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PHARMACOLOGICAL ANTAGONISM OF MAST CELL SECRETION  
IN HUMAN LUNG TISSUE

- BY -

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Thesis for the degree of Doctor of Philosophy of the  
University of Southampton.

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ABSTRACT

FACULTY OF MEDICINE

CLINICAL PHARMACOLOGY

Doctor of Philosophy

PHARMACOLOGICAL ANTAGONISM OF MAST CELL SECRETION IN HUMAN LUNG TISSUE

by Kevin Douglas Young

Sodium cromoglycate, salbutamol and chlorpromazine have been studied for their effects on antigen-induced histamine release from mast cells in passively sensitised human lung tissue. The tissue response to antigen and drugs was found to be extremely varied; only gross effects of drugs were detectable. All 3 agents inhibited histamine release but their characteristics of inhibition were quite different. Cromoglycate was a weak and partial antagonist to which the tissue became tolerant on prolonged exposure. Salbutamol was much more potent and effective and did not exhibit tolerance. Chlorpromazine was the least potent drug which also liberated histamine from the tissue if the dose range or length of pre-incubation was extended too far. These diverse characteristics, and the demonstration that salbutamol and chlorpromazine were fully effective in lung desensitised to cromoglycate, suggest that the 3 drugs inhibit secretion in lung mast cells via totally different mechanisms.

Cromoglycate acted neither as a stabiliser of the plasmalemma of mast cells nor as a chelator or antagonist of calcium. Its mode of action remains unknown. Tolerance to the drug seemed to involve hyperstimulation, e.g. of a membrane receptor or cellular process, and was difficult to reverse. Salbutamol inhibited secretion through stimulation of lung  $\beta$  receptors. Chlorpromazine antagonised secretion in a similar manner to the local anaesthetic, lignocaine. It acted directly on the plasmalemma of mast cells but did not seem to compete with calcium for a binding site.

The mechanistic and clinical implications of these findings are discussed.

AIMS OF PROJECT

Mast cells contain many inflammatory substances which they secrete in response to specific antigens. It is generally accepted that release of these substances in the lungs of susceptible individuals directly or indirectly leads to the clinical state of asthma (Austen and Orange, 1975). This has stimulated an intensive search for drugs which curb their release. However, the pharmacological mechanisms by which secretion in human lung mast cells may be inhibited are not fully understood. A sound knowledge of these mechanisms is essential to the development of future anti-asthmatic drugs.

Using fresh human lung tissue, the main aims of the project were

- a) to compare and contrast the effects of 3 different inhibitors of mast cell secretion i.e. the specific anti-allergic compound, sodium cromoglycate, the  $\beta$  adrenergic agonist, salbutamol, and the major tranquilliser, chlorpromazine.
- b) to investigate the mechanism of action of each drug.

PUBLICATIONS

The work gave rise to 2 society communications, both of which have been accepted for publication.

- 1) Tolerance to sodium cromoglycate in human lung fragments in vitro. Presented to the British Pharmacological Society at the University of Bristol in April 1980.  
(YOUNG KD and CHURCH MK, Br.J.Pharmac., 70, 122-123P).
- 2) Comparison of sodium cromoglycate derivatives, anti-histamines and  $\beta$ -stimulants on histamine release from human lung in vitro. Presented at the 13th symposium of the Collegium Internationale Allergologicum at Inselhotel, Konstanz in Germany, in July 1980.  
(CHURCH MK and YOUNG KD, Int.Archs Allergy appl.Immun., in press).

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CHAPTER I.   GENERAL INTRODUCTION

Asthma is a complex disease of the bronchi which is unique to man. Many generations have been aware of the symptoms of this disease; asthma itself was a term used by the ancient Greeks to mean "panting". Asthma is characterised by an increased responsiveness of the trachea and bronchi to various stimuli and is manifested by widespread narrowing of the airways which may reverse spontaneously or as a result of therapy (Vaughan, Eng, Mathison, Stevenson, Berman and Braude, 1973).

Episodes of asthma may be precipitated by many factors: inhalation of antigenic substances, bronchial infection, air pollution, laughing or coughing, exercise, emotional stress or by a conditioned response (Howell, 1977). Around 1190 A.D., the Jewish philosopher Moses Maimonides wrote "... asthma has many aetiological aspects and should be treated according to the various causes that bring it about. Physicians are well aware that this disease cannot be managed successfully without a full knowledge of the patient's condition as a whole ..." (cited in Boyd, 1980). Eight hundred years later, the situation has not altered. There is no single pathological change which accounts for this disease and no all-embracing treatment.

There is, however, substantial evidence that asthma frequently has an allergic basis. A medical practitioner named Blackley was the first to demonstrate this in 1873. In a study of patients who suffered from asthma and hay fever, he showed that application of pollen grains to abraded areas of the skin elicited an immediate wheal-and-flare reaction. When the same patients were given inhalations of pollen they developed symptoms of asthma and rhinitis (Blackley, 1873). In immunological terms, such subjects are said to exhibit immediate hypersensitivity, ie they develop an inflammatory response, called an anaphylactic reaction, immediately they come into contact with the substance to which they are sensitive. In young and middle-aged patients with asthma, there appears to be a high incidence of immediate hypersensitivity (Dodge and Burrows, 1980).

From many years of research, it has evolved that immediate hypersensitivity diseases are caused by an interaction between antigens and specific antibodies on the surface of tissue mast cells. This interaction causes the cells to release various substances which produce inflammation.

### The mast cell as a source of inflammatory substances

The mast cell was first described by Ehrlich in 1877. Under the light microscope, Ehrlich identified certain connective tissue cells which were packed with granules. He named them "Mastzellen", meaning well- or over-fed cells. In man, mast cells are found in the loose connective tissues of all organs except the brain (Riley, 1959).

The first inflammatory substance to be identified in mast cells was histamine. In 1941, Jaques and Waters showed that the liver of dogs undergoing anaphylactic shock contained fewer mast cells than normal, whilst the surviving cells appeared severely damaged. In canine anaphylaxis, the liver was thought to be the main source of the histamine released so these findings suggested the histamine originated from disrupted or damaged mast cells (Mongar and Schild, 1962). The presence of histamine in mast cells was later confirmed in 1953, in an extensive study conducted by Riley and West.

We now know that the mast cell contains and releases several substances which can cause tissue inflammation. In the resting cell, these substances are confined within the intracellular granules. As reviewed by several authors (recent reviews: Lewis and Austen, 1977; Kaliner, 1980), they may be divided into

- a) those which are loosely bound to the granule matrix and are therefore easily liberated ie histamine and chemotactic factors which attract eosinophils and neutrophils (ECF-A and NCF, respectively);
- and b) those which constitute the granule matrix and are less readily displaced ie heparin, proteolytic enzymes such as chymotrypsin, and various other enzymes.

When these substances are released from the mast cell, they interact with other cell-types in the tissue to generate secondary mediators of inflammation. This obviously exacerbates the inflammatory reaction. In human lung tissue for example, several metabolites of arachidonic acid are generated: products of the cyclo-oxygenase pathway - prostaglandins  $E_1$ ,  $E_2$ ,  $F_{2\alpha}$  and its keto-derivatives (Piper and Walker, 1973;

Adkinson, Newball, Findlay, Adams and Lichtenstein, 1980) and the lipoxigenase-derived hydroxyeicosatetraenoic acids (Hamberg, Hedqvist and Radegran, 1980) and leukotrienes (Lewis, Austen, Drazen, Clark, Marfat and Corey, 1980). Bradykinin is also generated due to the release of a mast cell kallikrein (Kaliner, 1980).

#### Role of mast cells and mediators in allergic asthma

When asthmatic subjects inhale substances to which they are sensitive, mast cell-derived mediators are released from their lungs into the arterial circulation. This is accompanied by a decrease in respiratory flow due to bronchoconstriction (Bhat, Arroyave, Marney, Stevenson and Tan, 1976; Atkins, Norman and Zweiman, 1978). It seems likely that the mediators are responsible for the bronchoconstrictor effect because, together with secondary mediators generated from other cell-types, they are capable of producing most, if not all, of the pathological changes seen in asthma. Histamine, the leukotriene SRS-A (slow-reacting substance of anaphylaxis), bradykinin and certain prostaglandins all contract smooth muscle, so they may be responsible for the bronchospasm. Studies of isolated bronchial tissue have provided good evidence for this. Antigen challenge of a chain of sensitised bronchial rings (Schild, Hawkins, Mongar and Herxheimer, 1951) or sensitised bronchial strips (Adams and Lichtenstein, 1979) causes a rapidly developing, prolonged contraction of the tissue. Histamine contributes to the early phase of the contraction whereas SRS-A, and leukotrienes in general, are responsible for its protraction (Adams and Lichtenstein, 1979; Dahlén, Hedqvist, Hammarstrom and Samuelsson, 1980).

Other changes seen in the lungs of asthmatics are: increased secretion of mucus from the bronchi, oedema and thickening of the basement membrane of the bronchial mucosa and infiltration of the mucosa by inflammatory cells, particularly eosinophils, neutrophils and macrophages (Vaughan *et al*, 1973). Histamine stimulates sensory receptors of the vagus nerve so this may account for the increased mucus secretion (Gold, 1980). Mucosal oedema may result from an increased capillary permeability induced by histamine, SRS-A and some prostaglandins; release of chemotactic factors from mast cells is almost certainly responsible for the cellular infiltrates of the bronchial tissue (Kaliner, 1980).

Given that mast cell-derived mediators are released in the lungs of patients with allergic asthma and that they are capable of causing the symptoms of the disease, inhibition of the release of these substances should prevent or alleviate the patients' asthma. The pharmacologist endeavours to find suitable drugs which will antagonise the release process. To do this, he must have a full understanding of how mast cells become sensitised to specific antigens, how they become activated and how they expel their content of mediators.

#### A. SENSITISATION OF THE MAST CELL WITH SPECIFIC ANTIBODIES

Studies of the mast cell and the related cell, the basophil, have shown that 2 types of immunoglobulin are capable of binding to these cell-types. The first is IgE (Ishizaka, Tomioka and Ishizaka, 1970; Tomioka and Ishizaka, 1971) and the second is a sub-class of IgG, which in humans is probably IgG<sub>4</sub> (Bach, Bloch and Austen, 1971a,b; Stanworth and Smith, 1973; Parish, 1974). The mechanism of binding of the 2 antibodies is similar in that they both attach via their Fc (fraction crystallisable) portion to specific receptors in the cell membrane (Conrad and Froese, 1976; 1978; Ishizaka, Sterk and Ishizaka, 1979). However, the extents and strengths of their binding are totally different. The proportion of IgE antibodies on the cell surface is much greater than that of IgG (Ishizaka, De Bernardo, Tomioka, Lichtenstein and Ishizaka, 1972). Moreover, the affinity of IgE for the cells is high and in consequence it binds strongly and persistently (Ishizaka and Ishizaka, 1975) whereas the affinity of IgG is relatively poor so its binding is weak and short-lived (Parish, 1973; Möller and König, 1980).

Not only are their differences in the binding of these antibodies, there are also differences in their abilities to mediate anaphylactic reactions. Combination of specific antigen or an anti-IgE antiserum with IgE bound to the surface of mast cells and basophils always causes the release of cell-derived mediators (Ishizaka and Ishizaka, 1975). This is not so with IgG. Exposure of cells sensitised with this type of antibody to antigen or an anti-IgG antiserum sometimes provokes mediator release (Grant and Lichtenstein, 1972; Vijay and Perelmutter, 1975) and sometimes does not (Ishizaka, Sterk and Ishizaka, 1979; van Toorenenbergen and Aalbeerse, 1979).

The frequency with which these antibodies occur in vivo is also different. Specific IgE antibodies are detected in the circulation of most allergic subjects (Ishizaka and Ishizaka, 1973). Anaphylactic IgG antibodies, ie those capable of binding to mast cells and mediating acute allergic reactions, have been found in patients sensitive to milk protein, horse serum, bacterial and avian antigens, grass pollen and the house dust mite Dermatophagoides pteronyssinus (Parish, 1973; Berry and Brighton, 1977; Bryant, Burns and Lazarus, 1973; 1975). However, in a recent survey of 84 patients with various allergies, Brighton found that the frequency of anaphylactic IgG towards common allergens was rare (Brighton, 1980).

There is no doubt that most acute allergic reactions in man are mediated by IgE antibodies. The significance of anaphylactic IgG appears to be limited because of its rare occurrence, poor affinity for mast cells and basophils and its irregular capacity to mediate the release of inflammatory substances. This does not preclude a significant role in individual subjects. Bryant (1977) has stated that whilst anaphylactic IgG is only found in a small number of patients with asthma, in such cases it is often associated with bronchial immediate allergic reactivity.

## B. TRIGGERING OF THE MAST CELL

Interaction of antigen with the Fab (fraction antigen-binding) region of cell-bound IgE is the immunological event which triggers mast cell secretion (Stanworth, 1973). Evidence outlined below shows that the triggering mechanism involves antigen bridging of adjacent IgE molecules which brings IgE receptors in the mast cell membrane into close proximity.

### Antigen bridging of IgE

In 1968, Levine and Redmond studied wheal-and-flare reactions in penicillin-sensitive individuals induced by a series of bivalent, oligo-valent and multivalent benzylpenicilloyl haptens. They found that wheal-and-flare activity required the formation of a complex between 2 or 3 membrane-bound IgE antibodies closely bridged together by the hapten. Ishizaka and Ishizaka obtained similar findings. In an analysis of pre-formed allergen-IgE complexes they found that complexes containing 2 or more IgE antibodies elicited skin reactions whereas complexes of 1 antibody and 2 antigen molecules failed to do so (Ishizaka and Ishizaka, 1968).

Proof that mast cells were activated by bridging of cell-bound IgE came from studies using anti-IgE, its  $F(ab')_2$  dimer and  $Fab'$  monomer fragments to stimulate the cells.  $F(ab')_2$  dimer fragments gave positive wheal-and-flare reactions in normal human skin and provoked histamine release from human leucocytes (Ishizaka and Ishizaka, 1969). They also released mast cell-derived mediators in monkey lung tissue (Ishizaka, Ishizaka, Orange and Austen, 1970). The reaction in each case was qualitatively similar to that of undigested anti-IgE.  $Fab'$  monomer fragments, however, provoked none of these reactions.

From this evidence and that of other studies, it is suggested that IgE which has been cross-linked to form dimers serves as the unit signal for triggering the mast cell (Segal, Taurog and Metzger, 1977).

### Transmission of the antigenic stimulus

Three main hypotheses have been submitted to explain how the antigenic "message" created by bridging of surface-bound IgE molecules is transmitted to the mast cell.

a) Polymerisation of cell-bound IgE

It has been suggested that antigen bridging causes polymerisation of IgE and thereby activates membrane-associated enzymes (Ishizaka, Ishizaka and Lee, 1970; Metzger, 1974). This hypothesis has since been rejected (Ishizaka, Ishizaka, Conrad and Froese, 1978).

b) Allosteric change in IgE

Stanworth proposes that combination of antigen with IgE triggers an allosteric transition within the IgE molecule. This causes the molecule to hinge about a flexible region and brings an activating site into contact with the mast cell membrane. The resultant activation of a membrane enzyme or pro-enzyme leads to mediator release (Stanworth, 1971). From results of studies with various peptides, Stanworth postulates that the activating site of IgE consists of a sequence of amino-acids located in the Fc region of the molecule (Stanworth, Kings, Roy, Moran and Moran, 1979; Roy, Moran, Bryant, Stevenson and Stanworth, 1980).

There are 2 main reasons for doubting this hypothesis. Firstly, a specific antigen is unlikely to initiate an allosteric change in cell-bound IgE because of the structural constraints which exist in immunoglobulins (Metzger, 1974). Secondly, mast cells are stimulated by activation of membrane receptors specifically associated with the Fc binding site on the IgE molecule (Conrad, Froese, Ishizaka and Ishizaka, 1978). In the allosteric model, however, it is suggested that the effector group and the target cell binding sites on IgE are distinct (Stanworth et al, 1979).

c) Receptor bridging hypothesis

The hypothesis which best explains how antigenic stimuli are transmitted to the mast cell is that the stimuli cause a redistribution of receptors in the cell membrane. In a study of human basophils, Becker and his colleagues found that challenge with anti-IgE caused the distribution of surface-bound IgE antibodies to change from a diffuse pattern to aggregated patches or caps. This was initiated by bridging of the antibody

molecules and was directly associated with histamine release from the cells (Becker, Ishizaka, Metzger, Ishizaka and Grimley, 1973). Ishizaka and Ishizaka found that this redistribution was accompanied by migration of IgE receptors in the cell membrane. They proposed that the change in membrane structure or interaction between adjacent receptors created by receptor migration was the event which triggered the cell (Ishizaka and Ishizaka, 1975).

This hypothesis was subsequently tested in a series of studies which utilised antibodies raised against the membrane receptor. It was found that anti-receptor antibodies and their  $F(ab')_2$  dimer fragments induced non-cytotoxic histamine release from rat mast cells. Monovalent  $Fab'$  fragments of the antibodies failed to provoke histamine release on their own but did induce release when cross-linked by a specific antiserum (Ishizaka *et al*, 1978). These results proved that bridging of IgE receptors in the cell membrane was the triggering event.

Membrane receptors for IgE have been extensively studied and have recently been characterised. Each receptor consists of a complex of at least 2 sub-units: a glycopeptide which protrudes at the membrane surface and binds IgE and a polypeptide which lies deeper in the membrane (Iversky, Mendoza and Metzger, 1977; Metzger, Kanellopoulos, Holowka, Goetze and Fewtrell, 1981). The glycopeptide sub-unit appears to be univalent with respect to the binding of IgE (Newman, Rossi and Metzger, 1977).

#### Stimulation of mast cells by complement components

$C_{3a}$  and  $C_{5a}$  anaphylatoxins, formed in serum by enzymatic cleavage of certain complement components (Hugli and Müller-Eberhard, 1978), are capable of triggering secretion in mast cells. There is evidence for this both *in vitro* and *in vivo*. Exposure of isolated mast cells to these substances causes histamine release (Mota, 1959; Dias da Silva and Lepow, 1967). *In vivo*, intradermal injection of anaphylatoxins into human skin causes massive mast cell degranulation and thereby elicits a severe wheal-and-flare reaction (Lepow, Willms-Kretschmer, Patrick and Rosen, 1970).

Anaphylatoxins also stimulate secretion in human basophils. In these cells, several workers have compared the mechanism of activation with that mediated by IgE. Hook and Siriganian found that histamine release induced by anaphylatoxins or by antigen-IgE interaction exhibited similar requirements for calcium and energy, modulation by cyclic AMP and involvement of microfilaments (Hook and Siriganian, 1977). However, Grant and his colleagues discovered that the rate of anaphylatoxin-induced release is much faster than that mediated by IgE antibodies (Grant, Dupree, Goldman, Schultz and Jackson, 1975). Others have found that desensitisation of basophils to one stimulus does not affect the response of the cells to the other (Petersson, Nilsson and Stalenheim, 1975). Hence, although anaphylatoxins and antigen-IgE interactions stimulate similar intracellular pathways, the membrane sites which they activate appear to be distinct.

Another complement-derived factor, probably  $C_{3b}$ , augments IgE-mediated reactions. This substance enhances antigen-induced release of histamine from human basophils (Thomas and Lichtenstein, 1979). Histamine release induced by anaphylatoxins is not enhanced by this factor, so its effect appears to be specifically linked to the antigen-IgE mechanism of cell activation (Thomas, Findlay and Lichtenstein, 1979).

Evidence for complement-induced mast cell activation in asthmatic disease is conflicting. There are reports that the total serum levels of complement decline during an asthma attack, suggesting that the system has been activated, but there are other reports that suggest the levels remain unchanged (eg Hutchcroft and Guz, 1978; Molina, Brun and Wahl, 1979).

#### Other mechanisms of mast cell activation

In addition to immunological and anaphylatoxic stimulation, mast cells may be activated by a wide variety of biological and chemical substances. As reviewed by Kazimierczak and Diamant (1978), the following may induce non-cytotoxic histamine release from the cells:-

- a) cationic proteins liberated from neutrophil leucocytes, eg the band II protein from rabbit neutrophils;

- b) animal toxins and venoms, eg extracts from coelenterates, cobra and bee venoms;
- c) lectins derived from plants, ie concanavalin A and phytohaemagglutinin;
- d) various drugs and chemicals. These include calcium ionophores, ATP, sodium fluoride, various amines (eg compound 48/80, chlorpromazine) and polysaccharides (eg dextran). Some workers have reported that acetylcholine is a powerful liberator of histamine from mast cells (Fantozzi, Moroni, Masini, Blandina and Mannaioni, 1978) but others cannot reproduce this (Kazimierczak, Adamas and Maśliński, 1980).

The fact that such a diversity of agents can trigger mast cell secretion must reflect the sensitivity of the cell membrane to chemical or physical disturbance. It is assumed that a temporary increase in the membrane permeability to calcium is the common mechanism of action of these agents. Cellular metabolic energy and calcium ions are essential for mediator release to occur and most types of release are modulated by cyclic nucleotides (Kazimierczak and Diamant, 1978).

### C. STIMULUS-SECRETION COUPLING IN MAST CELLS

As a result of IgE receptor bridging in the mast cell membrane, a sequence of energy-dependent, intracellular events is triggered which leads to the secretion of granule-associated mediators and to the synthesis of unstored mediators such as prostaglandin D<sub>2</sub> (Lewis, Holgate, Roberts, Oates and Austen, 1981).

