lewis Mk IV) and embedded in silicone jelly. The vagus nerve was stimulated with pulses (2 msec) of increasing voltage (0.5, 1.0, 1.5 and 2 volts) at 30 Hz for 30 seconds. It was not practicable to keep the voltage constant and alter the frequency, since below 5 Hz no response could be elicited and between 5 and 50 Hz transport rate was maximal (Fig 4).

2.1.7.2) Sympathetic chain stimulation

The paravertebral sympathetic chain was exposed, by pinning the overlying oesophagus at an angle to the midline (approximately 15 degrees). The most cephalad ganglia were placed over the electrodes of the stimulator and encased in silicone jelly. Ganglia were stimulated with pulses (2 msec) between 1 and 5 volts at 1 to 50 Hz.

2.1.7.3) Measurement of mucociliary transport during nerve stimulation

Particle transport rate was determined within 2 minutes of onset of stimulation. Stimulus-effect studies were repeated in some preparation in order to obviate any reduction in transport rate due to tissue deterioration or tachyphylaxis. Stimulus-effect relationships were determined in the absence and presence of drugs, using the same preparation.

2.1.8) Measurement of heart rate

2.1.8.1) Dissection

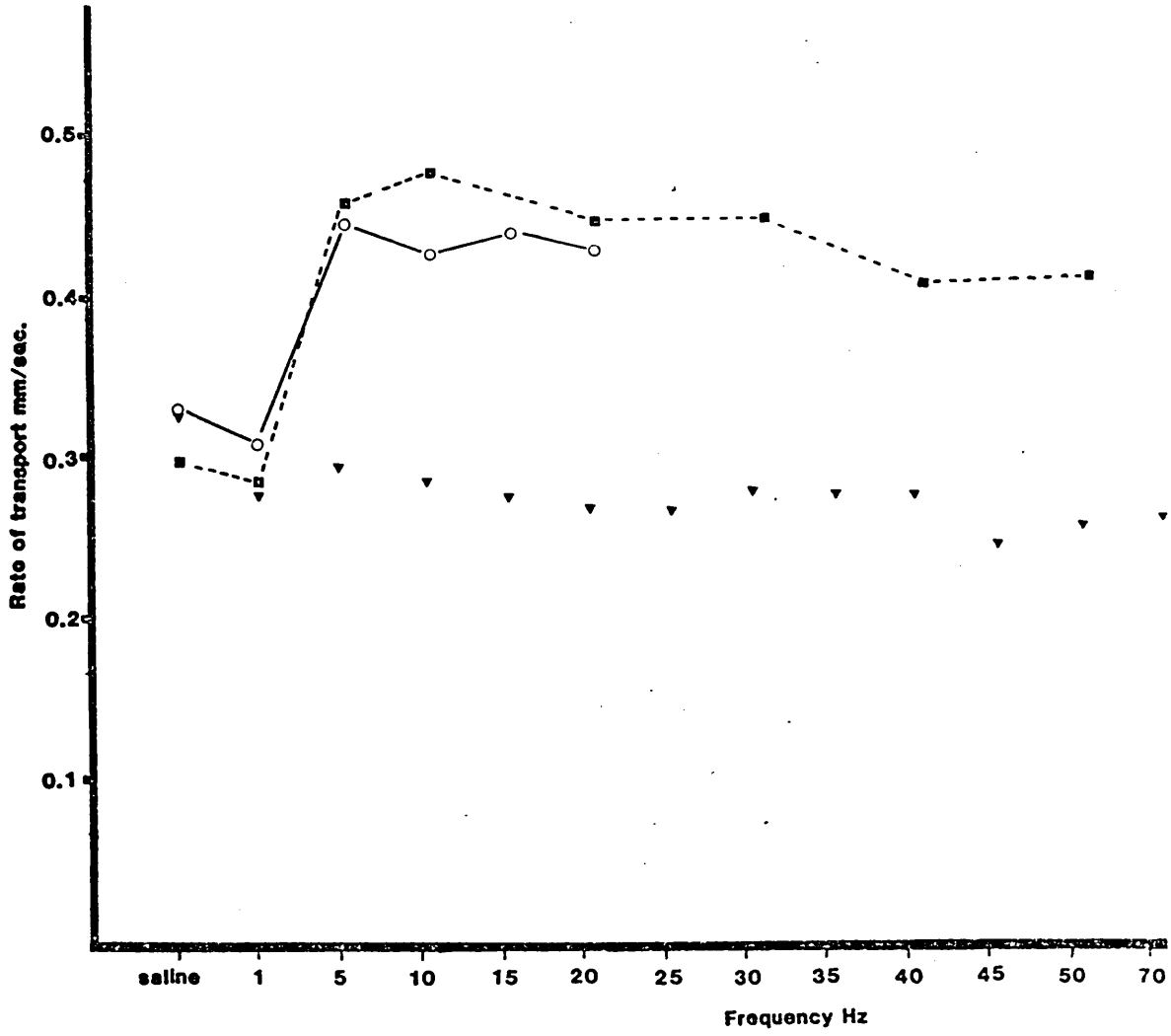


Figure 4. Effects of pulse width (msec) and frequency (Hz) on oesophageal mucociliary transport, during vagal stimulation at a constant voltage (2v). No increase in transport rate was evident with a pulse width of 0.2 msec (▼). Mucociliary transport was increased when the vagus nerve was stimulated with 5 Hz or more using a 2 msec pulse width (○ , ■).

The abdominal wall of a pithed frog was incised along the ventral midline. The heart was exposed by cutting through the pectoral girdle and folding back the two halves of the body wall. The apex of the heart was impaled with a small hook, attached to a tension transducer (Harvard Instruments) and balanced with a 1 gm load (Fig 5). Beat frequency and heart rate were recorded on a chart recorder (Ormed Engineering MX6).

2.1.8.2) Experimental protocol

After dissection and attachment of a heart to the tension transducer, 30 minutes elapsed before any treatment. FRS was dripped onto the heart throughout the experiment at 1 ml/min. Drugs were administered in FRS from a second reservoir, via a three-way tap (Fig 5).

2.1.9) Histology

Square sections (approximately 4 x 4 mm) of palate and oesophagus were obtained from five locations: fore-palate, mid-palate, pharynx, upper oesophagus and lower oesophagus and prepared for light microscopy. The excised tissues were fixed for 2 to 4 hours in glutaraldehyde (2.5% in cacodylate buffer) and post-fixed in osmium tetroxide (1% in cacodylate buffer), then deyhydrated serially in ethanol (70-100%). After treatment with propylene oxide (epoxypropane) for 30 minutes, tissues were immersed in a 1:1 mixture of propylene oxide and araldite and after 12 hrs embedded in araldite and incubated at 60 C for 48 hr. Sections (1 micron thick) were cut from tissue blocks using an ultramicrotome (LKB) with a glass knife and stained with toluidene blue

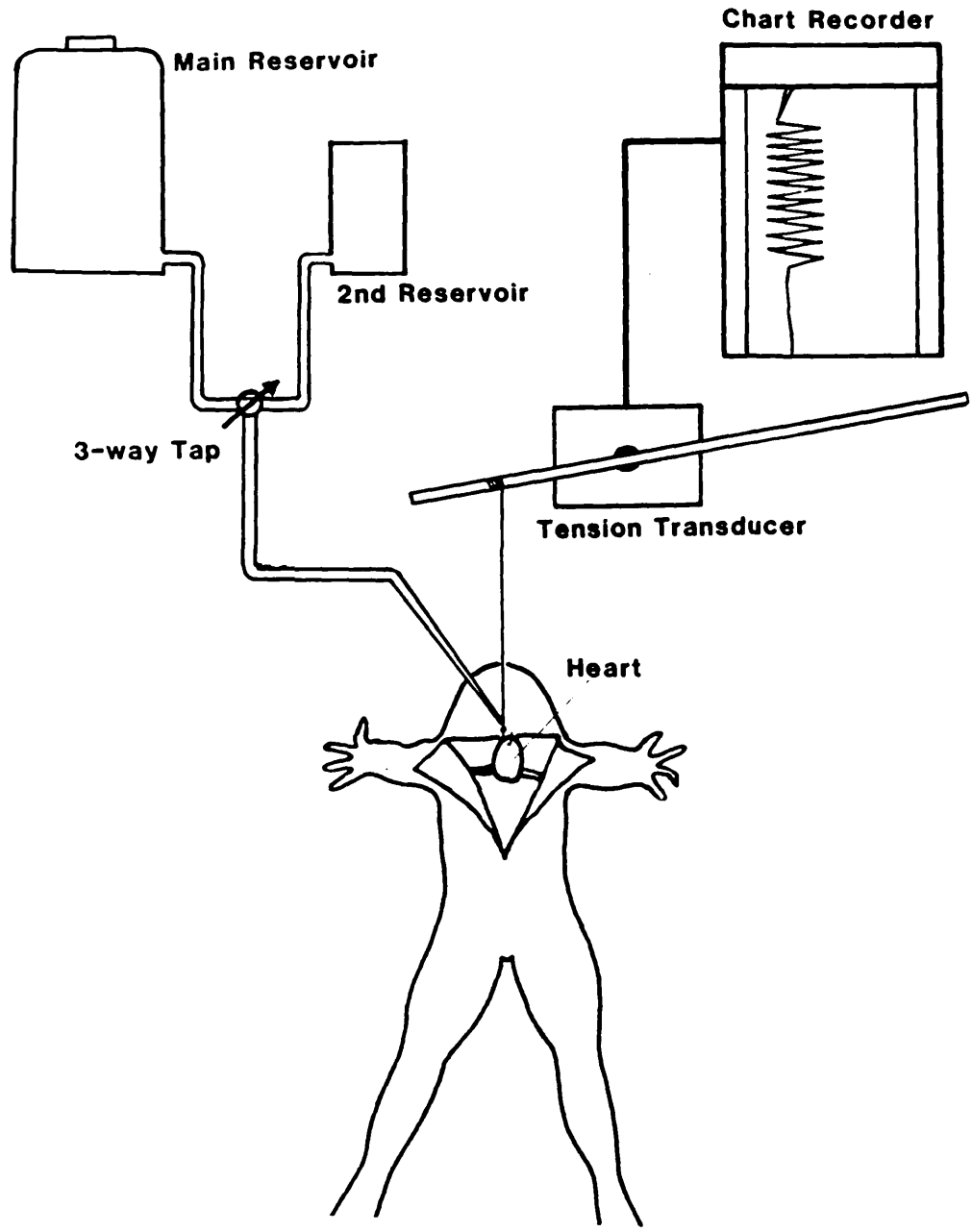


Figure 5. Diagrammatic representation of apparatus and method for measurement of frog heart rate.

(1% w/v) in borax (1% w/v).

2.1.10 Frog oesophageal mucus

2.1.10.1) Collection of mucus

Oesophageal mucus was collected from the chamber preparation (See section 2.1.2.2). Immediately after dissection, the preparation was placed into the chamber and flooded with FRS. As far as possible, all traces of blood were removed, since blood glycoproteins would interfere with the detection of glycoproteins in mucus. Fluid was removed from the chamber every 5, 10 or 15 minutes and replaced with fresh FRS. This procedure was repeated for up to 60 minutes (control period), after which drug solutions of increasing concentrations were introduced into the chamber, at intervals of 5, 10 or 15 min. Each sample was frozen for later analysis of sugar or protein content. After addition of any solution, MCT was measured within the first 2 minutes so as to permit correlation of MCT changes with those of protein secretion.

2.1.10.2) Protein assay

The protein content of collected samples was measured using the modified Lowry method (Markwell et al., 1978). The following reagents were prepared: Reagent A; 20% sodium carbonate; 0.4% sodium hydroxide; 0.16% sodium tartrate; 1% sodium dodecyl sulphate. Reagent B, 4.0% hydrated copper sulphate ($CuSO_4$, $5H_2O$). Folin-Ciocalteu phenol reagent was diluted 1:1 with distilled water. Reagents A and B were mixed in a ratio of 100:1 to produce reagent C. Standard solutions of bovine serumin albumin (BSA) (10-100 ug/ml) and

FRS (saline blank) were included in the test series. 1 ml aliquot of sample solution was added to 3 ml of reagent C and incubated at room temperature for approximately 20 minutes. After incubation, 0.3 ml of phenol reagent was added to the sample, which was vortex mixed and incubated for 45 minutes at room temperature.

The absorbance of samples at 660 nm was determined in a UV spectrometer (Gilford). Absorbance for the saline blank was subtracted from that of the sample solutions and a standard calibration curve was produced from the optical density of BSA solutions of known concentration (Fig 6). Protein contents of unknown samples were interpolated from this calibration curve and expressed as microgram equivalents of BSA.

2.10.3) Sugar content

The sugar content of frog oesophageal mucus was analysed using high pressure liquid chromatography (HPLC), by Dr C. Marriott (Brighton Polytechnic).

2.2) Mammalian studies

2.2.1) Animals and anaesthesia

2.2.1.1) Guinea-pigs

Male guinea-pigs (Dunkin-Hartley) weighing 450-750 g were used. Animals were housed in groups of 12. Food (autoclaved hay and food pellets) and water were available ad libitum. Animals were not used until at least three days after delivery. Urethane (7 ml/kg of a 25%

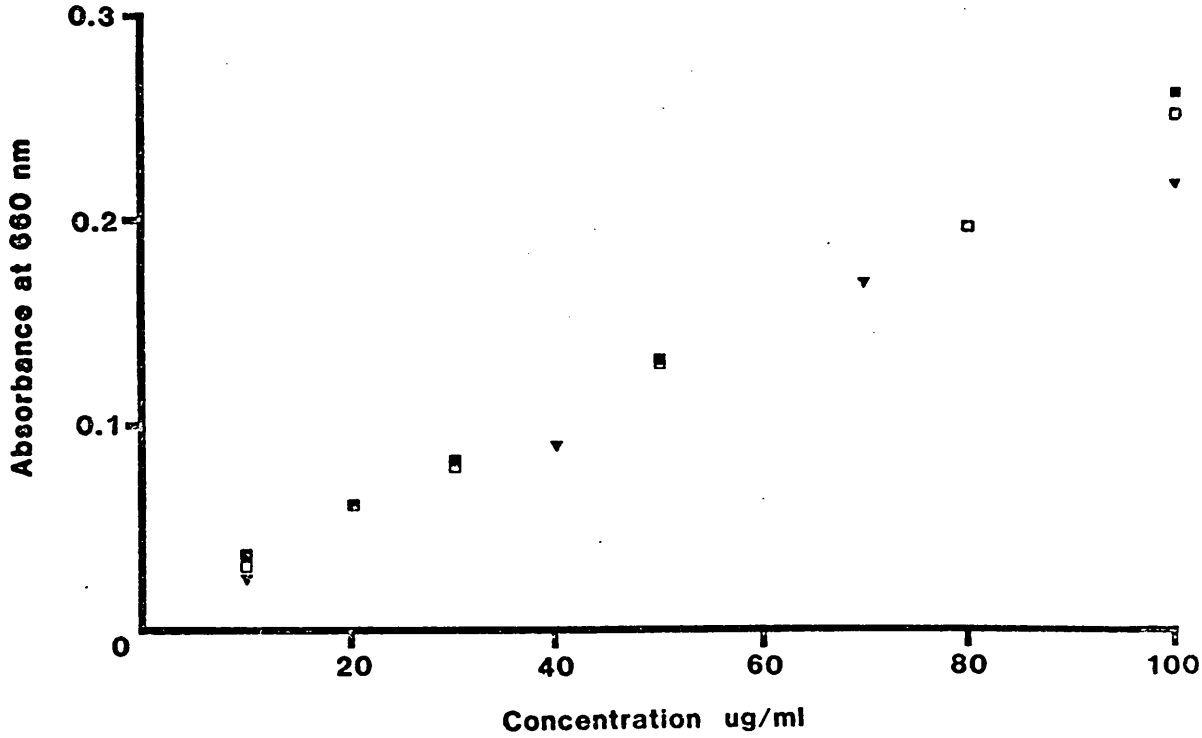


Figure 6. Calibration curve for measurement of protein content of unknown samples. Bovine serum albumin (BSA) is used as the standard protein and the amount of protein in unknown samples is expressed in terms of ug equivalent of BSA, by extrapolation from the standard curve. Symbols indicate individual calibration curves.

solution, i.p.) was used for induction of anaesthesia, after which maintenance doses of anaesthetic were not required.

2.2.1.2) Ferrets

Male and female ferrets (Mustela putorius furo) weighing 700-1600 g were obtained from a game-keeper and housed in separate cages. Upon arrival, oxytetracycline was administered in drinking water for 4 days and animals were not used until at least one week after arrival. Routine microbiological tests revealed no pathogens within lung tissue. Animals were fed on food pellets, cow liver and culled laboratory animals. Food and water were available ad libitum. Anaesthesia was induced with sodium pentobarbitone (Sagatal, 50 mg/kg i.p.); maintenance doses of 3 mg were administered intravenously, as required.

2.2.1.3) Rabbits

Male rabbits (New Zealand-White) weighing 2-3 kg were housed in separate cages. Food pellets and water were available ad libitum. Animals were sedated with fentanyl droperidol (Hypnorm, 0.5 ml/kg i.m.) and surgical anaesthesia was induced with intravenous urethane (5 ml/kg of a 25% solution). No maintenance doses were required after induction of anaesthesia.

2.2.2.) Erythrocyte labelling

Erythrocytes provided suitable marker particles when labelled with indium-111 oxine (111-In) or phosphorus-32 (32-P). Erythrocytes were convenient, since these cells were readily available, labelled

efficiently and a single batch could be used for several days (usually 5 days) after labelling.

2.2.2.1) Isotopes and buffer

Phosphorus-32 (74 MBq/ml, as orthophosphate solution) and indium-111 (37 MBq/ml, as indium oxine) were obtained from Amersham International.

The buffer solution used for labelling (Mollison, 1967) had the following composition:

trisodium citrate (dihydric)	28 g
sodium dihydrogen phosphate	0.15 g
glucose	2 g
water (distilled)	1000 ml

The pH of this solution was 7.4. After autoclaving at 15 lb/square inch for 30 minutes, caramelisation gave the solution an orange colour and the pH fell to 6.9. This autoclaved solution is referred to as "Cit-Ph buffer".

2.2.2.2) Labelling of guinea-pig erythrocytes with 32-P

Guinea-pigs were anaesthetised with ether and blood (4 ml) was collected by cardiac puncture into heparin (5 units/ml). Whole blood was centrifuged at 200 g for 10 minutes. 1 ml of packed erythrocytes was removed, resuspended in 9 ml of Cit-Ph buffer and centrifuged (200g) for 10 minutes. The supernatant was discarded and erythrocytes were resuspended in 4 ml of Cit-Ph buffer, together with 0.5 ml of 32-P

solution (18.5 MBq) and incubated at 37 degrees C for 30 minutes. After incubation, erythrocytes were centrifuged (200g; 10 minutes) and the supernatant discarded. Erythrocytes were washed twice in Cit-Ph buffer and stored at 4 degrees C for later use. Labelled cells were washed each day and could be used for up to five days since no frank haemolysis was evident during this period and since less than 1% of the total isotopic content was present in the supernatant on successive days.

2.2.2.3) Labelling of ferret erythrocytes with 32-P

Heparinised blood was obtained from pentobarbitone-anaesthetised ferrets by cardiac puncture. Ferret erythrocytes were labelled and stored using the procedure described for guinea-pig erythrocytes. However, ferret erythrocytes proved less stable, as indicated by colouration of the carrier solution and by the appearance of substantial (up to 15% of total) counts in the supernatant after 24 hr. This instability affected tracheal mucus velocity (see Results) and, hence, ferret erythrocytes were only used for preliminary experiments.

2.2.2.4) Labelling of guinea-pig erythrocytes with 111-In

The procedure for labelling erythrocytes with 111-In essentially paralleled that described for 32-P. 111-In (0.92 MBq) was used in place of 32-P and the incubation period was reduced to 5 min. 111-In labelled erythrocytes were also stable over 5 days and less than 1% of total activity could be detected in the supernatant solution on successive days.

2.2.3. Isotope monitoring equipment

Gamma radiation from 111-In was detected by sodium iodide (NaI) crystal scintillation probes and beta-particles from 32-P were detected by a solid state surface barrier detector. Amplified signals were logged by a common counting facility, which allowed continuous monitoring of pulses from the detectors and provided automated analysis and printing of this data.

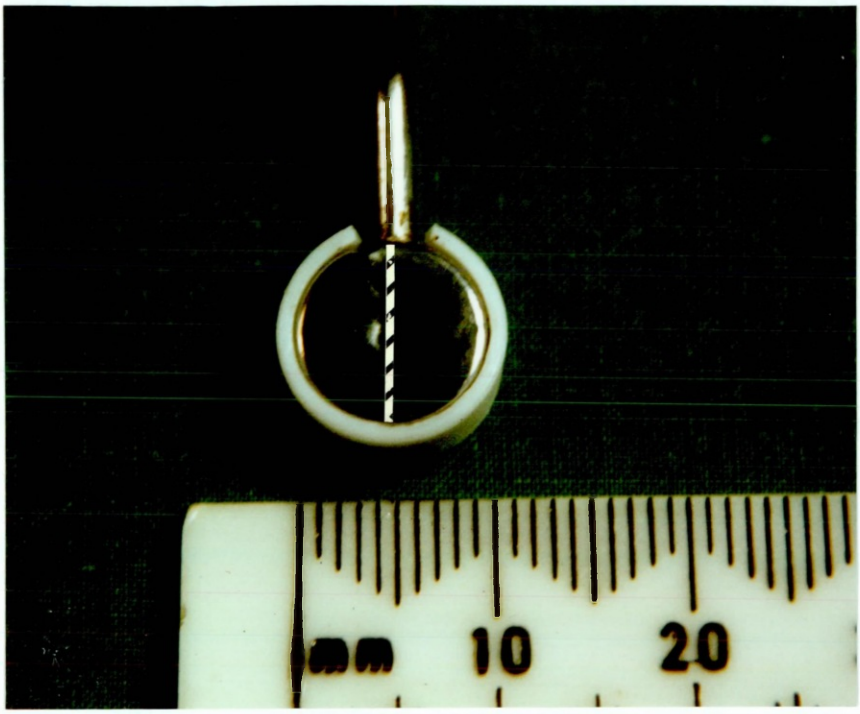
2.2.3.1) Crystal scintillation detectors

A Na-I crystal scintillation detector, 1 inch diameter (GP-6, Nuclear Enterprises), was used for detection of 111-In. Pulses from the detector were amplified and analysed by a spectrometer (NE 4630, Nuclear Enterprises). The detector was collimated by insertion of the crystal into a tubular lead cylinder with a centre aperture of 2.5 cm diameter. At a distance of 1 cm, activity could be detected over an area of 80 sq cm (approximately 5 cm radius).

2.2.3.2) Surface barrier detectors

Solid state beta-particle detectors (Harwell, AERE) were used for detection of 32-P. These detectors were encased in stainless steel for protection from light and water. The detector surface formed a circle of 8 mm diameter and the diameter of an encased detector was 10 mm, with a thickness of 4 mm (Plate 3a and b). A pre-amplifier was located within the handle of the detector, in order to reduce electronic noise due to stray capacitance between the detector and earth. A two-channel unit (Harwell, AERE) was used to apply a bias voltage (approx. 50 v)

a



b

Plate 3. Beta-particle detector, view from above (a) and in profile (b). In plate (a), the plastic cap (white ring) holds the lead collimator in position. The dotted lines indicate alignment of the collimator slit.

to each detector. Signals from the detector were amplified by an amplifier-analyser (NE 4630, Nuclear Enterprises). To increase resolution, the detector face was collimated using a lead disc of 1mm thickness which had a diametrical slit of 1 x 6 mm. A source of radiation was only detectable within a 10 mm radius from the centre of the collimator slit (Fig 7).

2.2.3.3) Counting apparatus

Counts were recorded continously using an automated isotope monitoring system. The system comprised: isotope detectors (gamma or beta) and appropriate amplifiers (NE 4630 or NE 4697), a microcomputer (North Star Horizon, fitted with a special interface counting board) and associated software (AIMS 8000), a visual display unit (VDU, Lyme 5000) and a dot matrix printer (Epson, MX-80 F/T III) (Fig 8). Pulses from the detectors were logged and total counts for successive steps (maximum 45) were stored in the computer. The duration of each step was pre-selected and could be varied between 1 sec and 2 hrs. The progress of an experiment was monitored in real time on the VDU. At the end of an observation period, both original and processed data were printed in tabular and graphical form, or stored on disc.

2.2.4) Measurement of mucociliary transport

Mucociliary transport was measured in small laboratory animals by detection of accumulation or clearance of radiolabelled erythrocytes. Three techniques were evaluated.

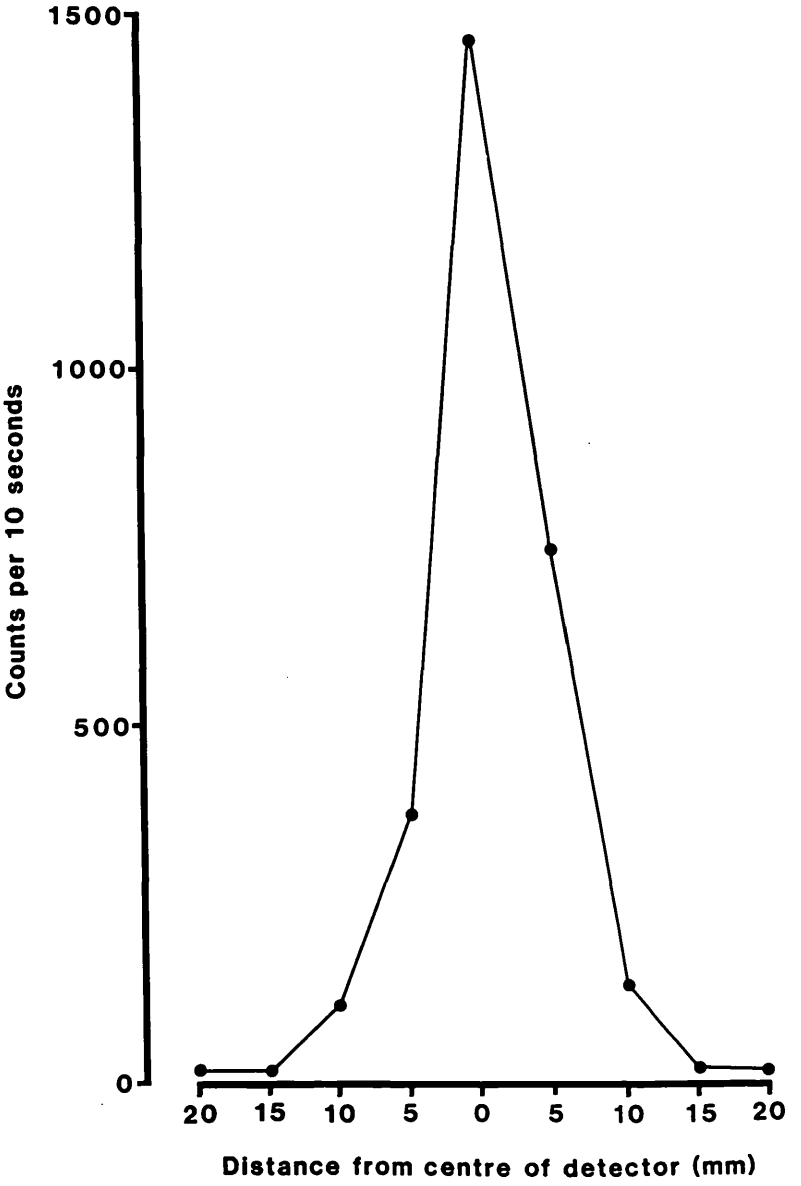


Figure 7. Resolution of the collimated beta-particle detector is measured by positioning a source of activity 5 mm directly beneath the collimator slit, and counting for 10 seconds. The source of activity is moved away from the centre of the detector in 5 mm steps, along an axis perpendicular to the collimator slit, whilst counting radioactivity for 10 seconds for each step. No counts above background are observed when the source is 15 mm away from the collimator.

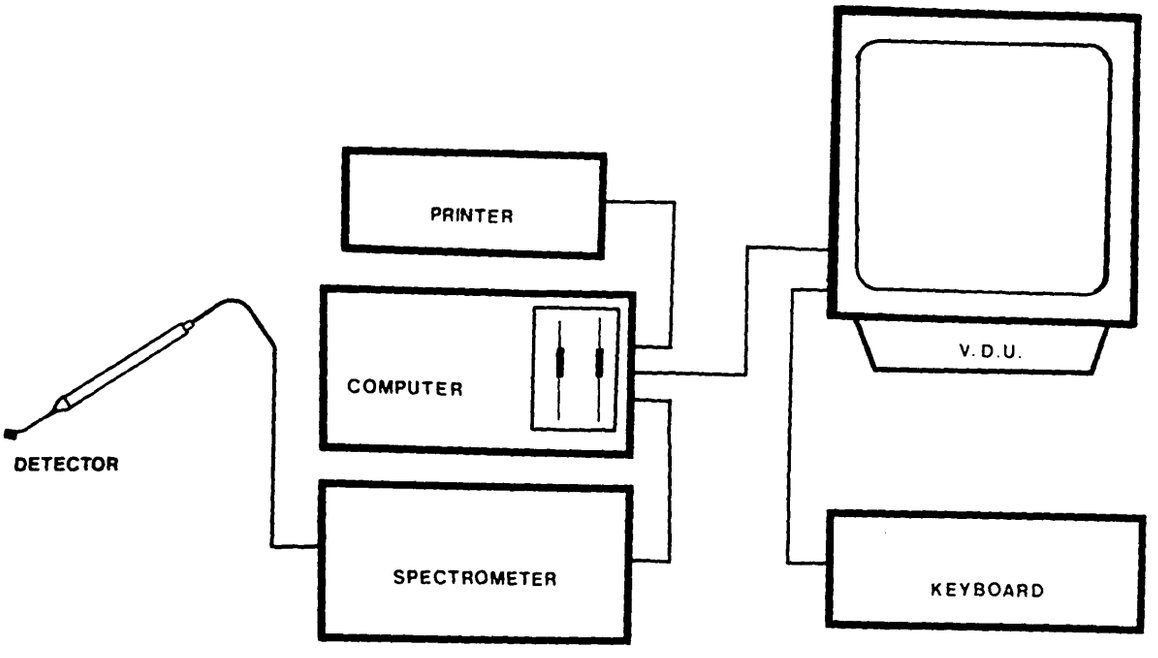


Figure 8. Diagrammatic representation of a beta-particle detector and automated isotope monitoring equipment. A crystal (NaI) scintillation detector is used for detection of gamma-radiation.

2.2.4.1) Monitoring of the stomach

An anaesthetised guinea-pig was placed in a prone position. A collimated crystal scintillation detector was held by a clamp over the abdomen, just below the xiphisternum to ensure that contents of the stomach would be monitored. An incision was made along the ventral midline of the neck and a small segment of trachea was exposed, close to the sternum. A Hamilton microsyringe (25 ul) was inserted into the trachea through a small aperture in the trachea and held by a clamp. A lead shield separated the microsyringe, containing 111-In labelled erythrocytes, from the detector (Fig 9). The animal was also shielded with lead bricks. The computer was programmed to count for 45 steps of 1 min duration. A 5 ul bolus of labelled erythrocytes was introduced into the airways and counting was initiated simultaneously.

Radiolabelled erythrocytes were transported from the airways to the pharynx and eventually swallowed. A progressive increase in the content of radioactivity of the stomach was detected. Accumulation of radiolabelled erythrocytes into the stomach was dependent upon regular swallowing, hence, radioactivity in the stomach was observed to increase in large steps, at irregular intervals (Fig 10).

2.2.4.2) Monitoring of the oropharynx

An anaesthetised guinea-pig was placed in a prone position. The trachea was exposed by a ventral midline incision along the neck. A Hamilton microsyringe (25 ul) containing 111-In labelled erythrocytes was inserted into the trachea. The tip of the microsyringe was positioned proximal to the site of insertion (10 mm) and clamped. This maneouvre increased the distance between the site of deposition of

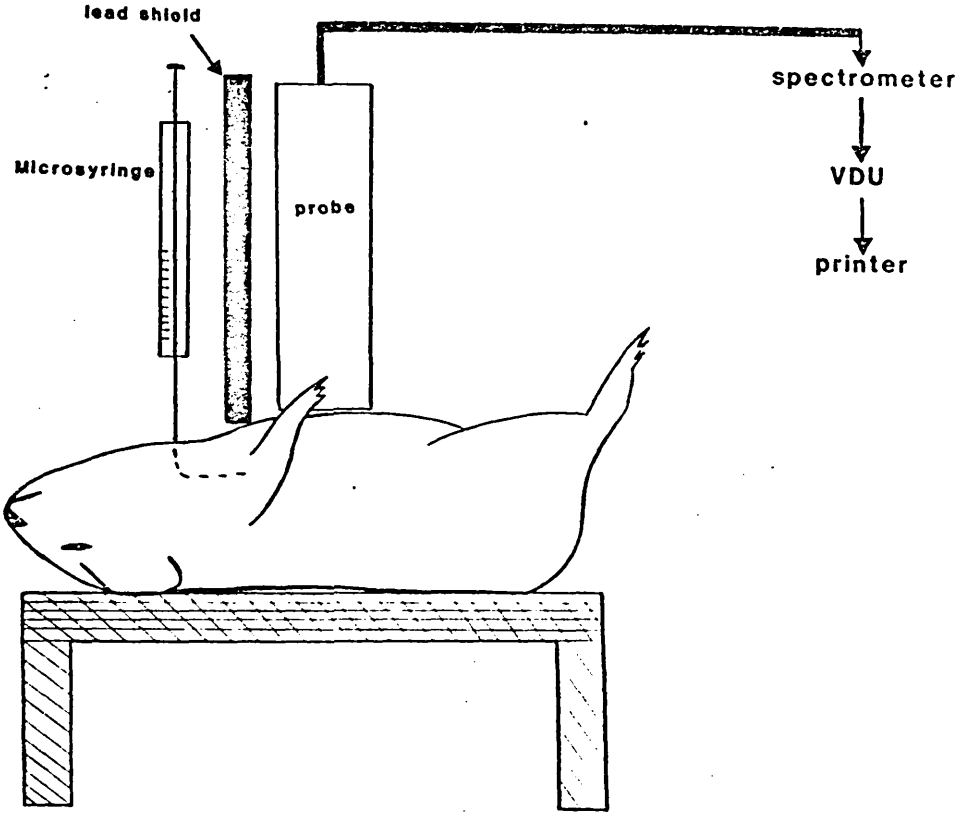


Figure 9. Diagrammatic representation of the method for measurement of mucociliary transport by monitoring accumulation of radiolabelled markers (III-In labelled erythrocytes) in the stomach. The microsyringe is inserted through the trachea and introduced into the airways, where 5 ul of labelled cells are introduced.