#### Morphology of the resting mast cell and changes which occur during secretion

Under the light microscope, the cytoplasm of a fully developed mast cell is dominated by closely-packed granules which stain metachromatically with Toluidine Blue. Electron micrographs of the mast cell have revealed the usual constellation of cell organelles: an eccentric nucleus, mitochondria, Golgi vesicles, centrioles, microtubules, microfilaments, ribosomes, endoplasmic reticulum and lysosomes (Lagunoff, 1972).

The cell membrane possesses surface folds or microvilli (Trotter and Orr, 1974; Caulfield, Lewis, Hein and Austen, 1980). A unit membrane encloses each mast cell granule, separating it from the cytoplasm. Although the perigranular membranes of neighbouring granules are closely opposed, there does not appear to be direct continuity between them in the resting cell (Röhlich, Anderson and Uvnäs, 1971). Bands of thin, contractile microfilaments are associated with both the perigranular and cell membranes (Röhlich *et al*, 1971; Trotter and Orr, 1973). In the human mast cell, intermediate filaments are distributed around the nucleus (Behrendt, Rosenkranz and Schmutzler, 1978; Caulfield *et al*, 1980).

Human mast cell granules have a well-developed crystalline structure which is characterised by whorls, scrolls, gratings and lattices. These crystalline lamellae often co-exist within the same granule. The lamellae probably represent the heparin proteoglycan core of the granule matrix to which all the pre-formed granule mediators are bound (Trotter and Orr, 1973; Caulfield *et al*, 1980).

The following sequence of ultrastructural changes is observed in human nasal, bronchiolar and skin mast cells undergoing degranulation (Trotter and Orr, 1973; 1974):-

- a) disappearance of the crystalline lamellae in granules,
- b) swelling of the area within the perigranular membrane,
- c) fusion between apposed perigranular membranes and fusion of perigranular and plasma membranes,
- d) perforation of fused perigranular and plasma membranes, forming labrynth or channels within the cell and, finally,
- e) extrusion of the granules.

These changes in morphology closely resemble those seen during secretion in rat mast cells (Röhlich et al, 1971) and in the cells of exocrine glands (Palade, 1975).

It appears that the granule contents in human mast cells are partially solubilised prior to their release since, after cell activation, each granule is progressively transformed from the resting crystalline appearance to a swollen amorphous state. Only granules with amorphous contents are seen discharging from the cell (Caulfield et al, 1980). The solubilisation is probably caused by the redistribution of ions which occurs when the mast cell is stimulated. Extracellular ions are transported across the perigranular membrane, probably by an active process. This sets up an osmotic gradient between the granule matrix, the cytoplasm and the extracellular space and thereby causes an influx of water into the granule (Lagunoff, 1972; Holgate, Lewis and Austen, 1981a). The importance of the process is that it frees all the pre-formed mediators from their storage sites (Holgate et al, 1981a).

Contraction of thin filaments associated with the perigranular and cell membranes probably facilitates the movement of each granule up to the cell membrane before it is extruded. On activation of the mast cell, cellular ATP is hydrolysed (Johansen, 1979) by calcium- and magnesium-dependent adenosine triphosphatases in the mast cell membrane (Batchelor, Cooper and Stanworth, 1979; Chakravarty, 1980). This process generates high energy phosphate groups which, together with calcium, are utilised

for contraction of the microfilaments. In human mast cells, intermediate filaments seem to be particularly involved in the intracellular translocation of granules. As a result of cell activation, the intermediate filaments redistribute from the perinuclear region to below the cell membrane surface, around discharging granules (Behrendt et al, 1978; Caulfield et al, 1980). They may interact with the sub-cortical thin filaments to promote exocytosis (Caulfield et al, 1980).

The morphology and distribution of microtubules do not change during secretion in mast cells. However, if microtubular function is altered by drugs, then the secretory process is affected accordingly. Thus colchicine, which binds to the protein sub-unit tubulin and thereby upsets the equilibrium within the microtubule which is essential to its function (Margolis and Wilson, 1977), inhibits the release of histamine from human leucocytes and human lung fragments (Gillespie and Lichtenstein, 1972; Orange, 1976). Conversely, deuterium oxide enhances the release of histamine from both tissues, under certain conditions, because of its capacity to increase the number of microtubules (Gillespie and Lichtenstein, 1972; Orange, 1976). In platelets, microtubules assist in the translocation of granules to the surface membrane by co-operation with contractile thin filaments (Crawford and Castle, 1976). Perhaps they perform a similar function in mast cells.

The membrane surface folds, or microvilli, of mast cells become noticeably elongated during granule discharge. Elongation of the microvilli increases the surface area of the cell membrane, facilitating the exchange of nutrients between the intracellular and extracellular milieu (Trotter and Orr, 1974; Caulfield et al, 1980). In conjunction with an increased permeability of the perigranular membrane, this process allows greater access of extracellular cations and water to the granule matrix during the early stages of secretion.

Fusion between adjacent perigranular membranes and between perigranular and cell membranes is the final secretory event before the granule contents are externalised from the cell. A pentalaminar membrane structure forms at the apposition of the two membranes. This then develops into a pore, bridged by a thin diaphragm. The diaphragm eventually ruptures and the granule matrix is extruded (Röhlich et al, 1971; Holgate et al, 1981a).

### Phospholipid changes in the mast cell membrane and their role in secretion

Changes in the phospholipid composition of the mast cell membrane during secretion have only been described in detail for rat mast cells. The plasma membrane of the mast cell is largely composed of phospholipids (Strandberg and Westerberg, 1976). As in most secretory systems, cell stimulation increases the turnover of a number of phospholipids including phosphatidyl inositol, phosphatidyl choline and the precursor, phosphatidic acid (Kennerly, Sullivan and Parker, 1979; Cockcroft and Gomperts, 1979). More specifically, bridging of membrane receptors for IgE triggers the conversion of phosphatidyl serine to the ethanolamine, which is then trans-methylated to phosphatidyl choline by two membrane-associated enzymes within 15 sec of cell activation (Hirata, Axelrod and Crews, 1979; Ishizaka, Hirata, Ishizaka and Axelrod, 1980). The first enzyme, which faces the cytoplasmic side of the membrane, converts phosphatidyl ethanolamine to its mono-methylated form; the second enzyme is orientated towards the outer surface and adds two methyl groups to form phosphatidyl choline (Hirata et al, 1979). Such spatial segregation of the methyltransferases in the cell membrane causes translocation of methylated phospholipid from the cytoplasmic site to the outer membrane surface (Hirata and Axelrod, 1978a). Simultaneously, the cell membrane becomes less viscous due to the accumulation of methylated phospholipids (Hirata and Axelrod, 1978b). Both of these events are thought to facilitate the formation of calcium channels and thus enhance calcium entry into the mast cell from the extracellular space (Ishizaka, Hirata, Ishizaka and Axelrod, 1981). In accord with this, the increased production of methylated phospholipids precedes calcium uptake by the mast cell and agents which inhibit methylation reactions also abolish calcium influx and histamine release (Ishizaka et al, 1980).

Re-orientation of methylated phospholipids to the external face of the cell membrane brings them into contact with several phospholipases (Hirata and Axelrod, 1979a) which are activated during mast cell secretion (Nakao, Buchanan and Potokar, 1980; Sullivan, 1981). Phospholipase A<sub>2</sub> cleaves fatty acids such as arachidonic, oleic and linolenic acid from the phospholipids, to form lysophospholipids (Sullivan and Parker, 1979). For example, lysophosphatidyl choline is generated from phosphatidyl choline

during secretion stimulated by the calcium ionophore, A23187 (Nakao et al, 1980) or by anti-Fc<sub>ε</sub> receptor antibodies (Ishizaka et al, 1981). Lyso-phosphatidyl choline may promote the uptake of calcium ions by the mast cell, by enhancing the activity of a calcium-dependent ATPase in the cell membrane (Sydbom and Uvnäs, 1976). Lysophospholipids in general and oleic acid have fusogenic properties (Maggio and Lucy, 1975), so their generation would facilitate fusion between perigranular and cell membranes during the secretion of pre-formed mediators.

In activated mast cells, phospholipase C converts phosphatidyl inositol and phosphatidyl choline to 1-2-diacylglycerol (Kennerly, Sullivan, Sylwester and Parker, 1979). This is further split into 1-monoacylglycerol and free fatty acid by the enzyme diglyceride lipase (Sullivan, 1981). Like lysophospholipids, mono- and diacylglycerol cause fusion of membranes, thereby facilitating exocytosis of granule-associated mediators (Kennerly, Sullivan, Sylwester and Parker, 1979).

Phospholipase D removes the polar head group from phospholipids to produce phosphatidic acid (Lehninger, 1975). Generation of phosphatidic acid begins within 8 sec of mast cell activation (Kennerly, Sullivan and Parker, 1979) and precedes the influx of calcium into the cell which peaks at 2 min (Ishizaka et al, 1980). In view of this, and because endogenously-produced phosphatidic acid acts as a calcium ionophore in neurosecretion (Putney, Weiss, Van de Walle and Haddas, 1980), it may assist in the transport of calcium ions into the mast cell.

Changes in the phospholipid turnover of the mast cell membrane which occur as a result of cell activation, have 3 main consequences. Firstly, accumulation of methylated phospholipids and phosphatidic acid early in the secretory process make the membrane environment suitable for calcium entry into the cell. Secondly, formation of lysophospholipids and glycerol-based cleavage products promotes the fusion between perigranular and cell membranes which occurs immediately before the granules are extruded from the cell. Thirdly, the fatty acids which are cleaved from phospholipids and diacylglycerol are used to synthesise secondary mediators such as metabolites of arachidonic acid.

### The role of cyclic nucleotides in mast cell secretion

In virtually every tissue which has been examined, cyclic nucleotides are affected by plasma membrane stimulation and are thought to function as intracellular mediators of external stimuli (Robison, Butcher and Sutherland, 1971). Therefore considerable attention has been focussed on the importance of these nucleotides, particularly cyclic AMP, in mast cell secretion.

Activation of the membrane-bound enzyme, adenylyl cyclase, increases intracellular levels of cyclic AMP by promoting hydrolysis of ATP (Robison et al, 1971). Cyclic AMP phosphorylates the regulatory sub-unit of various protein kinases within the cell, releasing the catalytic sub-unit of the kinase. This effect is counter-balanced by a protein phosphatase. Each free catalytic sub-unit of protein kinase phosphorylates an intracellular protein to produce the pharmacological effect (reviewed by Nimmo and Cohen, 1977).

#### a) Adenylyl cyclase, cyclic AMP and protein kinase activation in mast cells

Early studies suggested that cyclic AMP inhibits the immunological release of histamine from most tissues. Lichtenstein and his colleagues found that dibutyryl cyclic AMP, catecholamines, inhibitors of phosphodiesterases and prostaglandins of the E series, which increase cellular cyclic AMP by different mechanisms, all inhibited histamine release from human leucocytes (Lichtenstein and Margolis, 1968; Lichtenstein, Gillespie, Bourne and Henney, 1972). Subsequently, similar results were obtained in rat serosal cells and in human lung fragments (Orange, Austen and Austen, 1971; Tauber, Kaliner, Stechschulte and Austen, 1973; Sullivan, Parker, Eisen and Parker, 1975). In human lung fragments, the inhibitory potency of several agents correlated with their ability to increase the total tissue content of cyclic AMP (Orange, Kaliner, Larãia and Austen, 1971). Conversely,  $\alpha$ -adrenergic stimulation of immunologically-challenged lung fragments decreased the tissue levels of cyclic AMP and concomitantly enhanced histamine release (Kaliner, Orange and Austen, 1972).

These observations were based on the assumption that the pharmacological agents which were used specifically modified the intracellular levels of cyclic AMP. It is now known that phosphodiesterase inhibitors, in particular, have multiple effects on the mast cell (Fredholm, Guschin, Elwin, Schwab and Uvnäs, 1976; Fredholm and Sydbom, 1980) and that dibutyryl cyclic AMP non-specifically stabilises the mast cell membrane (Kazimierczak and Diamant, 1978). Moreover, when cyclic nucleotide levels were measured in these early studies, they were assessed in heterogeneous tissues rather than in a purified population of mast cells. Therefore, the changes in nucleotide levels which were recorded probably reflected the sum of changes in many different cell types.

More recent studies on highly-purified rat mast cells have revealed that different types of stimulation may cause diverse changes in cyclic AMP levels. For example, stimulation of the mast cells by compound 48/80 (Sullivan et al, 1975) or by an anti-rat light chain antibody (Kalinier and Austen, 1974a) produces a rapid decrease in cyclic AMP. In contrast, activation of the cells with a different anti-rat light chain antibody (Lewis, Holgate, Roberts, Maguire, Oates and Austen, 1979) or with concanavalin A (Sullivan, Parker, Kulczycki and Parker, 1976) causes an initial rise in cyclic AMP. Using various pharmacological agents to stimulate the cell, Johnson and co-workers could find no relationship between the intracellular levels of the nucleotide and histamine release (Johnson, Moran and Mayer, 1974).

There is, however, good evidence that bridging of surface-bound IgE molecules on purified rat mast cells is directly linked to an increase in cyclic AMP. This process triggers 2 independent, transient increases in the intracellular levels of the nucleotide, the first occurring within 15 sec of cell activation and the second occurring after 2-5 min (Sullivan et al, 1976; Lewis et al, 1979). The first peak is unaffected by inhibitors of prostaglandin synthesis such as indomethacin and is therefore not caused by prostaglandins generated during secretion (Lewis et al, 1979). Pharmacological stimulation of adenylyl cyclase potentiates both the initial rise in cyclic AMP and the release of granule-associated mediators (Holgate, Lewis and Austen, 1980a). Inhibition of adenylyl cyclase activity has the reverse effect (Holgate et al, 1980a). Hence, the initial rapid

elevation of cyclic AMP levels which is observed when surface-bound IgE molecules are bridged is probably caused by direct activation adenyl cyclase in the mast cell membrane, and this may be partly responsible for propagating the immunological stimulus within the cell.

Activation of adenyl cyclase alone does not induce the release of mediators from mast cells (Holgate et al, 1980a), so IgE-mediated reactions must stimulate at least one other event which is essential to the secretory process. The studies of Ishizaka and her colleagues have demonstrated the importance of membrane phospholipid methylation in secretion in rat mast cells (Ishizaka et al, 1980). An increase in phospholipid methylation occurs simultaneously with a rise in cyclic AMP in activated cells and, although these two events appear to be mutually independent (Ishizaka et al, 1981), together they probably ensure full activation of secretion.

In most tissues, cyclic AMP exerts its effects through activation of protein kinases (Nimmo and Cohen, 1977). Holgate and his colleagues have isolated 2 iso-enzymes of protein kinase from rat mast cells, both of which are cyclic AMP-dependent and whose activity is increased by immunological challenge of the cells (Holgate, Lewis and Austen, 1980b). As histamine release from mast cells was found to be directly proportional to the extent of protein kinase activation, these iso-enzymes probably mediate rather than modulate secretion. They may be activated by the rapid elevation of cyclic AMP levels which occurs during immunological challenge.

Similar characterisation of the inhibitory effects of cyclic AMP in mast cells has not been achieved. The second monophasic elevation of cyclic AMP levels, which occurs 2-5 min after cell activation, is blocked by indomethacin (Lewis et al, 1979) and appears to be caused by prostaglandins which are generated by the mast cell during secretion (Holgate, Lewis and Austen, 1981b). However, as indomethacin does not inhibit the immunological release of histamine from mast cells (Sullivan and Parker, 1979), and as histamine release is normally completed within 1 min of cell activation (Foreman, Hallett and Mongar, 1977), the rise in cyclic AMP

caused by endogenously-produced prostaglandins appears to have no effect on the secretory process. Nevertheless, in immunologically-activated cells, theophylline elevates the levels of cyclic AMP and also inhibits calcium uptake and histamine release (Foreman *et al*, 1977; Holgate *et al*, 1981b). Although these effects are not necessarily related, they suggest that cyclic AMP within the mast cell is capable of directly inhibiting secretion.

In an effort to explain the role of cyclic AMP in mast cells, Holgate and his colleagues propose that each cell possesses specific complexes of adenylyl cyclase which, on stimulation, are capable of generating cyclic AMP at specific intracellular loci. This leads to the activation of specific protein kinase iso-enzymes and subsequent phosphorylation of proteins (Holgate *et al*, 1981b). In accord with this, activation of purified rat mast cells increases the phosphorylation of 4 separate proteins, all of which are linked with the process of secretion (Sieghart, Theoharides, Douglas and Greengard, 1978).

Greengard (1978) considers that biological responses are governed more by the extent of protein phosphorylation than by the levels of second messengers such as cyclic AMP. If this is so, the main factors which influence whether cyclic AMP inhibits or enhances secretion in mast cells will be the site of its production, the protein kinases which it activates and the relative activity of the phosphatase which opposes its effects. By analogy with other secretory and contractile systems (Greengard, 1978; Nishikawa, Tanaka and Hidaka, 1980), cyclic AMP-dependent protein phosphorylation may regulate

- a uptake and intracellular distribution of calcium,
- b transport of ions and water across the perigranular membrane, and
- c actin-myosin interactions (through the enzyme myosin light chain kinase) thereby governing the contraction of microfilaments.

Studies of mast cells themselves have revealed that cyclic AMP controls the phospholipid changes which occur in the cell membrane after cell activation (Kennerly, Secosan, Parker and Sullivan, 1979; Ishizaka *et al*, 1981). Hence, it is conceivable that all stages in the stimulus-secretion coupling process in mast cells are under the control of cyclic AMP.

## b) Effect of cyclic GMP on mast cell secretion

Cyclic GMP, formed by the action of guanyl cyclase on GTP, may either stimulate or oppose the actions of cyclic AMP, depending on the tissue (Weiss and Fertel, 1977). As yet, there is no evidence to clearly show that secretion in mast cells is controlled by intracellular cyclic GMP.

Cholinergic agonists, which elevate cyclic GMP levels in some tissues (Greengard, 1978), and 8-bromo-cyclic GMP potentiate the immunological release of histamine from human lung fragments (Kalinin *et al*, 1972). However, direct measurement of cyclic nucleotide levels in human lung tissue indicates that histamine release is reduced when cyclic GMP levels are raised (Coulson, Ford, Marshall, Walker, Wooldridge, Bowden and Coombs, 1977). Few conclusions can be drawn from these data because they were obtained from studies of whole tissue rather than of purified mast cells.

In purified rat mast cells, bridging of surface-bound IgE molecules produces an early and late rise in the levels of cyclic GMP. Both of these increases are blocked by indomethacin and are therefore assumed to be caused by prostaglandins synthesised by the mast cell during secretion (Lewis *et al*, 1979). As indomethacin does not inhibit the immunological release of histamine from rat mast cells (Sullivan and Parker, 1979; Lewis *et al*, 1979), the changes in cyclic GMP caused by endogenously-produced prostaglandins do not seem to affect the secretory process.

## The role of calcium in mast cell secretion

In most types of secretion, the essential link between cell stimulation and exocytosis of granules from the cell is an increase in the free calcium concentration of the cytosol (Douglas, 1968). Calcium transmits the membrane stimulus mostly by binding to, and causing a conformational change in, an ubiquitous cellular protein called calmodulin. The calcium-calmodulin complex binds to the inactive precursors of various enzymes, thereby unleashing their catalytic activities (reviewed by Klee, Crouch and Richman, 1980).

All resting eukaryotic cells maintain low levels of free calcium in the cytosol (Kretsinger, 1977), through the action of adenosine triphosphatases in the mitochondrial and cell membranes which exchange calcium for magnesium (Larsen and Vincenzi, 1979). Cell activation increases the free intracellular calcium concentration either by promoting influx of calcium ions from the extracellular space or by mobilising intracellular calcium stores located in the mitochondria, endoplasmic reticulum and cell membrane (Brading, 1979). There is evidence to suggest that both of these processes occur in activated mast cells.

Histamine secretion from mast cells induced by antigen-antibody reactions on the cell surface requires the presence of calcium in the extracellular medium (Mongar and Schild, 1958; Foreman and Mongar, 1972a). Bridging of surface-bound IgE molecules (Foreman et al, 1977) or bridging of membrane receptors for IgE (Ishizaka, Foreman, Sterk and Ishizaka, 1979) stimulates the mast cells to take up calcium ions. This is mainly a passive process, but is not caused by simple diffusion of calcium into the cells during the discharge of granules (Foreman et al, 1977; Ranadive and Dhanani, 1980). There is a close relationship between calcium uptake and histamine release (Foreman et al, 1977; Ranadive and Dhanani, 1980). Also lanthanum, which blocks the movement of calcium ions across membranes, is a potent inhibitor of antigen-induced histamine release (Foreman and Mongar, 1972b).

Promotion of calcium influx by means other than antigen-antibody reactions also induces secretion in mast cells. This occurs when the cells are exposed to ionophores which directly transport calcium across the cell membrane (Foreman, Mongar and Gomperts, 1973) or when calcium ions are introduced via an intracellular micropipette (Kanno, Cochrane and Douglas, 1973). Calcium-loaded liposomes also provoke secretion in mast cells, by fusing with the mast cell membrane and releasing their contents within the cell (Theoharides and Douglas, 1978).