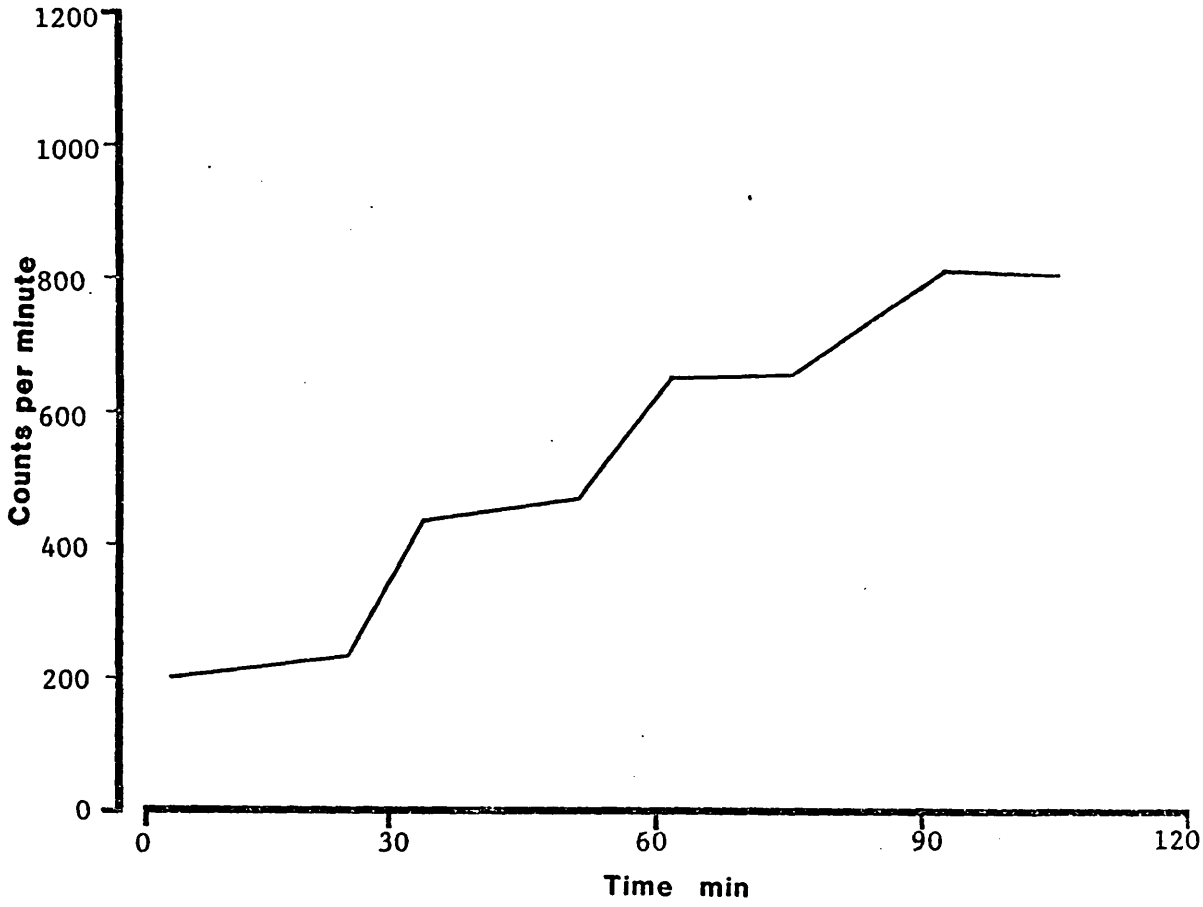


Figure 10. The graph shows stepwise accumulation of labelled erythrocytes into the stomach.

labelled erythrocytes and the detector. A gamma detector was positioned above the lower jaw, such that the centre of the detector was directly above the larynx. A lead shield separated the detector and the microsyringe (Fig 11). The animal was shielded on all sides with lead bricks. The computer was programmed to count for 45 steps of 1 min duration. A 5 ul bolus of labelled erythrocytes was injected into the airways and counting was initiated, simultaneously. In all experiments, the duration of monitoring required to complete two intratracheal instillations of labelled erythrocytes exceeded 45 min. At the end of each 45 min observation period, stored data was recorded on floppy discs and counting resumed by re-setting the computer, a procedure which could be completed within 15 seconds.

Accumulation of label into the oropharynx, prior to swallowing, was detected as a gradual rise in counts, reaching a plateau when most of the labelled erythrocytes had been cleared from the airway. When a new steady count rate was observed, a further bolus of labelled erythrocytes was injected into the airways and a rise in counts was observed (Fig 12). The slope of this graph, and the earliest detectable count above background, were considered as criteria for measurement of MCT. It was possible to obtain reproducible slopes with replicate injections of labelled erythrocytes into the airways (Fig 12). However, intermittent swallowing proved limiting. Figure 13 shows the effect of swallowing after counts have reached a steady level. In this instance a new, lower, count rate was observed. If the animal swallowed during the steep part of the slope, transport rate could not be estimated.

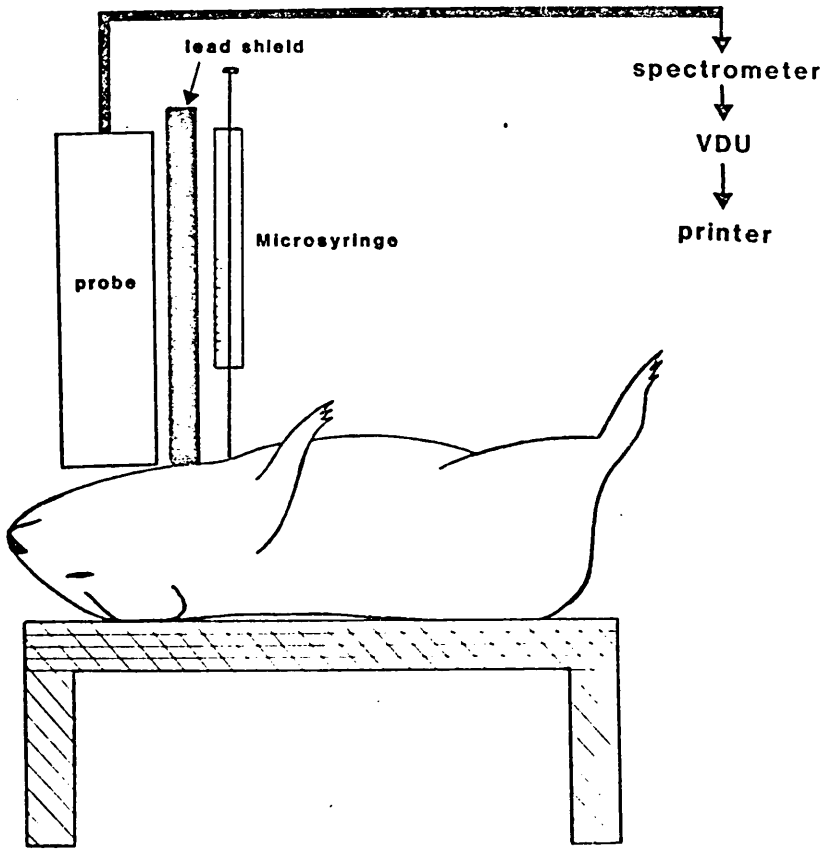


Figure 11. Diagramatic representation of the method for measurement of mucociliary transport by monitoring accumulation of radiolabelled markers (III-In labelled erythrocytes) in the oropharynx. The microsyringe is inserted into the trachea and 5 ul of labelled cells are injected for each estimation of mucociliary clearance.

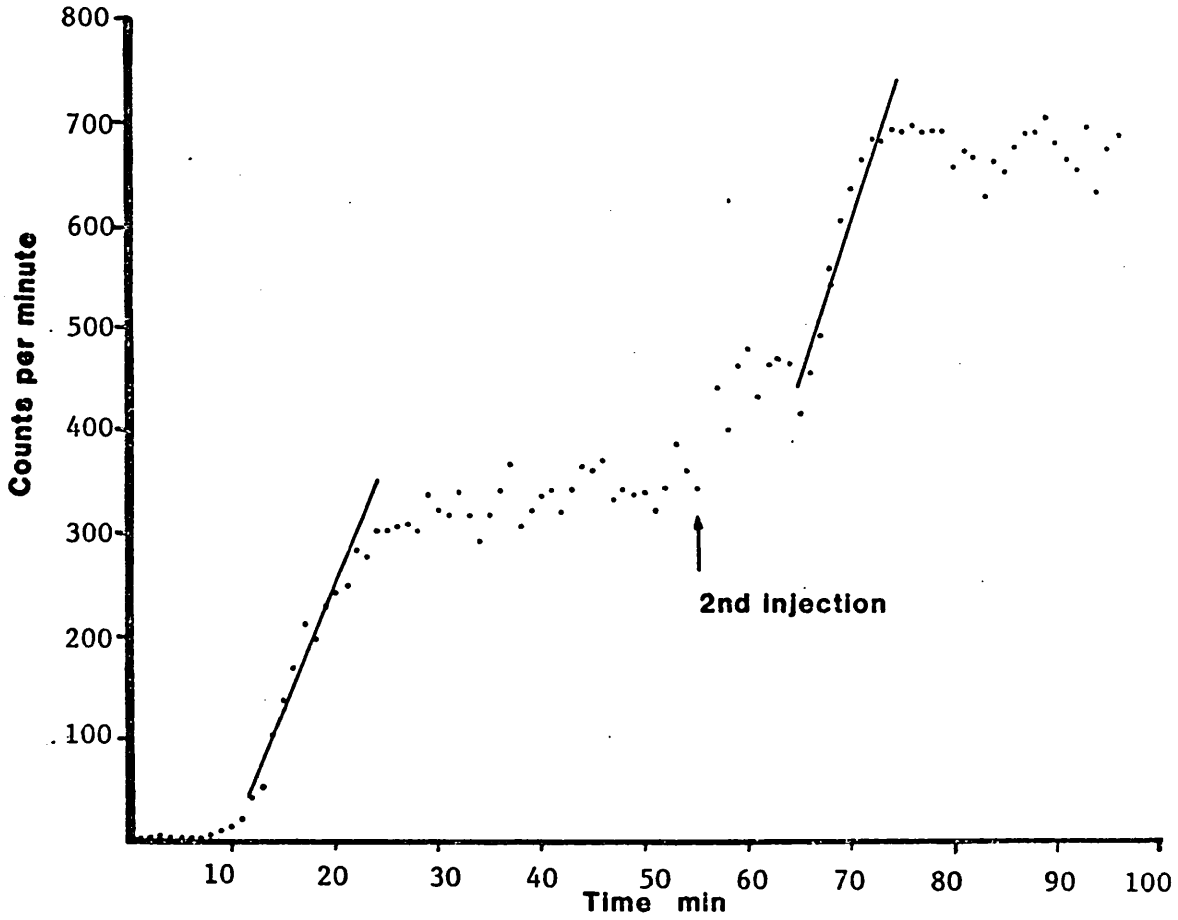


Figure 12. The graph shows continuous accumulation of labelled erythrocytes into the oropharynx after the 1st injection (5 ul). A plateau is observed in approximately 25 min, after which a 2nd injection of labelled cells produces a further rise in counts.

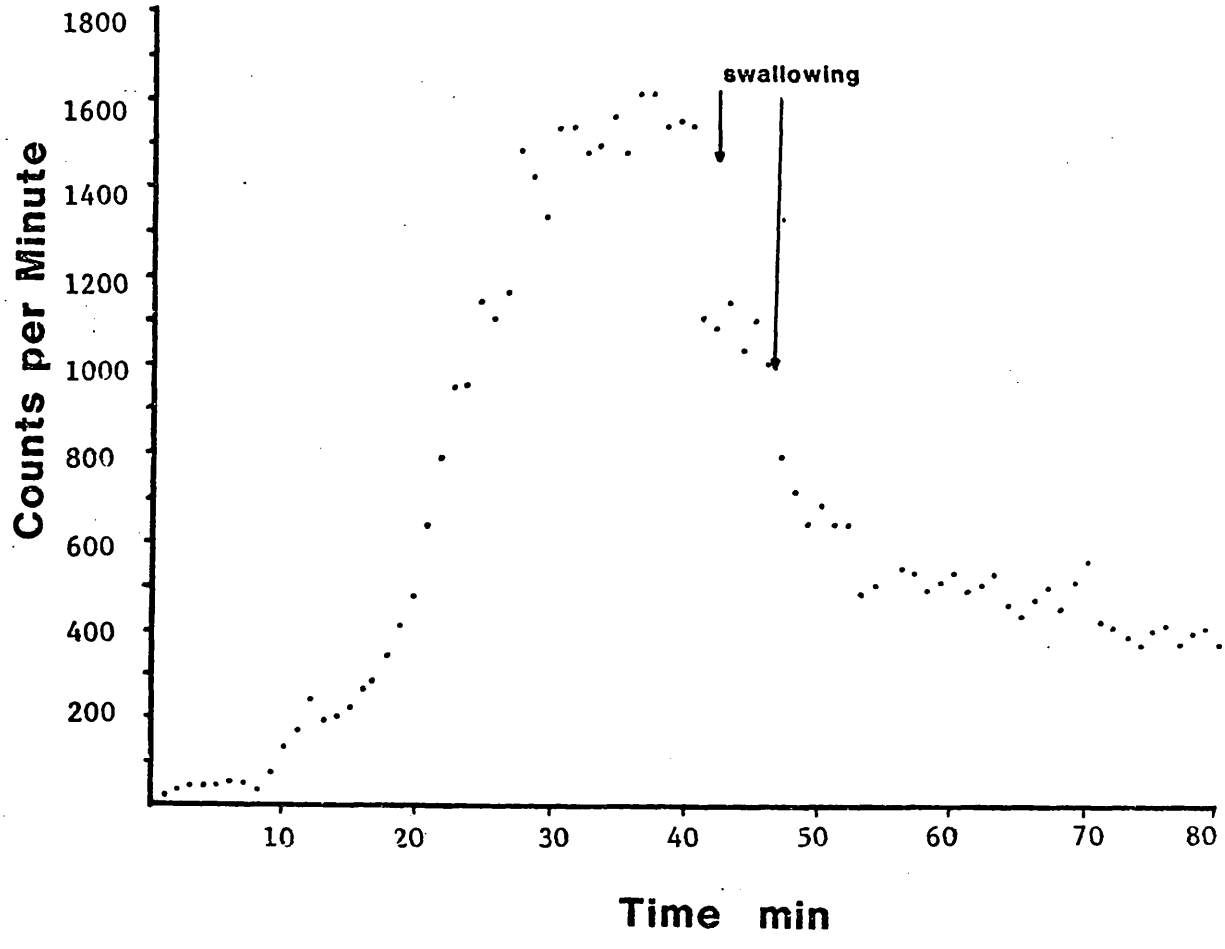


Figure 13. The graph shows continuous accumulation of labelled erythrocytes into the oropharynx. The plateau phase is interrupted by the animal swallowing (arrows).

2.2.4.3) Measurement of tracheal mucus velocity

Measurement of tracheal mucus velocity (TMV) in small animals was facilitated by employing compact, solid state beta-particle detectors and 32-P labelled erythrocytes. An anaesthetised guinea-pig was placed in a prone position and the trachea exposed by a ventral midline incision and retraction of sternohyoid muscles. The length of trachea from the top of sternum to the larynx was carefully cleared of connective tissue and fat. The ventral surface of the trachea, close to the sternum, was punctured using a large needle (19 G) and a Hamilton microsyringe (25 ul) containing 32-P labelled erythrocytes was inserted into the trachea through this aperture.

Two beta-particle detectors were placed over the trachea, positioning the collimator apertures at right angles to the tracheal axis. Separation between the centres of the detectors (corresponding to the collimator aperture) was 15 mm. The recording system was programmed to count for 45 steps of 10 second duration. A 2 ul bolus of labelled erythrocytes was injected into the trachea and counting initiated. The labelled cells were transported along the mucosal surface, successively passing the face of each detector. A maximum count rate was detected when the bolus of erythrocytes was directly beneath the aperture of a collimator. Count rates declined as the labelled cells moved away from the first detector and towards the second detector. At this point signals were registered by the second detector, eventually reaching a second maximum and then declining. The distance between the detectors and the time separation of the two maxima was used to calculate TMV in mm/min (Fig 14). Use of two detectors proved cumbersome; therefore, use of a single detector was evaluated.

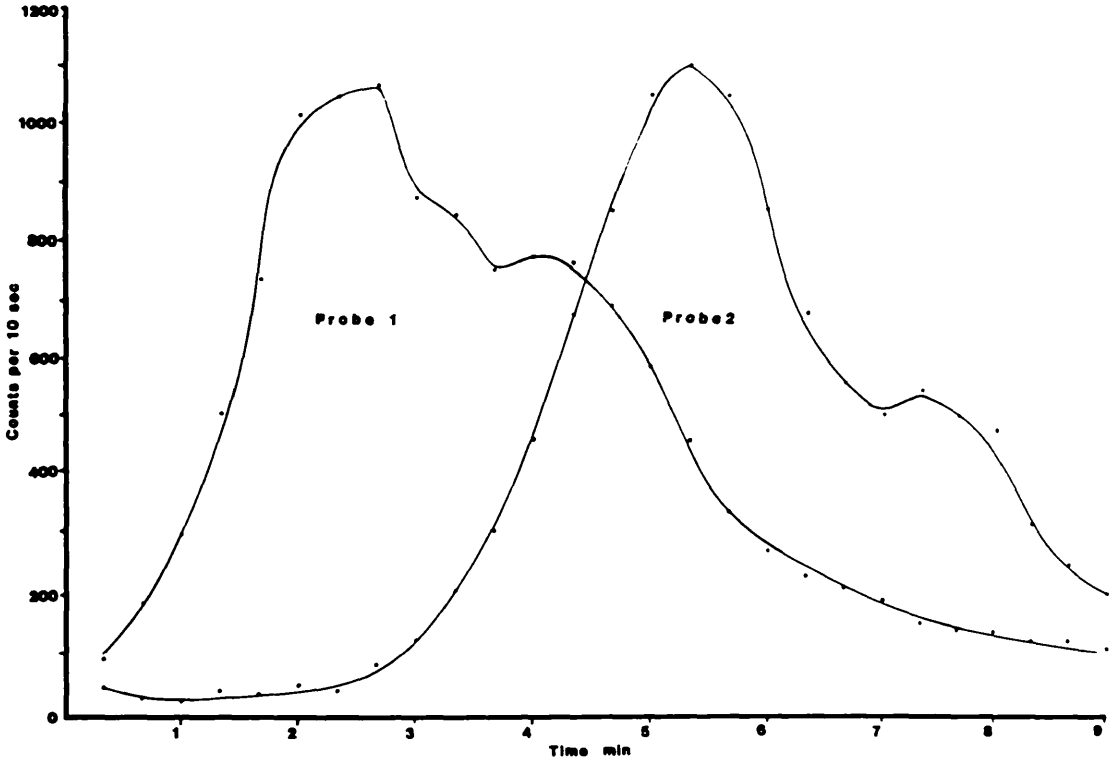
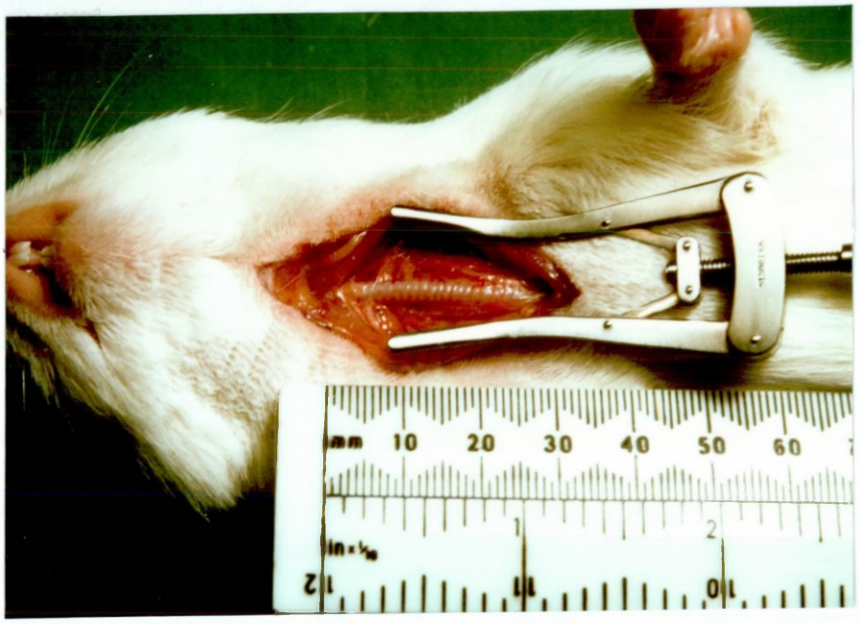


Figure 14. Measurement of tracheal mucus velocity (TMV) using two beta-particle detectors. The detectors are placed over the exposed trachea a known distance (15 mm) apart. 32-P labelled erythrocytes (1.5 ul) are introduced into the trachea and each detector registers the passage of marker cells. Time separation of maximum count rates is used to calculate TMV.

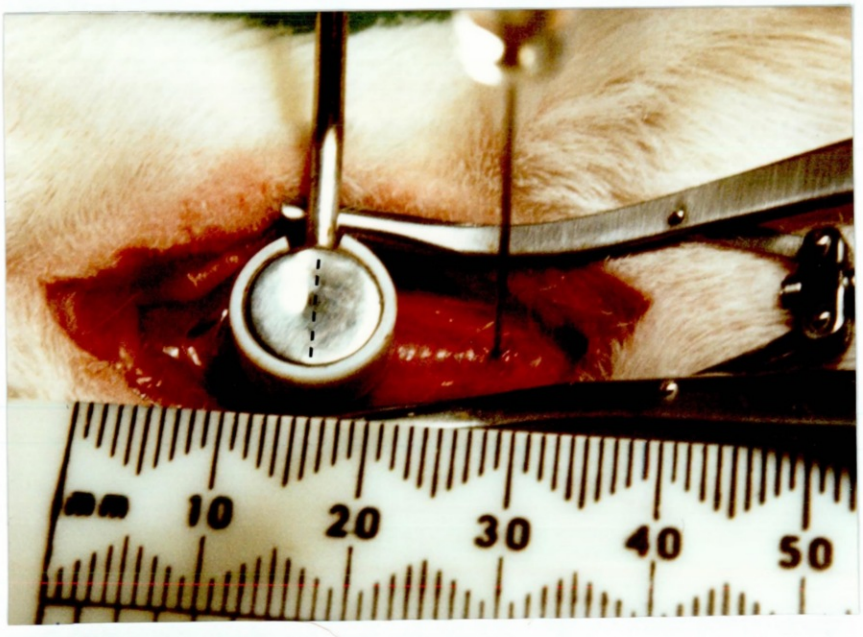
The trachea of an anaesthetised animal was exposed, as described above. A single detector was positioned just above the distal trachea with the slit of the collimator at right angles to the tracheal axis. The aperture for insertion of the microsyringe was located 15 mm proximal to the centre of the detector and held by a clamp (Plate 4a, b, c). The recording system was programmed to count for 45 steps of 10 second duration. A 1.5 ul bolus of labelled cells was injected into the trachea and counting was initiated. Figures 15 and 16 showed diagramatic representations of transport of a bolus of labelled erythrocytes and detection of radioactivity. In order to calculate TMV, the distance between the needle and the collimator slit was divided by the time which elapsed between injection of labelled cells and observation of a maximal count rate (Fig 17) and expressed in mm/min.

This method was preferred to other techniques and was used for drug studies. In each preparation, TMV was measured at 15 minute intervals, until 3 successive determinations with a coefficient of variation of less than 15% were obtained. Mean TMV calculated from these 3 determinations was taken as basal TMV. Drug effects were only determined in those animals in which a consistent basal TMV had been observed. This technique was used in guinea-pigs, ferrets (Plate 5) and rabbits. For ferrets and rabbits the distance between the needle and the detector was increased to 20 mm and the volume of labelled cells was increased to 2 ul and only 2 pre-treatment determinations with a coefficient of variation of less than 15% were used to calculate basal TMV.

a



b



c

Plate 4. Stages in the dissection and preparation of a guinea-pig prior to measurement of tracheal mucus velocity. The anaesthetised guinea-pig is placed in supine position (a) and the trachea is exposed by a midline incision along the neck and by retraction of the sternohyoid muscles (b). The detector is positioned above the distal trachea with the collimator slit (dotted line indicate orientation) at right angles to the tracheal axis. A microsyringe containing labelled erythrocytes is inserted into the trachea via a puncture hole 15 mm proximal to the collimator slit (c).

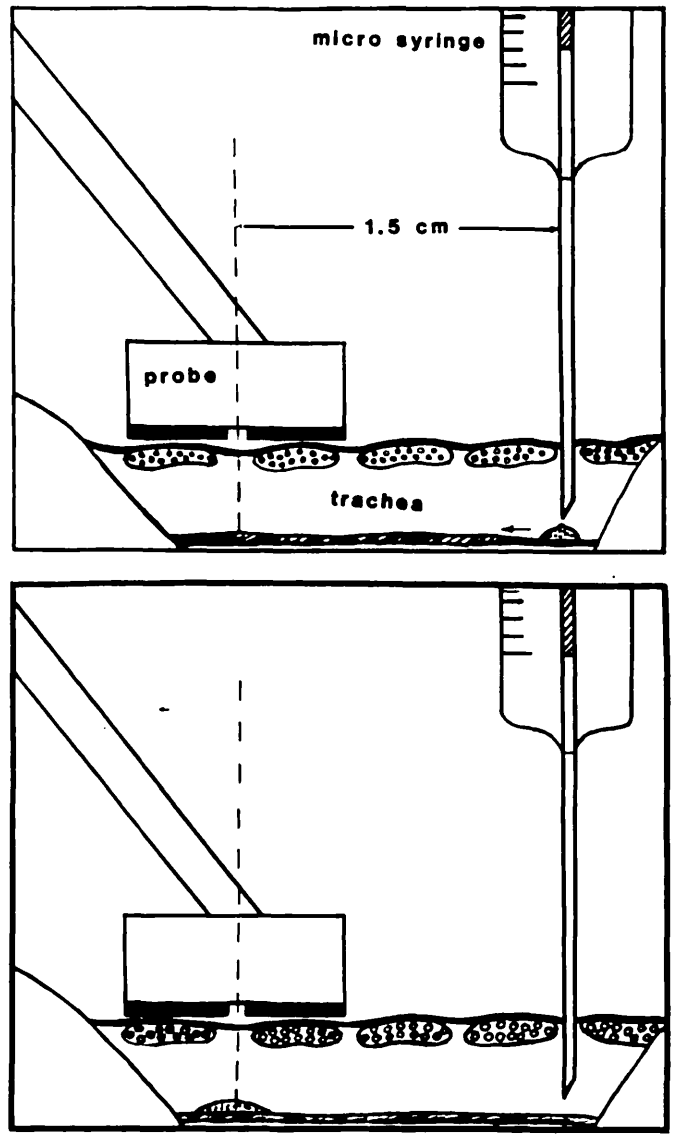


Figure 15. Diagramatic representation of the method for measurement of tracheal mucus velocity using one beta-particle detector (saggital section).

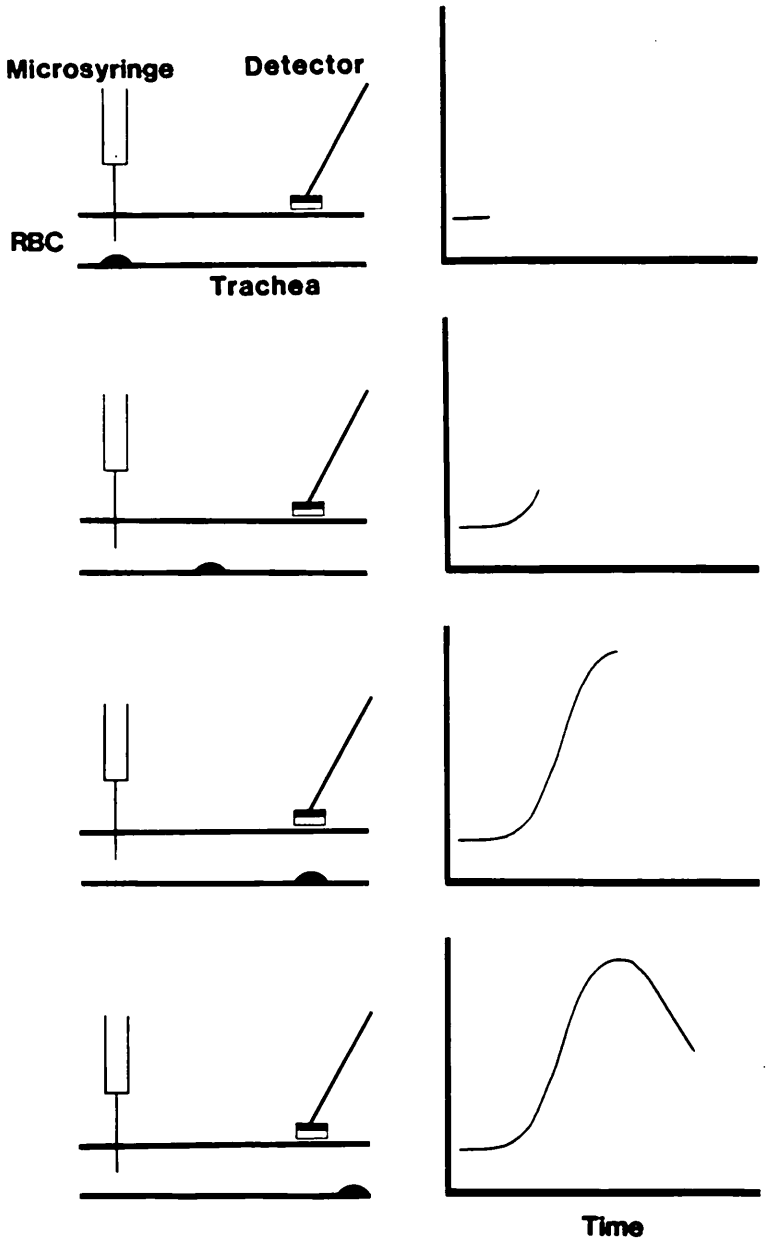


Figure 16. Diagramatic representation of the sequence of events during measurement of tracheal mucus velocity, using phosphorus-32 labelled erythrocytes. Labelled cells (1.5 or 2.0 ul) are introduced into the trachea, and counting is initiated simultaneously. As the labelled cells approach the detector, higher counts are registered. When the bulk of labelled cells are directly beneath the collimator slit, maximum count rates are observed. Counts gradually return to background levels as the labelled cells are removed from the trachea.

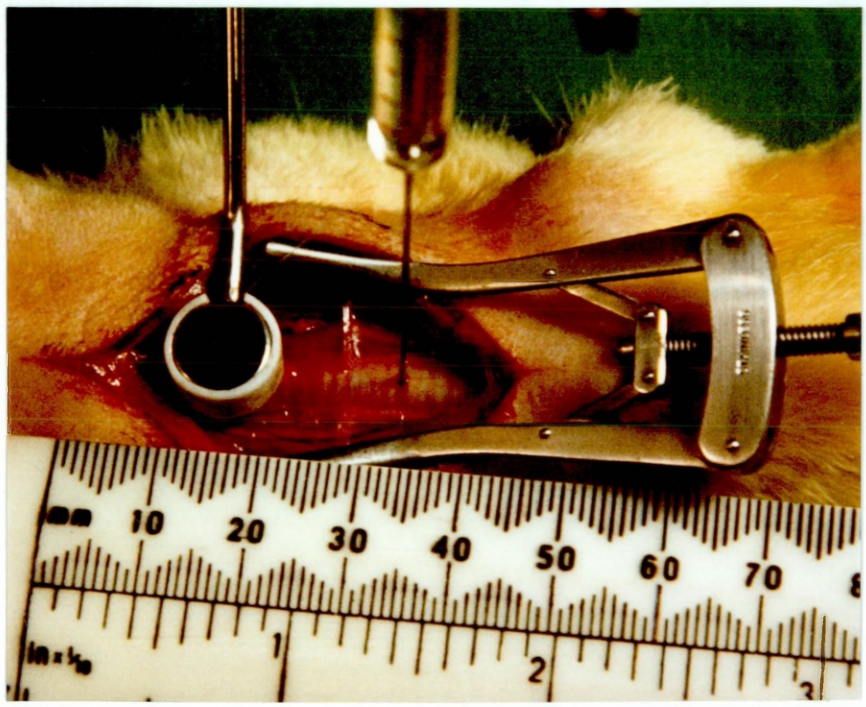

10
20
30
40
50
60
70
1
2

Plate 5. To measure tracheal mucus velocity in the anaesthetised ferret, the dissection procedure described for the guinea-pig has been used (Plate 4). In this instance, the distance between the microsyringe needle and the collimator slit has been increased to 20 mm.

2.2.5) Drug administration

Drugs were dissolved in phosphate buffered salt solution (PBS, Gibco) and were usually administered intravenously. A 25 gauge butterfly cannula (Abbott Laboratories) was inserted into a foot vein of the guinea-pig, the saphenous vein of the ferret or an ear vein of the rabbit. In some animals, solutions of drugs were applied topically to the exposed, outer (serosal) wall of the trachea.

2.2.6) Vagal stimulation

The effect of vagal stimulation was examined in guinea-pigs and ferrets. In both species, the vagus nerve on the left side was dissected from the carotid artery and tied at the distal end. The nerve was placed over nickel-coated copper electrodes of an electrical stimulator (Fenton Lewis MK IV) and embedded in petroleum jelly. Stimuli were pulses (2 msec) of 1 or 2 volts at 20 Hz for 1 min.

2.2.7) Measurement of blood pressure and heart rate

In the majority of animals, blood pressure and heart rate were measured in order to give an independent assessment of drug effects and to monitor the depth of anaesthesia. In guinea-pigs and ferrets, blood pressure and pulse rate were measured by inserting a plastic cannula (Portex, 1 mm O.D.) into the right carotid artery. The cannula was connected to a blood pressure transducer (PDCR 75, Druck) and both blood pressure and pulse rate were displayed on a chart recorder (MX6, Ormed Engineering). In rabbits, the ear artery was used for monitoring blood pressure and pulse rate in like manner.

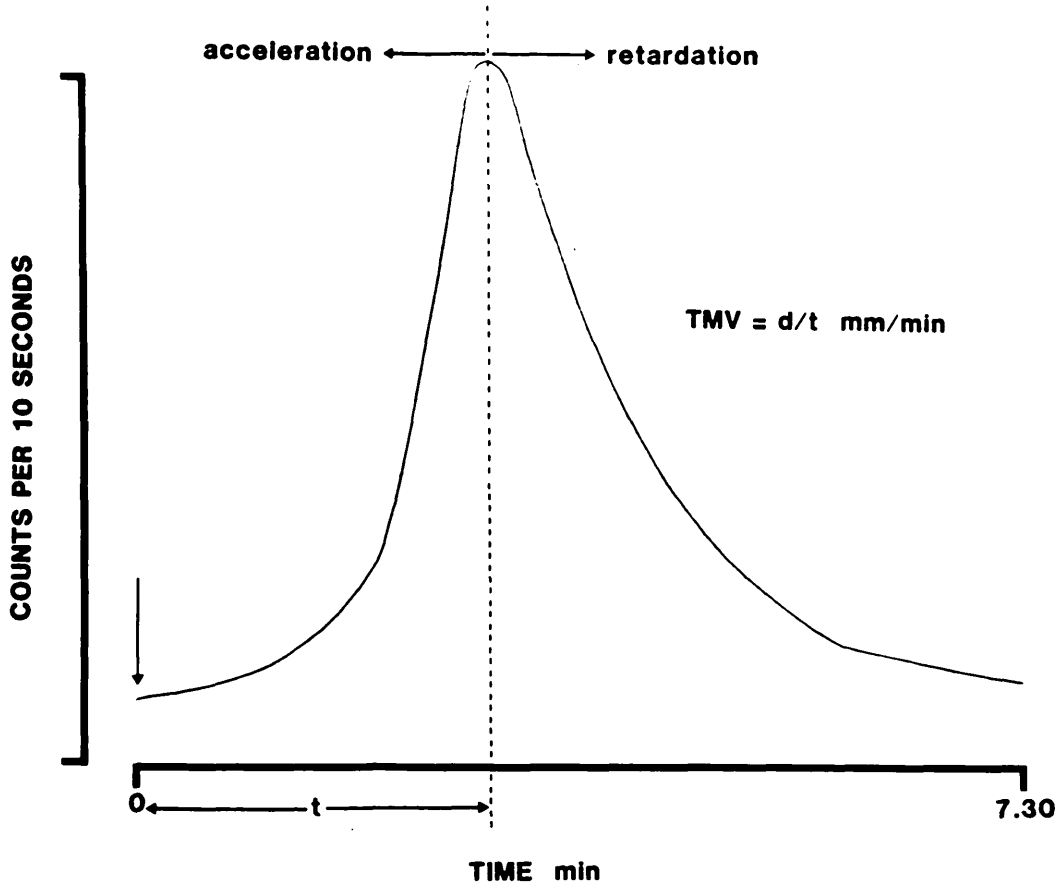


Figure 17. Isotope detection by beta-particle detector during passage of 32-P labelled erythrocytes along the trachea. Arrow indicates the point at which labelled erythrocytes are injected. Tracheal mucus velocity (TMV) is calculated by division of the distance between the microsyringe needle and the collimator slit (15mm) by the time which elapsed to observe maximal radioactivity and expressed in mm/min.

2.2.8) Measurement of bronchoconstriction

Bronchoconstriction was measured in a separate group of animals in order to ascertain the effect of stimuli which were used for mucociliary studies on bronchial tone. The trachea of an anaesthetised animal was exposed by a ventral midline incision along the neck. A low tracheostomy was performed by inserting a plastic cannula (3 mm o.d.) into the trachea. The tracheal cannula was connected to a small animal respirator (Harvard Instruments Ltd.) and an air pressure transducer (UP1, Ormed Engineering) (Fig 18). Animals were ventilated with 1 ml of air per 100 g body weight at 60 breaths per minute. Insufflation pressure was measured by the transducer and recorded on a chart recorder (MX6, Ormed Engineering). Drugs were administered intravenously and bronchoconstriction was measured as increased insufflation pressure.