Thus, antigen-antibody reactions and some other stimuli of mast cell secretion all increase the uptake of calcium ions by the cell and this process is causally related to the release of mediators. With respect to immunological activation, there are probably 2 mechanisms by which this occurs. Firstly, when the cell is stimulated, there is a rapid

change in the turnover of certain phospholipids which provides a suitable membrane environment for calcium entry (Kennerly, Sullivan and Parker, 1979; Ishizaka *et al*, 1980). Secondly, ATP is hydrolysed (Johansen, 1979) by a calcium/magnesium-dependent adenosine triphosphatase in the plasma membrane (Chakravarty, 1980), thereby supplying energy for calcium transport across the membrane. Cell stimulation may simply reverse the adenosine triphosphatase pump which normally maintains a low concentration of calcium in the cytosol of the resting cell (Larsen and Vincenzi, 1979), or alternatively may activate a different adenosine triphosphatase.

In rat mast cells, various agents evoke histamine release in the absence of extracellular calcium. This lead Douglas to propose that mast cells can utilise either cellular or extracellular calcium to effect the response depending on the nature of the secretory stimulus (Douglas, 1975a). Ennis and her colleagues suggest that the mast cell contains several different calcium pools which are in equilibrium. Calcium which is loosely bound to the outer membrane migrates into the cytosol in response to cell stimulation. In the absence of extracellular calcium ions this pool rapidly dissociates from the membrane and secretion under these circumstances is brought about by mobilisation of intracellular calcium stores. A third pool, which resides in the inner aspect of the cell membrane, regulates both calcium influx and mobilisation of intracellular calcium (Ennis, Truneh, White and Pearce, 1980). Support for this idea comes from the demonstration that calcium binds to cell membrane fragments isolated from rat mast cells (Tolone, Bonasera, Vitale and Pontieri, 1980) and that an analogous regulation of calcium movements occurs in smooth muscle cells (Van Breeman, Aaronson and Loutzenheiser, 1978).

However, compared with secretion in rat mast cells, secretion in both sensitised human basophils (Lichtenstein, 1971) and sensitised human lung mast cells (Kalinin and Austen, 1973) is much more dependent upon extracellular calcium. Even in rat mast cells, antigen-antibody reactions are more sensitive to depletion of extracellular calcium than are other stimuli (Ranadive and Dhanani, 1980). Therefore, it seems likely that the major effect of an immunological stimulus is to promote calcium uptake.

by the cell. Calcium entering the cell might interact with, or simply displace, intracellular stores of the ion. As a result, free calcium would be generated to regulate the activities of various enzymes involved in the secretory process, by binding to calmodulin.

It is not known whether calmodulin is present in mast cells, but this is likely since it has been found in almost every cell which has been examined (Klee et al, 1980). As calcium-calmodulin complexes participate in stimulus-secretion coupling in platelets (Nishikawa et al, 1980) and in the  $\beta$ -cells of the pancreas (Gagliardino, Harrison, Christie, Gagliardino and Ashcroft, 1980), it is reasonable to speculate on their role in mast cell secretion. Many enzymes are stimulated by the calcium-calmodulin complex including: phosphorylase kinase which regulates glycogen metabolism, the magnesium-dependent adenosine triphosphatase responsible for transport of calcium across membranes, and myosin light chain kinases which control the interaction between the muscle proteins, actin and myosin (Klee et al, 1980). Thus at least 3 processes which are essential to secretion in mast cells, ie energy production, the uptake of calcium ions and contraction of microfilaments, may be under calmodulin control. Additionally, calcium-calmodulin complexes stimulate adenyl cyclases and phosphodiesterases involved with cyclic AMP metabolism, as well as protein kinases which govern the phosphorylation of intracellular proteins (Klee et al, 1980). Hence, through binding to calmodulin, calcium could mediate secretion in mast cells by directly activating enzymes such as phosphorylase kinase, and modulate secretion by controlling the cyclic AMP-dependent protein phosphorylation system. Calcium ions themselves neutralise the negative charge possessed by membranes and in this way they facilitate the fusion of perigranular and cell membranes during exocytosis (Banks, 1966).

Immunologically-activated mast cells do not remain permanently permeable to calcium. The uptake of extracellular calcium ions increases during the first 30 sec of challenge, but thereafter it rapidly declines as the permeability of the cell returns to its resting level (Foreman et al, 1977). In accord with this, antigen-induced increases in the levels of cyclic AMP and certain membrane phospholipids, which are thought to promote calcium uptake by the mast cell, are also transitory (Holgate et al, 1981a).

### The sequence of biochemical events in mast cell secretion

Using pharmacological agents to inhibit certain cellular processes, Kaliner and Austen (1973) partially delineated the sequence of biochemical events which occurs in mast cells in passively sensitised human lung fragments when they are challenged with antigen. The initial event is calcium-dependent activation of a serine pro-esterase, which is inhibited by di-isopropyl-fluorophosphate (DFP). This is followed by an energy-requiring step which can be blocked by 2-deoxyglucose; then a second calcium-dependent step which leads to a cyclic AMP-inhibitable stage and finally to the release of mediators. Recent evidence from studies in purified rat mast cells indicates that the sequence needs reviewing to include initial increases in the levels of cyclic AMP and methylated phospholipids (Holgate et al, 1981a).

### Feedback regulation of mast cell secretion

Several of the inflammatory mediators which are released or generated during anaphylaxis are capable of influencing secretion in mast cells. However, it is difficult to assess their general significance in the regulation of secretion because their effects are sometimes tissue-specific and do not always equate with the effects of endogenously-produced mediators.

In human basophils, histamine inhibits its own release via an  $H_2$  receptor-mediated, cyclic AMP-dependent effect (Lichtenstein, 1976). This suggests that basophil secretion is subject to negative feedback regulation. However, histamine does not seem to influence secretion in rat serosal mast cells or in human lung fragments (Platshon and Kaliner, 1978), so the feedback mechanism appears to be specific to human basophils.

Most tissues generate many metabolites of arachidonic acid during anaphylaxis. In human lung tissue, several metabolites of the cyclo-oxygenase and lipoxygenase pathways are produced (Kalinier, 1980). Exogenously-added prostaglandins  $E_1$ ,  $E_2$  and  $F_{2\alpha}$  depress the release of histamine and SRS-A from human lung fragments challenged with antigen (Tauber et al, 1973). However, when the tissue cyclo-oxygenase is

inhibited by indomethacin to abrogate prostaglandin synthesis, antigen-induced histamine release proceeds unchanged (Walker, 1973). This implies that, in human lung tissue, endogenous production of prostaglandins does not form part of a feedback mechanism controlling secretion. Fatty acid hydroperoxides which are normally synthesised by the lipoxygenase pathway enhance anaphylactic release of histamine and SRS-A from perfused guinea pig lungs, by a direct effect on the lung mast cells (Adcock, Garland, Moncada and Salmon, 1978). Indeed, there is evidence to suggest that lipoxygenase products may be a necessary requirement for IgE-mediated histamine release (Marone, Kagey-Sobotka and Lichtenstein, 1979). Further work is required to clarify this effect.

During anaphylaxis, mast cells release a chemotactic factor (NCF) which attracts neutrophils to the site of invading antigen (Austen and Orange, 1975). In phagocytosing foreign particles, neutrophils release cationic proteins which stimulate further secretion in mast cells (Kazimierczak and Diamant, 1978). Thus, in vivo, NCF released from mast cells directly enhances the secretory process.

#### D. DRUGS WHICH ANTAGONISE MAST CELL SECRETION

##### 1) SODIUM CROMOGLYCAT

Sodium cromoglycate was introduced 14 years ago as a prophylactic treatment for asthma, after the identification of its protective effect on mast cells. Cromoglycate was first synthesised in 1965 during a study of the biological properties of khellin, a naturally-occurring chromone. Khellin had been found to relax smooth muscle but undesirable side-effects precluded its use in the treatment of asthma. In the course of a study arrived at improving the bronchodilator action and diminishing the side-effects of khellin, it was discovered that derivatives of chromone-2-carboxylic acid could inhibit antigen-induced bronchospasm without causing smooth muscle relaxation (Cox, 1967; 1970). This led to the testing of a series of bis-chromone derivatives which included a sodium salt of the dibasic acid 1,3-bis(2-carboxychromon-5-yloxy)-2-hydroxypropane, called sodium cromoglycate (Cox, 1967). Cromoglycate was found to be inactive on oral administration but it prevented symptoms of asthma in sensitised individuals when it was inhaled prior to antigen challenge. Administration of the drug after antigen challenge markedly reduced its efficacy (Altounyan, 1967; Howell and Altounyan, 1967). This indicated that cromoglycate has a different mechanism of action from other drugs used to treat asthmatic disease. Other studies have shown that cromoglycate is not a conventional anti-inflammatory agent, it has no corticosteroid-like effects, it is not a bronchodilator and is not a pharmacological antagonist of mediators of tissue reactions such as histamine and SRS-A (Cox, 1967; Cox, Beach, Blair, Clarke, King, Lee, Loveday, Moss, Orr, Ritchie and Sheard, 1970).

The findings that cromoglycate inhibits passive anaphylaxis in the skin of rats and monkeys and in the bronchi and parenchyma of isolated human lung tissue (Cox, 1967) suggested that the drug interferes with immunological secretion in mast cells. This was confirmed by the demonstration that it inhibits antigen-induced changes in the morphology of mast cells (Orr and Charles, 1970) and prevents the release of mediators (Orr, Hall, Gwilliam and Cox, 1971).

Cox (1971) has stated that blockade of mediator release from tissue mast cells is the primary action of cromoglycate. This is consistent with the findings that asthmatics only benefit when they receive the drug prophylactically (Altounyan, 1967) and that patients with strong evidence of extrinsic allergy are more likely to respond than those without (Brogden, Speight and Avery, 1974a). Moreover, cromoglycate is effective in other diseases caused by the release of mediators from mast cells, eg allergic rhinitis and vernal conjunctivitis (Brogden, Speight and Avery, 1974b). However, certain characteristics, such as its ability to relieve exercise-induced asthma (Anderson, Silverman, König and Godfrey, 1975), have lead to the belief that other mast cell-independent mechanisms are involved in its action.

#### Mode of action of cromoglycate on mast cells

Orr and his colleagues have shown that cromoglycate interferes neither with sensitisation of mast cells by antibodies nor with interaction between antigens and antibodies on the cell surface (Orr, Pollard, Gwilliam and Cox, 1970). Therefore, the drug appears to act directly on the cell, itself. The same investigators have obtained evidence that cromoglycate acts directly or indirectly at a stage following antigen-antibody interaction but prior to the release of granule-associated mediators (Orr *et al*, 1970). In addition to inhibiting antigen-induced release of mediators, cromoglycate inhibits release provoked by various other stimuli such as compound 48/80, the calcium ionophore A23187, dextran and phospholipase A (Orr, 1977). Thus, either the drug inhibits a secretory event which is common to all of these stimuli or else it produces a non-specific stabilisation of the cell.

At the commencement of this project, 2 main hypotheses had been submitted to explain the action of cromoglycate on mast cells. The first, and more popular hypothesis, proposes that cromoglycate inhibits the transport of calcium ions into the cell. This is based on evidence that the drug reduces both the uptake of radio-labelled calcium and the secretion of histamine by mast cells stimulated by several different mechanisms (Johnson and Bach, 1975; Foreman and Garland, 1976; Spataro and Bosmann, 1976). Cox has suggested that cromoglycate might influence the permeability

of the mast cell to calcium ions by altering the hydration sheath of the cell membrane, due to its profound affinity for water molecules (Cox, 1974). The second hypothesis proposes that the drug inhibits a specific phosphodiesterase involved with cyclic nucleotide metabolism. Whether the net result of this is an increase in cyclic AMP or cyclic GMP levels, or a change in the AMP/GMP ratio, is not clear (Taylor, Francis, Sheldon and Roitt, 1974; Bergstrand, Kristoffersson, Lundqvist and Shurmann, 1977; Coulson *et al*, 1977). A third but relatively minor hypothesis suggests that, in lung tissue, cromoglycate produces an inhibitory substance, eg a phosphodiesterase inhibitor or a nucleotide cyclase, which then acts on the mast cell (Taylor and Sheldon, 1977).

#### Other actions of cromoglycate

Single dose studies in human subjects which were either uncontrolled, single-blind or double-blind have proved that cromoglycate inhibits exercise-induced asthma if given a short time before the exercise (reviewed by Brogden *et al*, 1974a). Although mast cells may be involved in this type of asthma, stabilisation of the cells does not adequately explain the action of cromoglycate in this disease. From their studies in dogs, Jackson and Richards have submitted an explanation for the drug's effect. They have found that intravenous or inhaled cromoglycate inhibits bronchospasm induced by an histamine aerosol, but not that induced by electrical stimulation of the vagus nerve. With the evidence that cooling of the vagus also blocks the response to histamine, they suggest that cromoglycate reduces the activity of the afferent vagal pathway, probably by an action on irritant receptors in the lung (Jackson and Richards, 1977). This could explain the benefit of cromoglycate in exercise-induced asthma since the vagal reflex in bronchial smooth muscle is one of several mechanisms which precipitate the disease (McFadden and Soter, 1977).

In human provocation tests, Pepys and his colleagues have shown that inhalation of cromoglycate before antigen challenge inhibits the late-developing bronchial reactions which are assumed to be mediated by precipitin antibodies, in addition to inhibiting immediate responses which

are mediated by IgE. Pepys argues that this is not surprising since the late reaction in most patients seems to be a consequence of the immediate response. However, cromoglycate has been found to block late reactions in 2 patients with avian sensitivity in whom no immediate reaction takes place (Pepys, Hargreave, Chan and McCarthy, 1968; Pepys, 1973). To explain this and other anomalies of cromoglycate action, Orr suggests that the drug interferes with activation of the complement pathway. He cites the evidence of others who have found that cromoglycate blocks changes in the levels of circulating leucocytes induced by the steroidal anaesthetic, Althesin. This effect of Althesin seems to be caused by its activation of the alternate pathway of complement (Watkins, Clarke, Appleyard and Padfield, 1976; Orr, 1977).

Evidence has been submitted to suggest that cromoglycate mimics the action of the  $\alpha$ -adrenoceptor antagonist, phenoxybenzamine, in preventing certain types of bronchoconstriction. However, according to Church (1978), the evidence is largely circumstantial and is not reproducible.

#### Variable response to cromoglycate *in vivo* and *in vitro*

Since the initial reports on the activity of cromoglycate in asthma, numerous clinical trials have been conducted to establish its efficacy, the majority of which have been comprehensively reviewed by Brogden and his colleagues (1974a). The main feature of these trials is the variability in response to the drug.

The diverse reports of cromoglycate activity may be partly attributed to problems associated with the trials themselves. Clinical evaluation of any anti-asthmatic drug is difficult because of the variable and unpredictable nature of the disease and because it is precipitated by many factors. With cromoglycate there is the added problem that its activity may take several weeks to subside after cessation of treatment. This makes cross-over trials difficult to interpret. Also, patient compliance with cromoglycate is often difficult, particularly with young children, because subjects have to be trained to use the sp inhaler by which the drug is delivered and because the inhaled powder can cause irritation of the throat and trachea (Brogden et al, 1974a).

Despite these problems which plague the interpretation of clinical data, there appears to be much inter-patient variability in the activity of cromoglycate. The best responses are seen in the young, in patients with demonstrable allergy and in those with exercise-induced asthma. Neither atopy nor a history of allergy is a clear determinant of a good response. Some asthmatics with associated allergies have actually developed worse symptoms on administration of the drug (Lobel, Machtey and Eldror, 1972; Menon and Das, 1977). Brogden and his associates conclude that cromoglycate is of considerable benefit to a few patients and of some benefit to many.

The protective effect of cromoglycate against anaphylaxis in animals is also highly variable, as are responses to the drug in human and animal tissues in vitro (Church, 1978). For example, in models of anaphylaxis mediated by IgE antibodies in the rat, the relationship between drug dose and response of the tissue is often unpredictable. Several workers have reported that cromoglycate inhibits anaphylaxis in rats biphasically, with greater inhibition of mediator release at lower dose levels than at higher doses (Church, 1978). Conversely, others have demonstrated an augmentation of anaphylactic release by low concentrations of the drug, with inhibition only occurring at higher doses (Garland, 1973; Sung, Saunders, Krell and Chakrin, 1977a). Mast cell secretion induced by the chemical histamine liberator, compound 48/80, is inhibited linearly in vitro but in vivo it is inhibited in a bell-shaped manner (Orr *et al*, 1971). This could simply reflect a difference in responsiveness of mast cells in an isolated form compared with being in a tissue environment. However, as histamine release from isolated rat mast cells induced by the calcium ionophore A23187 is inhibited in a bell-shaped manner (Johnson and Bach, 1975), this is clearly not the case.

In human tissues, cromoglycate does not inhibit mediator release from sensitised leucocytes or sensitised skin, but does block the release from sensitised lung tissue (Assem and Mongar, 1970). In lung tissue, the extent of cromoglycate inhibition of histamine secretion has been described as: 29% with a dose of 100 nM (Morr, 1978), 29% at 20  $\mu$ M (Sheard and Blair, 1970) and 50-100% with a dose of 30  $\mu$ M (Assem and Mongar, 1970).

Having examined a range of drug concentrations, some authors have reported a lack of effect at higher dose levels (Orange and Austen, 1971; Church and Gradidge, 1978). Others have found that the efficacy is unchanged by increasing the drug concentration above a maximum inhibitory dose (Sharpe, Ross and Spicer, 1978).

#### Immunological dependence of cromoglycate activity

One factor which contributes to the diversity of cromoglycate inhibition is that its activity seems to be partly governed by the antibody which mediates the anaphylactic reaction. In animal studies, generally IgE mechanisms of mediator release are well antagonised whereas IgG mechanisms are not (Church, 1978). From studies of passive cutaneous anaphylaxis in rats, it has been reported that cromoglycate only blocks IgE-mediated responses (Mielens, Ferguson and Rosenberg, 1974), that it abrogates both IgE and IgG-mediated secretion but much higher doses are required to inhibit the IgG mechanisms (Miller and James, 1978), or that it blocks both types of reaction equally well (Orr, 1975). In isolated rat mast cells, cromoglycate inhibits secretion mediated by either antibody (Morse, Austen and Bloch, 1969). The drug is rarely effective in guinea pigs but where it does inhibit mediator release both IgE and IgG mechanisms may be antagonised, such as in lung tissue, or only IgE mechanisms are affected, as in the skin (Assem and Richter, 1971).

There is some evidence to suggest that a lack of response of some extrinsic asthmatics to cromoglycate may be associated with specific IgG antibodies in their serum. Bryant and his colleagues detected a short-term anaphylactic IgG antibody in the sera of some asthmatics whose bronchial immediate allergic reactions were not inhibited by cromoglycate (Bryant et al, 1973; 1975). Therefore, it was suggested that allergic asthma, in some patients who do not respond to treatment with cromoglycate, is mediated by such antibodies. More recently, however, Bryant reviewed his earlier work and found that the patients who did not respond to conventional cromoglycate doses demonstrated markedly high serum levels of allergen-specific IgE, as well as specific anaphylactic IgG antibodies (Bryant, 1977). Clinical improvement was achieved in a proportion of these patients by using higher doses of the drug. Since then,

Gwynn and his colleagues have shown that in a group of 12 children who possessed raised serum concentrations of IgG<sub>4</sub>, either alone or in conjunction with raised serum IgE, 9 had to be treated with steroids because cromoglycate failed to control their symptoms. In contrast, 13 of 16 patients with raised IgE and normal IgG<sub>4</sub> levels had been controlled successfully by cromoglycate alone (Gwynn, Morrison-Smith, Leon-Leon and Stanworth, 1978).

From results obtained in the human chopped lung model, Church and Gradidge (1978) submitted an explanation for these clinical findings. They showed that when lung was passively sensitised with allergic serum and then challenged with a pollen extract in the presence of increasing doses of cromoglycate, histamine release was inhibited in a bell-shaped fashion. The inhibitory part of the curve was due to blockade of IgE-mediated release whereas the apparent lack of efficacy at higher dose levels was due to potentiation of IgG-mediated release. Therefore, they suggested that the sensitivity of a patient's asthma to cromoglycate may depend on the predominant antibody mediating the disease.

Thus in the clinic it has been shown that a lack of response of patients with allergic asthma to cromoglycate may be associated with raised serum levels of an IgG sub-class. Using human lung *in vitro*, it has been demonstrated that an IgG-mediated release mechanism exists which can be potentiated by cromoglycate. This provided one of the purposes of the present work, ie to investigate the mechanisms underlying the drug-induced potentiation of IgG-mediated release.

#### Drug-induced tolerance and tachyphylaxis

One feature which distinguishes the action of cromoglycate in animal studies is that exposure of a tissue to one dose of the drug substantially reduces the effect of a second dose added some time afterwards. This capacity for self-inhibition, or tachyphylaxis, is not confined to its protective effect on mast cells. In fact, the phenomenon was first detected during studies of the cardiovascular effects of cromoglycate in marmosets and dogs. In anaesthetised marmosets, low doses of cromoglycate

given intravenously produce a transient but marked hypertension and tachycardia, via stimulation of the sympathetic nervous system. These effects are subject to tachyphylaxis when one dose is repeatedly administered at intervals of less than 30 min (Cox et al, 1970). Administration of cromoglycate to anaesthetised dogs precipitates a transient fall in blood pressure together with bradycardia and, in some cases, apnoea. The hypotensive effect and the bradycardia are thought to be caused by stimulation of cardiac vagal afferents because the response resembles the so-called Bezold-Jarisch effect which occurs after intravenous injection of 5-hydroxytryptamine (Douglas, 1975b; Jackson and Richards, 1977). In this model, intravenous infusion of cromoglycate blocks the normal activity of a subsequent bolus injection of the drug for 2-3 h (Cox et al, 1970).