2.2.9) Measurement of ciliary beat frequency

An animal was killed and the segment of trachea that had been used for measurement of TMV was excised. Ciliary beat frequency of an epithelial clump of excised trachea was measured photoelectrically, using the method of Yager et al. (1978), as modified by Rutland and Cole (1980). A cytological brush, 2 mm in diameter, was inserted into the trachea and epithelial material was removed by drawing the brush across the epithelial surface. Adherent cellular material was dislodged by brisk agitation in 2 ml of culture medium (medium 199 with Earl's salts, Flow Laboratories). Fragments of tracheal epithelium were deposited onto a microscope slide using a Pasteur pipette. A thin layer of silicone jelly was deposited around the droplet containing the epithelial fragments and a cover slip gently lowered onto the droplet.

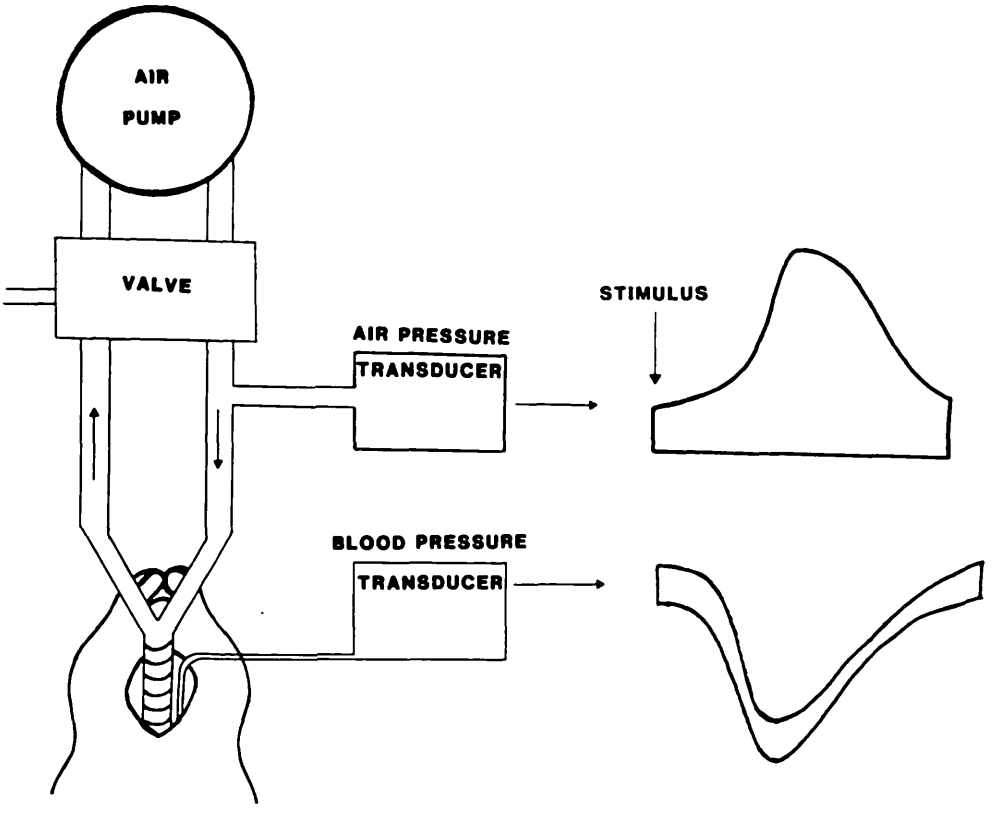


Figure 18. Diagramatic representation of measurement of bronchoconstriction and blood pressure.

Adhesion of the silicone layer to the cover slip provided a seal around the droplet. The preparation was examined on a warm stage (Microtec), mounted on a microscope (Leitz Dialux 20). Clumps of ciliated epithelium were observed at a magnification of 320 fold, by bright field illumination with the condenser at a low aperture. Variation in light intensity caused by ciliary beat was detected by a microscope photometer (Leitz MPV). The rectangular (1.5 x 5 um) field diaphragm of the photometer was oriented parallel to the long axis of the cilia, so that only two or three cilia were viewed. Ciliary beats produced variations in light intensity at the photometer, whose signals were filtered (cut off 40 Hz) and amplified for display on an ultra violet oscillograph (SE Labs 6150). This provided a permanent record of ciliary activity as an oscillating signal. Mean peak height separation was used to calculate CBF (Fig 19). CBF was measured in at least 10 clumps obtained from one trachea and mean CBF $\pm$ s.d. was calculated. CBF was measured in guinea-pigs, ferrets and rabbits and related to basal TMV of the donor animals.

2.3) Statistics

Student's t-test for paired observations was used to assess the significance of observed differences. Student's t-test for groups of equal or unequal size was used to assess statistical significance of observations made in groups of animals.

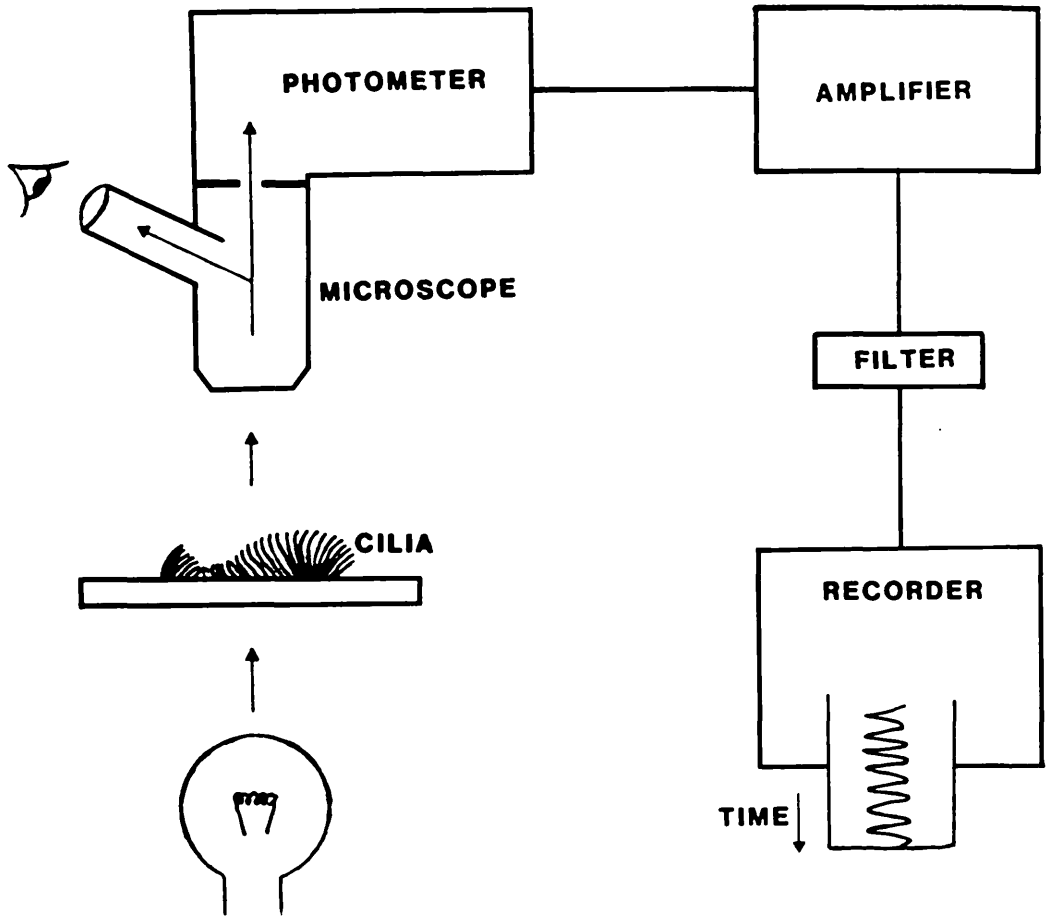


Figure 19. Diagramatic representation of the method for measurement of ciliary beat frequency from tracheal explants.

3) RESULTS

3.1) Histology

Tissue samples from palate and oesophagus of 6 frogs were examined. The epithelial layer of both areas contained ciliated columnar cells and goblet cells. However, only sections from the oesophagus showed glands in the submucosa (Plate 6a and b).

3.2) Frog oesophageal mucociliary transport

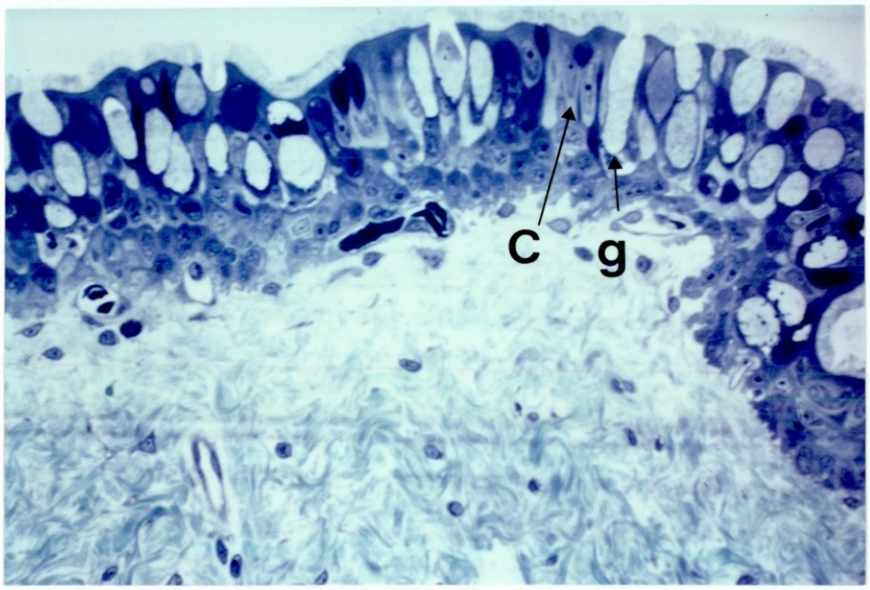
3.2.1) Site of measurement

In light of these histological findings, mucociliary transport was measured in those regions from which material for histology had been excised. In five animals, particle transport rate was measured at: fore-palate, mid-palate, pharynx, upper oesophagus and lower oesophagus. The pharyngeal region was observed to transport particles at a significantly ($P<0.001$) faster rate than all other regions, with fore-palate being the slowest (Fig 20). All subsequent experimental observations were made in the region of the pharynx and distal oesophagus.

3.2.2) Basal transport rates

Immediately after dissection, high rates of transport were observed. In 7 animals, particle transport rate was measured at regular intervals (5 or 10 min) after dissection. The rate of transport declined progressively until a relatively consistent value was achieved within 30 min of dissection. This effect was observed in both *in situ*

a



50 µ

b

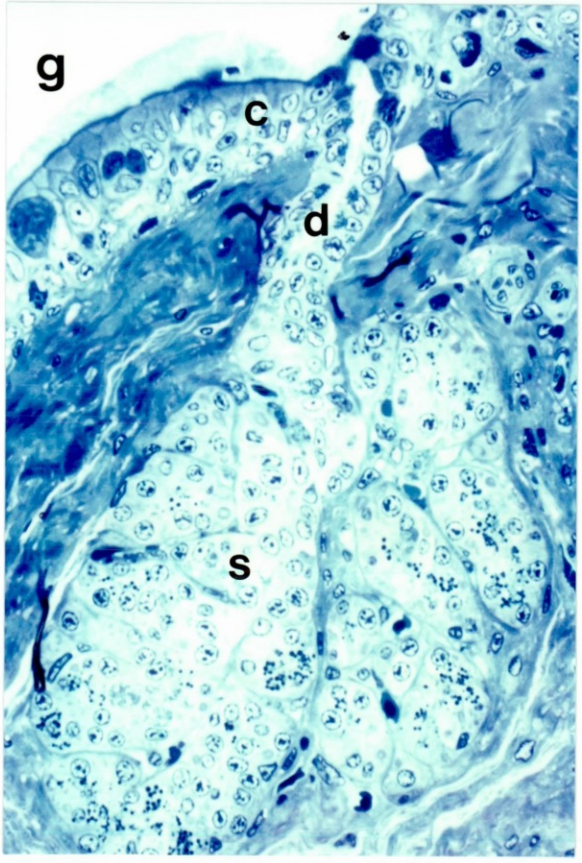


Plate 6. Transverse sections (1 um) through the palate (a) and oesophagus (b) of R. temporaria. Epithelial layers in both tissues contain ciliated columnar cells (c) and goblet cells (g). Submucosal glands (s) were observed only in the oesophageal sections. The duct of the gland (d) is clearly visible.

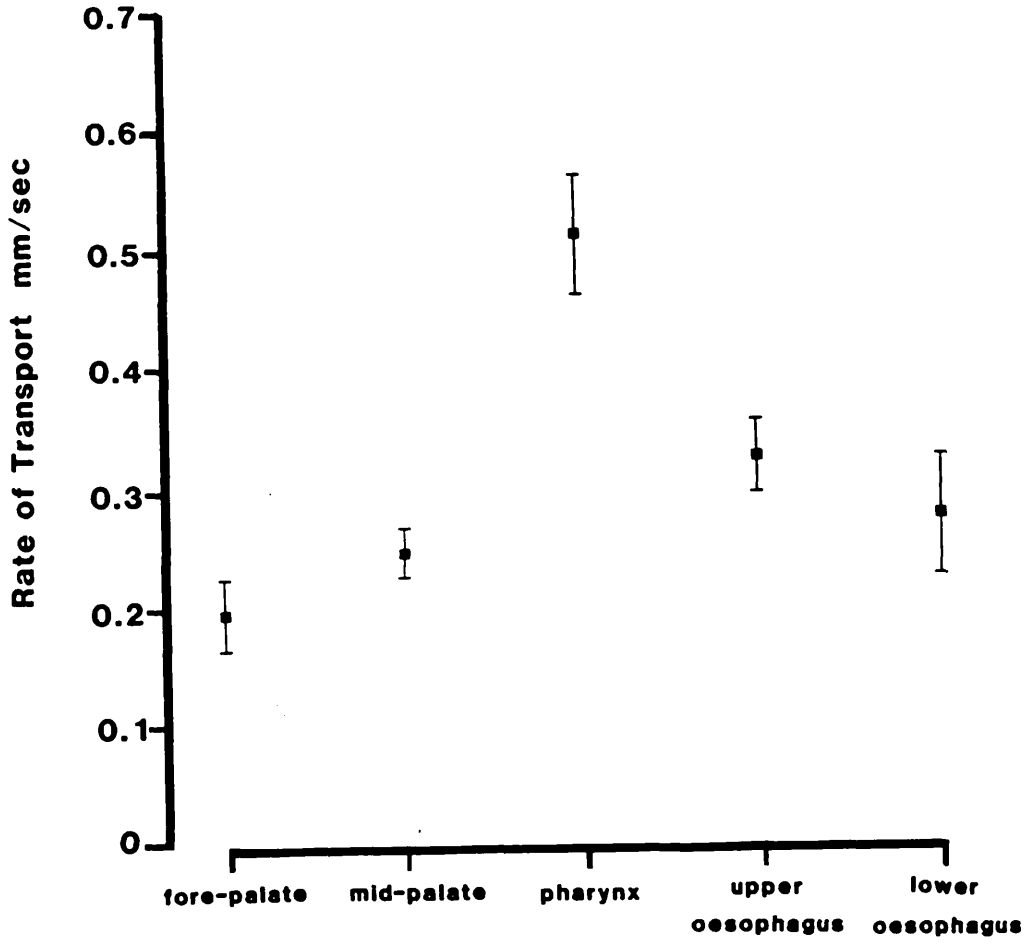


Figure 20. The graph shows the sites which were used to measure mucociliary transport along the frog palate and oesophagus (n=4). Particle transport rate is significantly faster in the pharynx, compared with fore-palate ($P<0.001$), mid-palate ($P<0.005$) and lower oesophagus ($P<0.001$)

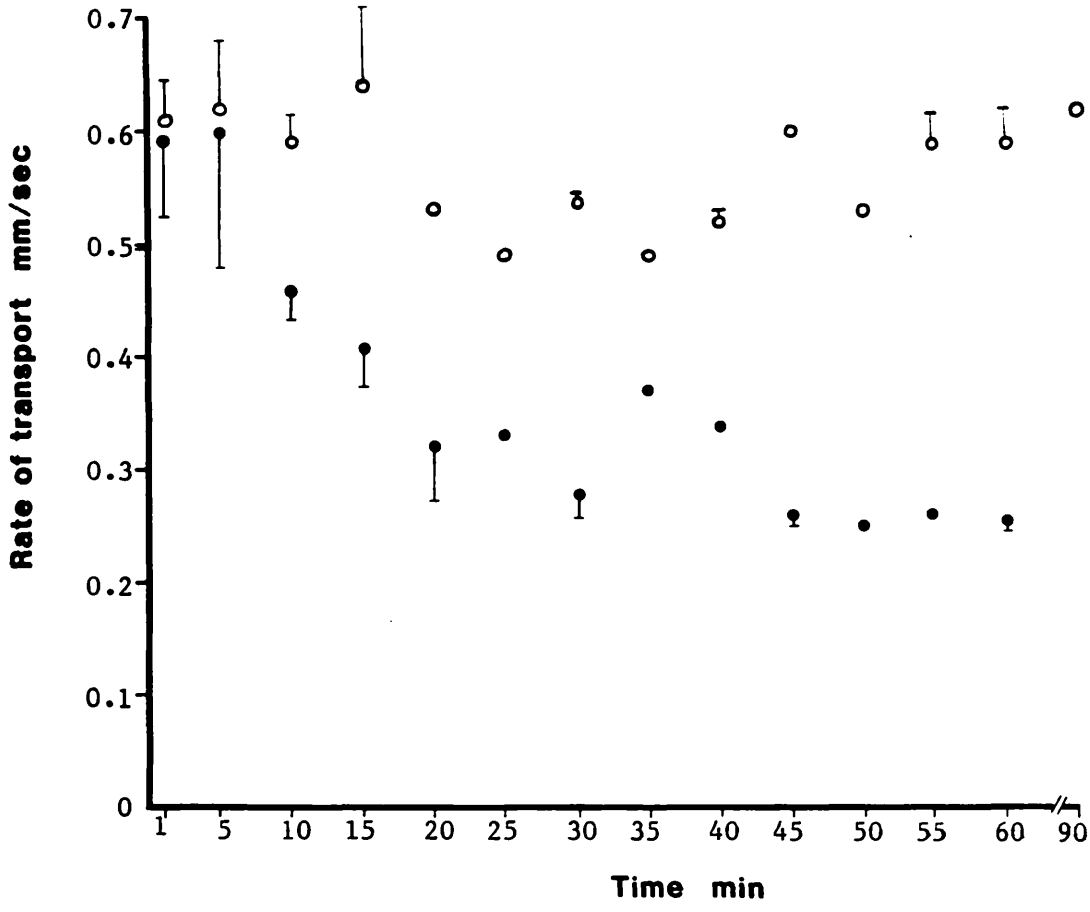


Figure 21. Comparison between in situ and chamber (●;n=7) methods with in vitro (○;n=4), mucociliary transport (MCT). In both, in situ and chamber methods, MCT declines to a steady basal level within 30 min after dissection; in vitro, MCT remains elevated for lengthy periods.

(section 2.1.2.1) and chamber (section 2.1.2.2) methods. In four of these animals, estimates of MCT in central and peripheral regions were made. No substantial difference was observed between central and peripheral transport rates. Nonetheless, all subsequent estimations of particle transport were made in the central region, where particles more commonly moved in a straight line, in contrast to the periphery where some particles followed the curvature of the cut edge and, hence, traversed a greater distance.

In 107 preparations, basal transport rate ranged between 0.17 and 0.41 mm/sec. Distribution of these estimates of basal transport rate approximated to normal, although there was a slight negative skew (Fig 22). The arithmetic mean was 0.30 $\pm$ 0.05 (s.d.) and the median was 0.31 mm/sec. The coefficient of variation of basal transport rates (mean of at least 3 determinations in each animal) was 8.4 $\pm$ 4.1% in a group of 30 animals (range 3.6 to 20%). In the *in vitro* preparation, particle transport rate did not decline with time. One hour after the first measurements had been made, particle transport rate was not dissimilar to the initial value (immediatly after dissection, 0.61 $\pm$ 0.03; 60 min after dissection, 0.60 $\pm$ 0.03 mm/sec $\pm$ s.e.m; n=4). Figure 21 shows the comparison between *in situ* and chamber methods with the *in vitro* method.

3.2.3) Vagal stimulation

Electrical stimulation of the vagus nerve consistently accelerated particle transport rate. This acceleration was voltage dependent. When pulses of increasing voltage (0.5, 1.0 and 1.5 volts) were used, the rate of particle transport increased from a mean basal rate of 0.32

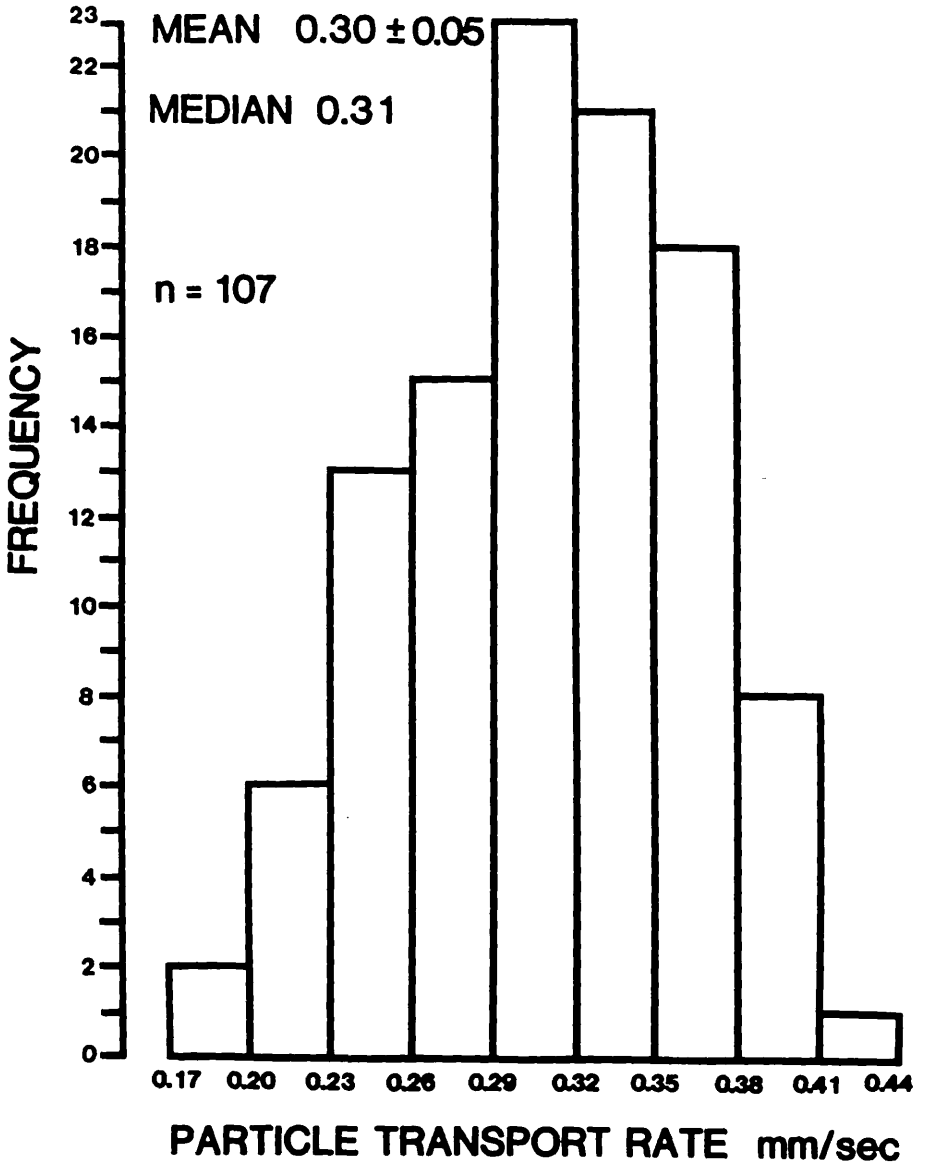


Figure 22. Distribution of basal oesophageal mucociliary transport rates from 107 frogs. Distribution approximates to normal with a slight negative skew. The mean is 0.30 ± 0.05 (s.d.) and the median 0.31 mm/sec.

$\pm$ 0.01 to 0.45 $\pm$ 0.01, 0.61 $\pm$ 0.02 and 0.76 $\pm$ 0.02 mm/sec $\pm$ s.e.m. respectively (n=30). Higher voltages caused further increase, but above 1.5 volts contractile activity of oesophageal smooth muscle precluded accurate measurement of particle transport rate. Repeated nerve stimulation did not produce tachyphylaxis (Fig 23a). Topical application of atropine (1 ug/ml) significantly inhibited the effect of vagal stimulation at all voltage levels (Fig 23b). Despite the reduced response to vagal stimulation after treatment with atropine, the responses to 1.0 and 1.5 volts were significantly ($P<0.05$) elevated, when compared with the saline control (Fig 23b). Topical application of hexamethonium bromide (1 ug/ml) significantly inhibited the effect of vagal stimulation at 0.5 and 1.0 volts although the reduction of response at 1.5 volts did not reach statistical significance (Fig 23c). After treatment with hexamethonium, particle transport rate due to nerve stimulation at 1.0 and 1.5 volts remained significantly ($P<0.025$ and $P<0.005$ respectively) greater than the saline control value (Fig 23c). Adrenaline (10 ug/ml), noradrenaline (10 ug/ml) and prostaglandin E2 (PGE2) (1 ug/ml) did not modify increased particle transport rate due to vagal stimulation. Eserine (0.1-100 ug/ml) was without effect on basal particle transport rate (Fig 24a) and, at 10 ug/ml, did not modify the increased particle transport rate due to vagal stimulation (Fig 24b). Lignocaine (1%, w/v) was able to reduce the effect of vagal stimulation, without altering basal transport rate or the increased particle transport rate after topical application of acetylcholine (Fig 25).

3.2.4) Parasympathomimetic agents

3.2.4.1) Acetycholine (ACh)

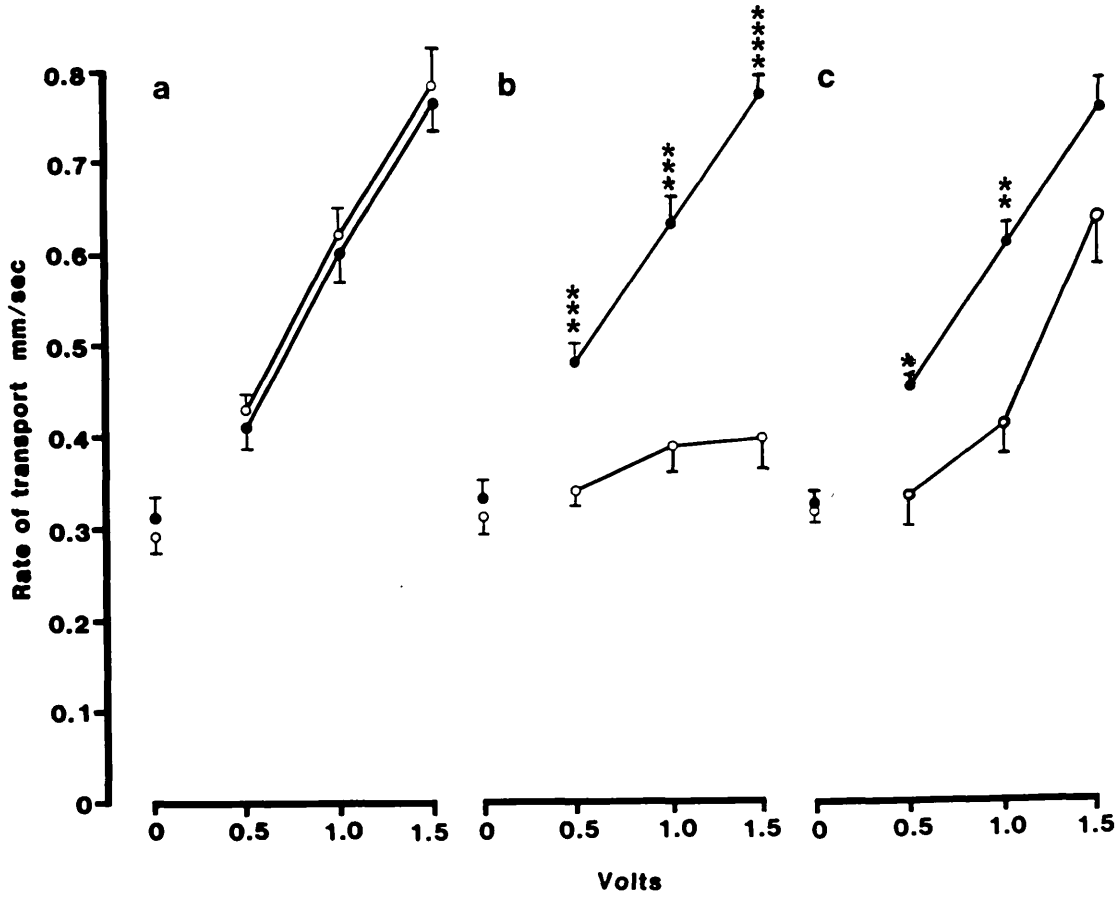


Figure 23. Effect of vagal nerve stimulation on frog oesophageal mucociliary transport. Effect of repeating vagal stimulation, within 15 minutes in the same animal (treatment 1 ●, treatment 2 ○); n=8 (a). Effect of vagal nerve stimulation before (●) and after treatment with atropine (○; 1 ug/ml); n=6 (b). Effect of vagal nerve stimulation before (●) and after treatment with hexamethonium bromide (○; 1 ug/ml); n=8 (c). Results are expressed as mean ± s.e.m. Student's t-test for paired observations was used to assess statistical significance of observed differences.

* $P<0.025$, ** $P<0.01$, *** $P<0.005$, **** $P<0.001$

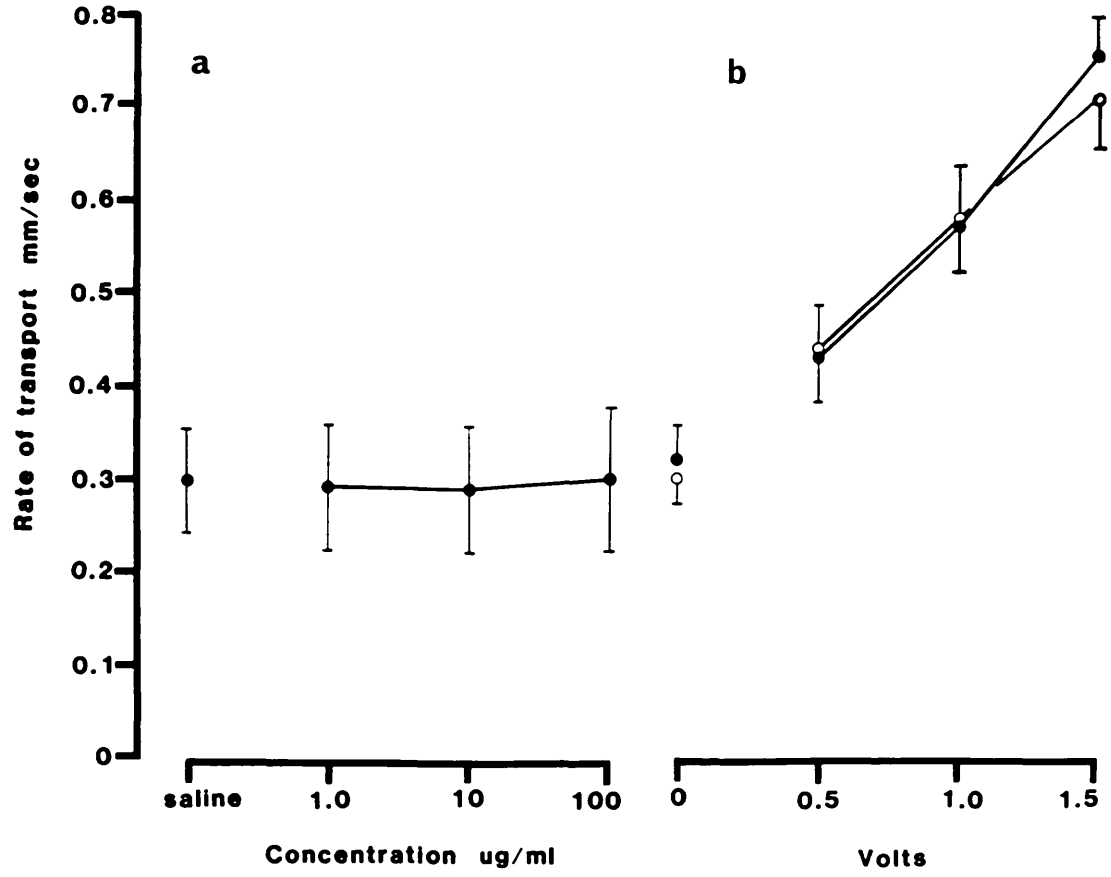


Figure 24. Effect of increasing concentrations of eserine sulphate (●; n=4) on oesophageal mucociliary transport (a). Effect of vagal nerve stimulation (n=4) prior to (●) and after treatment (○) with eserine sulphate (10 ug/ml) (b).

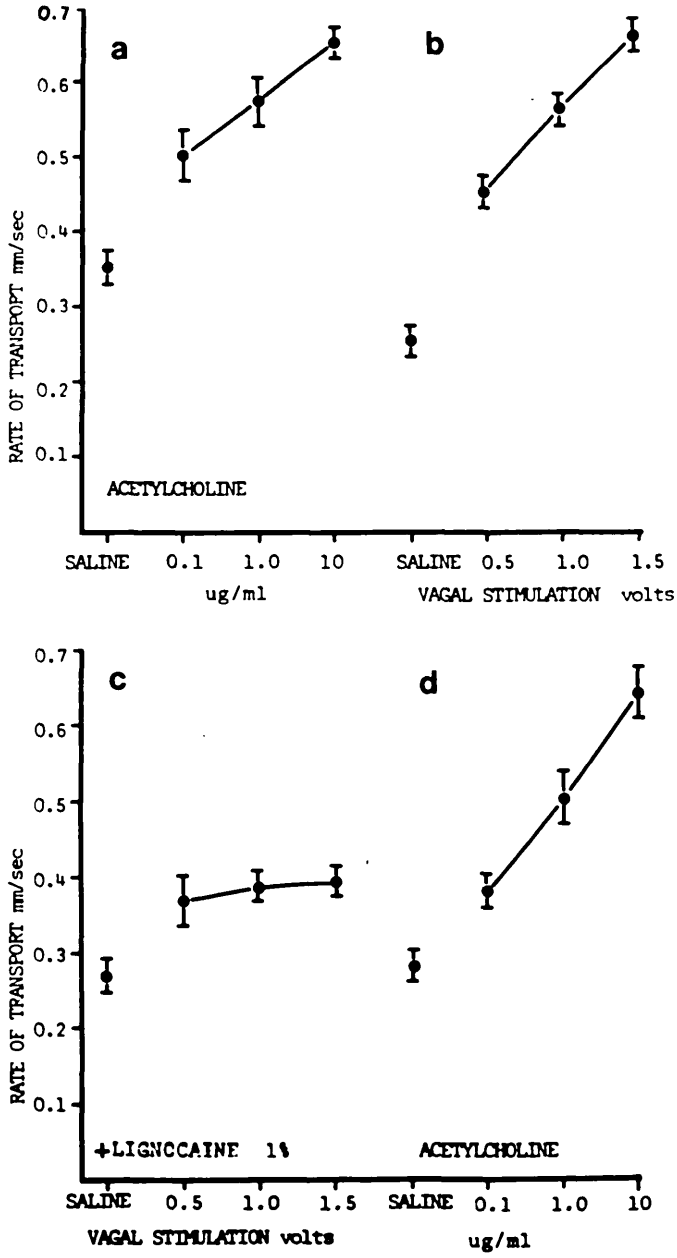


Figure 25. Effects of acetylcholine (a) and vagal nerve stimulation (b) on oesophageal mucociliary transport, before and after treatment with 1% lignocaine (c & d).