In studies performed on rat tissues both in vitro and in vivo, the anti-allergic activity of cromoglycate is highly dependent on the time of drug administration. Cromoglycate is maximally effective when given simultaneously with the antigen or other secretory stimulus but, as the pre-incubation time with the tissue is extended, it rapidly loses inhibitory activity (Orr et al, 1971; Thomson and Evans, 1973). This is due to the tissue becoming desensitised to the effects of cromoglycate rather than to a short duration of drug action because, under these conditions, addition of a fresh dose of cromoglycate has no effect (Kusner, Dubnick and Herzig, 1973; Thomson and Evans, 1973). The state of tolerance is not caused by gradual metabolism of the drug molecule (Kusner et al, 1973). Its onset depends on the concentration and contact time of the initial dose of cromoglycate and, after the drug has been removed, recovery from tolerance is slow (Thomson and Evans, 1973; Sung, Saunders, Lenhardt and Chakrin, 1977b).

Several drugs which act similarly to cromoglycate exhibit tachyphylaxis on repeated administration. Moreover, prior exposure of a tissue to these drugs desensitises it towards the effects of the parent compound (Herzig, Kusner, Fox and Kaplan, 1977). Therefore, tachyphylaxis and cross-tachyphylaxis are criteria by which the cromoglycate mode of action may be identified and investigated.

### Analogues of cromoglycate: effects in asthma

Because cromoglycate is inactive by oral administration and since it only benefits a proportion of asthmatics, there has been an intensive search for an orally active analogue which is more consistently effective and which retains the low toxicity of the parent compound. Many compounds have been synthesised which are more potent than cromoglycate in experimental models of anaphylaxis, but most are of little benefit clinically. Those which have demonstrated activity in clinical trials are inferior to cromoglycate (reviewed by Church, 1978). The reason why cromoglycate analogues are not fulfilling their predicted therapeutic efficacy is unknown.

### 2) DRUGS WHICH ELEVATE THE INTRACELLULAR LEVELS OF CYCLIC AMP

Several agents which relax bronchial smooth muscle are used in the treatment of asthma. Three major classes of drug are prescribed:  $\beta$  adrenergic agonists, methylxanthines and anti-muscarinic agents. Both  $\beta$  agonists and methylxanthines increase the intracellular levels of cyclic AMP in bronchial smooth muscle, the former by activation of adenylyl cyclase and the latter by inhibition of cyclic AMP-dependent phosphodiesterase. This is the principal mechanism behind the bronchodilator action of  $\beta$  agonists and may also partially explain the activity of methylxanthines (Rebuck, 1974). Additionally, both types of drug inhibit secretion in mast cells and this may contribute to their therapeutic effects, particularly in allergic asthma (Kalinin and Austen, 1974b).

### Methylxanthines

Theophylline compounds, particularly aminophylline, are the xanthines which are used clinically. Theophylline is a methylated derivative of xanthine which occurs naturally whereas aminophylline is synthesised by conjugation of theophylline with ethylenediamine (Ritchie, 1975). Methylxanthines inhibit immunologically-induced secretion of histamine in rat serosal mast cells (Foreman *et al*, 1977), human leucocytes (Lichtenstein and Margolis, 1968) and human lung fragments (Orange, Austen and Austen, 1971). The evidence that they increase the levels of cyclic

AMP in rat mast cells (Holgate et al, 1981b) and that they synergise with  $\beta$  agonists in inhibiting histamine release from human lung fragments (Kaliner and Austen, 1974b) suggests that their protective effect on mast cells is mediated by a rise in intracellular cyclic AMP.

However, methylxanthines are not specific in their action. In addition to inhibiting cyclic AMP-dependent phosphodiesterase they also inhibit the phosphodiesterase which metabolises cyclic GMP (Ritchie, 1975). They also affect the intracellular distribution of calcium (Ritchie, 1975) and antagonise the effects of adenosine by competing for an adenosine receptor (Fredholm and Sydbom, 1980; Welton and Simko, 1980). Therefore, the ability of methylxanthines to relax smooth muscle and to inhibit secretion in mast cells may result from a multiplicity of effects.

#### $\beta$ Adrenergic agonists

In 1936, Schild reported that adrenaline inhibits antigen-induced release of histamine from sensitised guinea pig lungs. This observation was extended in 1968, when it was demonstrated that a series of catecholamines inhibited histamine release from human leucocytes in an order of potency which suggested they acted through a  $\beta$  receptor (Lichtenstein and Margolis, 1968). The inhibitory potency of these agents correlated with their ability to increase the levels of cyclic AMP in the leucocyte preparation (Lichtenstein, 1976). Subsequently, Assem and Schild demonstrated that therapeutic concentrations of  $\beta$  agonists inhibited antigen-induced histamine release from passively sensitised human lung tissue. This did not occur in the presence of the  $\beta$  receptor antagonist, propranolol. Hence, they proposed that sympathomimetic amines may relieve symptoms of asthma partly by abrogating the release of pharmacological mediators from mast cells (Assem and Schild, 1969).

The anti-allergic activity of  $\beta$  agonists varies from tissue to tissue. For example, much higher doses of the drugs are required to inhibit antigen-induced histamine release from human leucocytes than are necessary to inhibit release from guinea pig and human lung tissue (Assem, 1974). Once mast cells are isolated from a tissue they become unresponsive to  $\beta$  agonists, so it seems that the cellular environment plays an important

part in the action of these drugs. For example, isoprenaline is a poor inhibitor of secretion in purified rat mast cells (Taylor *et al*, 1974) but incubation of the drug with rat lung tissue induces release of a factor which potentiates its activity (Taylor and Sheldon, 1977). Thus, at least part of the mechanism by which  $\beta$  agonists inhibit secretion in tissue mast cells appears to be indirect.

#### Tolerance and Tachyphylaxis to $\beta$ agonists

Several studies *in vivo* have demonstrated that repeated administration of  $\beta$  agonists may cause tachyphylaxis. Measuring changes in heart rate and blood pressure in cats, Atkinson and Rand (1968) showed that intravenous infusion of isoprenaline, adrenaline or orciprenaline decrease the  $\beta$  stimulant effect of a subsequent bolus injection of each drug. Similarly, in man and dogs, tachyphylaxis occurs to intravenously-administered isoprenaline and there is cross-tachyphylaxis between isoprenaline and other  $\beta$  agonists (Conolly, Davies, Dollery and George, 1971). Oral administration or inhalation of these drugs may also induce a state of tolerance. Holgate and coworkers showed that, after normal volunteers had inhaled salbutamol regularly for 4 weeks, the response of their airways to fresh inhalations of the drug was reduced by some 50% (Holgate, Baldwin and Tattersfield, 1977). Similarly, Greenacre and Conolly (1978) found that frequent administration of salbutamol, either in tablet or nebulised form, to normal subjects produced a reduction in the cyclic AMP response of their leucocytes to isoprenaline. However, studies in asthmatics and atopic subjects have failed to demonstrate tolerance to the bronchodilation (Harvey and Tattersfield, 1979) or change in leucocyte cyclic AMP (Gibson, Greenacre, König, Conolly and Pride, 1978) produced by salbutamol.

At the cellular level, chronic exposure to  $\beta$  agonists nearly always induces tolerance to their effects. This has been shown in frog erythrocytes, guinea pig macrophages, cells of the rat pineal gland and in human embryonic fibroblasts (Remold-O'Donnell, 1974; Mukherjee, Caron and Lefkowitz, 1975). Part of the present work was aimed at determining if tolerance develops to the effect of  $\beta$  agonists on mast cells.

### 3) ANTIHISTAMINES

Despite the poor clinical efficacy of the classical antihistamines in the treatment of asthma (Douglas, 1975b) there has recently been a resurgence of interest in their value as anti-allergic drugs. This is due to the development of 2 novel antihistamines, ketotifen and oxatomide, which in certain concentrations prevent mast cell degranulation in vitro and which give benefit on oral administration to some patients with asthma and allergic rhinitis.

Ketotifen, exhibits anti-allergic activity in several studies of anaphylaxis in animals. This derives from a combined ability to prevent the secretion of mediators from mast cells and to antagonise the effects of any histamine which is released (Martin and Römer, 1978). With claims that ketotifen improves lung function in asthmatic patients (Girard and Cuevas, 1977; Craps, Greenwood and Radjelovic, 1978), the drug has subsequently been introduced clinically. Oxatomide also inhibits secretion in mast cells and blocks smooth muscle contraction induced by histamine and other spasmogens. Oxatomide was found to be an effective treatment in a study of subjects with hay fever and therefore its therapeutic value is presently under further investigation (reviewed by Emanuel and Towse (1980a,b). However, both ketotifen and oxatomide demonstrate several pharmacological effects, so it is not yet possible to conclude whether any advantageous effect seen clinically may be accounted for by their mast cell stabilising action.

Mota and Dias da Silva were the first to observe that certain doses of histamine antagonists could inhibit the release of histamine from mast cells. They also found that, at relatively higher concentrations, the antihistamines actually provoked histamine release (Mota and Dias da Silva, 1960). These findings were later reproduced in human basophils by Lichtenstein and Gillespie (1975). On examining representatives of the 5 major classes of classical antihistamines, they observed that all 5 inhibited the immunological release of histamine and that 3 of the 5 representatives caused significant histamine release when they were added in high (mM) concentrations. The phenothiazines were clearly the most

potent class at inhibiting basophil secretion but the anti-allergic activity of the antihistamines was not related to their potencies as  $H_1$  receptor antagonists. Examination of other agents which possess a similar tricyclic structure to the phenothiazines showed that they too were effective inhibitors of secretion in basophils.

This prompted Church and Gradidge (1980a) to test a series of antihistamines for their inhibitory effects in passively sensitised human lung tissue. They found that all of the drugs, to a greater or lesser extent, inhibited antigen-induced histamine release. At high concentrations, most of the drugs liberated histamine. As in human basophils, the phenothiazines and other tricyclic compounds were the most potent inhibitors and the anti-allergic potencies of the antihistamines were not related to their  $H_1$  receptor blocking activities.

From their findings in human basophils, Lichtenstein and Gillespie suggest that drugs which are structurally similar to the phenothiazines should be developed for clinical testing. Since the mechanism by which antihistamines exert their anti-secretory effect is unknown, part of the present work was designed to establish the mode of action of the phenothiazine, chlorpromazine.

CHAPTER II.   MATERIALS AND METHODS

## II.1 MATERIALS

### TISSUES

- |                   |  |
|-------------------|--|
| Human lung tissue | - Western Hospital, Southampton.                         |
| Rat serosal cells | - from normal male Wistar rats, 150-300g<br>(Lions Lab.) |

### ALLERGIC SERA, ANTIGENS AND ANTISERA

- |                      |   |
|----------------------|---|
| Human allergic serum | - the selection of donors is described in<br>METHODS.   |
| Antigens             | - freeze-dried extracts of Timothy grass<br>pollen and <u>Dermatophagoides pteronyssinus</u><br>(house dust mite), donated by Miles Labs. |
| Antisera             | - goat serum containing anti-human IgE and<br>anti-human IgG (Miles Labs.)  |

### PHYSIOLOGICAL SALT SOLUTIONS AND BUFFERS

- |   |  |
|---|--|
| Tyrodes solution                          | - NaCl, 40.00; KCl, 1.00; MgCl <sub>2</sub> .6H <sub>2</sub> O, 0.50;<br>CaCl <sub>2</sub> .6H <sub>2</sub> O, 1.00; NaHCO <sub>3</sub> , 5.00; NaH <sub>2</sub> PO <sub>4</sub> ,<br>0.25 and Glucose, 5.00 grams dissolved<br>in 5 litres of distilled H <sub>2</sub> O (Reagents<br>from BDH Chemicals).              |
| Phosphate-buffered salt<br>(PBS) solution | - NaCl, 40.00; KCl, 1.00; MgCl <sub>2</sub> .6H <sub>2</sub> O, 0.50;<br>CaCl <sub>2</sub> .6H <sub>2</sub> O, 1.00; Na <sub>2</sub> HPO <sub>4</sub> , 5.75; KH <sub>2</sub> PO <sub>4</sub> ,<br>1.00 and Glucose, 5.00 grams dissolved<br>in 5 litres of distilled H <sub>2</sub> O (Reagents<br>from BDH Chemicals). |
| Tris buffer solution                      | - Tris (hydroxymethyl) methylamine plus HCl<br>(BDH Chemicals).  |

### DRUGS AND CHEMICALS

- |   |                 |
|---|-----------------|
| Chlorpromazine hydrochloride                      | - May and Baker |
| Compound 48/80                                    | - Wellcome      |
| *EGTA, ethyleneglycol-bis<br>(β-aminoethyl ether) | - Sigma         |
| N,N'-tetraacetic acid                             |                 |
| *Human albumin                                    | - Sigma         |

ICI 74,917 ("Bufrolin"),	- ICI
6-n-butyl-2,8-dicarboxy-	
4,10-dioxo-1,4,7,10-	
tetrahydro-1,7-phenan-	
throlin; disodium salt	
*Lanthanum chloride. 7H <sub>2</sub> O	- Sigma
Lignocaine hydrochloride	- Pharmacy Dept., Southampton General
(powdered form)	Hospital
Propranolol hydrochloride	- ICI
(racemate and + isomer)	
Salbutamol sulphate	- Glaxo-Allenburys
Sodium cromoglycate	- Fisons

\* Except for those denoted by an asterisk, the above compounds were gifts.

#### CHEMICALS FOR HISTAMINE ASSAY

n-Butanol  
 Heptane  
 Histamine acid phosphate  
 Hydrochloric acid  
 Methanol  
 O-phthaldialdehyde  
 Perchloric acid  
 Sodium chloride  
 Sodium hydroxide  
 Triton X-405

All the reagents were from BDH Chemicals, except for O-phthaldialdehyde (Sigma).

#### SPECIALISED EQUIPMENT

Aminco-Bowman spectrophotofluorimeter  
 Hewlett-Packard mini-computer  
 MSE minor, bench centrifuge  
 MSE major, refrigerated centrifuge  
 Technicon AAI sampler.

II. METHODSA. PASSIVE SENSITISATION AND CHALLENGE OF HUMAN LUNG IN VITROPassive sensitisation

Human lung tissue was passively sensitised and challenged according to a modified version of the procedure described by Sheard et al (1967). This is outlined below and in Fig. II.1. Any variations in the procedure which were required for individual experiments are mentioned in the text.

Specimens of human lung, resected mainly because of carcinoma, were collected as quickly as possible from the operating theatre and placed in Tyrodes solution. Macroscopically normal lung tissue was excised from the specimens, chopped with scissors into small fragments and dissected free of large bronchioles. The tissue fragments were then either sensitised as individual replicates (a), or sensitised in bulk (b) and divided into replicates before antigen challenge:-

a) The chopped lung tissue was washed well with Tyrodes solution and divided into 200 mg replicates. Each replicate was placed in a test tube containing allergic serum dissolved in 2 ml of freshly-carbonated Tyrodes. The test tubes were stoppered, and the lung left to sensitise for 18 h at room temperature followed by 1 h in a water bath at 37°C. Once sensitised, the lung replicates were each washed in 50 ml of PBS solution to remove unbound serum components, and then transferred to test tubes containing PBS.

b) The chopped tissue was washed with Tyrodes solution and divided into 3 g aliquots. Each aliquot was placed in a 25 ml perspex sample bottle which was then filled to the brim with allergic serum diluted with freshly-carbonated Tyrodes. The neck of the bottle was sealed with a screw-top. After 18 h at room temperature followed by 1 h at 37°C, the sensitised tissue was thoroughly washed with PBS solution and pooled, as required, with other tissue sensitised in the same way. The pooled tissue was divided into 200 mg replicates which were then placed in test tubes containing PBS.

The choice of sensitisation regimen depended on the nature of the experiment.

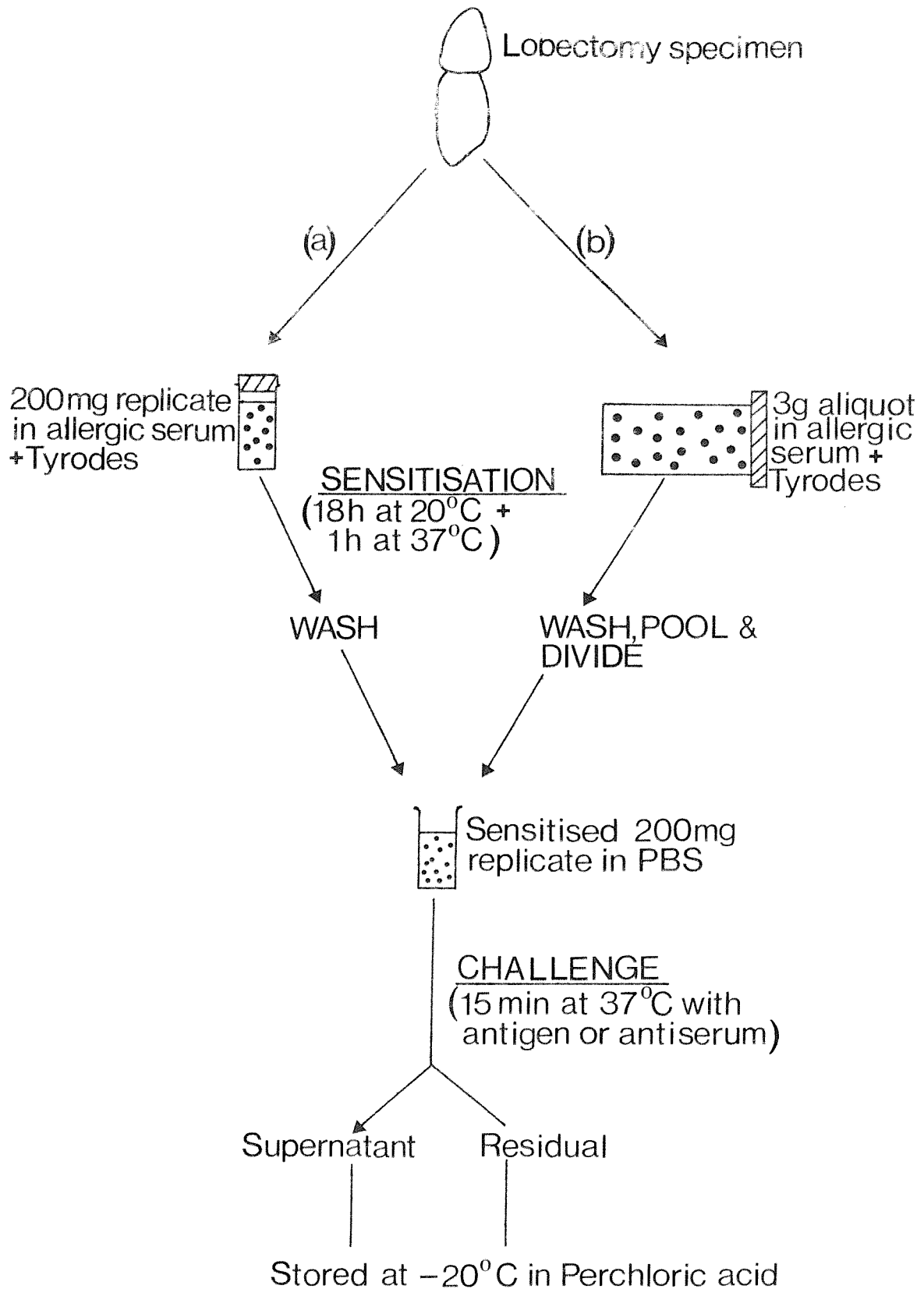


FIG. II.1. Passive sensitisation and challenge of human lung *in vitro*

### Challenge with antigen or antiserum

After the sensitised lung fragments had been transferred to fresh PBS solution, the test tubes were replaced in the water bath and left for 5 min for the temperature of the lung tissue to equilibrate to 37°C. Then drug solutions were added to appropriate samples, and all samples were subsequently challenged with antigen or antiserum in a final volume of 2 ml. The test tubes were shaken to ensure thorough mixing of their contents. After 15 min incubation, the samples were rapidly cooled to stop secretion in the lung mast cells. 1 ml of supernatant was removed and the tissue was frozen, then thawed, to rupture the mast cells and liberate the residual contents of their granules. Perchloric acid was added to both the supernatant and tissue to prevent chemical decomposition of histamine. Both were subsequently assayed for histamine, to measure the extent of secretion in terms of the original histamine content of the lung replicate. All samples were stored at -20°C until required for assay.

For ease of manipulation and accuracy, each experimental group was normally confined to a maximum of 30 replicates. This included 6 treated with antigen or antiserum alone, to establish the amount of stimulated histamine release, and 2 replicates to measure spontaneous release from lung which was left unchallenged. Most other treatments were performed in triplicate or quadruplicate.

### Choice of buffer solutions

In the original procedure of Sheard et al (1967), Tyrodes solution was used throughout. However, Tyrodes and other physiological salt solutions are bicarbonate-based buffers whose pH increases with time due to loss of CO<sub>2</sub> to the atmosphere. This can be avoided either by repeatedly gassing the solution with CO<sub>2</sub>, or by maintaining the solution in a sealed container in which the void space is minimal. For this reason, lung tissue was always sensitised in freshly-carbonated Tyrodes in a sealed container. It was impractical, however, to use sealed vessels at the challenge stage. Therefore, PBS solution was utilised, which is a phosphate-based buffer with a stable pH and which contains all the salts of Tyrodes solution, except for bicarbonate.

### Freshly-prepared materials

Tyrodes and PBS solutions were always prepared afresh. The Tyrodes was aerated for 15 min with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> and, where possible, it was subsequently gassed for 5 min every half-hour. The pH of the Tyrodes solution was 7.4 whereas that of PBS was 7.2.

Drugs were dissolved in PBS solution, approximately 15 min before they were added to the lung tissue. They were prepared in 10- or 20-fold excess concentrations so that they could be added in a small volume (0.2 and 0.1 ml, respectively) to the incubation medium (final volume, 2 ml).

### Preparation and storage of allergic serum, antigens and antisera

Donors of allergic serum were initially selected by their history of allergy and, in some cases, by history of positive skin reaction to a particular antigen. Each serum sample was prepared by clotting the blood in a sterile, glass beaker at 37°C for 3 h, after which the exudate was aspirated and centrifuged (750 g for 10 min) to obtain the cell-free serum. This was then tested for its ability to passively sensitise lung tissue, as indicated by the amount of histamine released on challenge with various antigens. Active sera with the same specificity, eg to grass pollen, were very often pooled. Pooled or individual sera were divided into aliquots and stored at -20°C until required. This was designed so that serum from the same batch could be used over several experiments in different lung tissues, without being repeatedly thawed and refrozen.

Antigens and antisera were dissolved in PBS solution at 10-fold higher concentrations than those required in the experiment. As with allergic serum, they were divided into aliquots and stored at -20°C until required. Then, on the day of the experiment, each was added as 0.2 ml to the incubation volume of 2 ml.

B. PREPARATION OF RAT SEROSAL MAST CELLS AND STIMULATION OF HISTAMINE RELEASE

Rat serosal mast cells were employed for a study which compared effects of drugs on 2 mechanisms of histamine release: firstly, release induced by activation of secretion with compound 48/80 and, secondly, release caused by cell lysis under hypotonic conditions. The methods by which the cells were harvested, washed and challenged are described below and summarised in Fig. II.2.

Preparation of the cells

A male Wistar rat weighing between 150 and 300 g was killed by cervical dislocation, and its abdomen exposed by removing a small piece of fur. 15 ml of PBS solution containing human serum albumin (PBS/HSA) was injected into the peritoneal cavity and the abdomen was vigorously massaged for 1-2 min. At the end of the period of massage, a small incision was made in the abdomen, avoiding the mid-line vasculature, and then the animal was carefully raised and inverted over a funnel so that fluid from the peritoneal cavity drained into a container. The funnel was washed with a small volume of PBS/HSA to collect any cells adhering to its surface. Then, the container was sealed and inverted several times to mix the suspension of cells. The suspension was divided into aliquots and centrifuged to yield 2 cell pellets ( $P_{1a}$  and  $P_{1b}$ ).

The cells of each pellet were washed twice before challenge. This entailed resuspension of the pellet in fresh buffer solution and centrifugation to separate the cells from their washings. Cells to be challenged with compound 48/80 were washed in PBS/HSA and recovered as a single pellet ( $P_{3a}$ ). Cells which were to be exposed to an hypotonic medium were washed in a solution of normal saline (0.9%), Tris buffer and HSA. They were divided into replicates after the first wash, and therefore recovered as individual pellets ( $P_{3b}$ ).

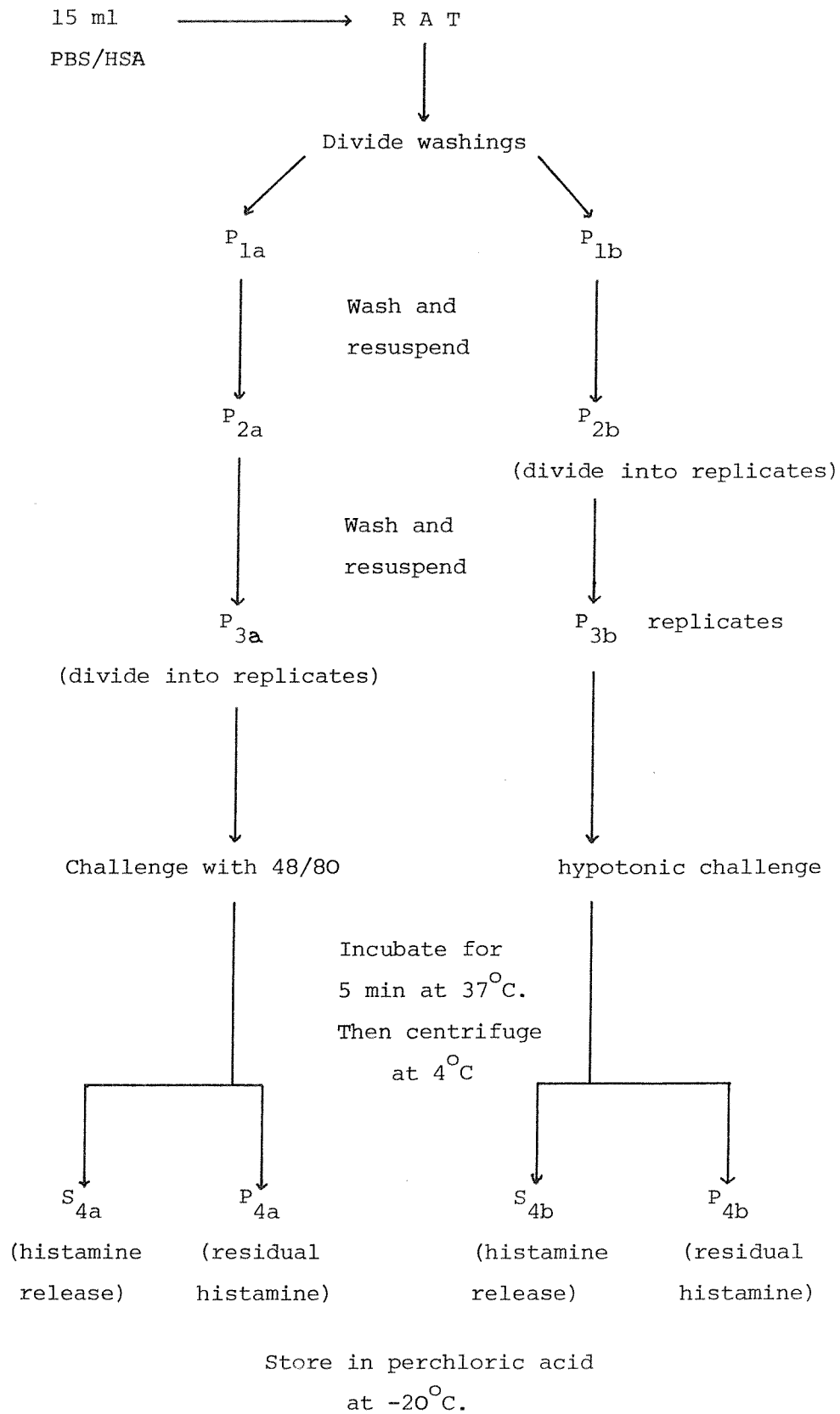


Fig. II.2. Collection and challenge of rat serosal mast cells

Challenge with compound 48/80

The single pellet of washed cells ( $P_{3a}$ ) was resuspended and diluted, as required, with PBS solution. The cell suspension was pipetted as 0.5 ml aliquots into test tubes. These were placed in a water bath at 37°C and left for 5 min for the temperature of the cells to equilibrate. Then drug solutions were added as 0.1 ml to appropriate samples, and each replicate of cells was challenged with compound 48/80 (0.1 ml) in a final volume of 1 ml. Incubation with 48/80 was continued for 5 min, and then the reaction with mast cells was stopped by immediately transferring the test tubes to a refrigerated centrifuge at 4°C. The test tubes were spun to separate the cells ( $P_{4a}$ ) from their supernatants ( $S_{4a}$ ). Using a micropipette, the supernatant of each tube was completely removed and dispensed into 0.1 ml of concentrated perchloric acid, to preserve the histamine content. The corresponding cell pellet was ruptured by addition of 1 ml of dilute perchloric acid. All samples were then stored at -20°C until their histamine content was assayed.

Cells incubated in PBS solution alone were used to correct for spontaneous histamine release. Cells incubated with drug in the absence of compound 48/80 controlled for effects of the drug on spontaneous release.

Hypotonic lysis

Each pellet of washed cells ( $P_{3b}$ ) was resuspended in 0.4 ml of a solution consisting of 0.5% saline and Tris buffer, which had been warmed to 37°C. Having placed the tubes in a water bath, 0.6 ml of Tris buffer, or Tris plus drug, was immediately added to appropriate samples. This gave a final saline concentration of 0.2%. After 5 min incubation at 37°C (to mirror the conditions for challenge with compound 48/80), histamine in the supernatant was separated from histamine retained in the mast cells by centrifugation, and both were stored in perchloric acid as previously described. Cells incubated in 0.5% saline and Tris buffer were used to correct for spontaneous histamine release. This saline concentration was not sufficiently hypotonic to cause lysis of mast cells (Chapter VI, section VI.I).

### Number of replicates

The number of cell replicates utilised from each rat was governed by the number of samples which could be accurately processed at the challenge stage rather than by the amount of histamine contained and released by the cells. An average-sized experiment involved 60 replicates. In each experiment, the variability between replicates was so small that duplicate observations were sufficient to obtain a mean drug response and a value for spontaneous histamine release. The mean level of histamine release induced by compound 48/80 or hypotonic lysis was calculated from 4 observations.

### Role of HSA

The purpose of including human serum albumin in the wash media was to maintain the reactivity and reduce the spontaneous degranulation of mast cells during their preparation (Uvnäs and Thon, 1961). HSA was omitted from the challenge media because most drugs bind to proteins and therefore the albumin would interfere with the reaction between drugs and mast cells.

### Preparation of materials

PBS solution was always prepared freshly on the day of the experiment. Tris buffer was taken from a 100 mM stock solution, prepared by dissolving 1.21 g of Tris (hydroxymethyl) methylamine in 100 ml of distilled water and titrating the pH to 7.2 with 4N hydrochloric acid. 7 ml of this solution was added to 93 ml of distilled water, to give a final Tris concentration of 7 mM. Then, 500 mg or 900 mg of sodium chloride was dissolved in the Tris buffer to yield saline concentrations of 0.5% and 0.9%, respectively. In wash media only, HSA was dissolved as a 0.1% solution. Drugs were dissolved in the appropriate challenge medium, approximately 15 min before being added to the cells.

### Equipment

Plastic apparatus was used throughout the experiment to minimise adherence of cells to foreign surfaces. Centrifugation of cell suspensions was performed either in an MSE minor bench centrifuge (150 g for 10 min) or in an MSE major, refrigerated centrifuge (150 g for 5 min). Cell pellets were resuspended by using a micropipette to mix the cells with fresh buffer solution, and a vortex mixer, run at slow speed, to produce an homogeneous suspension.

### C. MEASUREMENT OF HISTAMINE

Histamine was extracted from experimental samples and assayed spectrofluorimetrically, according to an automated version (Evans, Lewis and Thomson, 1973) of the procedure described by Shore, Burkhalter and Cohn, in 1959. The method is highly reproducible and is adequately sensitive and specific to measure the amounts of histamine contained and released by mast cells (Evans, Lewis and Thomson, 1973). A summary of the equipment used in the assay is given in Fig. II.3.

### Extraction of histamine and condensation with O-phthaldialdehyde (OPT)

Samples containing histamine in perchloric acid were initially centrifuged to discharge any precipitate. The supernatants were then loaded into cuvettes and sampled by an autoanalyser (Technicon AAII) every 2 min. The actual sampling time was 1 min and this was followed by a minute's wash in perchloric acid to ensure that histamine from successive samples did not become entrained. In the autoanalyser, histamine was first extracted from an alkaline medium into butanol, under a stream of air injected at regular intervals to improve the separation between the organic and aqueous phases. Then, on exposure to acidified heptane, it transferred back to the aqueous phase. The extracted histamine was condensed with OPT, in a strongly alkaline medium discretely bubbled with air. This produced a labile fluorophore which was stabilised by infusion of 4N hydrochloric acid.

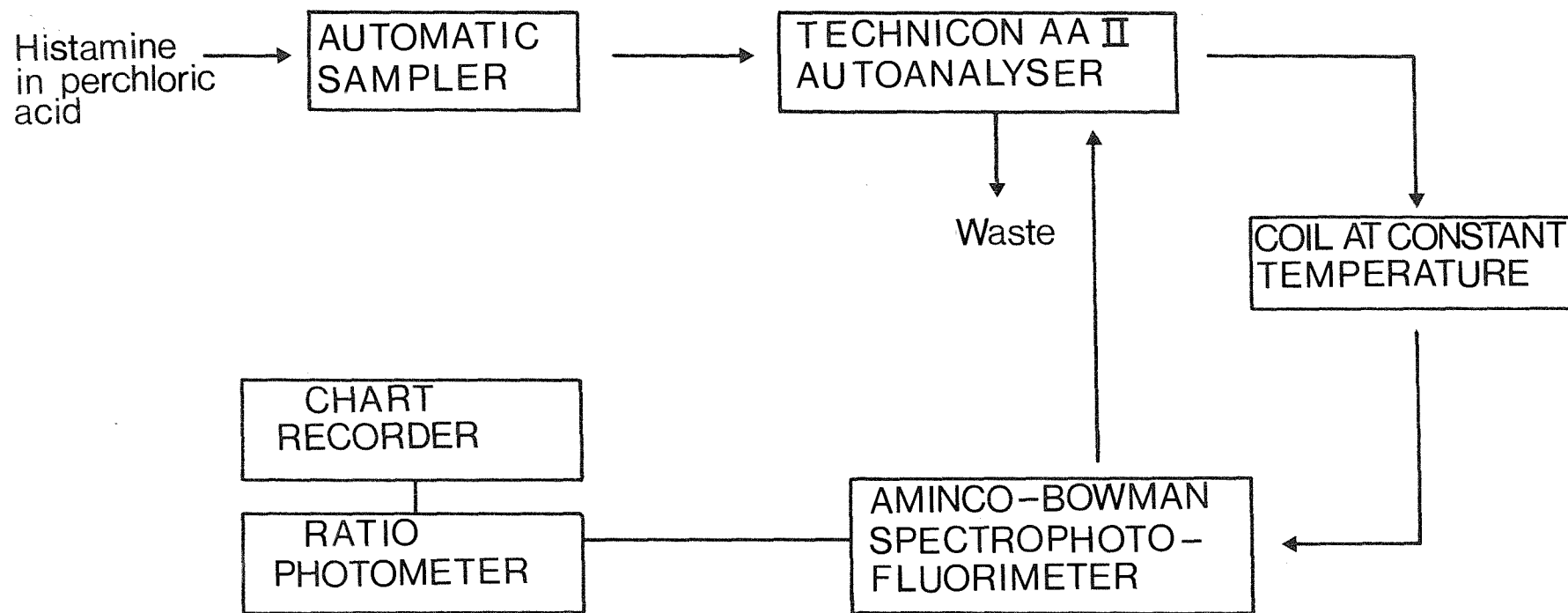


FIG. II.3. Summary of the stages and equipment for the histamine assay.

### Fluorimetric assay

The histamine-OPT fluorophore was immediately pumped through a coil at constant temperature and then into the flow-cell of a spectrophoto-fluorimeter. It was activated by a beam from a xenon lamp (wavelength, 350 nm) and its fluorescence measured at 465 nm by a ratio photometer. Deflections of the photometer were reproduced on a chart recorder so that the trace heights formed a direct measurement of the histamine fluorescence.

Standard solutions of histamine in perchloric acid were used to obtain a fluorescence/concentration plot from which the unknown concentrations of histamine were calculated. The histamine concentration was directly proportional to the relative fluorescent intensity, down to at least 25 ng/ml (Fig. II.4). Therefore, the size of experimental replicates was designed so that the amounts of histamine contained and released were always much greater than 25 ng/ml and, therefore, calculated from the linear region of the fluorescence/concentration graph. Each replicate of human lung contained so much histamine (mean content over 90 different lungs = 22.1 µg/g) that the tissue samples had to be diluted with perchloric acid before they were assayed. All drug solutions used in experiments were tested for effects on the fluorescence of standard histamine solutions. None of the drugs showed any interference with the histamine assay.

### Preparation of materials

The condensation reagent, OPT, was always prepared freshly on the day of assay. 50 mg of the reagent were dissolved in 6 ml of methanol and diluted to 25 ml with distilled water. As OPT decomposes in normal light, it was kept in a smoked-glass container during the assay.

All of the solutions, apart from butanol, were of analytical grade. Prior to the assay, butanol was washed with salt-saturated sodium hydroxide (250 g NaCl in 1 litre 0.1N NaOH). A "wetting agent", Triton X-405, was included at the condensation stage to ensure a continuous flow of extracted histamine through the glass coils of the autoanalyser.

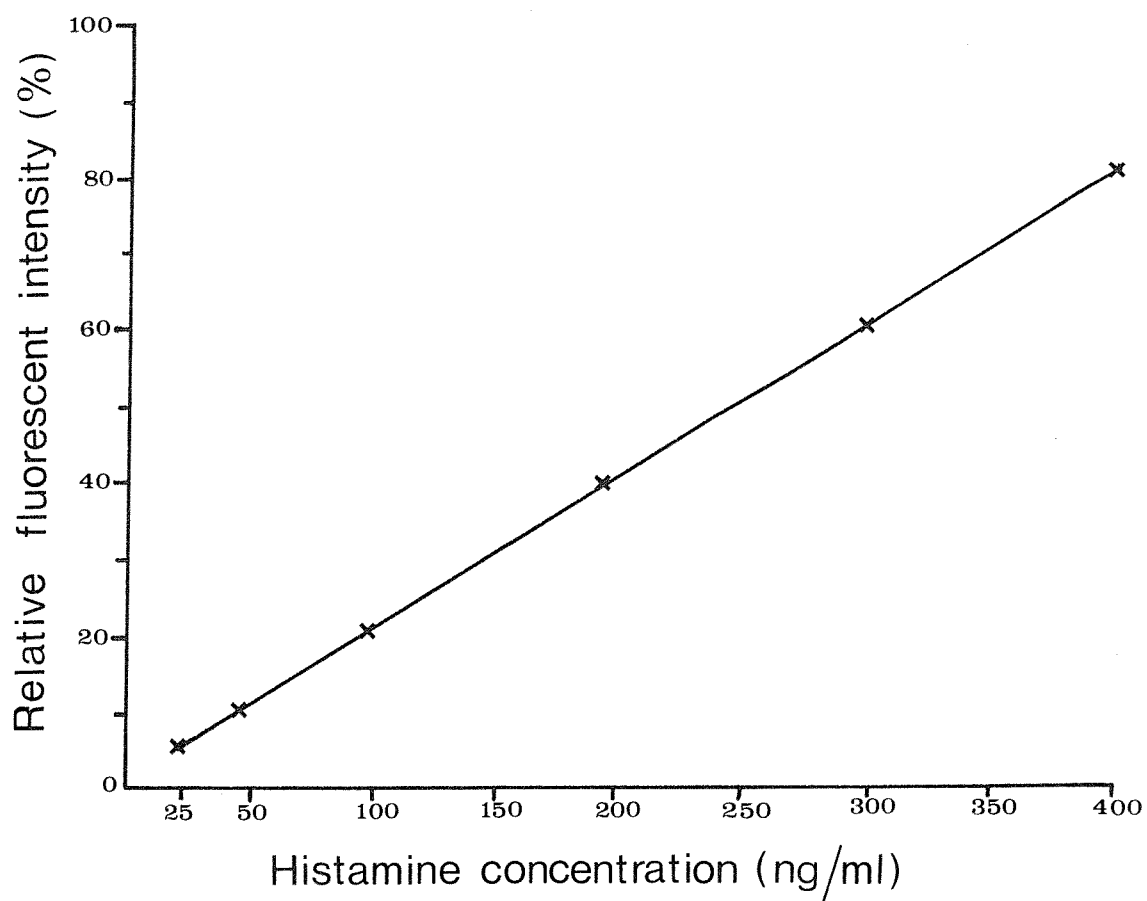


FIG. II.4. The fluorescence of standard histamine solutions prepared in perchloric acid, after condensation with o-phthaldialdehyde. The fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 465 nm. 0.4N perchloric acid solution was used as the blank. Each value is the mean of 2 observations.

#### D. ANALYSIS AND PRESENTATION OF DATA

##### Calculation of histamine release and the response to drug

In each tissue or cell sample, the amount of histamine release was expressed as a percentage of the original histamine content of the sample (1). This controlled for the variable amounts of histamine in different replicates. Stimulated release was corrected for spontaneous release by subtracting the average amount of histamine liberated from samples incubated in the absence of the stimulus (2):-

$$(1) \quad \% \text{ histamine release} = \frac{\text{histamine in supernatant}}{\text{histamine in supernatant} + \text{residual histamine}} \times 100\%$$

$$(2) \quad \begin{array}{l} \text{Corrected histamine} \\ \text{release} \end{array} = \text{stimulated release} - \text{mean spontaneous release}$$

The results from drug-treated samples were transformed into percentage inhibitions of the stimulated release (3), so that the response to drug could be compared over several experiments, ie in lung tissues from different patients or in cells from different rats.

$$(3) \quad \% \text{ inhibition of histamine release} = \frac{\text{Corrected release} - \text{Corrected release in presence of drug}}{\text{Corrected release}} \times 100\%$$

Any other transformations of the data are described in the text.