Topical application of ACh and other parasympathomimetic agents stimulated particle transport rate in R. temporaria, R. pipiens and Xenopus laevis. Figure 26 shows the effect of increasing concentrations of ACh (0.01-100 ug/ml) on MCT in the oesophagus of each species. Figure 27a shows the stimulatory effect of ACh (0.1-10 ug/ml) in R. temporaria (n=22). No tachyphylaxis was observed when a dose-response relationship for ACh was defined on successive occasions in individual animals (n=5; Fig 27b). Atropine (1 ug/ml) abolished the stimulatory effect of ACh (Fig 27c). Hexamethonium bromide (1 ug/ml) or eserine sulphate (10 ug/ml) were without effect on responses to ACh (Fig 28ab). The dose-response relationships for ACh (0.1-10 ug/ml) were compared, using in situ, chamber and in vitro methods. A dose-related increase in particle transport rate was observed in situ and in the chamber method (Fig 29). In the in vitro method, particle transport rate was elevated above the basal level, only with a very high concentration of ACh (100 ug/ml) (Fig 29).

3.2.4.2) Nicotine

Topical application of nicotine sulphate (0.1-10 ug/ml) increased particle transport rate in a dose-related manner from a basal value of 0.33 ± 0.01 to 0.45 ± 0.01, 0.61 ± 0.03 and 0.77 ± 0.02, respectively (n=14). Elevation of MCT was statistically significant at all concentrations ($P<0.001$). When a dose-response relationship was defined for nicotine in the same animal, no tachyphylaxis was observed (Fig 30a). Both atropine (1 ug/ml) and hexamethonium (1 ug/ml) inhibited responses to nicotine (0.1-10 ug/ml) (Fig 30bc). Significant stimulation of particle transport was observed after treatment with

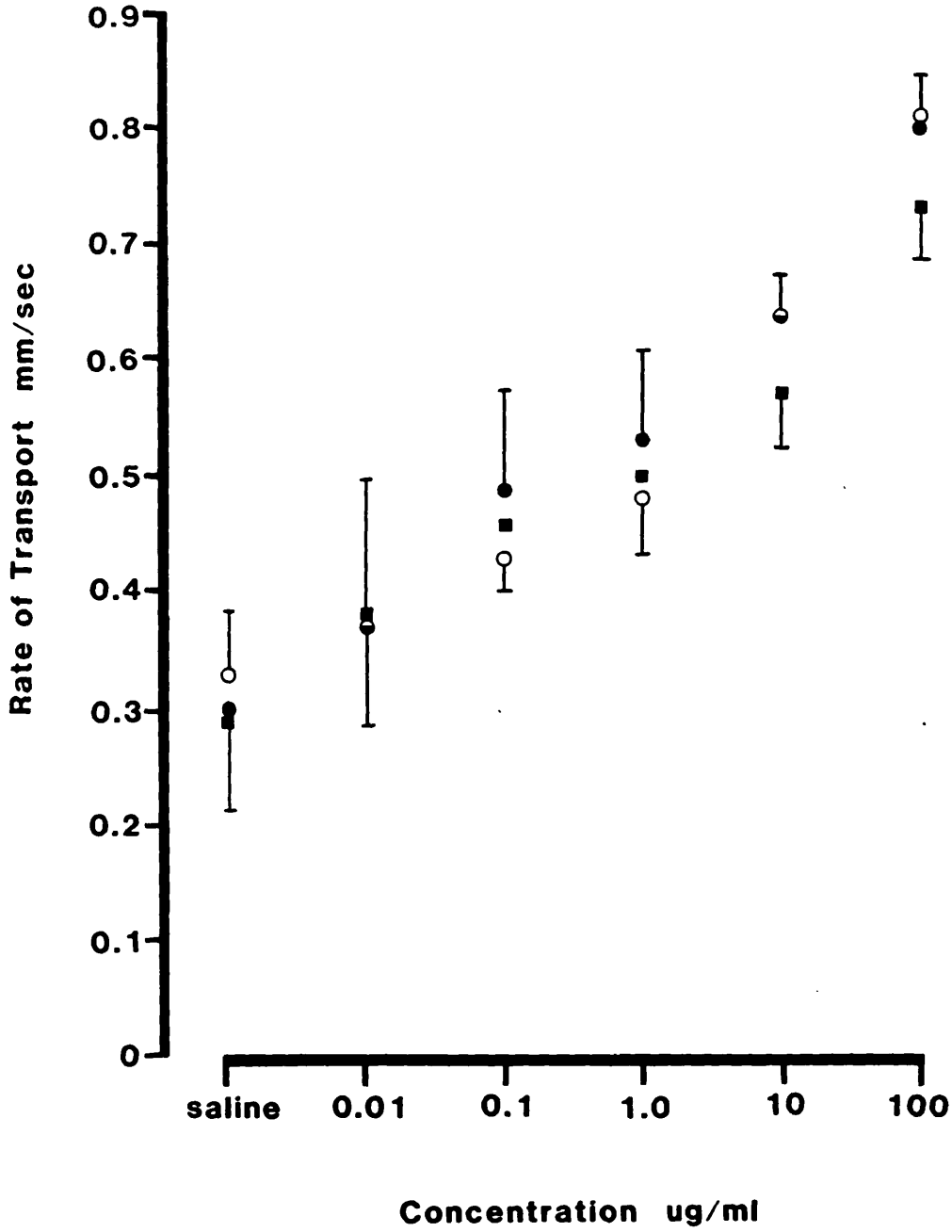


Figure 26. Effect of increasing concentrations of ACh on oesophageal mucociliary transport of R. temporia (○; n=4), R. pipiens (●; n=2) and X. Laevis (■; n=3).

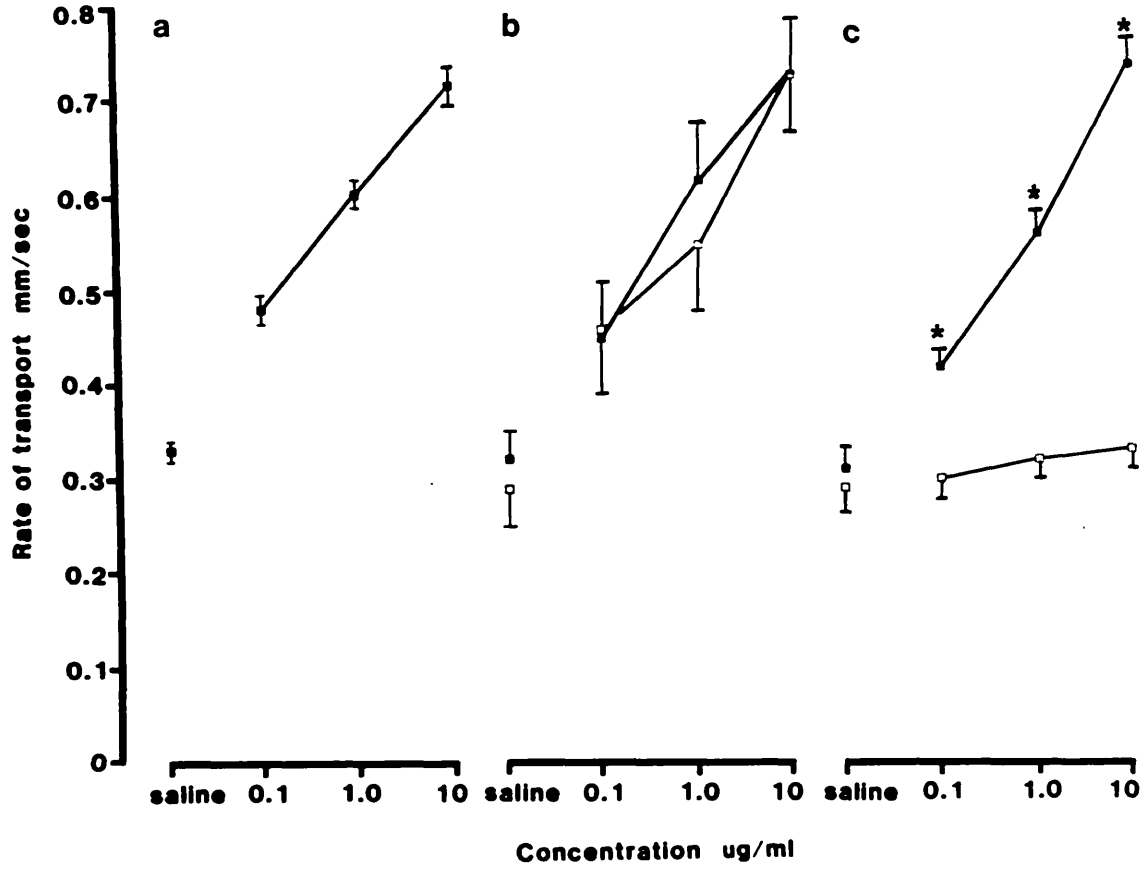


Figure 27. Effect of topical application of increasing concentrations of acetylcholine (ACh) on frog oesophageal mucocilioary transport (■); n=22 (a). Effect of repeating ACh dose-response relationships in the same preparations (treatment 1 ■, treatment 2 □); n=5 (b). ACh response before (■) and after (□) treatment with atropine sulphate (1 ug/ml); n=8 (c). Results are expressed as mean ± s.e.m. Student's t-test for paired observations was used to assess statistical significance of observed differences.

* P<0.001

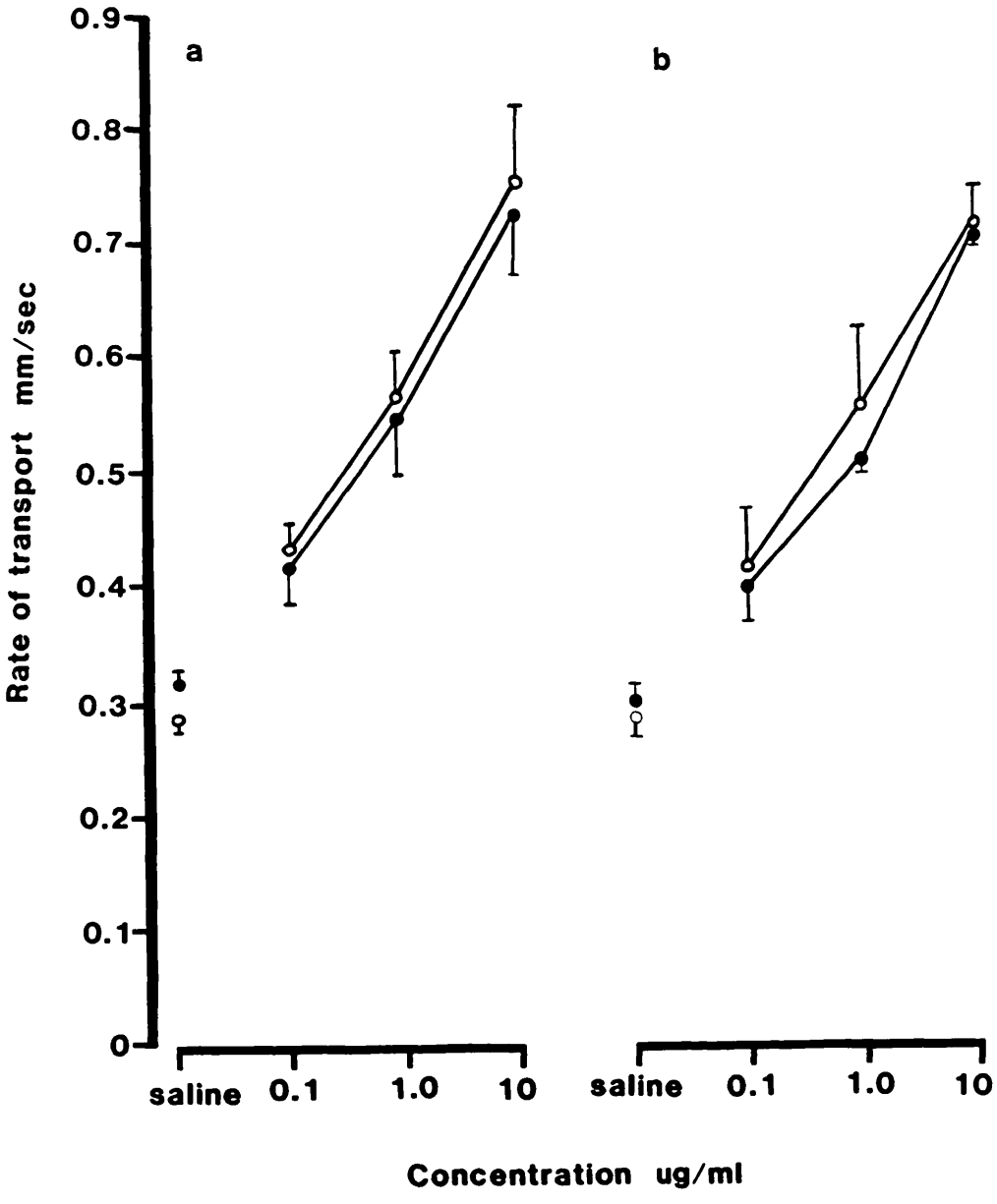


Figure 28. Effect of increasing concentration of ACh (n=3) on oesophageal mucociliary transport before (●) and after (○) treatment with hexamethonium (1 ug/ml) (a) or eserine sulphate (10 ug/ml) (b).

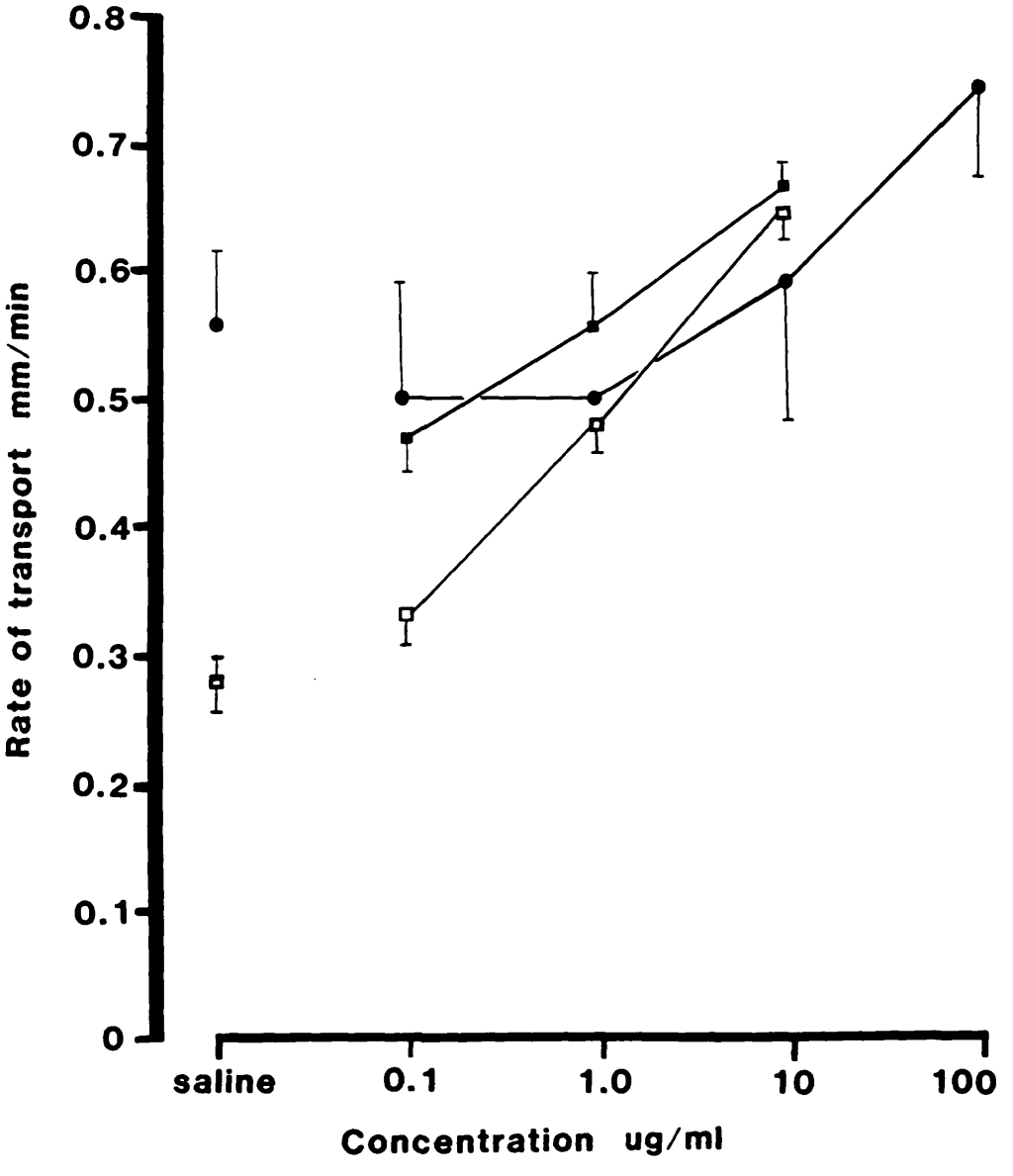


Figure 29. Comparison between ACh dose-response relationships on oesophageal mucociliary transport between in situ (■; n=4), chamber (□; n=4) and in vitro (●; n=4) methods.

hexamethonium, with the highest and lowest doses of nicotine ($P<0.05$ and $P<0.001$, respectively). In 2 experiments, nicotine (0.1-100 ug/ml) accelerated particle transport rate in the oesophagus of R. pipiens.

3.2.4.3) Atropine

Atropine (1 ug/ul) suppressed the enhancement of particle transport rate which accompanied vagal nerve stimulation, or topical application of ACh or nicotine. With respect to ACh, the inhibitory action was concentration dependent (Fig 31). However, atropine (1 ug/ml) did not alter basal particle transport rate to any noteworthy extent (saline control 0.31 ± 0.01; saline and atropine 0.28 ± 0.01; $n=21$; NS). Doses of atropine, up to 1 mg/ml did not suppress the elevated particle transport rate of the in vitro preparation.

3.2.4.4) Others

Other parasympathomimetic agents used in this preparation were pilocarpine, carbachol, butyrylcholine and propionylcholine. Table 2 lists the effects of these compounds.

3.2.5) Sympathetic nerve stimulation

The sympathetic paravertebral chain was stimulated with pulses (2 msec) of 1 to 5 volts and 1 to 50 Hz. Particle transport rate was not altered at any stimulus level (Fig 32). In frogs, this range of stimuli was without influence on the heart in situ, so that it cannot be presumed that the nerve was stimulated effectively.

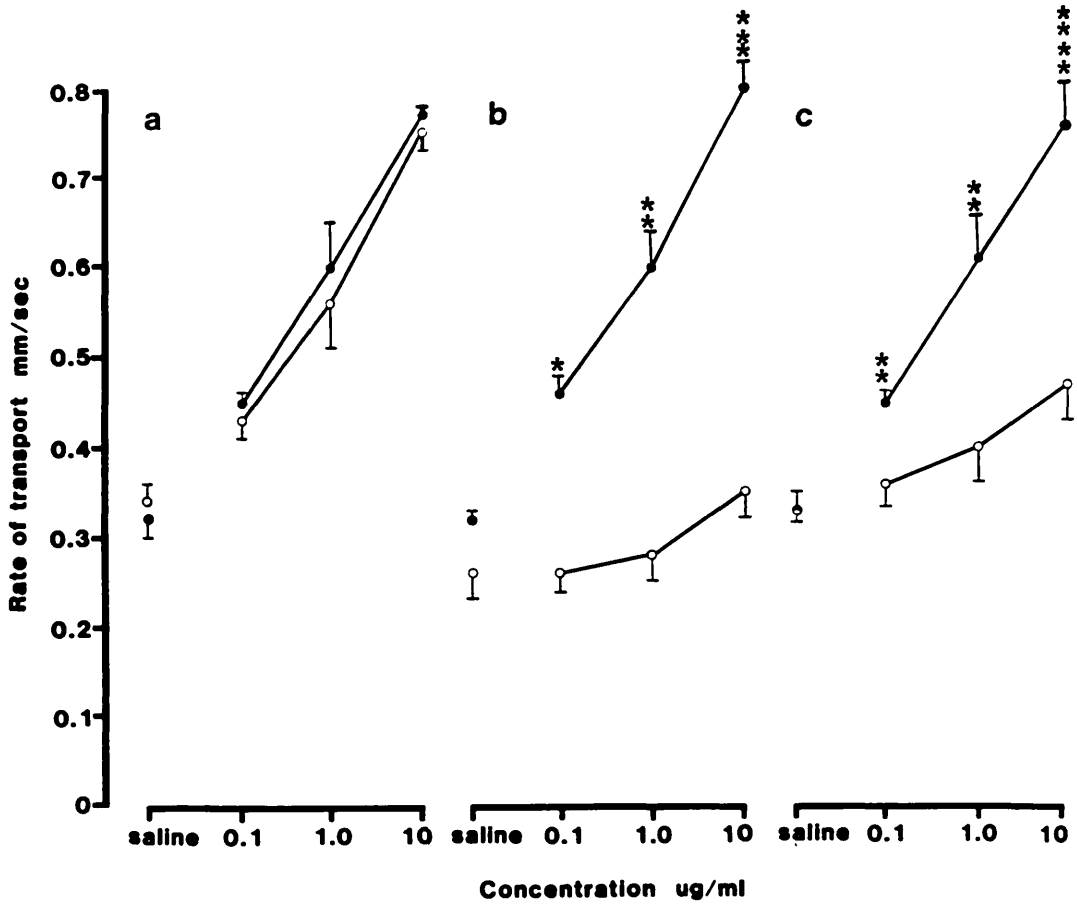


Figure 30. Effect of topical application of increasing concentrations of nicotine on frog oesophageal mucociliary transport. Effect of repeating nicotine dose-response relationships in the same preparation (treatment 1 ●, treatment 2 ○); n=3 (a). Response to nicotine before (●) and after (○) treatment with atropine (1 ug/ml); n=4 (b). Response to nicotine before (●) and after (○) treatment with hexamethonium bromide (1 ug/ml); n=7 (c). Results are expressed as mean ± s.e.m. Student's t-test for paired observations was used to assess statistical significance of observed differences.

* $P<0.025$, ** $P<0.01$, *** $P<0.005$, **** $P<0.001$

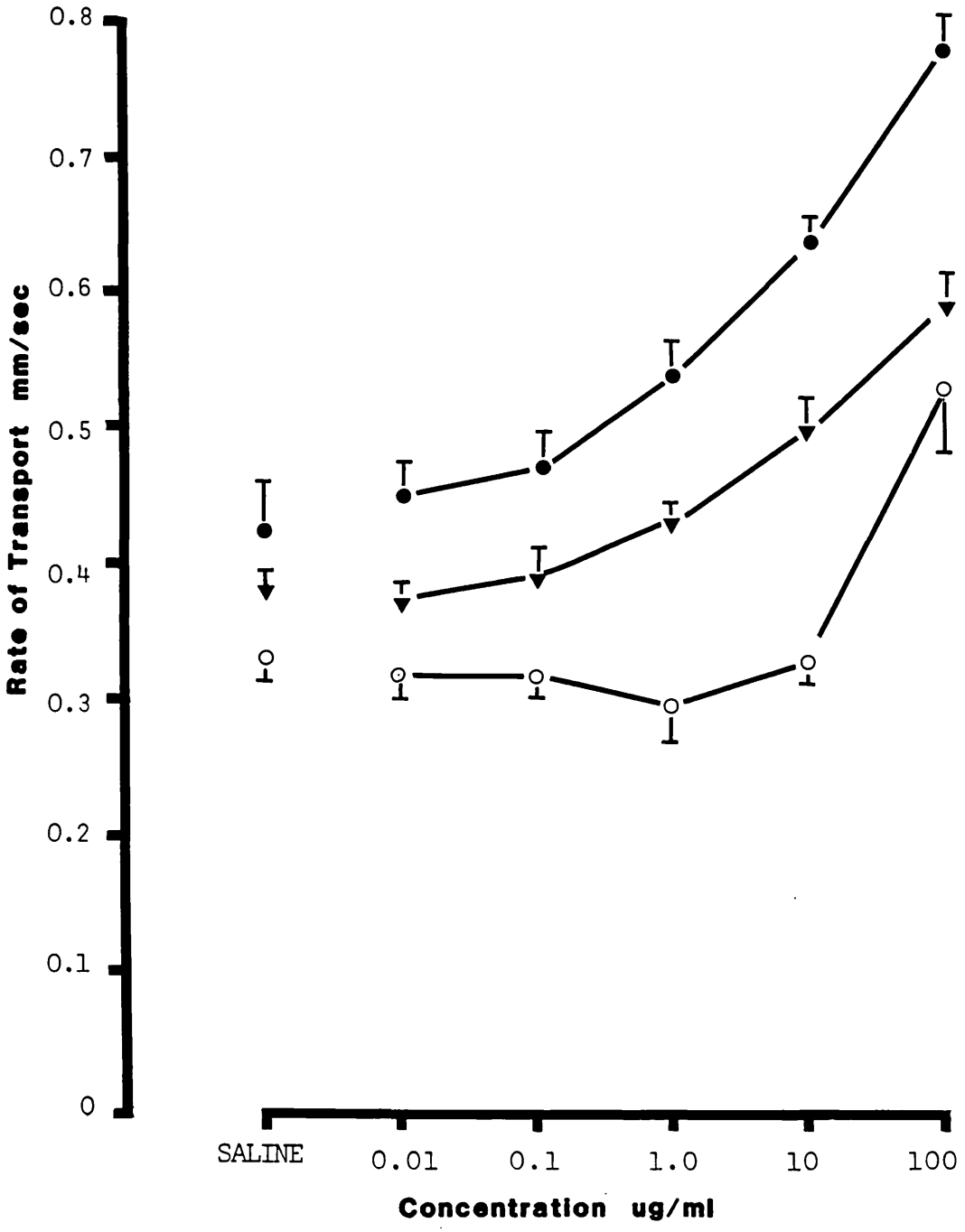


Figure 31. Effect of increasing concentrations of atropine sulphate (▼, 0.1 ug/ml; ○ , 1.0 ug/ml) on dose-response relationship to ACh (●) of oesophageal mucociliary transport.

Table 2. Effect of parasympathomimetic agents on frog oesophageal mucociliary transport.

AGENT	n	CONCENTRATION ug/ml			
		0*	0.1	1.0	10
ACETYLCHOLINE	22	0.33 ± 0.01	0.48 ± 0.01	0.60 ± 0.01	0.71 ± 0.02
BUTYRYLCHOLINE	3	0.26 ± 0.05	0.43 ± 0.09	0.53 ± 0.06	0.63 ± 0.09
PROPIONYLCHOLINE	2	0.28 ± 0.13	0.37 ± 0.12	0.45 ± 0.08	0.60 ± 0.09
MUSCARINE	2	0.26 ± 0.08	0.34 ± 0.10	0.40 ± 0.10	0.51 ± 0.01
PILOCARPINE	3	0.27 ± 0.01	0.45 ± 0.06	0.62 ± 0.06	0.79 ± 0.05
NICOTINE	14	0.33 ± 0.01	0.45 ± 0.01	0.61 ± 0.03	0.77 ± 0.02

Transport rates are expressed as mean ± s.e.m. in mm/sec; n= number of animals.

* Saline control.

3.2.6) Sympathomimetic agents

Sympathomimetic agents were essentially inactive in this preparation. Adrenaline, noradrenaline, isoprenaline and salbutamol, at doses between 0.1 and 100 ug/ml, failed to modify MCT (Fig 33; Table 3).

3.2.7) Methylxanthines

3.2.7.1) Theophylline and its analogues

Theophylline, proxyphylline and diprophylline were tested up to concentrations of 1 mg/ml. These drugs failed to alter basal particle transport rate in both R. temporaria and R. pipiens.

3.2.7.2) Iso-butylmethyl xanthine (IBMX)

Iso-butylmethyl xanthine increased MCT in a dose-related manner (1, 10 and 100 ug/ml). At doses of 10 and 100 ug/ml, responses were significantly higher than the basal value ($P<0.05$ and $P<0.005$ respectively) (Fig 34).

3.2.7.3) Caffeine

Caffeine (1 mg/ml) increased particle transport rate from a basal value of 0.26 ± 0.03 to 0.93 ± 0.01 ($P < 0.005$) in R. temporaria and 0.30 ± 0.02 to 0.86 ± 0.05 ($P < 0.001$), in R. pipiens. However, lower doses (1, 10 and 100 ug/ml did not significantly alter particle transport rate (Fig 34).

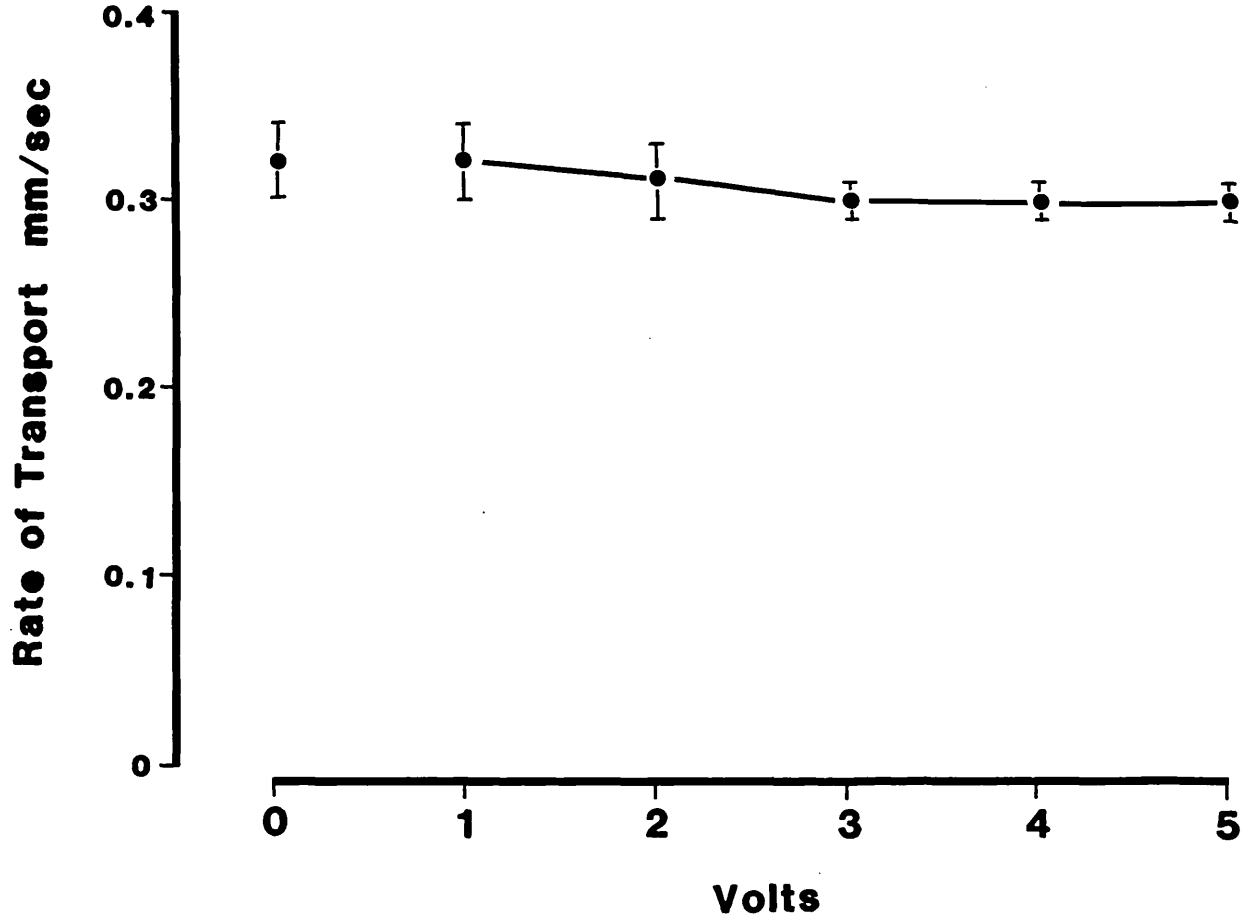


Figure 32. Effect of electrical stimulation of the paravertebral sympathetic chain (n=3) on oesophageal mucociliary transport. Frequency (50 Hz) and pulse width (2 msec) were constant.

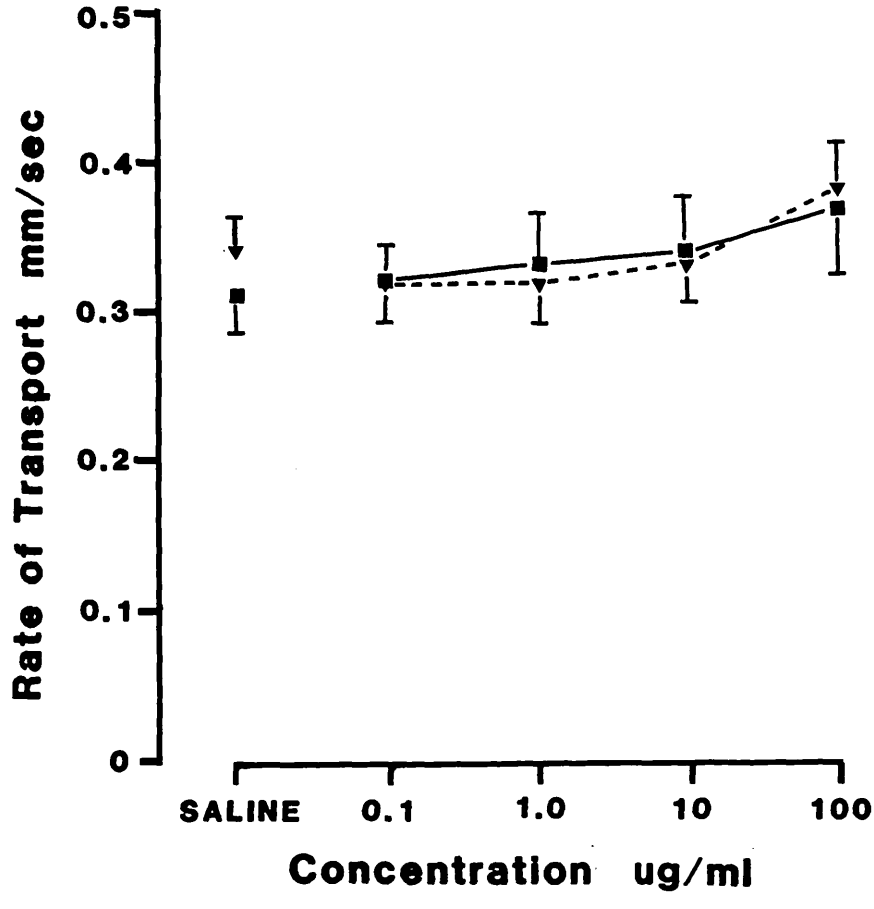


Figure 33. Effect of increasing concentrations of adrenaline (■; n=6) and isoprenaline (▼; n=6) on oesophageal mucociliary transport.

Table 3. Effect of sympathomimetic agents on frog oesophageal mucociliary transport.

AGENT	n	CONCENTRATION ug/ml				
		0*	0.1	1.0	10	100
ADRENALINE	6	0.34 ± 0.02	0.32 ± 0.02	0.32 ± 0.02	0.33 ± 0.02	0.38 ± 0.03
ISOPRENALINE	6	0.31 ± 0.02	0.32 ± 0.02	0.33 ± 0.03	0.34 ± 0.03	0.37 ± 0.04
NORADRENALINE	6	0.29 ± 0.06	0.31 ± 0.04	0.34 ± 0.07	0.34 ± 0.08	0.36 ± 0.06
SALBUTAMOL	6	0.32 ± 0.04	0.35 ± 0.03	0.35 ± 0.03	0.34 ± 0.06	0.24 ± 0.06

Transport rates are expressed as mean ± s.e.m. in mm/sec; n= number of animals.

* Saline control.

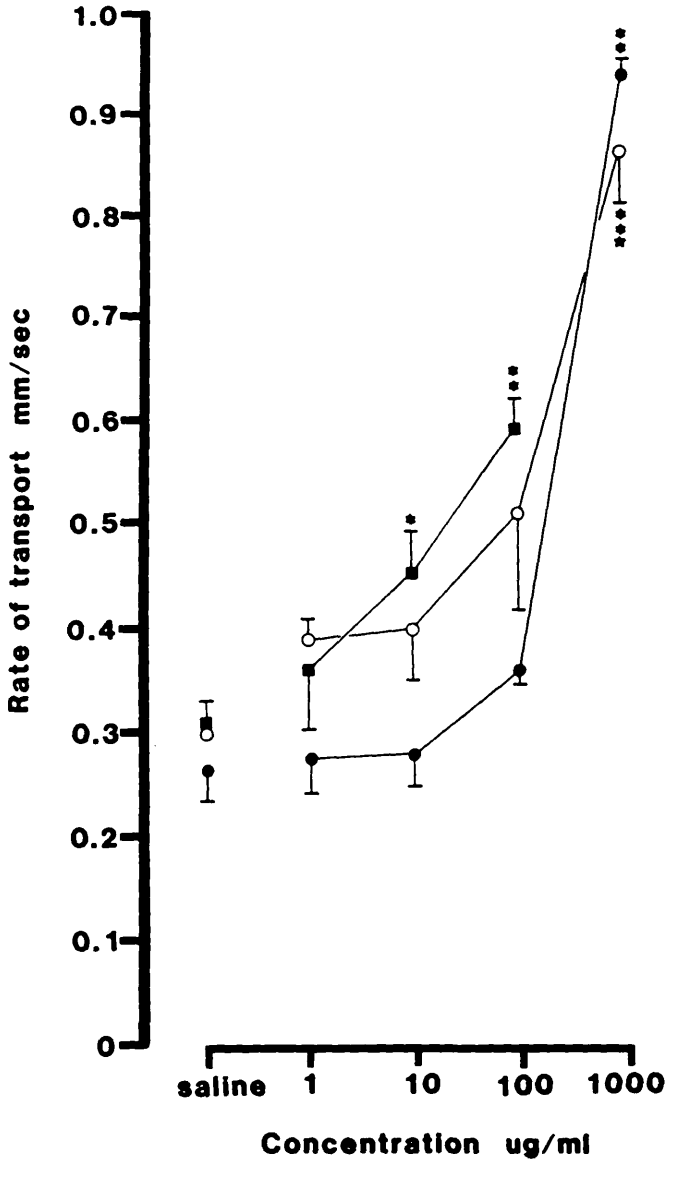


Figure 34. Effect of increasing concentrations of isobutylmethyl xanthine (■; n=3) and caffeine (○; n=3) in R. temporaria and caffeine (●; n=4) in R. pipiens.

* P<0.05, ** P<0.005, *** P<0.001

3.2.8) Mucolytics

Table 4 shows the effect of 7 mucolytic agents and one nasal decongestant, containing high concentrations of ATP (Rhin-ATP), on the frog oropharynx preparation. Whereas the cysteine derivatives, N-acetylcysteine (NAC), S-carboxymethylcysteine (SCMC) and Methylcysteine (MC) were ineffective, bromhexine and its analogue, ambroxol, stimulated MCT. Bromhexine was found to dissolve only at a low pH (4.0) and the low pH may have contributed to the stimulatory effect that was observed. Ambroxol was freely soluble, giving the drug solution a pH equal to that of FRS (7.3). The nasal decongestant was also a potent stimulant of MCT. To verify these observations, the effect of increasing concentrations (0.1, 0.5 and 1.0 mg/ml) of N-acetylcysteine, S-carboxymethylcysteine and ambroxol were tested in this preparation (Fig 35a). Ambroxol (0.1-1 mg/ml) increased particle transport rate in a dose-related manner; this stimulatory effect was not inhibited by atropine (1 ug/ml) (Fig 35b; Table 5a). Very low concentrations of NAC have been reported to increase ciliary beat frequency in the bronchi of the rat (Melville et al., 1980). NAC was tested at these low concentrations (0.1, 1.0 and 10 pg/ml) and found to be ineffective (Table 5b).

3.2.9) Histamine and 5-hydroxytryptamine (5-HT)

Histamine (n=3) and 5-HT (n=3) were tested in this preparation, using concentrations between 0.1 and 100 ug/ml. Neither agent altered particle transport rate (Table 6).

Table 4. Effect of topical application of mucolytic agents on frog oesophageal mucociliary transport.

Compound	Concentration mg/ml	Rate of transport mm/sec control	Rate of transport mm/sec treatment	% Change
N-acetyl cysteine	1	0.44	0.34	-23
	1	0.57	0.42	-23
Methyl cysteine	1	0.25	0.31	+24
Ethyl cysteine	1	0.43	0.43	0
	1	0.59	0.46	-22
S-carboxymethyl cysteine	1	0.36	0.56	+55
	1	0.64	0.33	-49
Rhin-ATP (Zyma SA)	10	0.32	0.88	+175
Bromhexine	1	0.36	0.64	+78
	2	0.30	0.68	+127
Ambroxol (Boehringer)	1	0.35	0.84	+140
	1	0.26	0.70	+169

Results represent the mean of 10 observations in single experiments and changes are expressed as a percentile of control value.

Table 5. Effect of NAC, SCMC and ambroxol on frog oesophageal mucociliary transport.

Drug	n	Drug Concentration (mg/ml)			
A		0*	0.1	0.5	1.0
N-acetyl-cysteine	4	0.31 ± 0.03	0.24 ± 0.03	0.27 ± 0.02	0.25 ± 0.02
S-carboxymethyl cysteine	4	0.40 ± 0.02	0.32 ± 0.04	0.34 ± 0.02	0.36 ± 0.02
Ambroxol	10	0.28 ± 0.02	0.30 ± 0.02	0.57 ± 0.04	0.80 ± 0.04
Ambroxol + Atropine (1 ug/ml)	5	0.28 ± 0.01	0.28 ± 0.02	0.52 ± 0.04	0.75 ± 0.05
B		Control	10	10	10
N-acetylcysteine	4	0.32 ± 0.06	0.30 ± 0.06	0.32 ± 0.06	0.34 ± 0.06

Transport rates are expressed as mean ± s.e.m. in mm/sec; n= number of animals.

* Saline control.

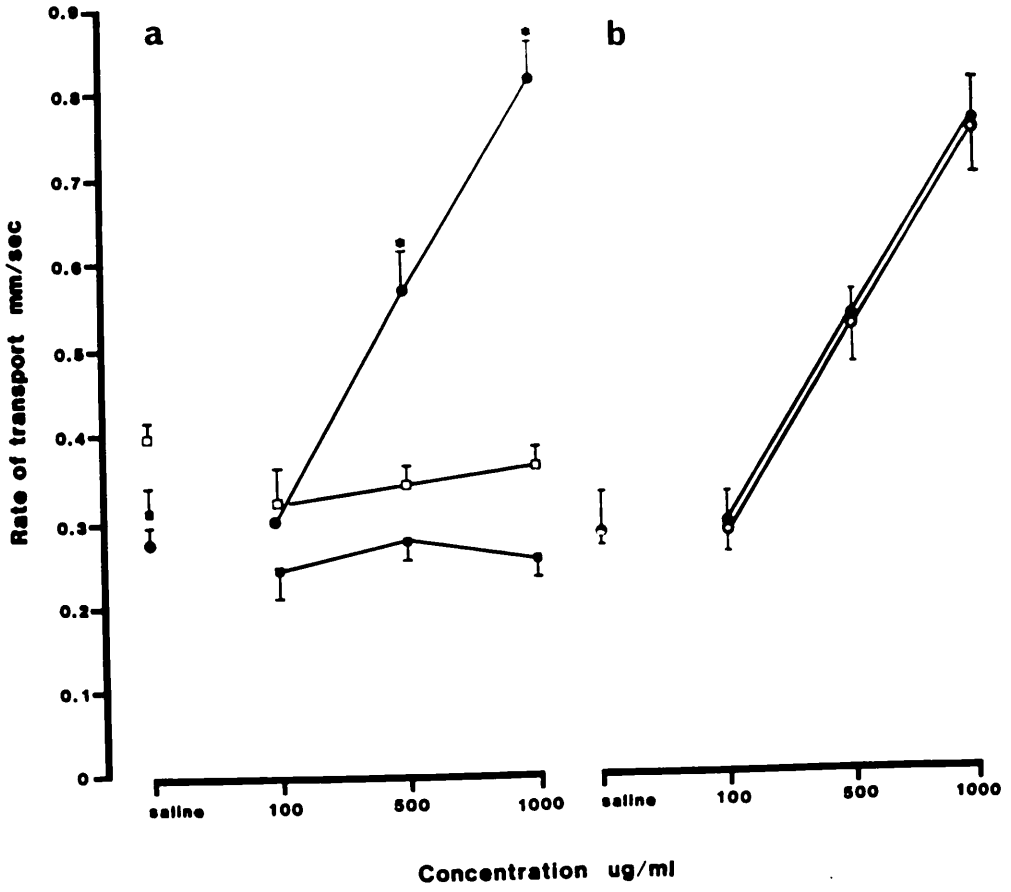


Figure 35. Effect of increasing concentration of ambroxol (●), N-acetyl cysteine (■) and S-carboxymethyl cysteine (□) on frog oesophageal mucociliary transport (a). Effect of increasing concentrations of ambroxol on frog oesophageal mucociliary transport before (●) and after (O) treatment with atropine (1 ug/ml), n=5 (b). Student´s t-test for paired observations was used to assess statistical significance of observed differences.

* P<0.001

3.2.10) Prostaglandins E1 and F2-alpha

Prostaglandin E1 (n=3) and prostaglandin F2-alpha (n=3) were applied to the oropharynx at 0.1, 1.0 and 2.0 ug/ml. There was a significant increase of MCT with all concentrations (Fig 36; Table 6); however, the acceleration resulting from application of these prostaglandins was small, in comparison with the effect of cholinergic stimuli.

3.2.11) Purines

Adenosine, guanosine and uridine tri-phosphates are potent accelerators of particle transport rate. These compounds were tested at concentrations ranging between 0.1 and 100 ug/ml. Diphosphate purines were inactive over the same dose-range, with the exception of uridine diphosphate (UDP). UDP was as potent a stimulant of MCT as the triphosphate purines. Figure 37 shows a comparison between these diphosphate purines. Mono-phosphate purines were inactive at all concentrations. Table 7 summarises the effects of these compounds.

3.2.14) Substance P

Substance P was tested over a wide range of concentrations. In one group (n=3), concentrations of 0.01-10 ng/ml were used, and in a second group (n=3) concentrations were increased one thousand fold (0.01-10 ug/ml). In both groups, mucociliary transport was elevated with all concentrations of substance P (Table 8). However, there was some evidence of tachyphylaxis, since, over the first minute, particle transport rate became visibly slower. In some animals, excessive mucus was produced.

Table 6. Effects of histamine, serotonin (5-HT), prostaglandins E2 and F2 alpha on frog oesophageal mucociliary transport.

AGENT	n	CONCENTRATION ug/ml 0*	0.1	1.0	10	100
HISTAMINE	3	0.33 ± 0.02	0.32 ± 0.03	0.32 ± 0.02	0.33 ± 0.02	0.34 ± 0.02
5-HT	3	0.32 ± 0.04	0.42 ± 0.05	0.36 ± 0.05	0.36 ± 0.05	0.38 ± 0.07
PGE 2	3	0.34 ± 0.01	0.44 ± 0.01	0.43 ± 0.01	0.43 ± 0.01#	
PGF 2 alpha	3	0.35 ± 0.02	0.41 ± 0.01	0.45 ± 0.01	0.46 ± 0.02#	

Results are expressed as mean ± s.e.m. in mm/sec; n= number of animals.

* saline control.

2 ug/ml.

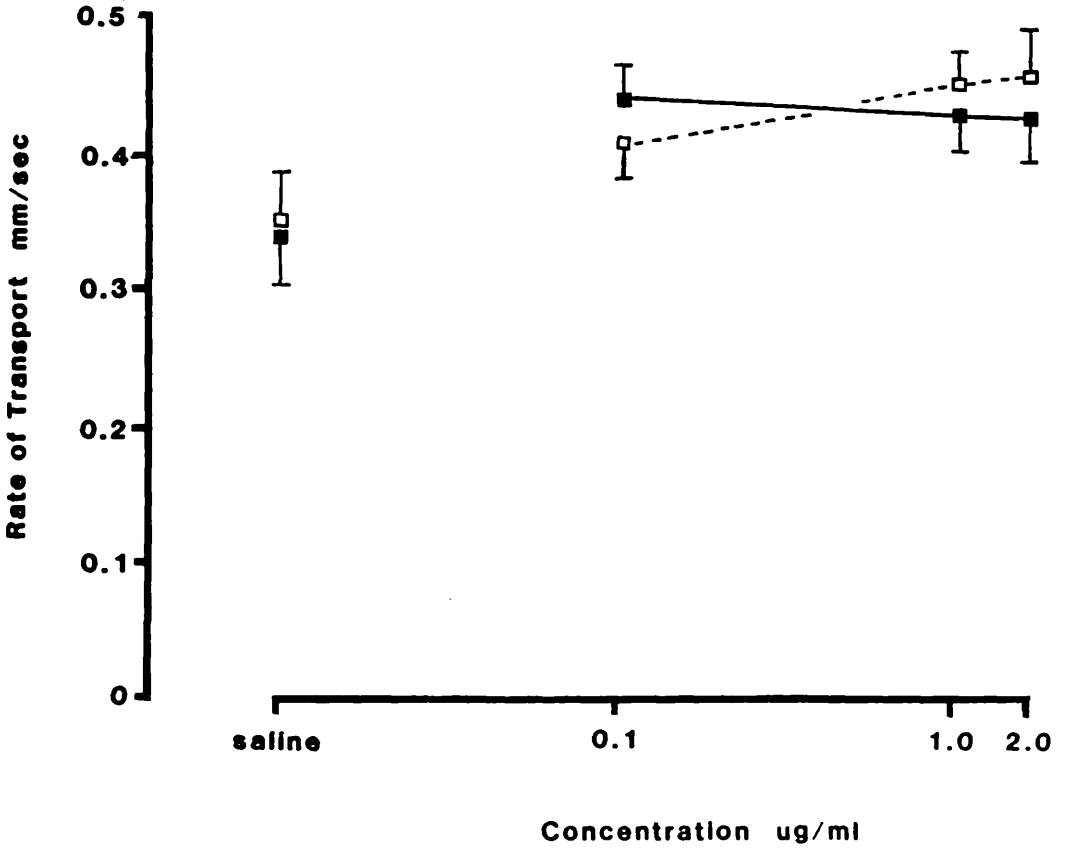


Figure 36. Effect of increasing concentrations of prostaglandins E2 (■; n=3) and F2 alpha (□; n=3) on oesophageal mucociliary transport.

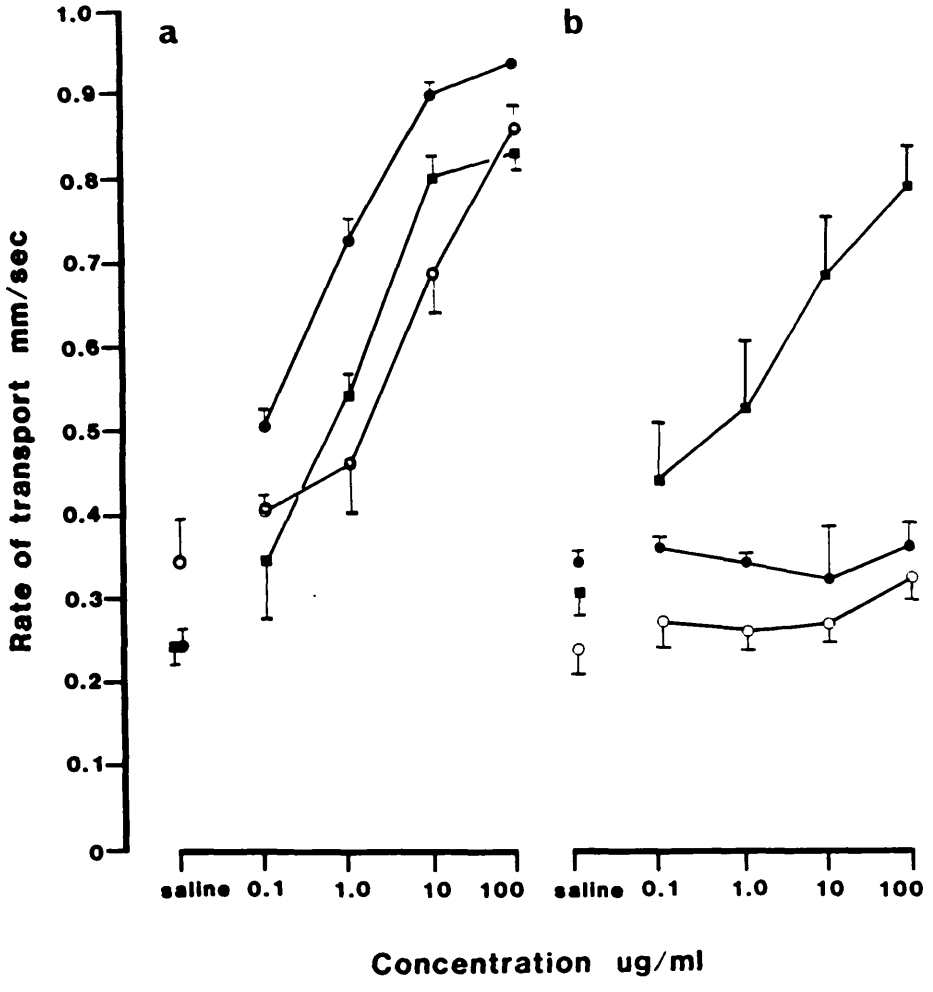


Figure 37. Effects of increasing concentrations of ATP (●; n=6), GTP (○; n=2) and UTP (■; n=2) (a), and ADP (●; n=2) GDP (○; n=2) and UDP (□; (n=4) (b) on oesophageal mucociliary transport.

Table 7. Effect of tri- and di-phosphate purines on frog oeseophageal mucociliary transport.

AGENT	n	CONCENTRATION ug/ml 0*	0.1	1.0	10	100
ATP	3	0.33 ± 0.03	0.48 ± 0.11	0.61 ± 0.16	0.77 ± 0.01	0.94
ATP (chamber)	3	0.24 ± 0.01	0.46 ± 0.10	0.61 ± 0.18	0.87 ± 0.03	-
GTP	2	0.35 ± 0.06	0.41 ± 0.01	0.46 ± 0.06	0.69 ± 0.04	0.86 ± 0.02
UTP	2	0.25 ± 0.03	0.35 ± 0.07	0.54 ± 0.03	0.80 ± 0.03	0.83 ± 0.01
ADP	2	0.35 ± 0.01	0.36 ± 0.01	0.34 ± 0.01	0.32 ± 0.08	0.36 ± 0.04
GDP	2	0.24 ± 0.03	0.27 ± 0.03	0.26 ± 0.01	0.27 ± 0.01	0.33 ± 0.03
UDP	4	0.31 ± 0.03	0.44 ± 0.07	0.53 ± 0.08	0.69 ± 0.07	0.79 ± 0.05

Results are expressed as mean ± s.e.m., in mm/sec; n= number of animals.

* saline control

All experiment carried out on the in situ preparation unless otherwise stated.

Table 8. Effect of Substance P on frog oesophageal mucociliary transport

	CONCENTRATION g/ml							
	0*	10^{-11}	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}
Gp 1	0.31 ± 0.01	0.38 ± 0	0.40 ± 0.01	0.40 ± 0	0.39 ± 0.02	-	-	-
Gp 2	0.26 ± 0	-	-	-	0.49 ± 0.12	0.48 ± 0.09	0.54 ± 0.05	0.54 ± 0.04

Results are expressed as mean ± s.e.m. in mm/sec; n=3 for Gps 1 and 2.

* saline control.

3.3) Frog oesophageal mucus

3.3.1) Comparison of protein secretion and mucociliary transport

In a preliminary study, an excised frog oesophagus was placed in the chamber and covered with FRS. After 30 minutes, particle transport rate was measured and the fluid withdrawn for analysis of protein content. FRS was introduced into the chamber and, after 5 minutes, contents of the chamber were aspirated; then increasing concentrations of ACh, ATP or pilocarpine were introduced into the chamber at 5 min intervals with a 5 minute control period between each dose-response study. MCT was measured within the first 2 min after application of each solution. Protein content of each sample declined steadily throughout the experiment, yet increases in particle transport rate due to drug application were observed. In the next experiment, FRS was introduced into the chamber immediately after dissection and removed every 5 minutes for 1 hr, after which increasing concentrations of pilocarpine were applied (0.01-100 ug/ml). Particle transport rate was measured after each addition. Protein content and MCT declined with time, reaching a steady basal level 30-40 min after dissection. However, on application of increasing concentrations of pilocarpine (0.01-10 ug/ml), protein content of samples did not increase, whereas MCT was elevated in a dose-related fashion. Since the contact time for drug solutions may have been insufficient to produce detectable amounts of protein, this experiment was repeated in another animal using a 15 min collection period. Results were similar to those obtained with 5 min collection periods. In a group of 3 animals, a collection period of 10 min was used. Four control samples (0-40 min) were collected after dissection, after which ACh (0.1-10 ug/ml) was introduced into

the chamber. Once again, protein content of samples remained unaltered, whilst MCT was elevated in response to ACh (Fig 38).

3.3.2) Sugar content of oesophageal mucus

Blood-free mucus was collected from 6 frogs over a period of 15 minutes. Sugar constituents were barely detectable, despite the presence of 6 to 14 mg of freeze-dried material from each frog.

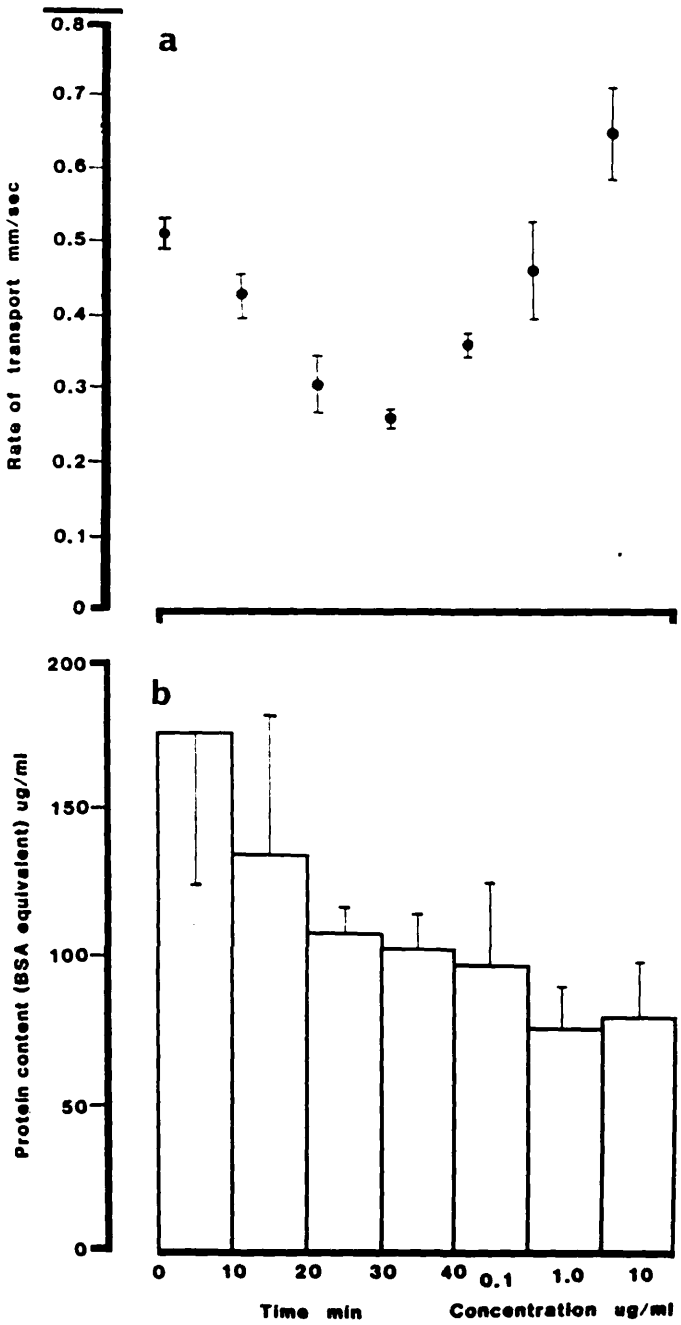


Figure 38. Decline of oesophageal mucociliary transport (a), and protein secretion (b), with time together with the effect of ACh on both parameters (n=3).

3.4) Measurement of mucociliary transport in guinea-pigs

3.4.1) Monitoring of the stomach

Stomach monitoring was performed in 6 animals. Only in one animal, was a steady stepwise increase in radioactive counts detected, over a long period (2 hr). More usually, the radioactivity was found to reside in the pharyngeal area, indicating that the animal had not swallowed. It was concluded that this method was not suitable for measurement of MCT.

3.4.2) Monitoring of the oropharynx

This method was more successful than monitoring of the stomach, in that MCT was manifest as a continuous rise in radioactivity in the oropharynx. Once the radioactive content of the lungs was removed, the count rate in the oropharynx remained stable, at a higher level, unless the animal swallowed. Once a stable count rate was observed, a second bolus of radioactive material was introduced into the airways and a second rise in counts was observed. This method permitted assessment of MCT by comparing the slopes obtained with successive injections of labelled erythrocytes. However, no drugs were tested using this method, since measurement of TMV using beta-particle detectors superseded all other methods in both convenience and reproducibility.

3.4.3) Measurement of tracheal mucus velocity

In three guinea-pigs, two detectors were used for estimation of TMV (see section 2.2.4.3). TMV was estimated as 5.8 mm/min in these animals. Measurement of TMV using a single detector proved to be less

cumbersome and was used for all subsequent measurements of TMV in guinea-pigs, ferrets and rabbits.

3.4.3.1) Basal TMV

Basal TMV was measured in 115 guinea-pigs; the distribution of these values approximated to normal (Fig 39). The arithmetic mean was 4.7 $\pm$ 1.4 (s.d.) with a median of 4.6 and a range of 2.4 to 7.8 mm/min. The coefficient of variation for three determinations, prior to any treatment in this group, was 12.4 $\pm$ 8.7% with a range between 0 and 46%.

When labelled ferret erythrocytes were used, mean basal TMV in 11 guinea-pigs was 9.4 $\pm$ 2.2 (s.d.) mm/min. TMV, as measured by labelled ferret erythrocytes, was significantly higher ($P<0.001$) than TMV measured using guinea-pig labelled erythrocytes (n=24). This difference was thought to be due to rapid haemolysis of ferret erythrocytes (evident as red colouration of the supernatant) and release of intracellular material.

3.4.3.2) Vagal stimulation

In eight animals, the vagus nerve was stimulated by 2 msec pulses of 1 volt at 20 Hz for 1 min. TMV was significantly greater 2, 15 and 30 mins after nerve stimulation (Fig 40a); 15 mins after nerve stimulation, TMV was fastest (7.7 $\pm$ 2.6 mm/min; $P<0.005$). Pretreatment with atropine (0.5 mg/kg) inhibited the stimulatory effect of vagal stimulation, so that TMV after nerve stimulation (4.2 $\pm$ 1.5), did not differ significantly from basal TMV (4.7 $\pm$ 1.8 mm/min; n=3)

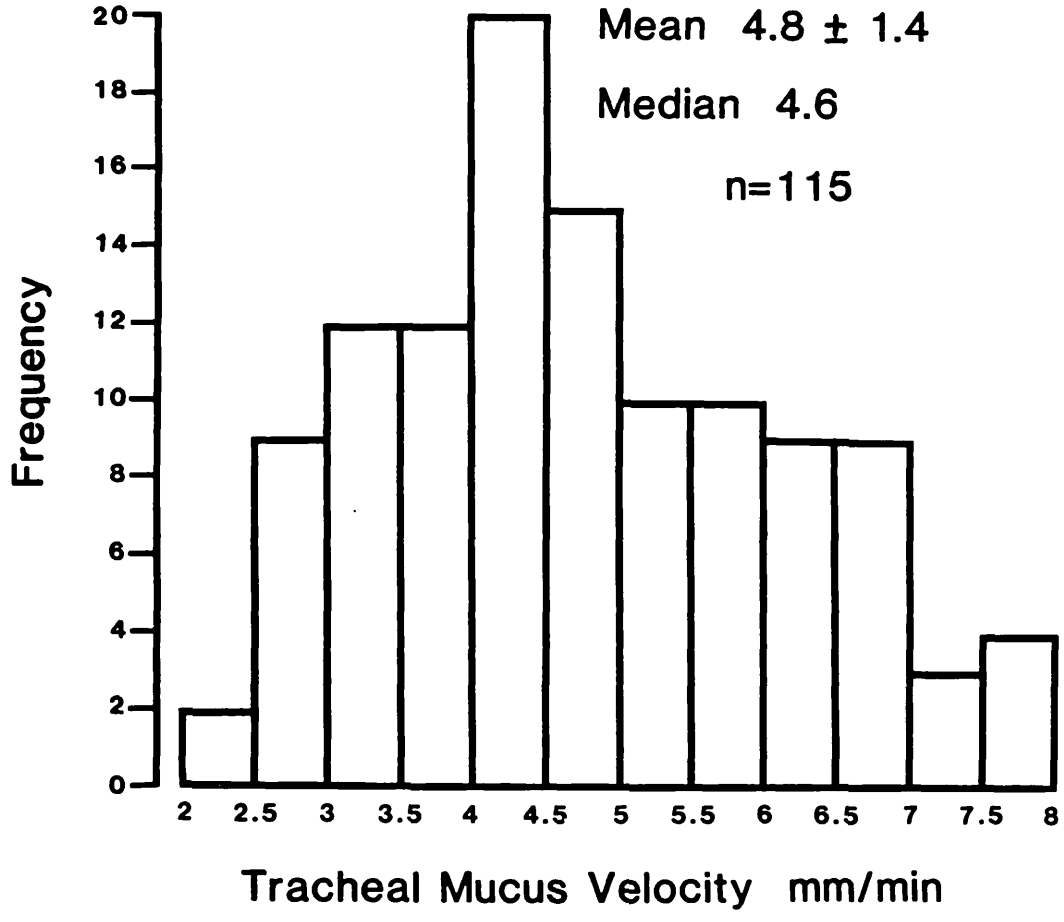


Figure 39. Distribution of basal tracheal mucus velocity (TMV) from 115 guinea-pigs approximates to normal with a mean of 4.8 ± 1.4 (s.d.) and a median of 4.6 mm/min.

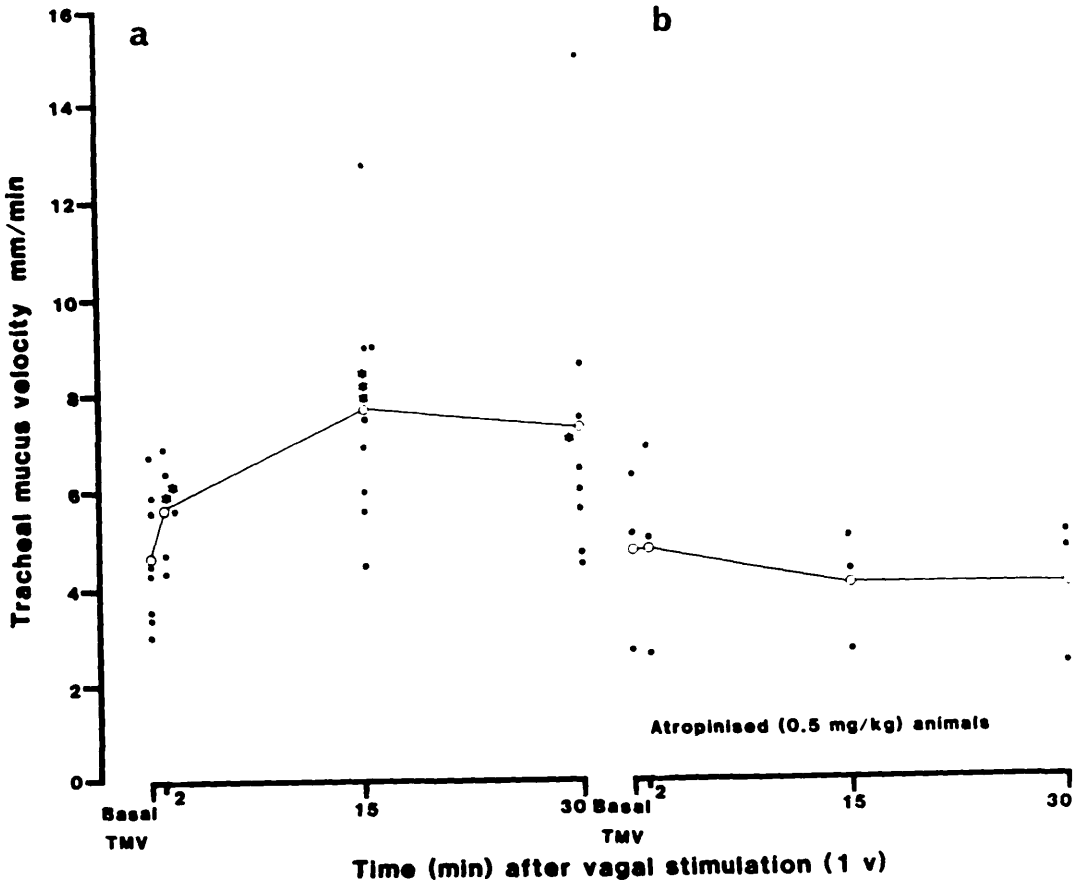


Figure 40. Effect of vagal nerve stimulation (1 v, 20 Hz, 2 msec for 1 min) on guinea-pig TMV. TMV is elevated significantly at 2, 15 & 30 minutes after stimulation (n=12) (a). In atropinised animals, no stimulation was evident (n=3) (b). Student's t-test for paired observations was used to assess statistical significance of observed differences.

* $P<0.05$, ** $P<0.01$, *** $P<0.005$.

Table 9. Effect of vagal nerve stimulation on tracheal mucus velocity in guinea-pigs.

Voltage*	n	Tracheal mucus velocity		P
		Basal	Post-stimulation	
1.0	8	4.6 ± 1.3	6.9 ± 2.3	<0.005
1.0 + ATROPINE (0.5 mg/kg)	3	4.7 ± 1.8	4.2 ± 1.5	-
2.0	2	6.0 ± 0.6	3.9 ± 1.1	-

Post-stimulation values are the mean of determinations, 2, 15 and 30 min after nerve stimulation.

Student's t-test for paired observations was used to assess statistical significance of observed differences.

Results are expressed as mean ± s.d. in mm/min; n= number of animals.

* The nerve was stimulated for 1 min at a constant frequency (20 Hz) and pulse width (2 msec).

(Fig 40b). When the vagus was stimulated by 2 msec pulses of 2 volts at 20 Hz for 1 min, basal TMV was reduced from 6.0 ± 1.0 to 3.9 ± 1.1 mm/min (n=2). At these voltage levels, no hypotension, bradycardia, bronchoconstriction or hyperventilation were observed on nerve stimulation. Table 9 summarises the effects of vagal stimulation.