##### Statistical analysis

All results were processed on a Hewlett-Packard mini-computer. The response to a certain treatment in a single experiment was calculated as the mean  $\pm$  one standard deviation, from the responses of several replicates. The response to the same treatment over repeated experiments was calculated as the mean  $\pm$  one standard error. Where the results are presented in a graphical form, the standard deviation or error about each mean is shown as vertical bars.

The 2-way analysis of variance or paired t-test was used to determine the significance of differences between means, with the significance limit set at the  $P = 0.05$  probability level. Formulae described by Moroney (1951) were used to calculate the probability values, as well as coefficients of variation, correlation and regression. In tables and graphs, the symbols \* and \*\* denote  $P < 0.05$  and  $P < 0.01$ , respectively.

CHAPTER III.    STANDARDISATION OF PROCEDURE  
WITH HUMAN LUNG AND VARIATION  
IN HISTAMINE RELEASE

## PART A. STANDARDISATION OF PROCEDURE

A series of preliminary experiments was performed to establish the optimal conditions for passive sensitisation and challenge of human lung tissue, so that a standard procedure could be used in drug studies.

### III.1 Concentration of allergic serum for sensitisation

The sensitising capacity of an allergic serum was initially tested by adding 2 ml of a 10% solution (ie 0.2 ml serum dissolved in 1.8 ml Tyrodes) to 200 mg replicates of lung, and subsequently determining whether the lung released significant amounts of histamine in response to antigen. Then, active sera were examined in serial dilutions to determine if they could be used at a more economical concentration. Examples of the ability of sera from 3 hay-fever sufferers to sensitize lung samples towards an extract of Timothy grass pollen (TPE) are shown in Fig. III.1. The results show that 2 of the sera (DJ, AC) sensitized the lung for 6 and 9% histamine release at the lowest serum concentration investigated, ie a 1% solution. As the concentration of serum was increased, more histamine release was obtained on challenge. The release reached a maximum after sensitizing with either a 5 or 10% solution, indicating that these serum concentrations were optimal for passive sensitization. The third serum (GF) was less potent at sensitizing lung fragments, since dilution of the serum beyond 1:10 produced a marked fall in the antigen-induced histamine release.

In general, relatively potent sera such as DJ and AC could be used in a 3 or 5% concentration whereas those which were less potent, such as GF, would only sensitize the lung sufficiently as a 10% solution or greater. When allergic sera were pooled, they were combined in proportion to their relative sensitizing potencies.

### III.2 Sensitisation volume

An experiment was performed to determine whether 1.8 ml of Tyrodes solution was sufficient to meet the physiological requirements of each lung replicate over the long sensitization period of 19-20 h. 200 mg replicates of lung were individually sensitized with the same serum,

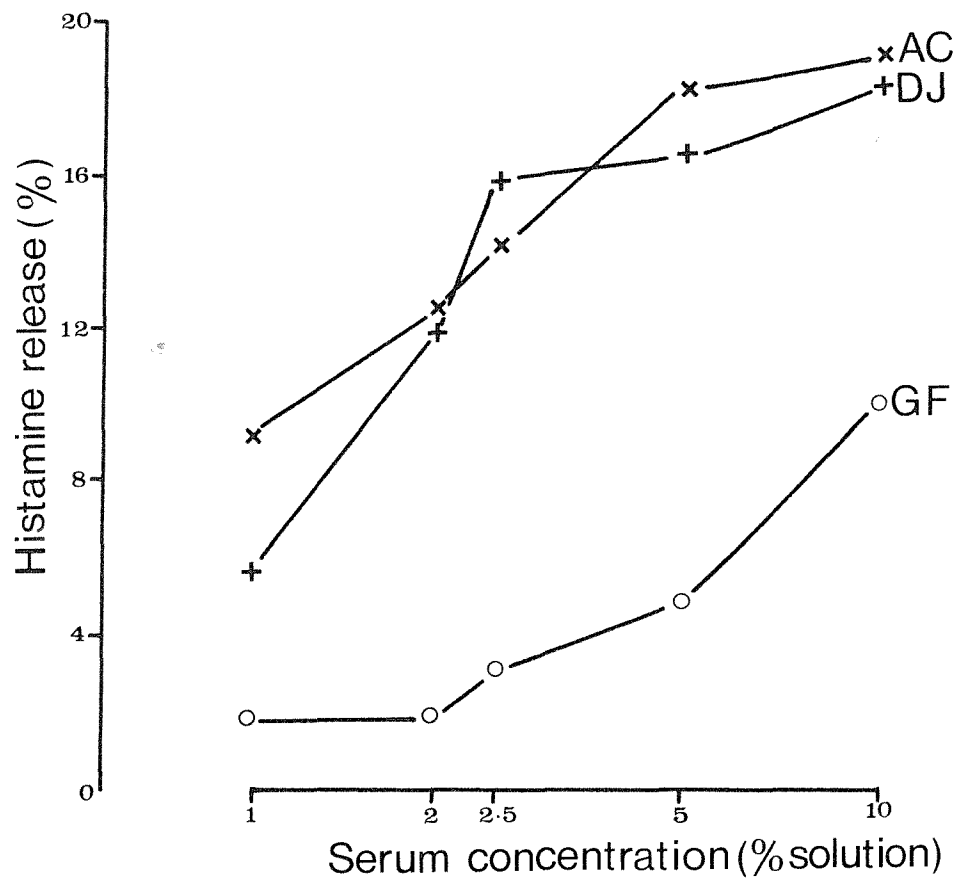


FIG. III.1. Histamine release from lung fragments sensitised with sera from 3 subjects with grass pollen allergy, on challenge with TPE (10  $\mu$ g/ml). Mean results are shown from one experiment with each serum.

at a sub-optimal concentration (0.1%), in a final volume of either 2 or 20 ml. The results in Table III.1 show that there was little difference in the spontaneous release of histamine from the two sets of samples, after they had been sensitised for 19-20 h. Histamine release stimulated by antigen was slightly greater in samples which had been sensitised in the larger volume (12.8% compared with 10.4%), but not significantly so ( $P > 0.1$ ). By contrast, stimulated release was trebled in tissue sensitised in a 2 ml volume with 10% rather than 1% allergic serum, demonstrating that sensitisation depended more on the concentration of serum than on the volume of buffer in which lung was suspended. These results showed that the 2 ml volume was sufficient to maintain the physiological responsiveness of mast cells in the lung, so this was used in all other experiments. When lung was sensitised in bulk, the sensitisation volume was calculated on the basis of 2 ml per 200 mg of tissue, and the amount of serum increased to keep the same concentration as that used for individual replicates.

### III.3 Concentration of antigens and antisera for challenge

Sensitised lung fragments were challenged with increasing amounts of antigen or antiserum to establish concentrations which were both economical and which regularly induced histamine release. Fig. III.2 illustrates the results obtained with the pollen antigen, TPE, and with an extract of the house dust mite, Dermatophagoides pteronyssinus (DPE). TPE caused a concentration-related release of histamine in the range 0.1 - 10  $\mu\text{g/ml}$ , with no greater release at higher concentrations. Therefore, in further experiments, TPE was used at 10  $\mu\text{g/ml}$ . Histamine release induced by DPE rose when the antigen dose was increased from 0.4 to 10  $\mu\text{g/ml}$ . DPE was subsequently used at 1  $\mu\text{g/ml}$  because only small amounts of the extract were available.

Also in Fig. III.2, results are shown from 3 experiments with an antiserum which contained anti-IgE. In 2 experiments, this caused a concentration-related release of histamine in the range 0.05 - 0.2%. Higher levels of release were obtained in the third experiment, in which the range of antiserum concentrations was extended to 2%. However, the 0.2% solution was selected for routine use to conserve the antiserum, and was shown in further experiments to provide an adequate stimulus for histamine release.

Serum concentration (% soln.)	Sensitisation volume	Spontaneous histamine release (%)	Stimulated histamine release (%)
1%	2 ml	2.0 $\pm$ 0.4	10.4 $\pm$ 3.2
1%	20 ml	1.5 $\pm$ 0.7	12.8 $\pm$ 3.6
10%	2 ml	1.9 $\pm$ 0.4	32.8 $\pm$ 5.8

TABLE III.1. Relative importance of the concentration of allergic serum and the incubation volume to sensitisation. Histamine release is expressed as a percentage of the total histamine content of each lung sample. Each spontaneous release was calculated from 2 replicates; stimulated release was calculated from 4 and, in each case, has been corrected for spontaneous release. The results were obtained in a single experiment in which lung replicates were challenged with TPE (10  $\mu$ g/ml).

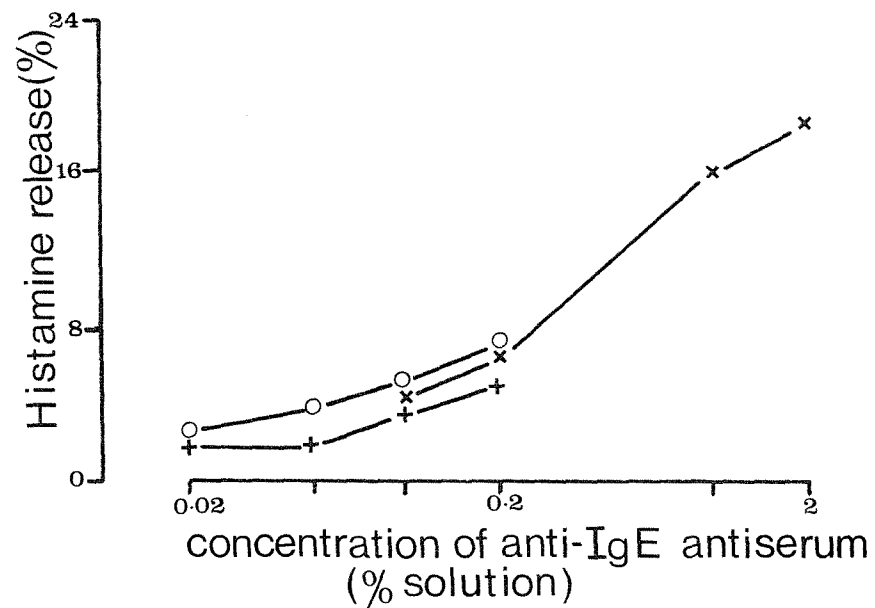
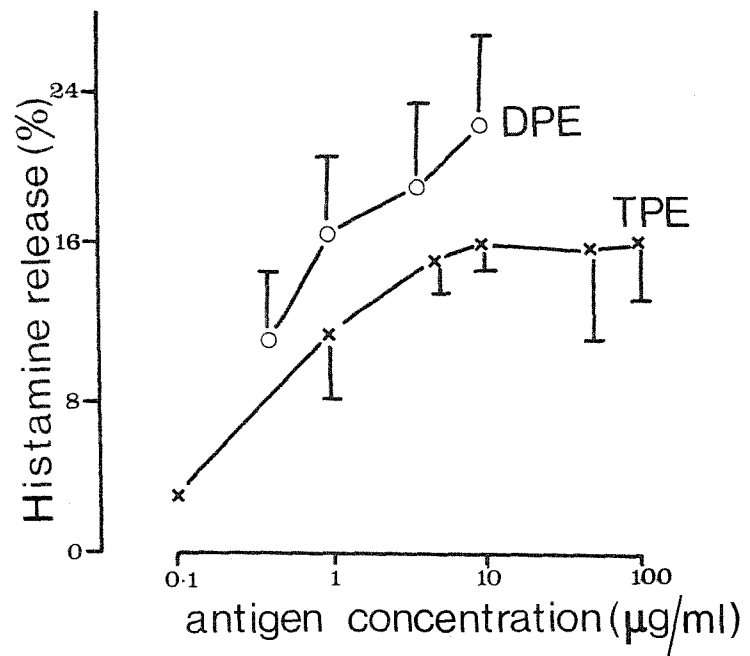


FIG. III.2. Histamine release from passively sensitised lung fragments on challenge with TPE, DPE or anti-IgE. Results for TPE and DPE are mean values from 3 or 4 experiments. With anti-IgE, the results from 3 experiments are plotted separately.

The other antiserum involved in the present studies, anti-IgG, was used as a 1% solution, to mimic the conditions described in a previous report (Church and Gradidge, 1978).

#### III.4 Incubation time for challenge

A study was made of the time course of histamine release from sensitised lung fragments challenged with TPE. The lung samples were incubated with TPE for periods ranging from 2½ to 60 min. The antigen was added at 30 sec intervals to successive replicates and each supernatant was aspirated exactly at the end of the stated incubation time. Histamine release stimulated by anti-IgE was studied under similar conditions, and the rates of secretion in the presence of TPE and anti-IgE were compared.

Spontaneous release of histamine from the sensitised tissue was minimal:  $0.7 \pm 0.2\%$  of the total histamine content over 30 min incubation at 37°C (11 experiments) and  $0.9 \pm 0.1\%$  over 60 min (4 experiments). Results from lung samples challenged with antigen or antiserum are shown in Fig. III.3. As the number of experiments performed to calculate the mean values of histamine release was variable, each result has been expressed as a percentage of the release obtained after 30 min incubation. On challenge with TPE, histamine release was detected after 2½ min ( $20.7 \pm 4.0\%$ ) reaching  $55.1 \pm 5.9\%$  by 5 min and  $81.5 \pm 6.8\%$  after 15 min. There was no difference between the results of the 30 and 60 min incubation which confirmed that the reaction had been completed by 30 min. Anti-IgE provoked an almost identical pattern of release, with  $24.0 \pm 6.2\%$  at 2½ min,  $47.5 \pm 7.0\%$  by 5 min and  $79.0 \pm 8.4\%$  after 15 min. Again, there was no further liberation of histamine between 30 and 60 min incubation. Interestingly, the rate of release declined between 5 and 10 min incubation with both TPE and anti-IgE, producing an inflexion in both curves in Fig. III.3. As such, the change was quite small and might be considered insignificant in the light of the experimental error. However, it occurred with both stimuli and has been documented by others (Platshon and Kaliner, 1978), so this appears to be a consistent observation.

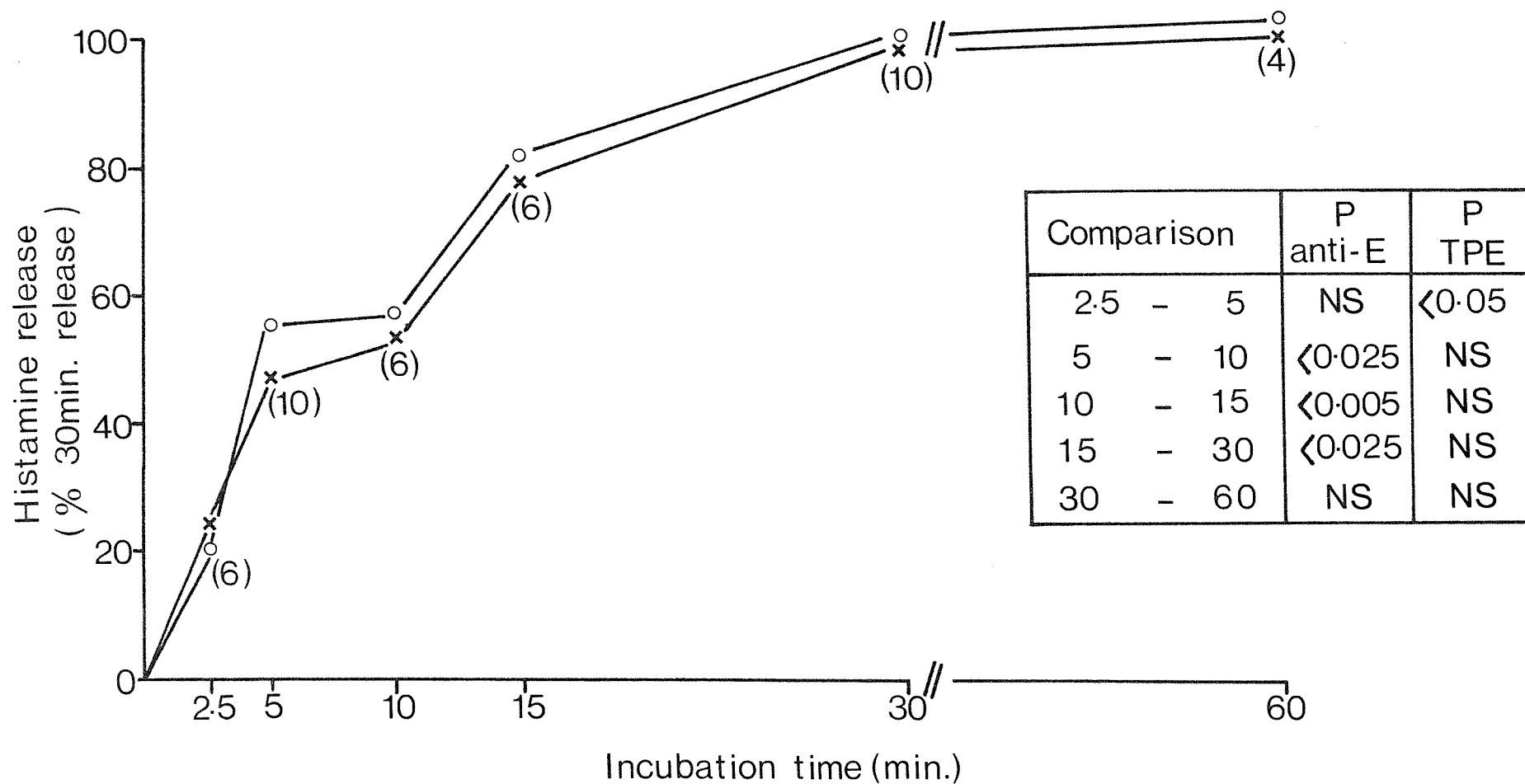


FIG. III.3. Time courses of histamine release from sensitised lung fragments challenged with TPE (O—O) or anti-IgE (x—x). Figures in parentheses indicate the number of experiments performed to calculate each mean. The table shows probability values obtained by analysis or variance testing (comparison of paired data only; NS - non-significant).

Thus, both types of immunological stimulus initially induced a rapid release of histamine from sensitised lung fragments which reached approximately 50% of maximum by 5 min. Between 5 and 10 min the rate of release fell, but showed a secondary increase after 10 min. By 30 min incubation the reaction was complete. Even after a complete reaction, only a certain proportion of the histamine in the tissue had been secreted. For example, histamine release after 30 min incubation with TPE was only  $22.0 \pm 3.7\%$  of the total histamine content (11 experiments).

### III.5 Selection of 15 min period of incubation

In most experiments, it was preferable to measure histamine release over a 15 min period rather than over 30 min, in order to mimic the conditions of incubation described in other reports. Then the effects of drugs on the release could be directly compared with data which has been previously documented. However, as histamine release had not reached a maximum by 15 min, it was conceivable that this might incur greater experimental error. To determine if this was so, the errors associated with measuring histamine release 15 and 30 min after challenge were compared in 4 experiments. The coefficient of variation for each mean estimate of histamine release is shown in Table III.2, to signify the percentage error. In the first experiment, the errors were similar for both estimates of histamine release ( $V = 29.2$  and  $30.7\%$ ). In 2 of the other 3 experiments, the error was in fact less when release was measured after 15 min, but in the fourth experiment it was greater. Hence, there was no consistent difference between the errors of the 2 estimates, and so measurement of histamine release after the shorter period of 15 min was not detrimental to the precision of the results.

## PART B VARIATION IN HISTAMINE RELEASE

### III.6 Index of secretion as a source of experimental error

The coefficients of variation in Table III.2 illustrate that there was often a substantial error associated with each mean estimate of histamine release. In this regard, Coleman (1980) has suggested that greater error is incurred when histamine release is calculated as a

Experiment	Histamine release (%)	Coefficient of variation, v (%)
1 a	8.9 $\pm$ 2.6	29.2
b	12.7 $\pm$ 3.9	30.7
2 a	19.4 $\pm$ 0.7	3.6
b	20.6 $\pm$ 2.6	12.6
3 a	6.8 $\pm$ 0.7	10.3
b	9.2 $\pm$ 1.6	17.4
4 a	13.0 $\pm$ 2.7	20.8
b	16.2 $\pm$ 2.1	13.0

TABLE III.2. Comparison of the errors associated with measuring histamine release after 15 min (a) or 30 min (b) reaction with antigen. Mean values for histamine release were obtained from 6 replicates. Sensitised lung samples were challenged with either TPE (10  $\mu$ g/ml; expt. 1 and 2) or with an anti-IgE antiserum (0.2%; expt. 3 and 4).

percentage of the total histamine contained in the lung, compared with when it is measured in nanograms. His evidence derives from a study of many replicates from a single lung, in which he found that there was a poor correlation between the histamine released by antigen and the total histamine. In the present studies, the correlation was investigated in 4 different lungs, using a large number of replicates from each. Also the coefficient of variation for release calculated as a percentage was compared with the coefficient for release measured as nanograms of histamine, to determine which index of secretion was more precise.

The 4 lungs which were investigated were deliberately selected for their wide-ranging secretion of histamine in response to antigen. Values for released and total histamine are shown with the coefficients of correlation ( $r$ ) and variation ( $V_R$ ,  $V_{R/T}$ ) in Table III.3. In contrast to the findings of Coleman, there was a significant correlation between the released and total histamine, regardless of whether the tissue responded mildly or strongly to antigen. This suggested that, in each lung, the amount released was dependent on the total histamine in the replicate, which validated the expression of release as a percentage. Moreover, the coefficient of variation was always less when release was expressed as a percentage, indicating that this was the more precise way of interpreting the data. Clearly, the experimental error was not increased by using this index of secretion.