3.4.3.3) Parasympathomimetic agents

Pilocarpine (100 ug/kg) was injected intravenously into 12 animals and TMV was measured 2 (in 7 of 12 animals), 15 and 30 minutes later. TMV determination 2 minutes after pilocarpine (5.7 ± 2.6 mm/min) was not increased when compared to pretreatment TMV (5.4 ± 2.0). However, in all animals, determinations of TMV 15 and 30 minutes after pilocarpine were significantly higher than basal TMV (basal TMV, 5.3 ± 1.6; after 15 min, 9.3 ± 3.1, $P < 0.001$; after 30 min, 9.2 ± 3.1 mm/min $P<0.001$) (Fig 41a). This dose of pilocarpine caused transient hypotension and bradycardia in all animals; copious salivation was frequently evident. In 5 animals pretreated with atropine (50 ug/kg i.v.), TMV was 5.5 ± 2.0 mm/min. In these animals, determinations of TMV at any time after intravenous pilocarpine (100 ug/kg) was not altered significantly when compared with basal TMV (basal TMV, 5.5 ± 2.0; post-treatment TMV, 4.7 ± 0.5; n=5; NS) (Fig 41b). This concentration of atropine was sufficient to prevent hypotension and bradycardia. Topical, extra-tracheal application of 100 ul of ACh (100 ug/ml) increased TMV significantly ($P<0.01$) in 6 animals (TMV 4.2 ± 1.1, post-treatment TMV 5.2 ± 1.1 mm/min) (Fig 42a). Topical application of 100 ul of PBS was without effect (pre-treatment TMV 4.3 ± 0.7, post-treatment TMV 4.5 ± 0.9 mm/min; n=5) (Fig 42b). In 10 animals, the effect of intravenous atropine (50 ug/kg) on basal TMV was studied. Basal TMV (5.6 ± 1.8)

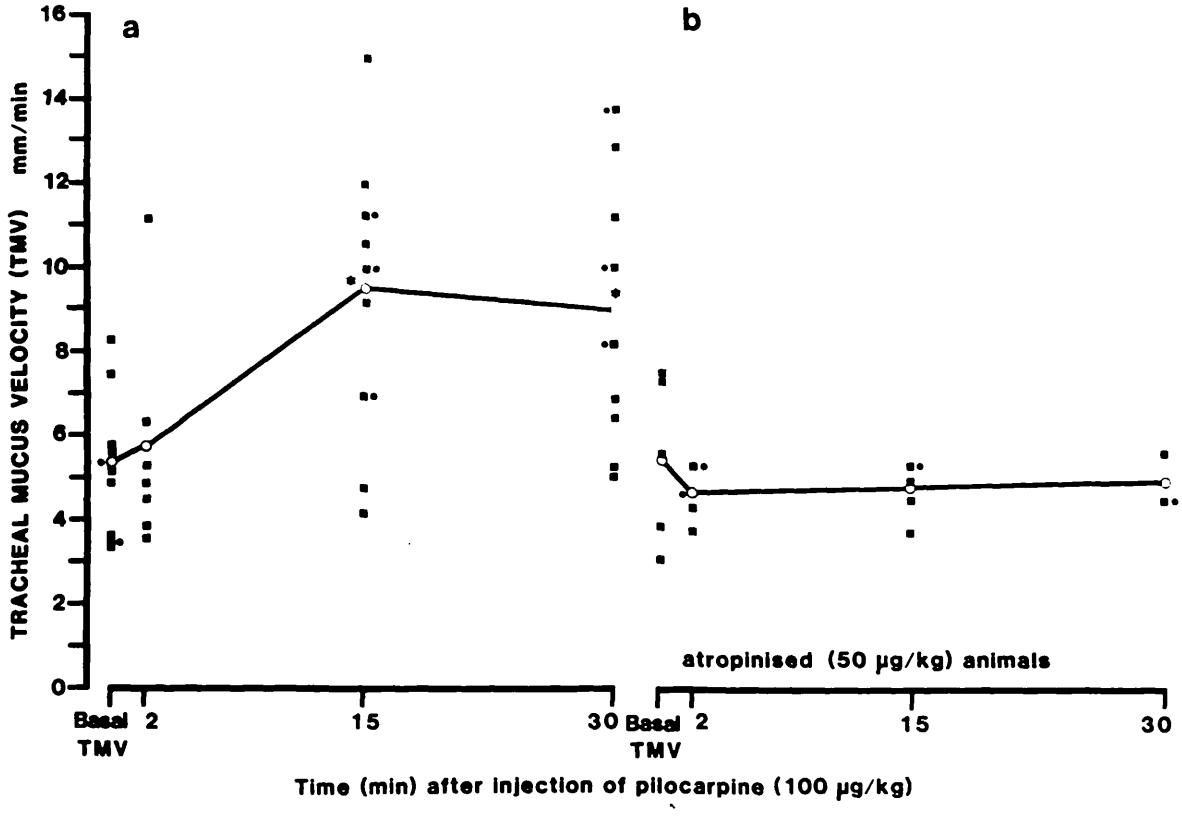


Figure 41. Effect of intravenous injection of pilocarpine (100 ug/kg; n=12) on guinea-pig TMV. TMV is unaffected 2 min post-injection, however, there was significant elevation of TMV at 15 & 30 min (a). In atropinised animals, no elevation of TMV was observed after injection of pilocarpine (b). Student's t-test for paired observations was used to assess statistical significance of observed differences.

• Indicate replication * $P<0.005$

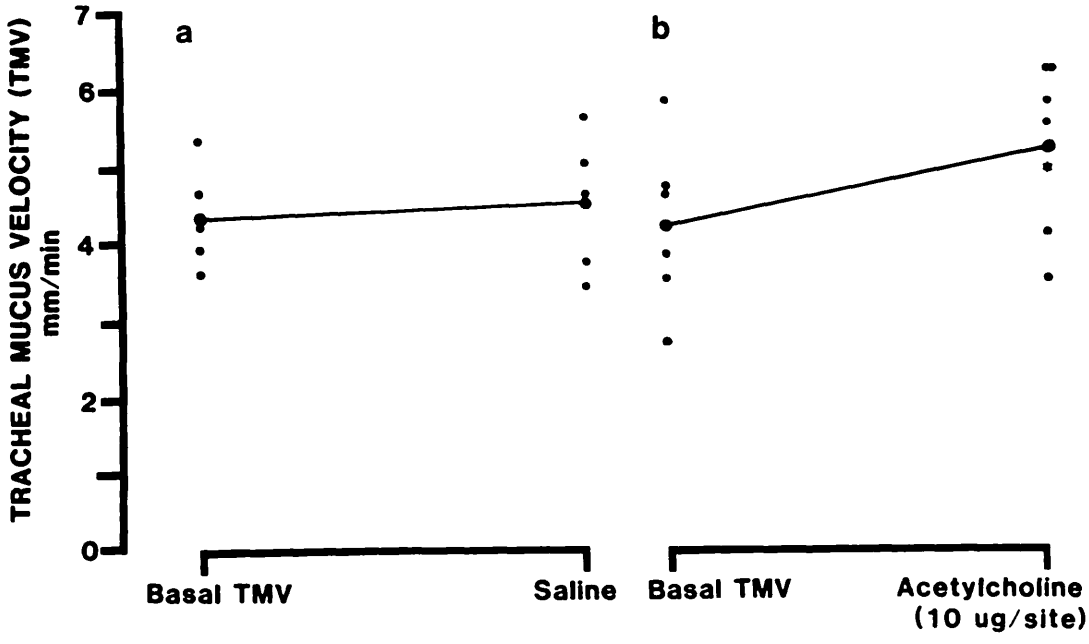


Figure 42. Effect of topical application of saline (n=5) (a) or ACh (10 ug/site; n=6 (b), to the external surface of exposed trachea of guinea-pigs. Student's t-test for paired observations was used to assess statistical significance of observed differences.

* P<0.01

was not significantly different when compared with post-treatment TMV (5.8 ± 1.8). The effects of administration of parasympathomimetic agents have been summarised in Table 10.

3.4.3.4) Sympathomimetic agents

Intravenous injection of isoprenaline (1 ug/kg) did not significantly alter basal TMV in 8 animals (pre-treatment TMV 4.5 ± 1.8; post-treatment TMV 3.6 ± 1.4 mm/min) (Fig 43a). Similarly, propranolol (1 mg/kg i.v.) was without effect (pre-treatment TMV, 5.2 ± 1.1; post-treatment TMV, 4.8 ± 1.3 mm/min; n=5) (Fig 43b). Both drugs produced substantial cardiovascular effects with tachycardia and hypertension following i.v. injection of isoprenaline and bradycardia and hypotension following i.v. injection of propranolol. Higher doses of isoprenaline (2 and 5 ug/kg) were used but pronounced systemic effects precluded measurement of TMV.

Intravenous injection of phenylephrine (10 ug/kg) stimulated TMV significantly ($P<0.05$) from 3.4 ± 1.0 to 5.9 ± 2.3 mm/min (n=6) (Fig 44a). This concentration of phenylephrine produced hypertension without affecting heart rate. Basal TMV in 4 animals treated with corynanthine (10 ug/kg) was 5.8 ± 1.0 mm/min. Intravenous injection of phenylephrine in these animals did not alter TMV significantly (post-treatment TMV, 5.0 ± 1.4 mm/min) (Fig 44b). Table 11 summarises the effects of sympathomimetic agents on TMV.

3.4.3.5) Histamine

Histamine (5 ug/kg) was injected intravenously in 5 animals. No

Table 10. Effects of parasympathomimetic agents on tracheal mucus velocity in guinea-pigs.

Agent	Dose (ug/Kg)	n	Tracheal mucus velocity Basal	Post-treatment	P
SALINE (topical)	-	5	4.3 ± 0.7	4.5 ± 0.9	NS
ACh (topical)	10*	6	4.2 ± 1.1	5.2 ± 1.1	<0.01
PILOCARPINE	100	12	5.2 ± 1.6	9.2 ± 3.0	<0.001
PILOCARPINE + ATROPINE	100 50	5	5.5 ± 2.0	4.7 ± 0.5	NS
ATROPINE	50	10	5.6 ± 1.8	5.8 ± 1.8	NS

Drugs were administered intravenously unless specified.

Post-treatment values are the mean of determinations 2, 15 & 30 min after drug administration.

Student's t-test for paired observations was used to assess statistical significance of observed differences.

Results are expressed as mean ± s.d. in mm/min; n= number of animals.
* 10 ug/site in 100 ul.

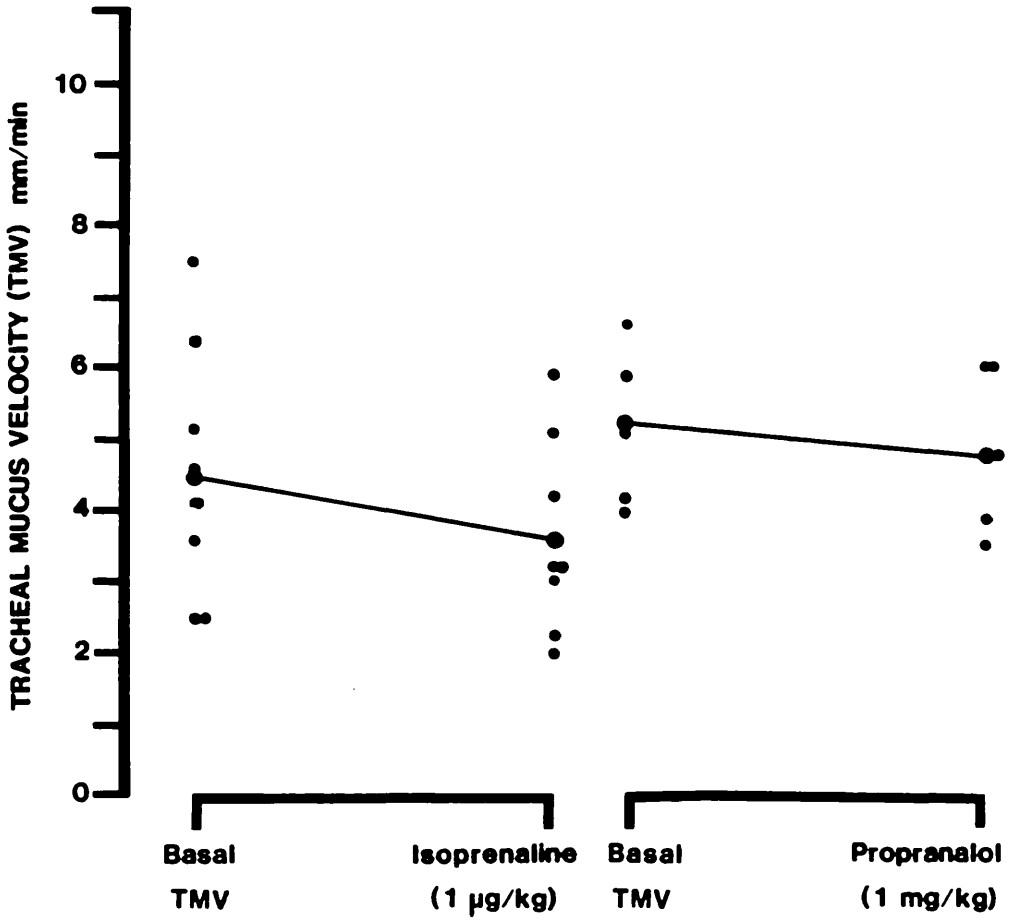


Figure 43. Effects of isoprenaline (1 ug/kg; n=8) and propranolol (1 mg/kg; n=5) on guinea-pig TMV.

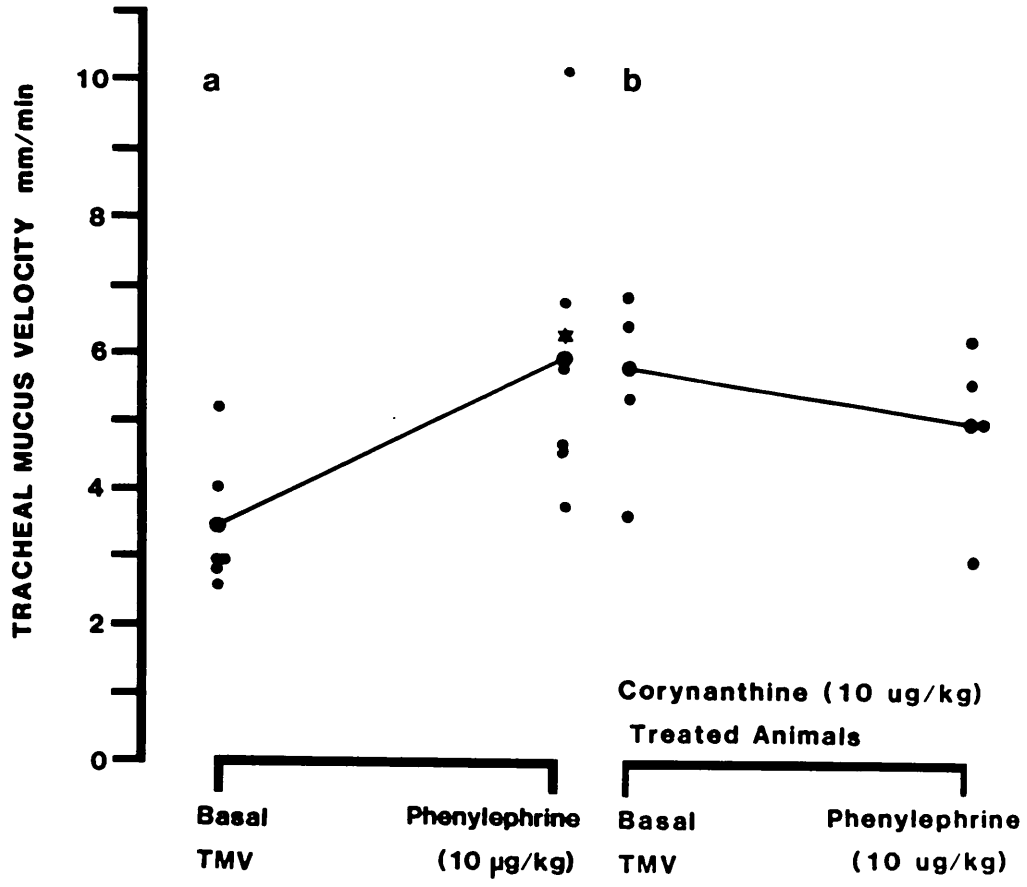


Figure 44. Intravenous injection of phenylephrine (10 ug/kg; n=6) increases TMV in guinea-pigs significantly (a). This effect is not observed in corynanthine (10 ug/kg; n=4) treated animals (b). Post-treatment values represent the mean of 2, 15 & 30 minute determinations. Student's t-test for paired observations was used to assess statistical significance of observed differences.

* $P<0.05$

Table 11. Effects of sympathomimetic agents on tracheal mucus velocity in guinea-pigs.

Agent	Dose (ug/Kg)	n	Tracheal mucus velocity Basal	Post-treatment	P
ISOPRENALINE	1	8	4.5 ± 1.8	3.6 ± 1.4	NS
PROPRANOLOL	1000	5	5.2 ± 1.1	4.8 ± 1.2	NS
PHENYLEPHRINE	10	7	3.4 ± 1.0	5.9 ± 2.3	<0.05
PHENYLEPHRINE + CORYNANTHINE	10 10	4	5.8 ± 1.0	5.0 ± 1.4	NS
CORYNANTHINE	10	2	5.9 ± 0	5.8 ± 0.8	NS

Drugs were administered intravenously unless specified.

Post-treatment values are the mean of determinations, 2, 15 and 30 min after drug administration.

Student's t-test for paired observations was used to assess statistical significance of observed differences.

Results are expressed as mean ± s.d. in mm/min; n= number of animals.

consistent pattern could be observed for post-treatment TMV determinations, within or between animals. Panting of varying intensity resulted from the histamine treatment and this appeared to influence TMV estimates. Mean basal TMV, prior to treatment, was 5.2 $\pm$ 0.5 compared with the mean post-treatment value of 4.9 $\pm$ 1.2 mm/min.

3.4.3.6) Substance P

Substance P (1 ug/kg) was injected intravenously in 6 animals. The results were inconclusive, since, in some animals, there was acceleration of TMV followed by retardation and, in some, an opposite effect was observed. All animals showed a hypotensive response. Higher concentrations of substance P produced bronchoconstriction and, hence, were not used for TMV estimation.

3.4.3.7) Ambroxol

The effect of a mucolytic agent, ambroxol, was tested on guinea-pig TMV. Two approaches were used: in the first series of experiments, the effect of intravenous administration (10 and 20 mg/kg) were studied; in the second, three groups of 12 animals were treated orally with either placebo, 2 mg/kg or 20 mg/kg of ambroxol, for five days. After this period, animals were anaesthetised and four determinations were made. The mean TMV for each group was calculated and compared with other groups using Student's t-test for groups of unequal size (as some animals in each group died). In the first group, animals were anaesthetised and TMV measured every 10 mins until a steady basal level was observed (coefficient of variation <15%). Ambroxol was injected (10 or 20 mg/kg) and TMV measured at 2, 10, 20 and 30 min

post-injection. Ambroxol at 20 mg/kg elevated TMV in 11 of 13 animals tested. Basal TMV in this group was 5.1 ± 1.4 (s.d.) mm/min, which was increased to 6.6 ± 2.3 (s.d.) mm/min ($P<0.005$). In this series, post-treatment values represent the mean of observations made at 2, 10 and 20 min after administration of ambroxol (Fig 45a). After a dose of 10 mg/kg, ambroxol did not noticeably enhance TMV in five animals. When the mean of post-treatment determinations at 10, 20 and 30 min. after injection (4.4 ± 0.31 mm/min) was compared with basal TMV (4.0 ± 0.34 mm/min), the difference was not significant. However, comparison of this basal TMV with determinations of TMV at 20 minutes after treatment (5.6 ± 0.8 mm/min) revealed an increase that was significant ($p < 0.01$) (Fig 45b).

In animals treated orally with ambroxol (2 or 20 mg/kg), TMV was observed to be 5.0 ± 0.40 (n=11) and 4.40 ± 0.20 (n=9) mm/min, respectively. Differences between TMV estimates for these groups and for animals treated with placebo (4.6 ± 0.25; n=11) were not significant.

3.4.3.8) Theophylline

Theophylline, injected intravenously into 3 animals at 3 mg/kg, was without cardiovascular effects; TMV was also unaffected (basal TMV, 4.2 ± 1.1; post treatment, 4.7 ± 3.1).

3.5) Measurement of TMV in ferrets

TMV was measured in the ferret trachea using guinea-pig labelled erythrocytes. As ferrets have a long trachea (approximately 10 cm from

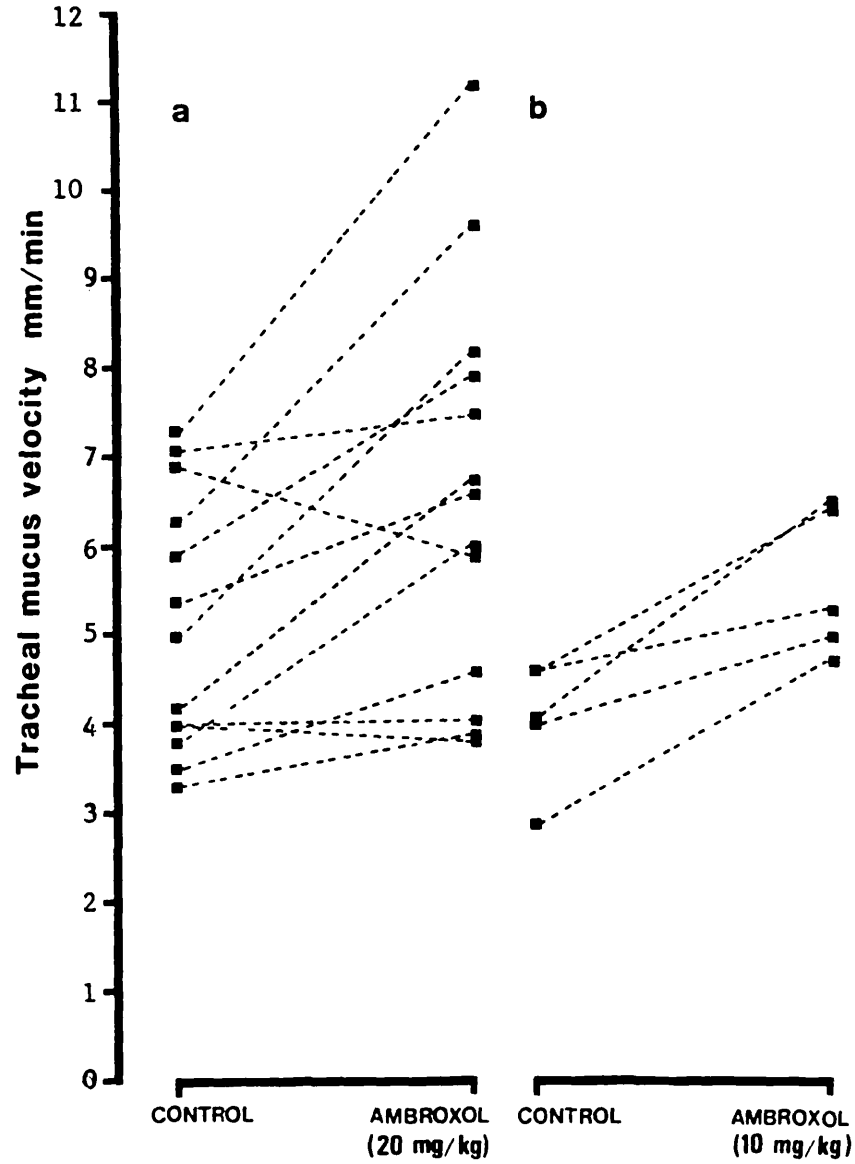


Figure 45. Effect of intravenous injection of ambroxol on guinea-pig tracheal mucus velocity (TMV). Intravenous injection of ambroxol (20 mg/kg) increased TMV significantly ($P<0.005$). Post-treatment TMV is the mean of, 2, 10 and 20 min determinations (a). Intravenous injection of 10 mg/kg of ambroxol increased TMV significantly ($P<0.01$) 20 min. post-injection (b). Pre and post-treatment values in a single animal are connected by dashed lines. Student's t-test for paired observations was used to assess statistical significance of observed differences.

the larynx to the carina), TMV could be measured over a 20 mm length of the trachea. In this species, care had to be taken whilst inserting the microsyringe into the trachea, since contact between the needle and the anterior mucosa caused bleeding in some animals and the resultant blood clot inhibited mucociliary transport.

3.5.1) Basal TMV

Basal TMV was measured in 71 ferrets; the distribution of these vales approximated to normal (Fig 46). The arithmetic mean was 7.9 $\pm$ 2.2 (s.d.) with a median of 7.8 and a range of 3.2 to 12.9 mm/min. In this group, the coefficient of variation of three determinations of TMV prior to any treatment was 9.2 $\pm$ 7.4% with a range of 0 to 55%.

3.5.2) Vagal stimulation

The vagus nerve was stimulated at 1 volt, 20 Hz and 2 msec for 1 min, in 2 animals. Basal TMV was unaltered (basal TMV, 6.6 $\pm$ 0; post-stimulation, 6.5 $\pm$ 2.2). When the vagus was stimulated at 2 volts (20 Hz, 2 msec, 1 min), TMV was reduced from a basal value of 6.6 $\pm$ 1.8 to 5.3 $\pm$ 2.6 mm/min; this was not statistically significant. At 2 volts, substantial bradycardia and hypotension were observed. These cardiovascular effects returned to normal on cessation of stimulation.

3.5.3) Parasympathomimetic agents

Intravenous administration of pilocarpine (100 ug/kg) elevated TMV. In

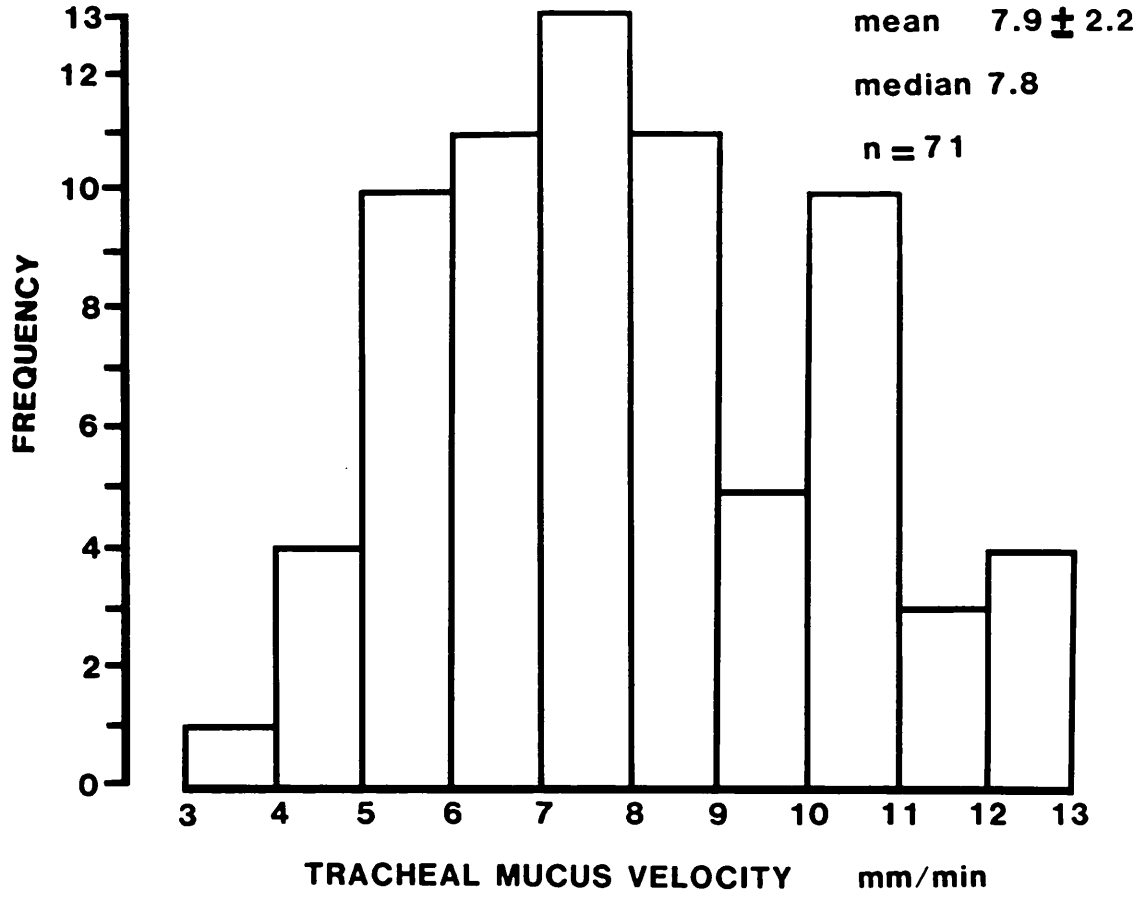


Figure 46. Distribution of basal TMV from 71 ferrets approximates to normal with a mean of 7.9 ± 2.2 and a median of 7.8 mm/min.

eleven animals, TMV was increased from a basal value of 7.7 $\pm$ 2.6 to 10.4 $\pm$ 4.0, 2 minutes ($P < 0.005$) and to 12.7 $\pm$ 5.1 mm/min, 15 min ($P < 0.005$) after injection (Fig 47a). This dose of pilocarpine produced no bradycardia; however, a transient hypotension was observed. In some animals, copious salivation and mucus production was sufficient to obstruct breathing eventually; in such animals TMV could not be measured after 20 min, for the trachea had become filled with froth. In animals pretreated with atropine (50 ug/kg), injection of pilocarpine (100 ug/kg) did not increase TMV significantly (pretreatment, 7.8 $\pm$ 1.7; post-treatment, 9.6 $\pm$ 4.1 mm/min n=5) (Fig 47b); the hypotensive effect of pilocarpine was abolished after this dose of atropine.

Topical, extratracheal application of 100 ul of ACh (100 ug/ml) increased TMV from a basal value of 8.5 $\pm$ 1.7 to 13.5 $\pm$ 5.0 mm/min ($P < 0.01$). Topical application of 100 ul of PBS was without effect (basal TMV, 9.8 $\pm$ 2.0; after PBS, 9.0 $\pm$ 1.2 mm/min; n=7 NS). The effect of ACh could be terminated by application of saline to the region to which ACh had been applied. In these animals, re-application of a second aliquot of ACh, after washing, increased TMV to the same extent as the first stimulation (Table 12).

Atropine (50 ug/kg) was injected intravenously in 7 ferrets. No alteration of basal TMV was observed (basal TMV, 7.9 $\pm$ 1.7, post-treatment, 7.4 $\pm$ 1.3 mm/min). This dose of atropine was

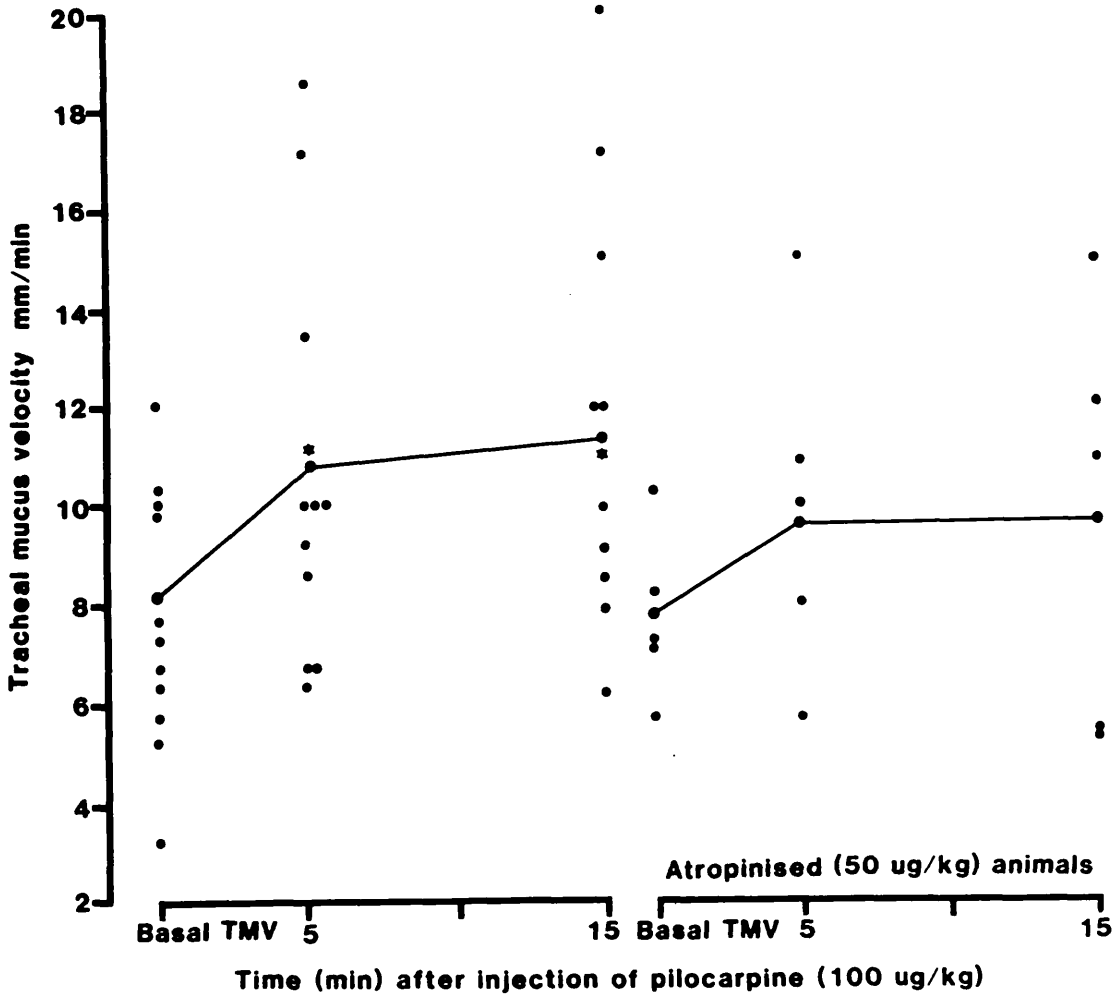


Figure 47. Effect of intravenous injection of pilocarpine (100 ug/kg) on ferret TMV. TMV is significantly elevated 5 and 15 min post-injection (n=11). Although in atropinised animals (n=5) there is an elevation of TMV 5 and 15 min post-injection, this increase does not have statistical significance (Student's t-test for paired observations).

* $P<0.001$

Table 12. Effects of parasympathomimetic agents on tracheal mucus velocity in ferrets.

Agent	Dose (ug/Kg)	n	Tracheal mucus velocity Basal	Tracheal mucus velocity Post-treatment	P
SALINE (topical)	-	7	9.8 ± 2.0	9.0 ± 1.2	NS
ACh (topical)	10*	7	8.5 ± 1.6	13.5 ± 5.0	<0.01
ACh (topical) 2nd application	10*	3	9.1 ± 0.7	16.0 ± 6.3	-
PILOCARPINE	100	11	8.1 ± 2.3	11.1 ± 3.5	<0.001
PILOCARPINE + ATROPINE	100 50	5	7.8 ± 1.7	9.6 ± 4.1	NS
ATROPINE	50	6	7.9 ± 1.7	7.4 ± 1.3	NS

Drugs were administered intravenously unless specified.

Post-treatment values are the mean of determinations, 5 and 15 min after drug administration.

Student's t-test for paired observations was used to assess statistical significance of observed differences.

Results are expressed as mean ± s.d. in mm/min; n= number of animals.

* 10 ug/site in 100 ul.

sufficient to inhibit hypotensive effects of pilocarpine (100 ug/kg) and methacholine (10 ug/kg). Results of parasympathomimetic agents have been summarised in Table 12.

3.5.4) Sympathomimetic agents

Intravenous injection of isoprenaline (1 and 2 ug/kg), propranolol (1 mg/kg) or phenylephrine (10 and 20 ug/kg) had no significant effect on basal TMV, despite prominent cardiovascular changes with all three agents. Table 13 summarises the effect of these compounds.

3.5.5) Substance P

Substance P (100 ng/kg and 1ug/kg) was injected intravenously. The lower dose was without effect on basal TMV (basal TMV, 8.3 $\pm$ 2.5; post-treatment, 7.1 $\pm$ 2.1; n=4; NS), but was sufficient to produce hypotension. The higher dose of substance P was a most potent stimulant of TMV. In 5 animals basal TMV increased from 7.0 $\pm$ 1.9 to 12.4 $\pm$ 3.1 mm/min; this increase was highly significant ($P < 0.005$). However, the most striking effect of substance P (at 1 ug/kg) was on salivation. In all animals, saliva had to be aspirated from the buccal cavity, after substance P injection. Approximately 1-2 ml of saliva was aspirated from each animal within the first minute. Table 14 summarises the effects of substance P on TMV in ferrets.

3.5.6) Theophylline

Theophylline (3 mg/kg) was injected intravenously into four animals; in two of these animal a higher dose of theophylline (10mg/kg) was also

Table 13. Effects of sympathomimetic agents on tracheal mucus velocity in ferrets.

Agent	Conc. (ug/kg)	n	Tracheal mucus velocity Basal	Post-treatment	P
ISOPRENALINE	1	5	9.4 ± 2.0	8.7 ± 1.9	NS
ISOPRENALINE	2	3	9.3 ± 2.6	8.2 ± 2.4	-
PROPRANOLOL	1000	2	6.4 ± 1.8	6.7 ± 0	-
PHENYLEPHRINE	10	3	8.0 ± 2.5	8.3 ± 2.4	-
PHENYLEPHRINE	20	4	8.3 ± 2.3	7.1 ± 1.4	NS

Drugs were administered intravenously unless specified.

Post-treatment values are the mean of determinations, 5 and 15 min after drug administration.

Student's t-test for paired observations was used to assess statistical significance of observed differences.

Results are expressed as mean ± s.d. in mm/min; n= nuber of animals.

Table 14. Effect of Substance P on tracheal mucus velocity in ferrets.

Concentration (ug/kg)	n	Tracheal mucus velocity		P
		Basal	Post-treatment	
0.1	4	8.3 ± 2.5	7.1 ± 2.1	NS
1.0	5	7.0 ± 1.9	12.4 ± 2.6	<0.005

Substance P was administered intravenously.

Post-treatment values are the mean of determinations, 5 and 15 min after drug administration.

Student's t-test for paired observations was used to assess statistical significance of observed differences.

Results are expressed as mean ± s.d. in mm/min; n= number of animals.

administered. The lower dose had no cardiovascular effects and did not alter TMV (basal TMV, 5.8 ± 1.0; post-treatment TMV, 5.4 ± 0.6). The higher dose produced a pronounced hypotension and TMV was reduced (basal TMV, 6.3 ± 0.1; post-treatment TMV, 3.9 ± 0.7; n=2).

3.5.7) Effect of anaesthetic

Most ferrets required maintenance doses of pentobarbitone (3 mg/kg, i.v.). Some animals appeared to be more sensitive to pentobarbitone, since hypotension was observed following injection of the anaesthetic. In five animals, TMV was substantially reduced after injection of a maintenance dose of pentobarbitone, but recovered within 15-20 min. In these animals, basal TMV was reduced from 5.4 ± 0.6 to 3.4 ± 0.6 in the first 10 min (n=5). This reduction was highly significant ($P < 0.001$).

3.6) Measurement of TMV in rabbits

Measurement of TMV was attempted in 15 animals, but in only 6 animals was mucociliary transport observed. Failures were associated with copious bleeding from the puncture hole in the trachea, which is highly vascularised when compared with guinea-pig or ferret trachea. When mucociliary transport was not detected, post-mortem examination of the trachea revealed large blood clots in the vicinity of the puncture. Basal TMV in 6 animals for which values could be obtained was 3.7 ± 1.2 mm/min.

3.7) Comparison of basal TMV in guinea-pigs, ferrets and rabbits

Basal TMV was compared in ferrets (n=22), guinea-pigs (n=30) and

rabbits (n=6). The ferret was observed to have the highest TMV (8.9 ± 2.2), followed by the guinea-pig (4.9 ± 1.1) and the rabbit (3.7 ± 1.2) (Fig 48). Student's t-test for groups of unequal size was used to assess statistical significance of differences observed. TMV in the ferret was significantly faster than the guinea-pig ($P < 0.001$) and the rabbit ($P < 0.001$); however, the difference between TMV values for the guinea-pig and the rabbit was not significant.

3.8) Measurement of ciliary beat frequency

Ciliary beat frequency (CBF) of clumps of ciliated tracheal epithelium was measured in guinea-pigs, rabbits and ferrets. In each instance, basal TMV was measured prior to tissue sampling from that region of the trachea.

3.8.1) Comparison of CBF and TMV in guinea-pigs, ferrets and rabbits

CBF was compared in ferrets (n=11), guinea-pigs (n=7) and rabbits (n=6). The ferret was observed to have the fastest CBF (16.5 ± 2.5, s.d.) followed by the guinea-pig (15.6 ± 1.8, s.d.) and the rabbit (12.7 ± 1.7, s.d.) (Fig 49). Student's t-test for groups of unequal size was used to assess statistical significance of differences observed between CBF of each species. CBF in the ferret was significantly higher than in the rabbit ($p < 0.005$); however, the differences observed between CBF values of the ferret and the guinea-pig or the rabbit and the guinea-pig were not significant. Table 11 shows the values of CBF and TMV for each animal.

No correlation between basal TMV and CBF could be shown for ferrets

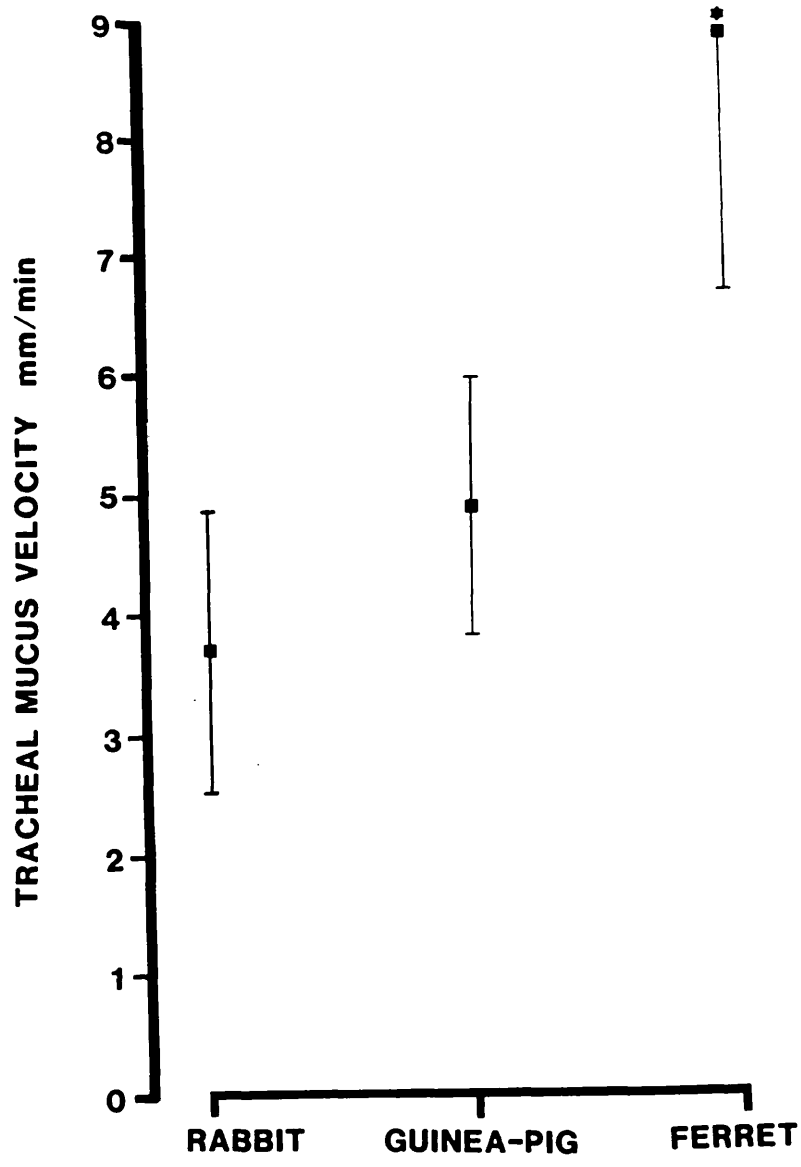


Figure 48. Comparison of basal TMV from ferrets (n=24), guinea-pigs (n=30) and rabbits (n=6). TMV in ferrets is significantly higher than both the guinea-pig and the rabbit. Bars indicate standard deviation. Student's t-test for groups of unequal size was used to assess statistical significance of observed differences.

* P<0.005

(r=0.013), guinea-pigs (r=-0.32) or rabbits (r=-0.27).

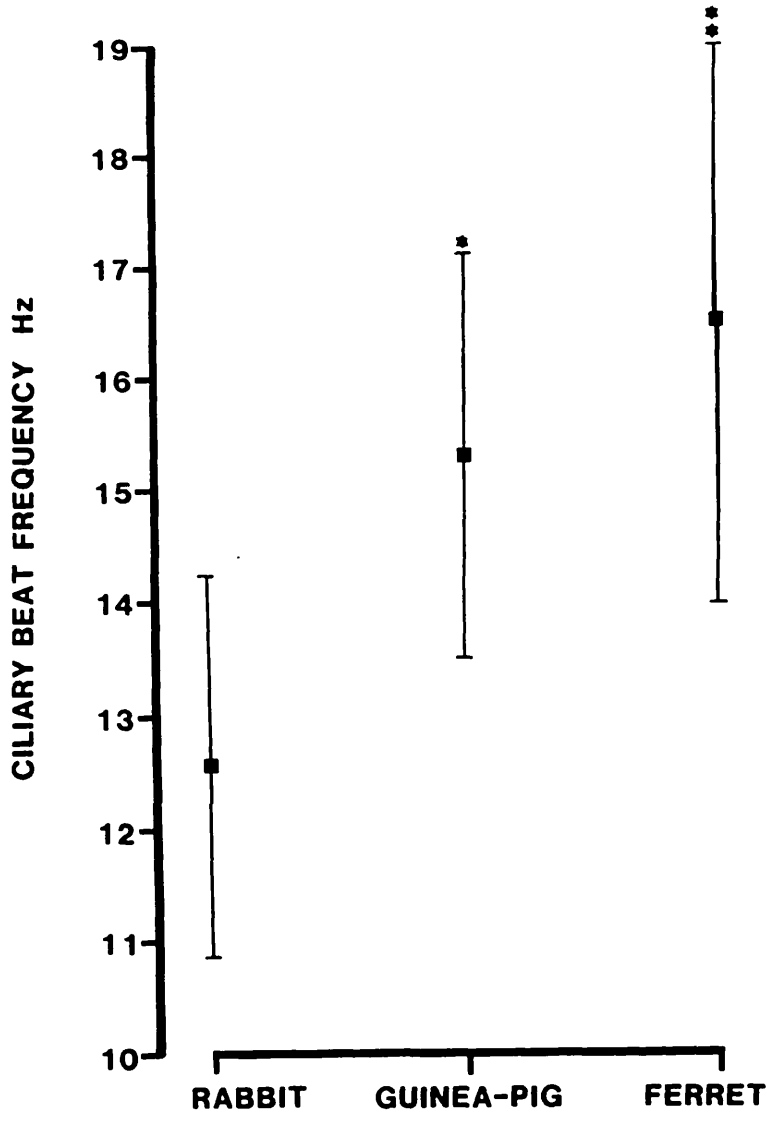


Figure 49. Comparison of ciliary beat frequency (CBF) of tracheal explants from ferrets (n=11), guinea-pigs (n=8) and rabbits (n=6). CBF of the rabbit was significantly slower than those of the ferret and the guinea-pig. Student's t-test for groups of unequal size was used to assess statistical significance of observed differences. Bars indicate standard deviation.

* P<0.01, ** P<0.005

4 DISCUSSION

In this thesis, two types of preparation for measurement of mucociliary transport in small laboratory animals have been developed and evaluated.

4.1) Measurement of mucociliary transport

The frog oesophagus provides a simple method for repeated measurement of mucociliary transport. The dissection procedure is rapid, and preparations can be used within thirty minutes of pithing a frog. Preparations are robust and give consistent results over periods of several hours. Basal transport rates and responses to stimuli show reproducibility, both within and between animals and no tachyphylaxis was observed with any of the stimuli. No obvious seasonal variation was apparent.

More usually, measurement of mucociliary transport in the frog has relied upon use of the isolated palate (Sade et al., 1970; King et al., 1974). However, intrusions of the orbital hemispheres distort the pathway for mucus transport, so that estimates of path distance are only approximate; also, the palate lacks submucosal glands and, hence, preparations deteriorate rapidly. For certain purposes, an absence of endogenous mucus can be advantageous, as in the investigation of exogenous mucus (e.g. sputum samples), whose rheological properties may determine transport rate in diseased patients (Puchell et al., 1973). From the present study, it has become evident that the frog oesophagus preparation has a clear advantage over the palate, especially for study of drug effects on mucociliary transport, since

experiments using the oesophagus were carried out over long periods (several hours), whilst the palate preparations ceased to transport effectively after only 40 min. The absence of submucosal glands, may contribute to the transient mucociliary transport observed in the palate. The present method was developed in order to allow repeated determinations of mucociliary transport, prior to and after treatment with a number of pharmacological agents and physiological stimuli. Although the frog oesophagus has been used by many investigators for similar studies (McDonald et al., 1928; Kordik et al., 1952; Hill, 1957; Burn and Day, 1958; Milton, 1959), certain additions to the apparatus have made measurement of MCT more accurate and less exacting. Thus, the video recording equipment allows a large number of experiments to be executed without the delay that would be needed for timing the passage of individual particles during an experiment. Usually, a series of experiments were recorded on magnetic tape and the results were analysed on a subsequent occasion; since a permanent record of an experiment was kept, it was possible to refer back to an experiment for a further examination. In previous investigations, the path length of particles was determined by placing a ruler alongside the oesophagus or by using an eye-piece graticule (Kilburn, 1967). Use of a T.V. monitor which has superseded direct observation, offers further advantages, including accurate measurement of path length and large magnification of the area under investigation (x 60).

Mucociliary transport in mammalian airways has been measured using a variety of visual, isotopic or roentgenographic methods (see 1.4.1). However, these techniques are more readily applicable to large animals and man. In small animals, methods exist for visualisation of particle transport, and the technique most extensively used for assessment of

drug activity was developed by Iravani (1967). In this method, ciliary activity and mucociliary transport can be observed by trans-illumination of the airways. The method is disadvantaged by requiring extensive dissection and careful maintenance of temperature and humidity of the *in vitro* tracheobronchial preparation. A less invasive method was used by Dalham (1956), in which a small window was cut into the trachea of an anaesthetised rat and transport of air bubbles and cell debris was observed using a microscope. Such preparations are critically dependent upon a good seal between the trachea and the microscope objective lens. Blood-free surgery is also mandatory, since some constituents of plasma (e.g. blood clotting factors) are known to inhibit ciliary activity (Battista *et al.*, 1980); indeed, in the present study, the bleeding associated with needle entry was sufficient to impair tracheal transport in 9 of 15 rabbits.

Large crystal detectors are usually employed for detection of gamma radiation; however, large amounts of lead shielding are required to collimate the detector and to isolate the area under observation. These factors severely limit the use of gamma-emitting isotopes for measurement of mucociliary transport in small animals. However, a method has been described (Patrick and Sterling, 1977), in which a droplet containing isotopic marker is deposited in the trachea of sedated rats and the subsequent reduction in radioactivity over the site of deposition used as an index of mucociliary transport. A similar method was later used in, guinea-pigs and rabbits (Felicetti *et al.*, 1981).

Measurement of lung clearance, analogous to clinical experiments, has been attempted (Kenoyer *et al.*, 1980) in rats. However, slow clearance

(weeks) and uncertain path length severely limit the use of this technique for evaluation of a large number of pharmacological agents. When gamma-emitting isotopes are used in small animals, only one observation per animal can be made, since residual activity after the first experiment introduces a large background signal, which interferes with subsequent estimations of mucociliary transport. This restriction necessitates inter-animal comparison.

In the present study, problems imposed by gamma-emitting isotopes have been circumvented by using a beta-emitting isotope. Beta-particles have limited penetration in air (maximum of 70 cm, more usually 6-7 cm) and are readily absorbed by very thin sheets of lead. In addition, beta-particle detectors are very compact, which makes them suitable for use with small animals. Resolution is enhanced by collimation with a small amount of lead sheet. In simulated experiments, the highest count rates were observed when a source of activity (2 ul of labelled erythrocytes) was directly under the collimator slit and activity beyond a radius of 10 mm could not be detected. Phosphorus-32 provided a suitable and inexpensive isotope for use in these experiments. The choice of guinea-pig erythrocytes for marker particles was fortuitous, since they labelled effectively with 32-P and were very stable over a 5 day period, in contrast to ferret erythrocytes.

The experimental procedure was greatly simplified by using a computerised data collection system. The computer incorporated a special application board for logging of pulses generated by the detectors. The software programme permitted measurement of radioactivity for variable duration (pre-set by investigator), using 45 steps or less. The data could be presented in tabular or graphical

form, which made estimation of tracheal mucus velocity very simple. The time interval between starting an experiment and observation of maximal radioactivity, together with the distance between the detector and site of deposition of marker particles, allowed calculation of rate of transport (TMV) in mm/min.

TMV measured by the method described is an estimate of the most frequent rate of transport of the population of applied particles. For physiological studies it would be more conventional to employ transit time (i.e. the time elapsed for 50% of particles to pass the detector). In these experiments, the distribution of particle transport time was frequently symmetrical so that transit time and time to peak were identical. Selection of the most frequent transport time (i.e. time to peak) is based solely on operator convenience, since calculation of transit times by normal methods would be inordinately protracted, without any evident benefit.

A notable disadvantage of this system is the use of anaesthetic, since anaesthesia has been shown to reduce mucociliary transport (Landa et al., 1975). The present study supports this view, especially when pentobarbitone was used for anaesthesia. Anaesthetic effects were apparent in only a small proportion of animals used. Since experiments could not be performed in conscious animals, the effect of anaesthetics was not assessed.

4.2) Physiological regulation in the frog

Physiological regulation of mucociliary clearance in the frog has been studied intermittently throughout this century. The frog palate and

oesophagus have provided convenient and simple preparations for measurement of mucociliary transport, yet attempts to define mechanisms of physiological control using this type of preparation have yielded conflicting data. McDonald et al. (1928) suggested that sympathetic nerves controlled ciliary activity in the frog palate and pharynx, whereas in a later study (Lucas, 1935), it was reported that cholinergic, rather than adrenergic nerves were involved. This view seems more plausible, since ACh has consistently been reported to accelerate oesophageal MCT (Plattner and Hou, 1931; Blaich and Klar, 1950; Kordik et al., 1952; Milton, 1959).

In the present investigation, electrical stimulation of the vagus nerve has been observed to increase particle transport along the frog oesophagus, an effect that was suppressed by atropine, or by hexamethonium bromide. Such results are consistent with cholinergic parasympathetic regulation of mucus transport in the frog oesophagus. The inability of eserine to affect particle transport rate during vagal stimulation may be construed as contrary to this interpretation. However, such discrepancies have been reported previously at sites of cholinergic transmission. Anti-cholinesterase drugs failed to prolong the time-course of the excitatory post-synaptic potentials in the superior cervical ganglia of the rat (Eccles, 1964; Gisiger, Gautron and Dunant, 1977; Gisiger and Vigny, 1977); this could be explained if the action of ACh were terminated by diffusion of transmitter from the synapse, rather than by the action of choline esterase. A similar explanation may apply to the frog oesophagus, even though high levels of acetylcholine-esterase have been demonstrated in the palatal mucosa of the toad (Satoh, 1959). Topical application of ACh, or other parasympathomimetic agents, accelerated mucociliary transport in a

concentration-related fashion and these effects were abolished by atropine. These observations further support the conclusion that this tissue is subject to cholinergic parasympathetic regulation, consistent with previous reports (Plattner and Hou, 1931; Kordik et al., 1952; Milton, 1959), who also used an in situ preparation. It is likely that the conflicting conclusion of Hill (1957), who observed frog oesophageal transport to be insensitive to ACh, reflects the use of an excised decentralised preparation; an observation that has been confirmed in the present study. It is possible that the elevated rate of transport observed in isolated oesophagus pre-empts stimulation by ACh. In contrast to previous studies, no inhibitory effect was observed with high concentrations of ACh. At these concentrations (10-4 g/ml), oesophageal smooth muscle contracted; an effect which altered the path length over which particles were transported. No interspecies difference was observed in response to ACh, nor was there a difference between the in situ and chamber methods.

In contrast with the effect of cholinergic agents, topical application of adrenaline, noradrenaline, isoprenaline or salbutamol consistently failed to alter particle transport rate in the frog oesophagus. Such observations are in agreement with an earlier report of Blair and Woods (1969) that isoprenaline did not affect ciliary beat frequency or MCT in a frog oesophageal preparation. It has previously been reported that certain species of toad secrete saliva in which adrenaline is present at a concentration of 4% w/v (Fischer and Lecomte, 1950). Such phenomena might be expected to preclude sympathomimetic regulation of mucociliary clearance in the mouth and oesophagus of these amphibia. The failure of adrenaline, or noradrenaline, to alter the stimulatory effect of vagal stimulation implies that presynaptic inhibitory

mechanisms, mediated through adrenergic agents, are absent.

Inflammatory mediators, such as histamine and 5-HT, have been tested in the frog oesophagus with no apparent effect on basal mucociliary transport. However, PGE2 and PGF2 alpha produced significant, though modest, stimulation. It has been suggested that noradrenaline exerts a stimulatory effect by increasing PGE synthesis conc omitant with a rise in cyclic-AMP levels (Yamamura et al., 1984); hence, a stimulatory effect with inhibitors of phosphodiesterase might be anticipated; however, theophylline did not affect basal MCT. Caffeine was also without effect in both R. temporia and R. pipiens until a concentration of 1 mg/ml was reached, at which point there was a dramatic increase in MCT. On the other hand, isobutyl methylxanthine, a more potent inhibitor of cAMP-phosphodiesterase, stimulated MCT in a dose-dependent fashion.

ATP and other tri-phosphate purines have been reported to increase ciliary beating and particle transportation in the frog oesophagus (Vorhous and Deyrup, 1958). In the present study, topical application of ATP, UTP and GTP accelerated MCT in a dose-related fashion. All di-phosphate purines were inactive, with the exception of UDP, which was equipotent with the tri-phosphate compounds, for which there is no explanation at the present.

In a few experiments, substance P was applied in a wide range of concentrations and was found to stimulate MCT. These results are interesting, in that they indicate that peptidergic innervation may influence mucociliary transport. This may be a property of other

peptides for, in a single experiment, bombesin was also tested (0.01 to 10 ug/ml) and was found to stimulate MCT strongly (results not shown).

A range of mucolytics have been tested on the MCT of frog oesophagus. All cysteine derivates were ineffective. These compounds are used to reduce viscosity of abnormal mucus, and hence promote expectoration; therefore, they may not be effective in influencing normal mucus or its rate of transport. However, other mucolytics affect MCT in normal animals and, of all mucolytics tested, ambroxol, an analogue of bromhexine, was the most potent stimulant of MCT. The mechanism of action of this compound remains unclear. The stimulation caused by ambroxol cannot be attributed to an effect on cholinergic nerves, since atropine was ineffective: nor can this stimulation be attributed to a sympathetic action, since sympathomimetic agents are not active in this preparation.

Since mucus contains a protein backbone, it is possible to detect the protein constituent, using the modified Lowry method (Markwell et al., 1978). A close correlation existed between mucus secretion and MCT from the onset of an experiment, with both parameters declining to a basal level after 30 to 40 min. After this period, addition of pilocarpine or ACh, both of which would be expected to increase mucus secretion, increased MCT in a dose-related fashion but were without effect on protein secretion. These results suggest that increased MCT is due to an increase in ciliary beat frequency; however, the possibility that mucus with a more favourable rheological property may be secreted, although unlikely, cannot be excluded.

In R. temporaria, oesophageal mucociliary transport appears to be regulated mainly by cholinergic parasympathomimetic nerves.

4.3) Physiological regulation in mammalian species

Physiological regulation of mucociliary transport is poorly defined in mammals. There is evidence of cholinergic regulation in dogs (Wanner et al., 1975) and man (Camner et al., 1974), since cholinergic agonists increased TMV and whole lung clearance. Atropine has been reported to reduce TMV in dogs (Sackner et al., 1977) and man (Foster et al., 1976; Yeates et al., 1975). However, this view is not universally held, since Chopra (1978) reported atropine to have no inhibitory effect on TMV in dogs; on the contrary, an enhancement was reported. Camner et al. (1974) also reported no decrease in mucociliary clearance in subjects treated with methylscopolamine nitrate, despite a decrease in saliva production. In addition to stimulating mucociliary transport, cholinergic agents also stimulate mucus secretion (Florey et al., 1935; Gallagher et al., 1975). Under normal conditions, a close correlation between mucus secretion and ciliary activity may be expected, since an increase in mucus secretion not matched by an increase in ciliary activity, may result in stagnation of mucus in the airways, leading to obstruction by mucus plugging. If mucus secretion and mucociliary transport are linked, it may be anticipated that vagal stimulation would increase mucociliary transport in cats, since vagal stimulation increases mucus secretion in this species (Gallagher et al., 1975; Ueki et al., 1980). There is a reduction in mucociliary transport in dogs, following lung transplant, which is thought to be due to transection of sympathetic and parasympathetic nerves (Edmunds et al., 1969). In, man no difference in mucociliary clearance was observed between normal subjects and patients with chronic autonomic failure; therefore, it was suggested that the autonomic nervous system was not an important regulatory mechanism in man (Jenkins et al., 1980).

However, since denervation may have been incomplete and the mucociliary system may have adjusted over the course of this chronic disease, some doubt remains. In experimental animals, beta-adrenoceptor agonists have been reported to increase mucociliary transport (Melville et al., 1976; Blair and Woods, 1969; Sackner et al., 1976). In man, conflicting data exists. Thus, enhancement (Camner et al., 1976; Foster et al., 1976), or no effect (Santa Cruz et al., 1974) of beta-adrenoceptor agonists, in normal subjects, has been reported.

The present investigation provides unequivocal evidence that cholinergic stimuli will promote mucociliary transport in the trachea of guinea-pigs and ferrets. In both species, intravenous pilocarpine (100 ug/kg) or topical application of ACh to the serosal surface of the trachea produced significant stimulation of TMV. In guinea-pigs, an enhancement of TMV was first observed 15 min after treatment with pilocarpine and was present at 30 min post-treatment. A similar latent period was not observed in ferrets. The difference may be attributed to the secretory elements in the trachea of these animals; ferrets have prominent submucosal glands (Basbaum et al., 1980) whereas in guinea-pigs, submucosal glands are scarce. If enhancement of mucociliary transport depends upon a concomitant enhancement of ciliary activity and mucus secretion, it may explain the latency observed in guinea-pigs, for secretion of mucus in significant quantity may take longer than in the ferret. Stimulation of TMV due to pilocarpine was inhibited by atropine, although, in the ferret, the mean TMV after pilocarpine was higher than the control group but did not reach statistical significance. Pilocarpine produced salivation in some animals and excessive mucus secretion, as evidenced by wheezing which, in some cases, was accompanied by respiratory distress. Cardiovascular

changes were always present following intravenous injection of pilocarpine, in both species. Treatment with atropine (50 ug/kg) fully inhibited cardiovascular effects of pilocarpine and ACh. Pilocarpine was chosen as the cholinergic agonist on the basis that, in the frog oesophagus, it was a potent stimulant and, at the concentrations used, it produced no bronchoconstriction or panting in either species, and it was not destroyed as rapidly as ACh.

Having observed a stimulatory effect of ACh and pilocarpine, the effect of vagal stimulation on TMV was explored. Vagal stimulation was expected to improve TMV, since such an effect was observed in the frog oesophagus and cholinergic agents had increased TMV. Using a low voltage (1 v, 20 Hz) setting, no bronchoconstriction or cardiovascular changes were observed in the guinea-pig and TMV was significantly increased in all animals. Although, 2 min after nerve stimulation, TMV was significantly enhanced, the most pronounced effect was observed at 15 min, which is similar to the effect of pilocarpine. Atropine inhibited the effect of vagal stimulation. In two animals, stimulation of the vagus at 2 volts caused a reduction in TMV, which is difficult to explain. In ferrets, vagal stimulation at 1 volt had no effect on basal TMV, whereas at 2 volts, a reduction in TMV was observed, which was similar to that seen in the guinea-pig. However, this reduction was not significant, even though severe bradycardia was observed. It appears that, despite the effect of cholinergic agents on ferret TMV, vagal stimulation is without effect, although investigation of a full stimulus frequency range may produce different results.

In both species, atropine prevented stimulation of TMV in response to cholinergic stimuli; however, atropine did not alter basal TMV in

either species. These results are at variance with reports in the dog (Sackner et al., 1977). The explanation may be that, in the present study and an earlier report in the dog (Chopra et al., 1979), where enhancement was observed, atropine was administered intravenously, whereas Sackner et al. (1977) administered this drug by aerosol.

Intravenous administration of isoprenaline or propranolol in ferrets and guinea-pigs produced pronounced cardiovascular changes; however, neither drug affected TMV. Once again, the route of administration may be an an important determinant in the activity of these compounds. In dogs (Sackner et al., 1976) and sheep (Sackner et al., 1979), inhalation of beta-adrenoceptor agonists produced an increase in TMV. Topical application of isoprenaline to the cat trachea (Blair and Woods, 1969) was also observed to increase ciliary activity and TMV. Incubation of ferret tracheal rings with isoprenaline (10-5 M) is reported to increase mucus secretion (Peatfield et al., 1983). If, in the present investigation, isoprenaline had been administered intratracheally, it would have been possible to achieve drug levels comparable to those used in vitro. Since an intravenous route was chosen, it was not possible to attain similar concentrations to the in vitro study. Indeed, to achieve such a concentration intravenously, a dose of 2 mg of isoprenaline would be required for an animal weighing 700 g (1000 fold above the limit of tolerance). Validity of extrapolating from in vitro observation to in vivo events in mucociliary clearance has yet to be established.

Alpha-adrenoceptor agonists have been reported to be potent stimulants of mucus secretion in cats (Phipps et al., 1980) and ferrets (Borson et al., 1980). Intravenous administration of phenylephrine (10 ug/kg)

elevated TMV in guinea-pigs significantly; this stimulation was inhibited by corynanthine. However, in ferrets, phenylephrine at 10 or 20 ug/kg had no effect on TMV, despite substantial cardiovascular effects with the high concentration and despite the observation, *in vitro*, that tracheal secretions are enhanced in the ferret.

More recently, considerable attention has been directed to peptidergic nerves as sources of potential neuro-transmitters. Vasoactive intestinal peptide (VIP) and substance P have been demonstrated in mammalian lung (Uddman *et al.*, 1978; Nilson *et al.*, 1977) and there is functional evidence of peptidergic innervation to the guinea-pig lung, since capsaicin will inhibit a proportion of the bronchoconstrictor response to vagal stimulation (Tomiak, 1984). The action of peptidergic transmitters on secretion and transport of mucus has received limited attention. Such substances are vasoactive, as indicated by persistent hypotension, and are secretagogues in dogs (Baker *et al.*, 1977) and ferrets (Peatfield *et al.*, 1983). In the cat, it has recently been reported that tracheal mucus production, due to vagal stimulation, is resistant to inhibition by atropine (Peatfield and Richardson, 1983a), and in ferrets, mucus secretion due to electrical field stimulation was unaffected by muscarinic, alpha- and beta-adrenoceptor antagonists (Borson *et al.*, 1982). Therefore, peptidergic transmitters may be considered as potential determinants of mucus secretion or mucociliary transport in such circumstances. In the present experiments, substance P accelerated mucociliary transport in the ferret consistently, although results in the guinea-pig proved inconclusive.

3.4) Therapeutic implications

Effective mucociliary clearance depends upon active ciliary activity and production of mucus with optimal rheological properties (Sade et al., 1970). In respiratory diseases, such as cystic fibrosis (Polgar and Denton, 1962; King, 1981), chronic bronchitis (Charman and Reid, 1972) and bronchial asthma (Keal and Reid, 1975), abnormal mucus is produced, hence mucociliary transport may be expected to be impaired. In such diseases, impairment of mucociliary transport is further aided by destruction of epithelial cells, leading to a reduced incidence of ciliated cells and an increase in the number of goblet cells (Dunnill et al., 1969). Accordingly, impaired mucociliary transport has been reported for all these conditions (Santa Cruz et al., 1974; Wood et al., 1975; Foster et al., 1978; Bateman et al., 1979). Patients with genetic ciliary defects have also been reported to have impaired mucociliary transport (Eliasson et al., 1977; Hendry et al., 1978; Sturgess et al., 1979), which may lead to development of obstructive lung disease (Mossberg et al., 1978). Impairment of mucociliary transport, coupled with formation of viscous and tenacious mucus, leads to plugging of the airways and death in status asthmaticus (Rigler and Koucky, 1938; Hilding, 1943; Dunnill, 1960). Occlusion of small bronchi in both severe and mild asthma was demonstrated as early as 1938 by Rigler and Koucky.

Beta-adrenoceptor agonists have been proposed to improve mucociliary transport in a variety of diseases. Thus, Santa Cruz et al. (1974) reported a significant improvement of TMV after treatment with terbutaline in patients with chronic obstructive airways disease, although TMV following treatment remained substantially less than

normal TMV. Terbutaline has also been reported to increase mucociliary transport in cystic fibrosis (Wood et al., 1975). Conversely, Mossberg et al. (1976) and Sackner et al. (1979) reported no effect of terbutaline or fenoterol in chronic bronchitis. More recently, further doubt has been cast on the efficacy of terbutaline as a stimulant of mucociliary transport in asthmatic patients (Bateman et al., 1979).

Mucociliary transport has been measured in experimental models of allergic asthma (Wanner et al., 1975; Allegra et al., 1983), where antigen challenge caused significant reduction of TMV. In sheep, this effect persisted for up to 8 days after challenge (Allegra et al., 1983). In dogs (Wanner et al., 1975), reduction of TMV due to antigen challenge was inhibited by FPL 55712, which is regarded as a selective antagonist of SRS-A (Augstein et al., 1973). Similar experiments have been performed in asthmatic patients with ragweed hypersensitivity, where TMV is significantly reduced following antigen challenge. Pre-treatment with disodium cromoglycate (DSCG) had no effect on basal TMV but prevented reduction of TMV after antigen challenge; it may be noted that TMV was significantly enhanced after antigen challenge, whereas reduction of specific airways conductance following exposure to allergen was not inhibited (Mezey et al., 1978). In a later study, FPL 55712 was employed (Ahmed et al., 1981), and results similar to those obtained with DSCG were observed. In an attempt to differentiate the role of cilia and mucus during antigen challenge, ciliated cells were obtained from sensitised sheep and challenged in vitro (Maurer et al., 1982). Ciliary activity was increased after challenge; an effect which was inhibited by DSCG. The discrepancy between TMV (reduction) and CBF (increase) is difficult to explain; it implies that ciliary function may not determine a decrease in TMV following antigen

challenge.

Theophylline is widely used in the treatment of asthma and there have been a few reports of the effect of theophylline or aminophylline on mucociliary transport. All investigators have reported an increase in mucociliary transport, associated mainly with the large airways (Sutton et al., 1981; Serafini et al., 1976; Matthys and Kohler, 1980). In the present investigation, intravenous theophylline did not increase TMV in guinea-pigs or ferrets. In ferrets, the high dose of theophylline (10 mg/kg) reduced TMV.

Mucolytics form a large group of drugs used for a variety of respiratory diseases. These compounds liquefy mucus and, hence, many promote expectoration (Webb and Jackson, 1962). Most of these compounds are cysteine derivatives and many have been tested on mucociliary transport, in experimental animals and man. N-acetylcysteine and S-carboxymethylcysteine are not effective stimulants of mucociliary transport in rats (Iravani et al., 1975); or man (Thomson et al., 1975; Goodman et al., 1978), despite an improvement in respiratory parameters (Edwards et al., 1976). In the present study, no increase in mucociliary transport was observed in the frog oesophagus, using a range of cysteine derivatives. Bromhexine and its more soluble analogue, ambroxol, have been reported to increase CBF, mucus secretion and mucociliary transport in rats (Iravani and Melville, 1974; Melville et al., 1980) and man (Seyfarth, 1964; Thomson et al., 1974). In the present investigation, both compounds increased mucociliary transport in the frog oesophagus. However, only ambroxol was extensively used, since bromhexine was soluble only at pH4. The stimulatory effect of ambroxol was not affected by atropine,

which implies that ambroxol may act directly on the ciliated cells, rather than by a secondary action mediated through cholinergic or adrenergic nerves. An effect on peptidergic nerves cannot be excluded, although it may be noted that the magnitude of response caused by ambroxol was greater than that caused by substance P. Ambroxol was also tested in guinea-pigs, and acute administration at 10 or 20 mg/kg produced significant stimulation of TMV. With the lower dose, there was a latent period such that TMV was only significantly elevated at 20 min, post-treatment. With the higher dose, no latency was observed. A similar latent period is observed with pilocarpine, which may indicate that ambroxol acts by increasing mucus secretion and by increasing CBF, as has been reported in rats (Iravani and Melville, 1974). A course of 5-day treatment *per os* with ambroxol produced no changes in TMV when treated animals were compared with placebo treated animals. It may be more appropriate to conduct such chronic studies with animals that have been exposed to pollutants, which are known to impair mucociliary transport, such as cigarette smoke (Wanner *et al.*, 1973) or SO2 (Spiegelman *et al.*, 1968).

The introduction of drugs as stimulants of mucociliary clearance has been largely empirical. The results of such therapy in diseases such as asthma and bronchitis are not readily apparent, as is evident by the disparity of results in clinical trials. The availability of a relatively simple reproducible test system can aid the preclinical evaluation of such compounds and, hence, assist with detection of more potent drugs for clinical evaluation.

REFERENCES

Ahmed T, Greenblatt DW, Birch S, Marchette B, Wanner A. Abnormal Mucociliary Transport in Allergic Patients with Antigen-induced Bronchospasm: Role of Slow Reacting Substance of Anaphylaxis. Am Rev Respir Dis 1981; 124: 110-114.

Aiello EL. Factors affecting ciliary activity on the gill of the mussel Mytilus edulis. Physiol Rev 1960; 33: 130-135.

Aiello E, Guideri G. Nervous control of ciliary activity. Science 1964; 146: 1692-1693.

Aiello E, Guideri G. Distribution and function of the bronchial nerve in the mussel. Biol Bull 1965; 129: 431-438.

Aiello E, Guideri G. Distribution between 5-hydroxytryptamine and nerve stimulation of ciliary activity. J Pharmacol Exp Therap 1966; 154: 517-523.