### III.7 Variation in histamine release due to the tissue itself

As the errors associated with determinations of histamine release were not created experimentally, it appeared that the variability was inherent in the tissue. Not surprisingly, there was considerable variation in the amount of secretion from different lungs. This is illustrated in Fig. III.4 which is a frequency distribution of antigen-induced histamine release from 83 lungs. Each tissue was passively sensitised with allergic serum from 2 pollen-sensitive donors and subsequently challenged with TPE. In 3 lungs (ie 3.6% of those examined), histamine release in the presence of antigen was no greater than spontaneous release. The mean release from those tissues which responded to antigen was 16.3% of the total histamine content, but the responses were distributed over a large range, ie 1-45%.

n	Histamine release (ng) R	Total histamine (ng) T	(%) release R/T	r	$v_R$ (%)	$v_{R/T}$ (%)
13	1053 $\pm$ 165	4267 $\pm$ 555	24.6 $\pm$ 3.2	0.62*	15.7	13.0
18	960 $\pm$ 187	2740 $\pm$ 458	34.9 $\pm$ 4.2	0.77**	19.5	12.0
15	448 $\pm$ 148	3278 $\pm$ 740	12.9 $\pm$ 1.8	0.91**	33.0	13.7
12	196 $\pm$ 52	2898 $\pm$ 526	6.8 $\pm$ 1.2	0.66*	26.5	18.1

TABLE III.3. Validation of the index for secretion. The table shows the correlation (r) between the amount of histamine released in response to antigen (R) and the total histamine contained in the lung (T). n refers to the number of replicates investigated in each tissue. The table also shows the coefficient of variation associated with histamine release measured in nanograms ( $v_R$ ) or as a percentage ( $v_{R/T}$ ). All lung replicates were challenged with TPE ( $10 \mu\text{g/ml}$ ).

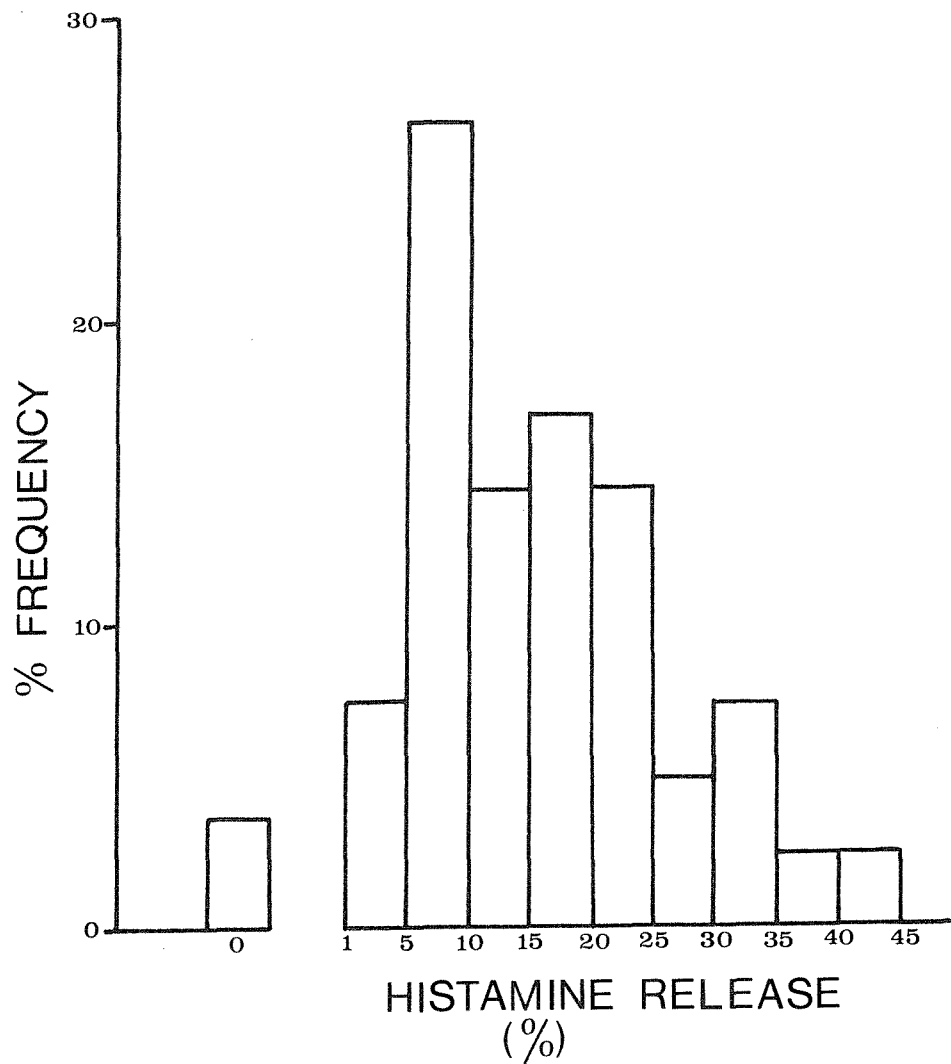


FIG. III.4. Frequency distribution of antigen-induced histamine release. 83 different lungs were investigated. The tissues were sensitised with AC or DJ serum, or pooled AC/DJ serum, and then challenged with TPE (10  $\mu\text{g}/\text{ml}$ ). The mean release from those lungs which did respond ( $n = 80$ ) =  $16.3 \pm 1.1\%$ .

One factor which must have contributed to this marked variation in response was that the majority of tissues were obtained from elderly patients suffering from lung cancer. In a survey of many donors the average age was 62 years and, apart from one donor who was only 22 years old, the majority were between 50 and 70 years of age (Table III.4). Virtually all of the patients reviewed had received surgery because of lung carcinoma. This varied from a localised tumour in the main bronchus or lobar regions, to general metastases throughout the lung. The degenerative changes caused by age and disease must have affected the passive sensitisation of the tissue mast cells and the ability of the cells to respond to antigen.

Another source of variation was that the majority of lung tissues were already sensitised with IgE antibodies, ie they were actively sensitised. This was revealed by incubation of chopped lung fragments with anti-IgE. Histamine release ensued and, over 45 lungs, ranged from 0.4 to 21.8% of the total histamine contained in the tissue (Table III.4). The endogenous antibodies must have interfered with passive sensitisation, by occupying IgE binding sites on mast cells and thereby blocking the uptake of antigen-specific IgE antibodies from the allergic serum.

#### SUMMARY AND IMPLICATIONS FOR STUDIES WITH DRUGS

Despite standardisation of the experimental conditions for passive sensitisation and challenge, there was considerable variation in the secretion of histamine from human lung. This was particularly noticeable in lungs from different patients but was also evident in different replicates from the same lung. Inherent factors such as the aged and diseased state of the tissue, and its pre-existing sensitisation with IgE antibodies, must have contributed to the variability.

Because of the diversity in secretion, results from drug-treated samples were transformed into percentage inhibitions of the stimulated histamine release so that they could be meaningfully compared (formula outlined in METHODS). When the release of histamine was poor, it was impossible to assess the inhibitory effects of drugs. Therefore an arbitrary limit of 5% was set, so that results were discarded if the stimulated release was less than this figure.

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<u>AGE</u>	Mean $\pm$ sd	=	62 $\pm$ 11 yrs.
(n=39)	Range	=	22 - 79 yrs.
 <u>CARCINOMA</u>			43 Carcinoma, 2 Bronchiectasis
(n=45)			
 <u>HISTAMINE RELEASE BY ANTI-IgE (%)</u>	Mean $\pm$ sd	=	6.5 $\pm$ 5.7%
(n=45)	Range	=	0.4 - 21.8%

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TABLE III.4. Age, disease and active sensitisation of human lung.  
 n refers to the number of donors or lungs studied;  
 sd = standard deviation. Endogenous sensitisation  
 with IgE was assessed by incubating lung replicates  
 for 15 min at 37°C with anti-IgE antiserum (0.2% solution).  
 In each lung, the histamine released by anti-IgE was  
 measured in duplicate.

The same allergic serum was used throughout each series of experiments with drugs, to minimise the variability involved with passive sensitisation. Unless otherwise stated, the serum was from pollen-sensitive subjects and lung sensitised with the serum was challenged with TPE.

CHAPTER IV.    THE EFFECT OF SODIUM CROMOGLYCATE  
ON IgE- AND IgG-MEDIATED SECRETION  
OF HISTAMINE

There is evidence that patients with extrinsic asthma respond much better to sodium cromoglycate if their allergy is mediated by IgE rather than IgG antibodies (Bryant, 1977; Gwynn *et al*, 1978). Church and Gradidge (1978) have suggested that this is because the drug antagonises secretion in lung mast cells when it is mediated by IgE, but enhances secretion mediated by IgG. This inspired a full investigation into the effects of the drug on the 2 types of secretion.

Background of the investigation - summary of the results of Church and Gradidge (1978)

The results which Church and Gradidge presented to demonstrate that the effect of cromoglycate on lung mast cells is antibody-dependent are summarised in Fig. IV.1. Drug responses are expressed as percentages of the stimulated release of histamine. When sensitised fragments of human lung were challenged with the pollen antigen, TPE, in the presence of increasing doses of cromoglycate, histamine release was inhibited in a bell-shaped manner, ie there was good inhibition with the lower doses of drug but a loss of activity at higher concentrations. In contrast, release initiated by anti-IgE was inhibited in a linear fashion by the same range of doses. Anti-IgG released only a small amount of histamine in the absence of cromoglycate, but this release was enhanced by increasing doses of the drug. Moreover, when lung samples were challenged with a combination of anti-IgE and anti-IgG, cromoglycate inhibited histamine release in a bell-shaped manner, similar to that observed in samples challenged with TPE.

Additional experiments were performed by Church and Gradidge to demonstrate that the results with anti-IgG were due neither to aggregation of non-mast cell bound IgG, nor to a non-specific action of the commercial preparation. In these experiments, lung was sensitised with allergic serum which had previously been heated at 56°C for 1 h to destroy the ability of IgE to bind to mast cells, without affecting IgG (Ishizaka, Ishizaka and Menzel, 1967). When challenged with TPE, these samples released no histamine in the absence of drug, but gave increasing amounts of release as the concentration of cromoglycate was increased.

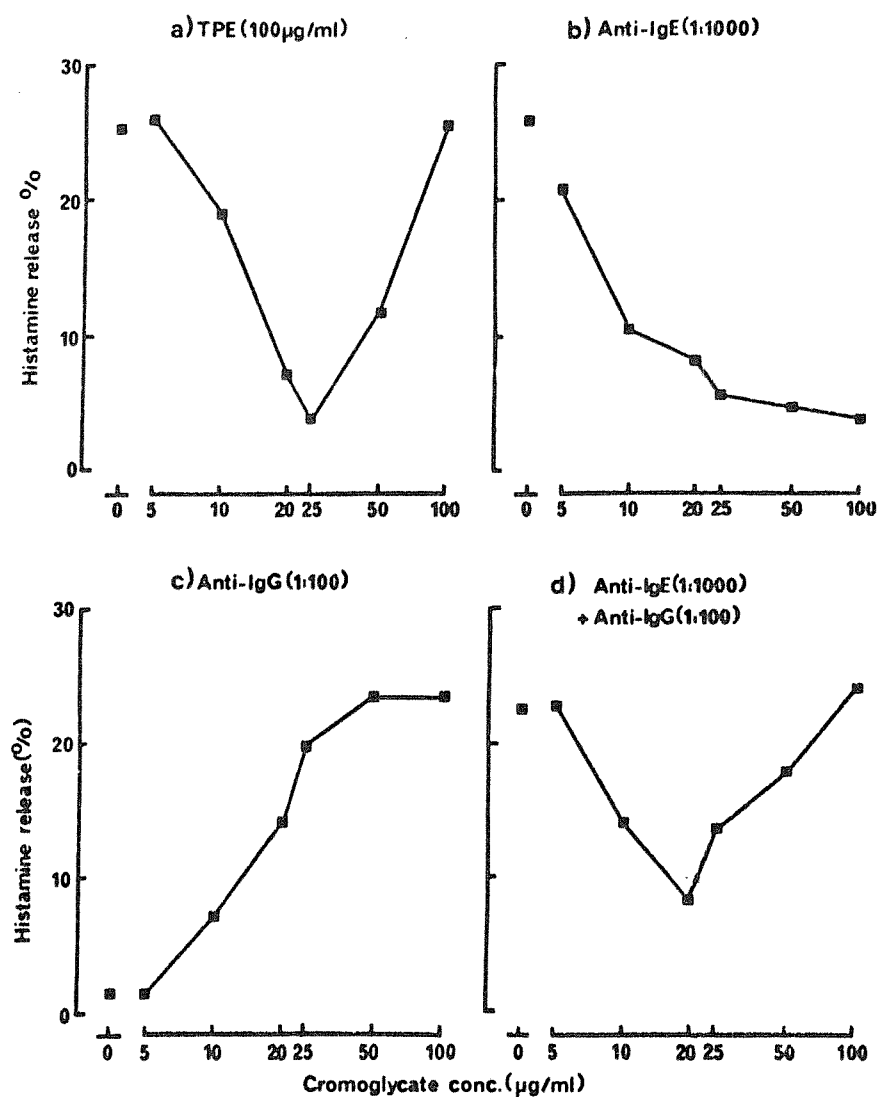


FIG. IV.1. Effect of cromoglycate on histamine release from human lung fragments induced by TPE, anti-IgE or anti-IgG. (Reproduced from Church and Gradidge, 1978). Each point is the mean of 4 replicates from 1 experiment.

The authors concluded that histamine may be secreted by human lung through 2 different mechanisms. IgE-mediated secretion, the dominant mechanism in the absence of drug, is inhibited by cromoglycate. The second mechanism, probably IgG-mediated secretion, is of minor importance in the absence of drug but is potentiated by cromoglycate.

Some of this work was repeated, using the same serum for sensitisation and the same antigen, antiserum and drug concentrations as Church and Gradidge. Very different results were obtained and, in particular, the potentiation of an IgG-mediated release of histamine was not reproduced. The following section gives details of some of these experiments and of the attempts which were made to identify the IgG mechanism of release.

#### IV.1 Dose-response study of cromoglycate against histamine release induced by TPE and anti-IgE

The first observation which contrasted with the published report was that cromoglycate produced similar inhibition of antigen- and anti-IgE-induced histamine release. The drug was examined in the concentration range 0.2 - 200  $\mu\text{M}$ , which encompassed the range of doses used by Church and Gradidge. Each dose was added to sensitised lung fragments 30 sec before they were challenged. Results from 9 experiments are shown individually in Table IV.1, as percentage inhibitions of histamine release. In lung challenged with TPE, inhibition by cromoglycate either increased or was maintained at the higher concentrations. In only 1 experiment was there a significant loss of inhibitory effect (expt. 6) suggestive of the bell-shaped relationship documented by Church and Gradidge. Surprisingly, there was more evidence of this relationship in lung samples challenged with anti-IgE. In 3 of the 9 experiments with anti-IgE (expts. 4, 6 and 7), there was a marked decline in inhibitory activity when the concentration of cromoglycate was increased to 200  $\mu\text{M}$ .

The mean inhibition of histamine release in these experiments was calculated for each dose from the data in Table IV.1. Then dose-response curves were constructed for the inhibition of the 2 types of release. These are shown in Fig. IV.2. At each dose level, cromoglycate was equally effective against TPE and anti-IgE. Drug inhibition increased with dose in the range 0.2 - 20  $\mu\text{M}$ , and even at the highest concentration investigated (200  $\mu\text{M}$ ) there was no significant loss of activity.

## Inhibition of histamine release (%)

Experiment	CROMOGLYCATE ( $\mu$ M)			
	0.2	2	20	200
1	57.6	54.0	58.1	51.2
2	-3.9	29.5	28.9	25.2
3	-6.9	6.4	7.1	15.5
4	-4.0	19.4	32.8	36.8
5	-	17.0	34.7	39.0
6	-	-1.6	27.0	16.0
7	-	12.5	35.2	46.1
8	-1.9	10.7	19.2	28.7
9	-0.9	35.7	40.0	38.7
<hr/>				
1	22.5	31.1	31.3	26.1
2	11.7	4.5	67.3	56.2
3	0.8	23.7	43.2	44.5
4	4.1	34.5	12.3	2.5
5	-	5.7	37.6	38.4
6	-	10.2	29.4	6.8
7	-	7.0	33.5	19.6
8	-0.6	3.6	5.9	-6.5
9	10.8	34.2	60.3	57.0

TABLE IV.1. Dose-response study of cromoglycate against histamine release induced by TPE (upper table) and anti-IgE (lower table). Individual results are shown from 9 experiments; each value is expressed as a percentage inhibition of the stimulated release and is calculated from triplicate observations.

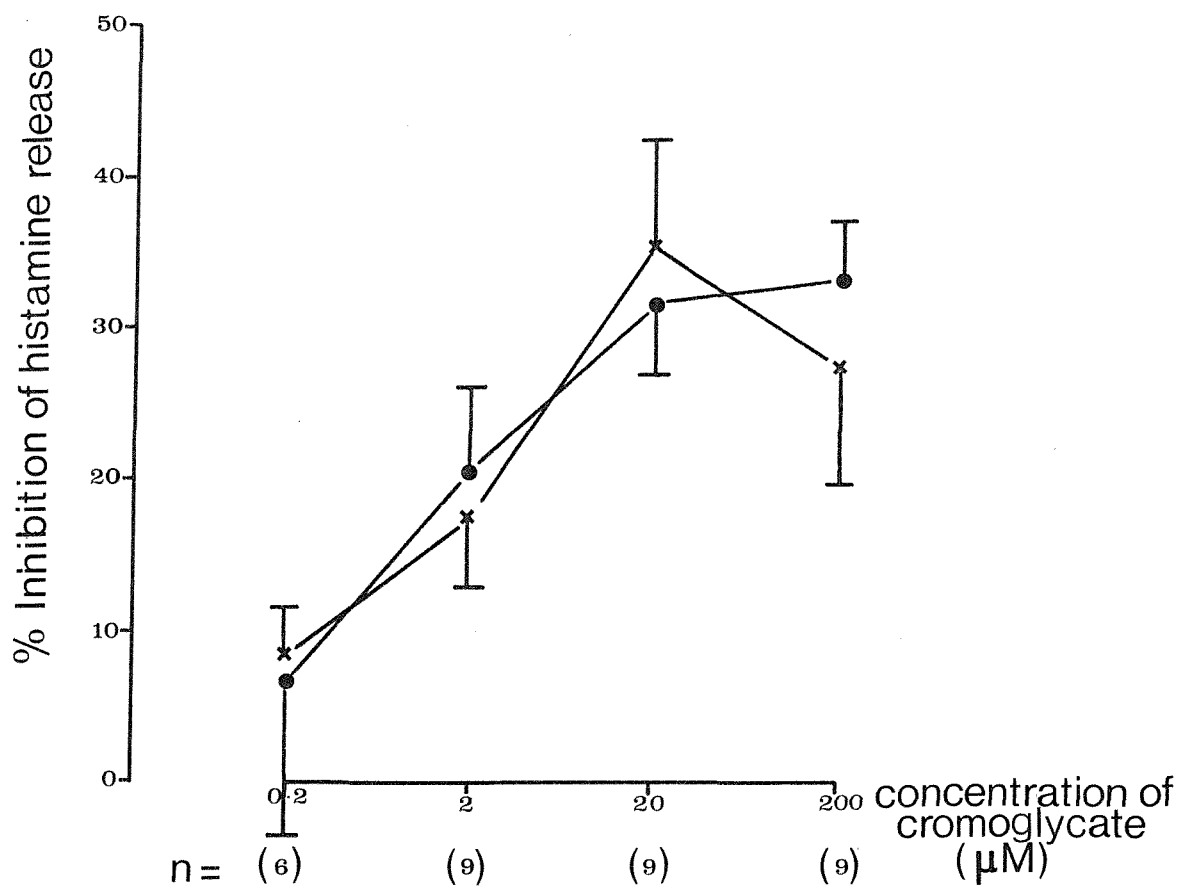


FIG. IV.2. Cromoglycate inhibition of histamine release stimulated by TPE (●—●) or anti-IgE (×—×). The number of experiments performed to calculate each mean is shown in parenthesis. Cromoglycate significantly inhibited both types of release at dose levels of 2  $\mu$ M and above ( $p < 0.01$  in all cases).

Hence, there was some evidence from individual experiments that cromoglycate inhibited histamine release in a bell-shaped manner. However, this occurred more frequently in lung samples challenged with anti-IgE, indicating that loss of activity at the higher drug concentrations was not due to an enhancement of an IgG mechanism of release. The overall dose-response relationship for inhibition of release was linear, and was the same regardless of whether lung tissue was challenged with TPE or anti-IgE.

#### IV.2 Studies with anti-IgG and heated serum

In preliminary studies, anti-IgG did not stimulate histamine release in sensitised lung samples, either in the absence or presence of cromoglycate. Similarly, TPE did not cause release from lung sensitised with heated serum. Several parameters were varied, eg the concentration of sensitising serum, concentrations of anti-IgG and TPE, but under no condition was drug-induced potentiation of an IgG mechanism of release observed.