Albert RE, Arnett LC. Clearance of radioactivity dust from the lung. Arch environm Health 1955; 12: 99-106.

Allegra L, Abraham WM, Chapman GA, Wanner A. Duration of mucociliary dysfunction following antigen challenge. J Appl Physiol 1983; 55: 726-730.

Asmundsson T, Kilburn KH. Mucociliary Clearance Rates at Various Levels in Dog Lungs. Am Rev Respir Dis 1970; 102: 388-397.

Augustein J, Farmer JB, Lee TB, Sheard P, Tattersall ML. Selective inhibition of slow-reacting substance of anaphylaxis. Nature 1973; 245: 215-217.

Aurnhammer W, Konietzko N, Matthys H. Problems in evaluating the effect of secretolytic agents on the mucociliary system by means of radioactive particles. Respiration 1977; 34: 92-99.

Azzopardi A, Thurlbeck WM. This histochemistry of non-ciliated bronchiolar epithelial cell. Am Rev Respir Dis 1969; 99: 516-525.

Baetjer AM, Bates LM. Measurement of ciliary activity in the intact trachea. Am Rev Respir Dis 1966; 93: 79-82.

Baker AP, Hillegass LM, Holden DA, Smith WJ. Effect of kallidin, substance P, and other basic polypeptides on the production of respiratory macromolecules. Am Rev Respir Dis 1977; 115: 811-817.

Barnett B, Dulfano MJ. Sputum visco-elasticity I: new methodology. Am Rev Respir Dis 1970; 101: 773-776.

Basbaum CB, Ueki I, Berzina L, Nadel JA. Tracheal submucosal gland serous cells stimulated in vitro with adrenergic and cholinergic agonists. Cell Tissue Res 1981; 220: 481-498.

Bateman JRM, Lennard-Jones AM, Pavia D, Clarke SW. Lung mucociliary clearance in normal subjects during selective and non-selective beta blockade. Clin Sci 1980; 58: 6P.

Bateman JRM, Newman SP, Duant KM, Pavia D, Clarke SW. Regional lung clearance of excessive bronchial secretions during chest physiotherapy in patients with stable chronic airways obstruction. Lancet 1979; 1: 294-297.

Bateman JRM, Agnew JE, Sheahan NF, Pavia D, Clarke SW. Tracheobronchial clearance and ventilatory function after medium dose oral corticosteroid therapy in stable asthmatics. Prog Resp Res 1980; 14: 145-149.

Bateman JRM, Pavia D, Sheahan NF, Clarke SW. Clearance of lung secretions and ventilatory function in patients with stable asthma after inhaled terbutaline sulphate via pressurized metered-dose inhalers. Clin Sci 57: 15P.

Battista SP, Mione P, Lapey A, Gori G, Metcher W. Role of clotting factor(s) in the etiology of lung disease related to ciliostasis. Arch environm Health 1980; 35: 239-246.

Bayer G, Wense T. Die Beeinflussung der Acetylcholinwirkung durch Histamin. Arch Ex Path Pharmakol 1936; 182: 533-536.

Blaich W, Klar G. Der Einflub des Penicillins auf die Flimmerbewegung der Rachenschleimhaut des Frosches. Arch Ex Path Pharmakol 1950; 211: 67-75.

Blair AMJN, Woods A. The effects of isoprenaline and disodium cromoglycate on ciliary motility and mucous flow measured in vivo in cats. Brit J Pharmacol 1969; 35: 379-380.

Boat TF, Cheng PW, Iyer RN, Carlson DM, Polony I. Human respiratory tract secretion. Mucus glycoproteins of non-purulent tracheobronchial secretions, and sputum of patients with chronic bronchitis and cystic fibrosis. Arc Biochem Biophys 1976; 177: 95-104.

Boat TF, Kleinerman JI. Effect of cholinergic and adrenergic agents on in vitro release of protein and mucous glycoprotein. Chest 1975; 67: 32-34.

Boat TF, Matthews LW. Chemical composition of human tracheobronchial secretions. In: Sputum: fundamentals and clinical pathology (Dulfano M.J., ed.) pp.243-274. Thomas, Springfield, III. 1973.

Borson DB, Charlin M, Gold BD, Nadel JA. Nonadrenergic, noncholinergic nerves mediate secretion of macromolecules by tracheal glands of ferrets. Fedn Proc 1982; 41: 1754P.

Borson DB, Chinn RA, Davis B, Nadel JA. Adrenergic & cholinergic nerves mediate fluid secretion from tracheal glands of ferrets. J Appl Physiol 1980; 49: 1027-1031.

Boyd EM, Jackson S, Ronan M. The effect of sympathomimetic amines upon the output of respiratory tract fluid in rabbits. Am J Physiol 1943; 138: 565-568.

Braga P. Macrophotographic evidence of the activity of Sobrerol on mucociliary transport in the frog's palate. Clinical Trials Journal 1981; 18: 30-38.

Breeze RG, Wheeldon EB. The cells of the pulmonary airways. Am Rev Respir Dis 1977; 116: 705-777.

Brogan TD. The high molecular weight components of sputum. Biochem J 1959; 71: 125-131.

Bulbring E, Burn JH. Action of acetylcholine on rabbit auricles in relation to acetylcholine synthesis. J Physiol 1949; 108: 508-524.

Bulbring E, Burn JH, Shelley FRS, Shelley H. Acetylcholine and ciliary movement in the gill plates of Mytilius Edulis. Proc Roy Soc B 1953; 141: 445-466.

Bulbring E, Lourie EM, Pardoe AV. Presence of acetylcholine in Trypanosoma rhodesiense and its absence from Plasmodium gallinacean. Brit J Pharmacol 1949; 4: 290-

Burn JH. A discussion on the action of local hormones. Proc Roy Soc B 1950; 137: 281-320.

Burn JH, Day M. The action of tubocurarine and acetylcholine on ciliary movement. J Physiol 1958; 141: 520-526.

Calvert J. Effect of drugs on the secretion from tracheal mucous membrane. J Physiol 1896; 20: 158-164.

Camner P, Helstrom P, Philipson K. Carbon dust and mucociliary transport. Arch environm Health 1973; 26: 294-296.

Camner P, Strandberg K, Philipson K. Increased mucociliary transport by cholinergic stimulation. Arch environm Health 1974; 29: 220-224.

Camner P, Strandberg K, Philipson K. Increased mucociliary transport by adrenergic stimulation. Arch environm Health 1976; 31: 79-82.

Chakrin JW, Baker AP, Christian P, Wardell JR. Effect of cholinergic stimulation on the release of macromolecules by canine trachea in vitro. Am Rev Respir Dis 1973; 108: 69-76.

Charman J, Reid L. Sputum viscosity in chronic bronchitis, bronchiectasis, asthma and cystic fibrosis. Biorheology 1972; 9: 185-199.

Chopra SK. Effect of atropine on mucociliary transport velocity in aenesthetized dogs. Am Rev Respir Dis 1978; 118: 367-371.

Chopra SK, Taplin GV, Elam D, Carson SA, Golde D. Measurement of tracheal mucociliary transport velocity in humans - smokers versus nonsmokers (preliminary findings). Am Rev Respir Dis 1979; 119: 205P.

Chopra SK, Taplin GV, Simmons DH, Elam D. Measurement of mucociliary transport velocity in the intact mucosa. Chest 1977; 71: 155-158.

Clarke SW, Lopez-Vidriero MT. The effect of a beta 2 agonist on the activity of human bronchial cilia in vitro. J Physiol 1983; 336: 40P.

Corssen G, Allen CR. Acetylcholine: its significance in controlling ciliary activity of human respiratory epithelium in vitro. J Appl Physiol 1959; 14: 901-904.

Creeth JM. Constituents of mucus and their separation. Br Med Bull 1978; 34: 17-24.

Dalham T. Mucus flow and ciliary activity in the trachea of healthy rats and rats exposed to respiratory irritant gases. Acta physiol scand 1956; 36: 1-161.

Dalham T. The determination in vivo of the rate of cil iary beat in the trachea. Acta physiol scand 1960; 49: 242-250.

Dalham T, Rylander R. Frequency of ciliary beat measured with a photo-sensitive cell. Nature 1962; 196: 592-593.

Dawson H, Douglas RB, Pavia D, Reeves E, Short MD, Thomson ML. An inexpensive automatic two detector lung scanner. Phys Med Biol 1971; 16: 691-692.

Dulfano MJ, Adler KB. Physical properties of sputum, VII. Rheological properties and mucociliary transport. Am Rev Respir Dis 1975; 112: 341-347.

Dunnill MS. The pathology of asthma, with special reference to changes in the bronchial mucosa. J Clin Path 1960; 13: 27-33.

Dunnill MS, Massarella GR, Anderson JA. A comparison of the

quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. Thorax 1969; 24: 176-179.

Eccles JC.. In: The Physiology of Synapses. Springer Verlag, Berlin. 1964.

Edmunds LH, Stallone RJ, Graf PD, Sagel SS, Greenspan RH. Mucus transport in transplanted lungs of dogs. Surgery 1969; 66: 15-22.

Edwards PAW. Is mucus a selective barrier to macromolecules. Br Med Bull 1978; 34: 55-56.

Edwards GF, Steel AE, Scott JK, Jordan JW. S-carboxymethylcysteine in the fluidification of sputum and treatment of chronic airway obstruction. Chest 1976; 70: 506-513.

Eliasson R, Mossberg B, Camner P, Afzelius BA. The immotile cilia syndrome. A congenital ciliary abnormality as an etiologic factor in chronic airway infection and male sterility. New Engl. J. Med 1977; 297: 1-6.

Fawcett DW, Porter KR. A study of the fine structure of ciliated epithelia. J Morphology 1954; 94: 221-264.

Feldberg W, Lin RCY. Synthesis of acetylcholine in the wall of the digestive tract. J Physiol 1950; 111: 96-118.

Felicetti SA, Wolff RK, Muggenburg BA. Comparison of tracheal mucous

transport in rats, guinea pigs, and dogs. J Appl Physiol 1981; 51: 1612-1617.

Florey H, Carleton HM, Wells AQ. Mucus secretion in the trachea. Br J Exp Pathol 1932; 13: 269-284.

Forbes AR. Humidification and mucus flow in the intubated trachea. Br J Anaes 1973; 45: 874-878.

Foster WM, Langenback E, Bohning D, Bergofsky EH. Quantitation of mucus clearance in peripheral lung and comparison with tracheal and bronchial mucus transport velocities in man: adrenergics return depressed clearance and transport velocities in asthmatics to normal. Am Rev Respir Dis 1978; 117: 337P.

Foster WM, Bergofsky EH, Bohning DE, Lippman M, Albert RE. Effect of adrenergic agents and their mode of action on mucociliary clearance in man. J Appl Physiol 1976; 41: 146-152.

Friedman M, Stott FD, Poole DO, Dougherty R, Chapman GA, Watson H, Sackner MA. A new roentgenographic method for estimating mucous velocity in airways. Am Rev Respir Dis 1977; 115: 67-72.

Gallagher JT, Hall RL, Jeffery PK, Phipps RJ, Richardson PS. The nature and origin of tracheal secretions released in response to pilocarpine and ammonia. J Physiol 1978; 275: 36P.

Gallagher JT, Kent PW, Passatore M, Phipps RJ, Richardson PS. The composition of tracheal mucus and nervous control of its secretion in

the cat. Proc Roy Soc B 1975; 192: 49-76.

Gamsu G, Weintraue RM, Nadel J. Clearance of tantalum from airways of different caliber in man evaluated by a roentgenographic method. Am Rev Respir Dis 1973; 107: 214-224.

German VF, Corrales R, Ueki IF, Nadel JA. Reflex stimulation of tra cheal mucus gland secretion by gastric irritation in cats. J Appl Physiol 1982; 52: 1153-1155.

German VF, Ueki IF, Nadel JA. Micropipette measurement of airway submucosal gland secretion: Laryngeal reflex. Am Rev Respir Dis 1980; 122: 413-416.

Gibbons IR, Rowe AJ. Dynein: a protein with adenosine triphosphatase activity from cilia. Science 1965; 149: 424-426.

Gibson LE. Use of water vapour in the treatment of lower respiratory disease. Am Rev Respir Dis 1974; 110: 100P.

Gisiger V, Gautron J, Dunant Y. Differences in acetylcholinesterase of neuro-muscular and sympathetic junctions. Experientia 1977; 33: 804P.

Gisiger V, Vigny M, Gautron J, Rieger F. Acetylcholinesterase of rat sympathetic ganglion: molecular forms, localization and effects of denervation. J Neurochem 1978; 30: 501-516.

Goco RV, Kress MB, Brantigan OC. Comparison of mucus glands in the

tracheobronchial tree of man and animals. Ann NY Acad Sci 1963; 106: 555-571.

Goldstein SF, Holwill MEJ, Silvester NR. The effects of laser microbeam irradiation on the flagellum of Crithidia (strigomonas) oncopelti. J Exp Biol 1970; 53: 401-409.

Goodman RM, Yergin BM, Landa JF, Golinvaux MH, Sackner MA. Relationship of smoking history and pulmonary function tests to tracheal mucous velocity in nonsmokers, young smokers, ex-smokers and patients with chronic bronchitis. Am Rev Respir Dis 1978; 117: 205-214.

Goodman RM, Yergin BM, Sackner MA. Effects of S-carboxymethylcysteine on tracheal mucus velocity. Chest 1978; 74: 615-618.

Gray J. In: Ciliary Movement, Cambridge University Press, Cambridge. 1928.

Havez R, Degrand P, Roussel P, Randoux A. Mode d´action biochemique des derive´s de la cysteine sur le mucus bronchique. Poumon Coeur 1970; 26: 81-90.

Havez R, Roussel P, Degand P, Biserte G. Etude des structures fibrillaires de la secretion bronchique humaine. Clin chim acta 1967; 17: 281-295.

Havez R, Roussel P, Degand P, Biserte G. In: Protides of the biological fluids: Proceedings of the Sixteenth Colloquium, Bruges,

1968 (Peeters H., ed.) pp. 343-360. Pergamon Press, Oxford and New York. 1968.

Hendry WF, Whitfield HN, Stansfeld AG, Prys-Davies J, Ryder TA, Knight RK, Pavia D, Bateman JRM, Clarke SW. Defects in Young's syndrome and Kartagener's syndrome. Lancet 1978; 2: 1152.

Hilding AC. The relation of ciliary insufficiency to death from asthma and other respiratory diseases. Ann Oto Rhin Laryn (St Louis) 1943; 52: 5-19.

Hill JR. The influence of drugs on ciliary activity. J Physiol 1957; 139: 157-166.

Hirsch JA, Tokayer JL, Robinson MJ, Sackner MA. Effects of dry_{λ}^{air} and subsequent humidification on tracheal mucus velocity in dogs. J Appl Physiol 1975; 39: 242-246.

Hoffman-Berling H. Adenosintriphosphat als Betreibstoff von Zellbewegung. Biochim biophys Acta 1954; 182: 182-194.

Hoffman-Berling H. Geisselmodelle und Adenosintriphosphat. Biochim biophys Acta 1955; 16: 146-154.

Inoue S, Hogg JC. Intercellular junctions of the tracheal epithelium in guinea-pigs. Lab Invest 1974; 31: 68-74.

Iravani J. Flimmerbewegung in den intrapulmonalen Luftwegen der Ratte. Pflugers Archiv ges Physiol 1967; 297: 221-227.

Iravani J, Melville GN. Mucociliary function of the respiratory tract as influenced by drugs. Respiration 1974; 31: 350-357.

Iravani J, Norris Melville G. Mucociliary Activity in the Respiratory Tract as Influenced by Prostaglandin E1. Respiration 1975 (a); 32: 305-315.

Iravani J, Melville GN. Wirkung von Pharmaka und Milieuanderungen auf die Flimmertatigkeit der Atemwege. Respiration 1975 (b); 32: 157-164.

Iravani J, Melville GN, Horstman G. N-acetylcysteine and mucociliary activity in mammalian airways. Drug Res 1978; 28: 250-257.

Jeffery PK. Structure and function of mucus-secreting cells of cat and goose airway epithelium. In: Respiratory tract mucus. Ciba Foundation Symposium 54 (new series). (Ed. R. Porter). Elsevier, New York. 1978; pp 5-20.

Jeffery PK, Reid L. New observations of rat airway epithelium: A quantitative and electron microscopic study. J Anat 1975; 120: 295-320.

Jenkins P, Pavia D, Bateman JRM, Clarke SW, Citron KM, Bannister R. Mucociliary clearance in patients with chronic autonomic failure. Thorax 1980; 35: 690-693.

Keal EE, Reid L. Neuraminic acid content of sputum in chronic bronchitis. Thorax 1972; 27: 643-653.

Kenoyer JL, Phalen RF, Davis JR. Particle clearance from the respiratory tract as a test of toxicity: Effect of ozone on short and long term clearance. Exp Lung Res 1981; 2: 111-120.

Kent PW, Widdicombe JG. Neurophysical control of glycoprotein secretion in the tracheal epithelium. Biochem Soc Trans 1977; 5: 414-417.

Kilburn KH. Mucociliary clearance from bullfrog (Rana cantesbiana) lung. J Appl Physiol 1967; 23: 804-810.

King M. Viscoelastic properties of airway mucus. Fedn Proc 1980; 39: 3080-3085.

King M. Is cystic fibrosis mucus abnormal. Pediat res 1981; 15: 120-122.

King M, Gilboa A, Meyer FA, Silberberg A. On the transport of mucus and its rheologic stimulants in ciliated systems. Am Rev Respir Dis 1974; 110: 740-745.

Kinosita H, MuraKami A. Control of ciliary Motion. Physiol Rev 1967; 47: 53-82.

Kokin P. Pflugers Arch ges Physiol 1896; 63: 622-630.

Konietzko N, Klopfer M, Adam WE, Matthys H. The influence of beta-adrenergic stimulation on the mucociliary system of the human lung. Pneumologie 1975; 152: 203-208.

Kordik P, Bulbring E, Burn JH. Ciliary movement and acetylcholine. Brit J Pharmacol 1952; 7: 67-79.

Korhonen LK, Holopainen E, Paavolainen M. Some histochemical characteristics of tracheobronchial and pulmonary neoplasms. Acta Histochem 1969; 32: 57-73.

Landa JF, Hirsch JA, Lebeaux MI. Effects of topical and general anaesthetic agents on tracheal mucus velocity of sheep. J Appl Physiol 1975; 38: 946-948.

Lierle DM, Moore PM. Further study of the effects of drugs on ciliary activity: a new method of observation in the living animal. Ann Oto Rhin Laryn (St Louis) 1935; 44: 671-684.

Lopez-Vidriero MT, Das I, Picot R, Smith AP, Reid L. Bronchial secretions from normal human airways after inhalation of prostaglandin F2 alpha, acetylcholine, histamine and citric acid. Thorax 1977; 32: 734-739.

Lopez-Vidriero MT, Reid L. Bronchial mucus in health and disease. Br Med Bull 1978; 34: 63-74.

Lucas AM. Coordination of ciliary movement; methods of study and relation of ciliary coordination to ciliary inhibition. J Morph 1932; 53: 243-263.

Lucas AM. Comparison of ciliary activity under in vitro and in vivo conditions. Proc Soc Exp Biol Med 1933; 30: 501-506.

Lucas AM. Neurogenous activation of ciliated epithelium. Am J Physiol 1935; 112: 468-476.

Malanga CJ, Yong MC, O´Donnell JP. Pharmacological characterization of the dopaminergic stimulation of mucociliary particle transport by Mytilus edulis gill. Gen Pharmac 1981; 12: 139-144.

Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Analyt. Biochem 1978; 87: 206-210.

Maruyama I. Conflicting effects of noradrenaline on ciliary movement of frog palatine mucosa. Eur J Pharmacol 1984; 97: 239-245.

Masson PL, Heremans JF. Sputum proteins. In: Sputum: fundementals and clinical pathology (Dulfano MJ ed.), pp.404-468, Thomas, Springfield, Illinois, USA. 1973.

Matthys H, Kohler D. Effect of theophylline on mucociliary clearance in man. Eur J Respir Dis 1980; 61: 98-102.

Maurer DR, Sielczak M, Oliver W, Abraham WM, Wanner A. Role of ciliary motility in acute allergic mucociliary dysfunction. J Appl Physiol 1982; 52: 1018-1023.

McDonald JF, Leisure CE, Lenneman EE. Newly discovered controls of ciliary activity. Am J Physiol 1928; 85: 395P.

Melville GN, Ismail S, Sealy C. Tracheobronchial function in health

and disease, effect of mucolytic substances. Respiration 1980; 40: 329-336.

Messerklinger W. Die Schleimhaut der oberen Luftwege im Blickfeld neuerer Forschung. Arc Ohr Nas Kehlk 1958; 173: 1-12.

Meyrick B, Reid L. Ultra structure of cells in the human bronchial submucosal glands. J Anat 1970; 107: 281-299.

Meyrick B, Sturgess JM, Reid L. A reconstruction of the duct system and secretory tubules of the human bronchial submucosal gland. Thorax 1969; 24: 729-736.

Mezey RJ, Cohn MA, Fernandez RJ, Januszkiewicz AJ, Wanner A. Mucociliary Transport in Allergic Patients with Antigen-Induced Bronchospasm. Am Rev Respir Dis 1978; 118: 677-684.

Milton AS. The action of tubocurarine on ciliary movement. Br J Pharmacol 1959; 14: 323-326.

Mollison PL. Labelling of red cells with radioactive phosphorus. In Blood Transfusion in Clinical Medicine, 4th edition pp. 722-724. Oxford and Edinburgh: Blackwell Scientific Publications. 1967.

Morley J, Sanjar S. Parasympathetic regulation of mucociliary transport in the frog oesophagus. J Physiol 1983; 334: 45-46P.

Morson BC. Intestinal metaplasia of gastric mucosa. Br J Cancer 1955; 9: 365-376.

Mossberg B, Strandberg K, Philipson K, Camner P. Tracheobronchial clearance and Beta-Adrenoceptor stimulation in patients with chronic bronchitis. Scand J Resp Dis 1976; 57: 281-289.

Mossberg B, Afzelius BA, Eliasson R, Camner P. On the pathogenesis of obstructive lung disease. A study on the immotile-cilia syndrome. Scand J Resp Dis 1978; 59: 55-65.

Nelson DJ, Wright EM. The distribution, activity, and function of the cilia in the frog brain. J Physiol 1974; 243: 63-78.

Nilsson G, Dahlberg K, Brodin E, Sundler F, Strandberg K. Distribution and constrictor effect of Substance P in guinea pig tracheobronchial tissue. In: `Substance P´ Nobel Symposium 37 (Ed. von Euler, U.S. and Pernow, B. pp. 75-81. New York: Raven Press). 1977.

Oliver RE, Davis B, Marin MG, Nadel JA. Active transport of Na and Cl and chloride across the canine tracheal epithelium in vitro. Am Rev Respir Dis 1975; 112: 811-815.

Palmer KNV. Reduction of sputum viscosity by a water aerosol in chronic bronchitis. Lancet 1960; 1: 91.

Palmer KNV, Ballantyne D, Diament ML, Hamilton WFD. The rheology of bronchitic mucus. Br J Dis Chest 1970; 64: 185-191.

Paparo A, Aiello E. Cilio-inhibitory effects of branchial nerve stimulation in the mussel, Mytilus edulis. Comp Gen Pharmac 1970; 1: 241-250.

Patrick G, Stirling C. Measurement of mucociliary clearance from the trachea of conscious and anesthetized rats. J Appl Physiol 1977; 42: 451-455.

Pavia D, Bateman JRM, Clarke SW. Deposition and clearance of inhaled particles. Bull europ Physiopath resp 1980; 16: 335-366.

Pavia D, Bateman JRM, Sheahan NF, Clarke SW. Clearance of lung secretions in patients with chronic bronchitis: effect of terbutaline and ipratropium bromide aerosols. Eur J Respir Dis 1980; 61: 245-253.

Pavia D, Thomson ML. Inhibition of mucociliary clearance from the human lung by hyoscine. Lancet 1971; 27: 449-450.

Pavia D, Thomson ML, Clarke SW. Enhanced clearance of secretions from the human lung after the administration of hypertonic saline aerosol. Am Rev Respir Dis 1978; 117: 92-96.

Peatfield AC, Barnes PJ, Bratcher C, Nadel JA, Davis B. Vasoactive intestinal peptide stimulates tracheal submucosal gland secretion in ferret. Am Rev Respir Dis 1983; 128: 89-93.

Peatfield AC, Piper PJ, Richardson PS. The effect of leukotriene C4 on mucin output into the cat trachea. J Physiol 1982; 325: 56-57P.

Peatfield AC, Richardson PS. The control of mucin secretion into the lumen of the cat trachea by alpha- and beta-adrenoceptors, and their relative involvement during sympathetic nerve stimulation. Eur J

Pharmacol 1982; 81: 617-626.

Peatfield AC, Richardson PS. Evidence for non-cholinergic, non-adrenergic nervous control of mucus secretion into the cat trachea. J Physiol 1983 (a); 342: 335-345.

Peatfield AC, Richardson PS. The action of dust in the airways on secretion into the trachea of the cat. J Physiol 1983 (b); 342: 327-334.

Perry WF, Boyd EM. Method for studying expectorant action in animals by direct measurement of output of respiratory tract fluid. J Pharmacol Exp Therap 1941; 73: 65-77.

Peterson M, Leblond C. Synthesis of complex carbohydrates in the Golgi region, as shown by auto-radiography after injection of labelled glucose. J Cell Biol 1964; 21: 143-148.

Phipps RJ, Nadel JA, Davis B. Effect of alpha-adrenergic stimulation on mucus secretion and ion transport in cat trachea in vitro. Am Rev Respir Dis 1980; 121: 359-365.

Phipps RJ, Richardson PS. The effect of irritation at various levels of the airway upon tracheal mucus secretion in the cat. J Physiol 1976; 261: 563-581.

Phipps RJ, Richardson PS. The nervous and pharmacological control of tracheal mucus secretion in the goose. J Physiol 1976; 258: 116-117.

Phipps RJ, Richardson PS, Corfield A, Gallagher JT, Jeffery PK, Kent PW, Passatore M. A physiological, biochemical and histological study of goose tracheal mucin and its secretion. Phil Trans R Soc London B Biol Sci 1977; 279: 513-543.

Plattner F, Hou CL. Zur Frage des Angriffspunktes vegetativer Gifte. Versuche am Embryonalherzen und am Flimmerepithel. Pflu Arch ges Phys 1931; 228: 281-294.

Pohle K. Zur Frage der nervosen Regulation des Flimmerepithels bei Wirbelteiren. Arch Ex Path Pharmakol 1931; 159: 452-462.

Polgar G, Denton R. Cystic Fibrosis in adults. Am Rev Respir Dis 1962; 85: 319-327.

Proetz AW. Motion picture demonstration of ciliary action and other factors of nasal physiology. Trans Amer Laryng Assoc 1932; 54: 264-273.

Puchelle E, Girard F, Zahm JM. Rheology of bronchial secretions and mucociliary transport. Bull europ Physiopath resp 1976; 12: 771-779.

Querinjean P, Mason PL, Heremans JF. Molecular weight, single-chain structure and amino acid composition of human lactoferrin. Eur J Biochem 1971; 20: 420-425.

Reid L. Measurement of bronchiole mucous gland layer: A diagnostic yardstick in chronic bronchitis. Thorax 1960; 15: 132-141.

Reid L, Clamp JR. The biochemical and histochemical nomenclature of mucus. Br Med Bull 1978; 34: 5-8.

Rhodin JAG. The ciliated cell: Ultrastructure and function of human tracheal mucosa. Am Rev Respir Dis 1966; 93: 1-15.

Richardson PS, Phipps RJ, Balfre K, Hall RL. The roles of mediators, irritants and allergens in causing mucin secretion from the trachea. Respiratory tract mucus Ciba foundation symposium Elsevier. 1978; 54: pp 111-131.

Rigler L, Koucky R. Roentgen studies of the pathology of bronchial asthma. Am J Roentgenol 1938; 39: 353-362.

Roberts GP. Isolation and characterisation of glycoproteins from sputum. Eur J Biochem 1974; 50: 265-280.

Roberts GP. Chemical aspects of respiratory mucus. Br Med Bull 1978; 34: 39-41.

Rozansky N. Russ Fiziol 1926; 9: 556.

Rutland J, Cole PJ. Non-invasive sampling of nasal cilia for measurement of beat frequency and study of ultrastructure. Lancet 1980; 564-565.

Sackner MA, Chapman GA, Dougherty RD. Effects of nebulized ipratropium bromide and atropine sulphate on traceal mucous velocity and lung mechanics in aenesthetized dogs. Respiration 1977; 34: 181-185.

Sackner MA, Epstein S, Wanner A. Effect of betaadrenergic agonists aerosolized by freon propellant on tracheal mucus velocity and cardiac output. Chest 1976; 69: 593-598.

Sackner MA, Reinhart M, Arkin B. Effects of beclomethasone dipropionate on tracheal mucous velocity. Am Rev Respir Dis 1977; 115: 1069-1070.

Sackner MA, Rosen MJ, Wanner A. Estimation of tracheal mucous velocity by bronchofiberscopy. J Appl Physiol 1973; 34: 495-499.

Sackner MA, Yergin BM, Brito M, Januszkiewicz A. Effect of adrenergic agonists on tracheal mucus velocity. Bull europ physiopath resp 1979; 15: 505-511.

Sade J, Eliezer N, Silberberg A, Nevo AC. The role of mucus in transport by cilia. Am Rev Respir Dis 1970; 102: 48-51.

Sanchis J, Dolovich M, Chalmers R, Newhouse M. Quantitation of regional aerosol clearance in the normal human lung. J Appl Physiol 1972; 33: 757-762.

Santa Cruz R, Landa J, Hirsch J, Sackner. Tracheal mucous velocity in normal man and patients with obstructive lung disease; effects of terbutaline. Am Rev Respir Dis 1974; 109: 458-463.

Satoh K. Studies of cholinesterase activity of palatal ciliated mucosa in toads. The Hirosaki Med J 1959; 10: 196-201.

Satoh K. Cholinesterase activity of palatal ciliated mucosa in toad. The Hirosaki Med J 1959; 10: 9-19.

Serafini SM, Michaelson ED. Length and distribution of cilia in human and canine airways. Bull europ Physiopath resp 1977; 13: 551-559.

Serafini SM, Wanner A, Michaelson ED. Mucociliary transport in central and intermediate size airways : effect of aminophyllin. Bull europ Physiopath resp 1976; 12: 415-422.

Seyfarth KE. Accelerated elimination of bronchographic contrast media by means of a mucolytic preparation. Praxis Pneumol 1964; 18: 803-810.

Sharpey W. Cilia are found in all animal phyla except nematoda. In: "Cyclopedia of Anatomy and Physiology" (R.B. Todds, ed.) pp.606-638. Longman, Brown, Green, Longmans and Roberts, London. 1835.

Sheffner AL, Medler EM, Jacobs LW, Sarett HP. The in vitro reduction in viscosity of human tracheo-bronchial secretions by acetylcysteine. Am Rev Respir Dis 1964; 90: 721-729.

Shih CK, Litt M, Khan MA, Wolf DP. Effect of nondialyzable solids concentration and viscoelasticity on ciliary transport of tracheal mucus. Am Rev Respir Dis 1977; 115: 989-995.

Short MD, Dowsett DJ, Heaf PJD, Pavia D, Thomson ML. A comparison between monodispersed Tc-99m labelled aerosol particles and Kr-81m for the assessment of lung function. J Nuc Med 1979; 20: 194-200.

Sleigh MA. In: The Biology of Cilia and Flagella, Pergamon Press, Oxford. 1962.

Spiegelman JR, Hanson GD, Lazarus A, Bennett RJ, Lippman M, Albert RE. Effect of acute sulfur dioxide exposure on bronchial clearance in the donkey. Arch environm Health 1968; 17: 321-326.

Sturgess JM, Chao J, Wong J, Aspin N, Turner JAP. Cilia with defective radial spokes. A cause of human respiratory disease. New Engl J Med 1979; 300: 53-56.

Sturgess J, Reid L. An organ culture study of the effect of drugs on the secretory activity of the human bronchial submucosal gland. Clin Sci 1972; 43: 533-543.

Sutton PP, Pavia D, Bateman JRM, Clarke SW. The effect of oral aminophylline on lung mucociliary clearance in man. Chest 1981; 80: 889-891.

Thomson ML, Pavia D, Gregg I I, Stark JE. Bromhexine and mucociliary clearance in chronic bronchitis. Br J Dis Chest 1974; 68: 21-27.

Thomson ML, Pavia D, Jones CJ, Mcquiston TA. No demonstrable effect of S-carboxymethylcysteine on clearance of secretions from the human lung. Thorax 1975; 30: 669-673.

Thurlbeck WM, Malaka D, Murphy K. Goblet cells in the peripheral airway in chronic bronchitis. Am Rev Respir Dis 1975; 112: 65-69.

Tomiak RHH. An investigation of vagal bronchoconstriction in the guinea-pig. J Physiol 1984; 346: 99P.

Uddman R, Alumets J, Densert O, Hakanson R, Sundler F. Occurrence and distribution of VIP nerves in the nasal mucosa and tracheobronchial wall. Acta Otolaryngol 1978; 86: 443-448.

Ueki I, German VF, Nadel JA. Micropipette measurement of airway submucosal gland secretion. Am Rev Respir Dis 1980; 121: 351-357.

Vorhaus EF, Deyrup IJ The effect of adenosinetriphosphate on the cilia of the pharyngeal mucousa of the frog. Science 1953; 118: 553-554.

Wanner A, Hirsch JA, Greeneltch DE, Swenson EW, Fore T. Tracheal mucous velocity in beagles sfter chronic exposure to cigarette smoke. Arch environm Health 1973; 27: 370-372.

Wanner A, Maurer D, Abraham WM, Szepfalusi Z, Sielczak M. Effects of chemical mediators of anaphylaxis on ciliary function. J Allergy Clin Immunol 1983; 72: 663-667.

Wanner A, Zarzecki S, Hirsch J, Epstein S. Tracheal mucous transport in experimental canine asthma. J Appl Physiol 1975; 39: 950-957.

Wardell JR, Chakrin LW, Payne BJ. The canine tracheal pouch: a model for use in respiratory mucus research. Am Rev Respir Dis 1970; 101: 741-745.

Warner FD. New observations on flagellar fine structure. The

relationship between matrix structure and microtubule component of axoneme. J Cell Biol 1970; 47: 159-182.

Webb WR, Jackson MD. Clinical evaluation of a new mucolytic agent, acetylcysteine. J Thorac Cardiovasc Surg 1962; 44: 330-343.

Weiss T, Dorow P, Felix R. Effects of a beta adrenergic drug and a secretolytic agent in regional mucociliary clearance in patients with COLD. Chest 1981; 80: 881-885.

Widdicombe JG. Action potentials in parasympathetic and sympathetic efferent fibres to the trachea and lungs of cats and dogs. J Physiol 1966; 186: 56-88.

Widdicombe JG. Control of secretion of tracheobronchial mucus. Br Med Bull 1978; 34: 57-61.

Widdicombe JH, Walsh MJ. Ion transport by dog tracheal epithelium. Fedn Proc 1980; 39: 3062-3066.

Wood RE, Wanner A, Hirsch J, Farrell P. Tracheal mucociliary transport in patients with cystic fibrosis and its stimulation by Terbutaline. Am Rev Respir Dis 1975; 111: 733-738.

Yager J, Chen T-M. Measurement of frequency of ciliary beats of human respiratory epithelium. Chest 1978; 73: 627-633.

Yager JA, Ellman H, Dulfano MJ. Human ciliary beat frequency at three levels of the tracheobronchial tree. Am Rev Respir Dis 1980; 121: 661-665.

Yeates DB, Aspin N, Levison H, Jones MT, Bryan AC. Mucociliary tracheal transport rates in man. J Appl Physiol 1975; 39: 487-495.

Foster WM, Langenback E, Bergofsky EH, Lung clearance and central airway transport of mucus in asymptomatic smokers and age-matched healthy subjects : comparison of control and $beta_2$-adrenergic clearance. Amer Rev respir Dis 1979 119: 309P

Spektor DM, Pitt BR, Yeates DB. Changes in regional mucociliary transport in the human lung resulting from systemic $beta_2$-adrenergic stimulation. Amer Rev respir Dis 1979 119: 236P.

Wong JW, Keens TG, Wannamaker EM, Douglas PT, Crozier N, Levison H, Aspin N. Effects of gravity on tracheal mucus transport rates in normal subjects and in patients with cystic fibrosis. Pediatrics 1977 60: 146-152.