#### IV.3 Investigation of different sera for passive sensitisation

The most logical explanation for the inability to reproduce the findings of Church and Gradidge was that there had been a change in the nature or levels of specific antibodies in the serum used for passive sensitisation. Serum levels of pollen-specific IgE and IgG (mainly IgG<sub>4</sub>) are maximal during or immediately after the pollen season, after which they gradually decline (Platts-Mills, von Maur, Ishizaka, Norman and Lichtenstein, 1976; van der Giessen, Homan, van Kernebeek, Aalbeerse and Dieges, 1976). In view of this seasonal variation in the titre of pollen-specific antibodies, experiments were performed using serum

- a) collected at different times of the year from 2 subjects with grass pollen allergy (2 of the 4 donors enlisted by Church and Gradidge) and
- b) taken from subjects with sensitivity to the house dust mite, Dermatophagoides pteronyssinus, an allergy in which exposure to antigen is continuous rather than seasonal.

- a) Effect of cromoglycate on histamine release from lung sensitised with sera collected at different times of year from 2 subjects with grass pollen allergy

Serum samples were collected before, during and after the hayfever seasons of 1978 and 1979. A small amount of serum remained from the samples donated in June 1976 for the studies of Church and Gradidge, so this was also used to passively sensitise lung tissue. Lung replicates were sensitised with unheated serum and then challenged with TPE or anti-IgG. Other replicates were sensitised with serum which had previously been heated ( $56^{\circ}\text{C}$  - 1 h) and these were challenged only with TPE. The effects of cromoglycate were examined by adding a  $200\text{ }\mu\text{M}$  dose 30 sec before challenge.

A summary of the results is shown in Table IV.2. TPE always induced histamine release from lung replicates sensitised with unheated serum. Cromoglycate mainly inhibited this release, though in some experiments it had little effect or produced a potentiation. These differences in effect were unrelated to the time of year in which the sensitising serum was collected. Anti-IgG induced significant histamine release (5.1%) on one occasion, when lung was sensitised with 1 of the original sera from 1976. However, this IgG-mediated release was inhibited by cromoglycate. Cromoglycate had no effect on the poor secretion of histamine in the other tissues challenged with anti-IgG. TPE liberated histamine from 2 lungs sensitised with heated serum (underlined in Table IV.2). However, the release was not mediated by IgG antibodies because there was no reaction in corresponding lung replicates challenged with anti-IgG. Cromoglycate inhibited the release on both occasions, and had no effect on the poor secretion of histamine in the other 6 experiments.

Thus, none of the sera sensitised lung for an IgG-mediated release of histamine which could be potentiated by cromoglycate. Lung exposed to serum which had been drawn from donor AC during the pollen season of 1976 did release histamine in response to anti-IgG, suggesting that it had been sensitised with IgG antibodies. However, the release was inhibited rather than enhanced by cromoglycate.

		Histamine release (%)					
Serum donor, Date of sampling		TPE v US		a-IgG v US		TPE v HS	
		-	cg	-	cg	-	cg
<u>DONOR AC</u>							
June	1976	18.1	25.7	<u>5.1</u>	<u>0.5</u>	-	-
Dec.-Feb.	1978	23.6	14.7	0.8	0.5	-	-
Dec.-Feb.	1978	20.5	11.8	-	-	1.3	0.0
Dec.-Feb.	1978	7.3	9.0	0.8	0.0	-	-
Aug.	1978	20.6	9.4	2.0	2.2	1.4	1.2
Feb.	1979	12.4	6.9	1.6	1.4	-	-
March	1979	15.4	7.5	1.4	1.7	-	-
July	1979	17.6	15.5	0.2	0.0	0.0	0.3
<u>DONOR DJ</u>							
June	1976	17.0	22.3	1.0	1.0	<u>12.9</u>	<u>9.6</u>
June	1976	5.5	5.3	0.1	0.0	1.5	0.8
April	1978	3.7	3.2	0.7	1.1	0.0	0.0
Sept.-Nov.	1978	25.8	22.3	0.6	0.6	0.4	0.5
July	1979	40.9	21.4	0.0	0.0	2.8	0.2
<u>AC/DJ POOL</u>							
Aug.-Sept.	1978	19.1	14.3	0.0	0.0	<u>14.7</u>	<u>1.3</u>

TABLE IV.2. Summary of the histamine release obtained from lung fragments sensitised with sera sampled at different times during the period 1976-1979, from 2 grass pollen-sensitive subjects. Lung sensitised with unheated serum was challenged by TPE (TPE v US) or anti-IgG (a-IgG v US). Fragments sensitised with heated serum were challenged by TPE only (TPE v HS). All treatments were performed in the absence or presence (cg) of 200  $\mu$ M cromoglycate. Histamine release is expressed as a percentage of total content; each value represents the mean from 1 experiment. Values underlined indicate release which may have been mediated by an IgG mechanism.

b) Effect of cromoglycate on histamine release from lung sensitised with sera from patients with house dust mite allergy

Serum samples were collected with the help of Dr. R. Godfrey from some patients who had attended his asthma clinic during the period 1974-1976. At that time they all presented with a history of allergy to house dust and gave a positive skin reaction to an extract of the house dust mite, Dermatophagoides pteronyssinus (DPE). Most had low total serum IgE, but all of them possessed relatively high levels of IgE specifically directed against DPE. From passive sensitisation studies in primate tissues, DPE-specific, short-term sensitising IgG antibodies had been detected in the sera of some of these subjects.

Seven of the 14 patients who were contacted attended the hospital and gave a blood sample. Five had been receiving steroids for their asthma (prednisolone, beclomethasone, or triamcinolone) over periods ranging from 1 month to 27 years. Other treatments included regular inhalation of salbutamol or sodium cromoglycate. One patient (FW) was particularly interesting from the point of view of this study in that he had stopped cromoglycate therapy because it worsened his asthma.

Each of the sera was used to sensitise lung replicates for challenge with DPE, anti-IgG or anti-IgE. Additional replicates were sensitised with heated serum and challenged with DPE. Cromoglycate was added before challenge, in a concentration of either 20 or 200  $\mu$ M.

The combined results (1 experiment for each serum sample) are presented graphically in Figs. IV.3a and IV.3b. For ease of interpretation the responses in the absence and presence of drug have been connected. Fig. IV.3a shows the histamine release obtained from lung challenged with DPE. Even though all 7 sera used for passive sensitisation were from DPE-sensitive subjects, their sensitising potencies were diverse. Three sera did not sensitise the lung towards the antigen at all, since the amount of histamine released on challenge with DPE was no greater than the release from unchallenged controls. Antigen-induced release from lung sensitised with the other 4 sera ranged from 3% (FW) to 28% (ES). Cromoglycate inhibited the release but in 2 cases (ES and CB) the 200  $\mu$ M

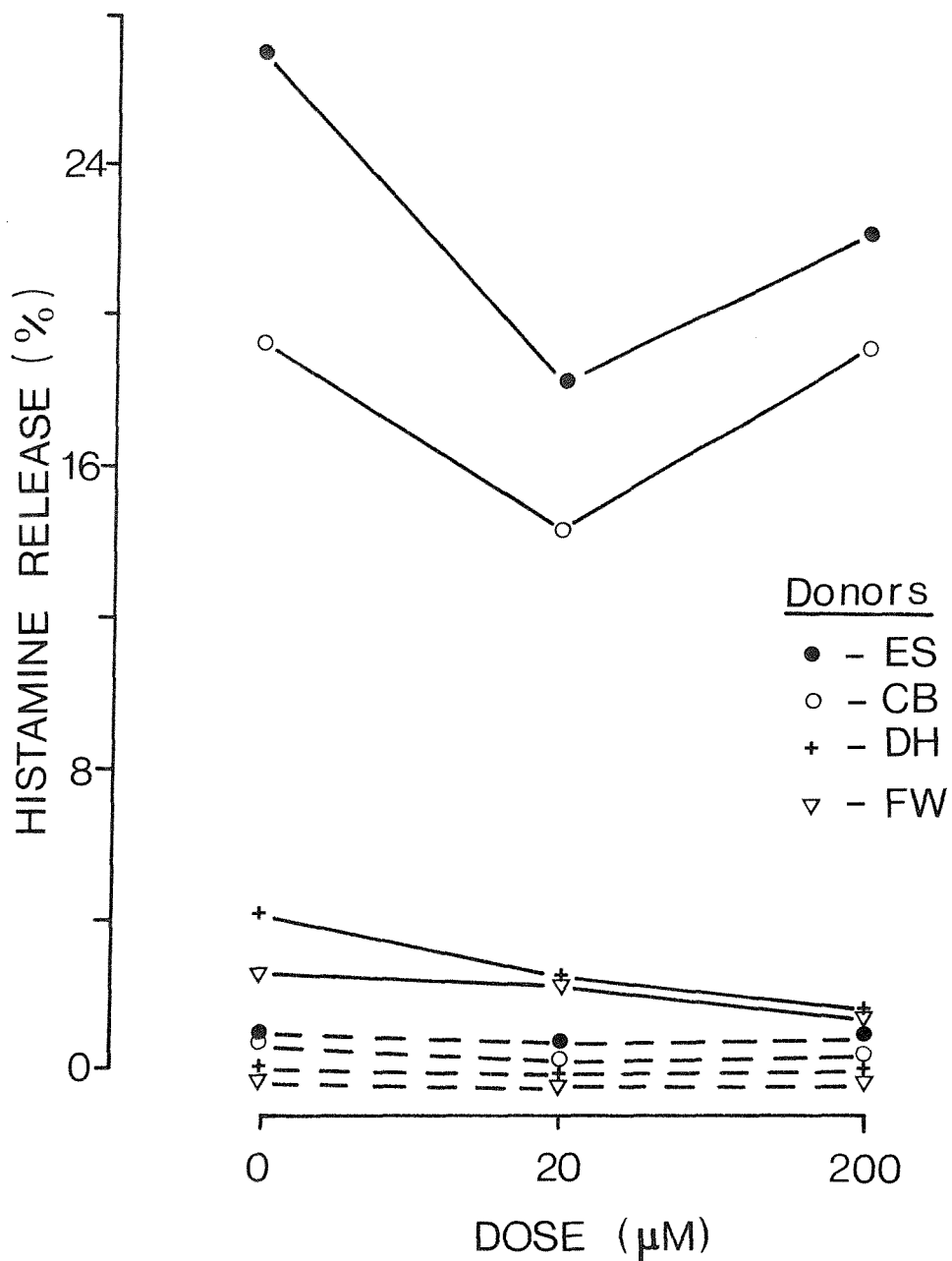


FIG. IV.3a. Effect of cromoglycate on DPE-induced histamine release from lung sensitised with sera from patients with house dust mite allergy. Only 4 of 7 unheated sera sensitised lung fragments for DPE challenge. Results with these sera are plotted above (solid lines) together with results from lung sensitised by aliquots of the same sera which had been heated at 56°C for 2 hours (broken lines). Histamine release from lung sensitised with the other 3 sera was no greater than spontaneous release. Each result represents the mean from paired observations.

concentration produced less inhibition than the 20  $\mu$ M dose. This did not appear to be caused by potentiation of an IgG-mediated release of histamine because the drug had no effect on release from lung which had been sensitised with heated serum.

Fig. IV.3b shows the histamine release obtained from samples challenged with anti-IgG or anti-IgE. Regardless of which serum was used to sensitise the lung, anti-IgG induced less than 2% release of histamine. This was unaffected by both doses of cromoglycate. In contrast, significant release was obtained in all 7 experiments with anti-IgE. The fact that anti-IgE also released histamine from unsensitised lung suggested that this was not entirely caused by interaction with passively sensitised tissue. With 1 exception, release induced by anti-IgE was inhibited by both concentrations of cromoglycate.

To summarise, as all of the sera used in this study were from DPE-sensitive subjects but only 4 out of 7 sensitised lung tissue towards DPE, it appears that the clinical symptoms of allergy and positive skin reactions do not guarantee that a subject's serum will passively sensitise human lung. Though the donors had previously been shown to possess specific IgG antibodies directed against DPE, none of their sera sensitised lung for cromoglycate-dependent, IgG-mediated release of histamine.

#### IV.4 Effect of cromoglycate on histamine release from lung sensitised with allergic serum for only 2 h

In studies of passive sensitisation of monkey skin, Parish found that human, anaphylactic IgG antibodies sensitised the tissue over a period of between 2 and 4 h. However, the sensitivity they produced was only short-lived. In contrast, specific IgE antibodies required a much longer latent period for sensitisation (24 h) but their effect was persistent (Parish, 1973). Therefore, a study was performed in which the duration of sensitisation of human lung was shortened to 2 h, in order to improve the binding of anaphylactic IgG antibodies to the tissue. Sera from 4 donors were used in the study. Two donors were selected from the group who were sensitive to DPE, whilst the other 2 were pollen-sensitive donors whose sera had been used in the investigation by Church

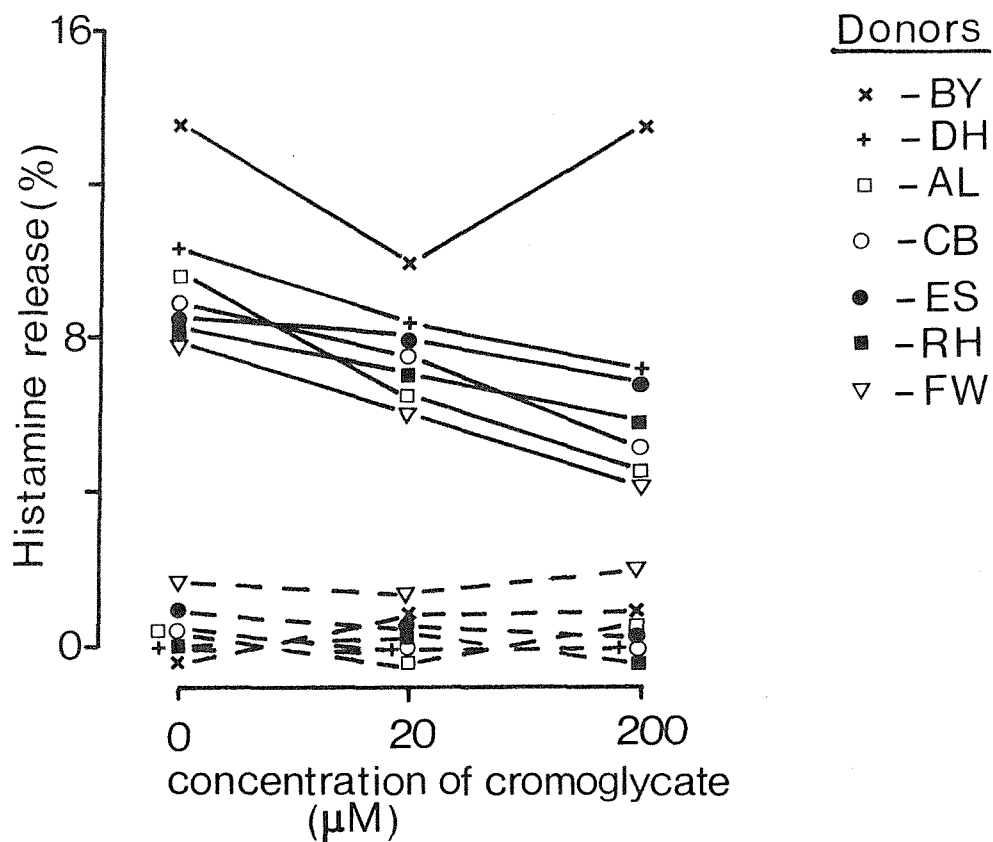


FIG. IV.3b. Effect of cromoglycate on histamine release induced by anti-IgE (solid lines) and anti-IgG (broken lines), from lung sensitised with sera from 7 patients with house dust mite allergy. Each result is the mean of 2 replicates. Sera ES, CB and RH were examined together in one lung whilst sera AL, DH, FW and BY were investigated in another. Both lungs were actively sensitised with IgE antibodies because anti-IgE provoked histamine release (2.7% and 7.8%, respectively) when neither tissue had been exposed to allergic serum.

and Gradidge. Lung replicates were exposed to each serum for 2 h (1 h at 20°C + 1 h at 37°C) and then subsequently challenged with anti-IgG or specific antigen. Additional replicates were exposed to heated serum for 2 h and then challenged with antigen only. Control groups were included in which lung was sensitised for the full 19 h. Histamine release from all samples was compared in the absence and presence of 200 µM cromoglycate.

The results, shown in Table IV.3, illustrate that anti-IgG did not stimulate histamine release from lung tissue, regardless of whether the tissue was sensitised for 2 or 19 h. Cromoglycate did not augment the release. Antigen challenge of lung sensitised with unheated sera for 2 h gave histamine release ranging from 2.5 to 15.8%. There was much greater release from the corresponding 19 h samples, suggesting that the longer period of contact with serum had increased the sensitisation of the tissue by specific IgE antibodies. The response of both 2 h and 19 h samples was mostly inhibited by cromoglycate. There was no significant reaction on antigen challenge of samples sensitised with heated serum, except for a small release of histamine (2.8%) from those sensitised with DJ serum for 19 h. On no occasion did cromoglycate increase the release of histamine from these samples.

Hence, whether or not the shortened period for sensitisation improved the binding of IgG antibodies to the lung tissue, it did not lead to identification of a cromoglycate-dependent, IgG mechanism of histamine release.

#### SUMMARY

From studies of human lung in vitro, Church and Gradidge (1978) have suggested that cromoglycate has a dual effect on antigen-induced mast cell secretion, ie it antagonises secretion mediated by IgE antibodies but augments that mediated by IgG. In the present studies, only the antagonistic effect of the drug was observed. In individual experiments, the dose-response relationship for antagonism of antigen-induced histamine release was sometimes bell-shaped but, generally, cromoglycate inhibited release in a linear fashion. Release induced by anti-IgE was inhibited in an almost identical manner which indicated that the drug was acting solely by interfering with IgE-mediated secretion.

		Histamine release (%)					
		Anti-IgG		TPE/DPE v US		TPE/DBE v US	
		-	cg	-	cg	-	cg
<u>Serum 2h</u>							
a)	AC	0.2	0.0	4.7	4.1	0.1	0.0
b)	DJ	1.1	1.4	4.1	1.8	0.0	0.0
c)	ES	0.4	0.3	15.8	8.2	0.4	0.7
d)	CB	0.0	0.0	2.5	1.5	0.0	0.0
<u>Serum 19h</u>							
a)	AC	0.2	0.0	17.6	15.5	0.0	0.3
b)	DJ	0.0	0.0	40.9	21.4	2.8	0.2
c)	ES	0.4	0.6	23.2	22.5	0.8	1.0
d)	CB	0.0	0.0	16.3	9.9	0.0	0.2

TABLE IV.3. The effect of cromoglycate (200  $\mu$ M) on histamine release from lung fragments exposed to allergic serum for 2 or 19 h. Sera from 4 donors (AC, DJ, ES and CB) were used to passively sensitise the lung. Histamine release was compared in the presence (cg) and absence of cromoglycate. One experiment was performed with each serum. Release is expressed as a percentage of total histamine content.

Not once did cromoglycate augment histamine release from lung challenged with anti-IgG. Neither did it affect release from lung which was sensitised with heated serum and challenged with specific antigen. This was true whether the tissue was

- a) sensitised with sera collected at different times of the year from 2 subjects with grass pollen allergy,
- b) sensitised with sera taken from 7 asthmatics who were allergic to house dust, or
- c) sensitised for a shortened period with serum from either pollen- or house dust-sensitive donors, to improve the binding of anaphylactic IgG antibodies to the tissue.

CHAPTER V.   COMPARISON OF THE ANTI-ALLERGIC EFFECT  
OF SODIUM CROMOGLYCATE WITH THE EFFECTS  
OF SALBUTAMOL AND CHLORPROMAZINE.

Sodium cromoglycate, salbutamol and chlorpromazine were investigated for their inhibitory effects on antigen-induced histamine release from human lung. Their dose-response relationships were compared, to estimate their relative potencies and efficacies. Then 1 dose of each drug was selected and its inhibitory effect examined after acute and prolonged administration. This was to determine if tolerance developed to any of the drugs, since it is a feature of the action of cromoglycate and  $\beta$ -adrenergic agonists, such as salbutamol, in other tissues (Mukherjee *et al*, 1975; Sung *et al*, 1977b). In the first part of this chapter, details are given of the dose-response curves, relative potencies and variable effects of the 3 drugs. Following this, the studies of tolerance are described.

#### V.1 Dose-response studies

Cromoglycate and salbutamol were added in increasing doses to replicates of sensitised lung, 30 sec before the addition of antigen. This allowed sufficient time to mix each dose with the tissue but limited the possibility of tolerance developing. As chlorpromazine is lipophilic (Seeman, 1972), 30 sec pre-incubation was considered to be insufficient time for the drug to distribute equally throughout the tissue. Therefore, chlorpromazine was added 5 min before antigen. The 3 drugs were examined in several different lungs, which revealed that not all tissues were susceptible to their inhibitory effects. When there was no response to a particular drug at any of the concentrations investigated, the results were excluded from the dose-response analysis.

The inhibitory dose-response curve for each drug is shown in Fig. V.1. Cromoglycate appeared to give dose-related inhibition of antigen-induced histamine release in the concentration range 0.2 to 20  $\mu$ M, though the response to the 0.2  $\mu$ M dose and the slope of the curve were not significant. Maximally effective concentrations of the drug inhibited release by only 30-35%. Cromoglycate was much less potent and less effective than salbutamol. Inhibition by salbutamol was linearly related to dose over the range 33 nM to 1  $\mu$ M. Calculation of the regression line over this range gave a significant slope of 34.1, ie a 10-fold increase in the dose produced an extra 34.1% inhibition of histamine release. Maximum inhibition

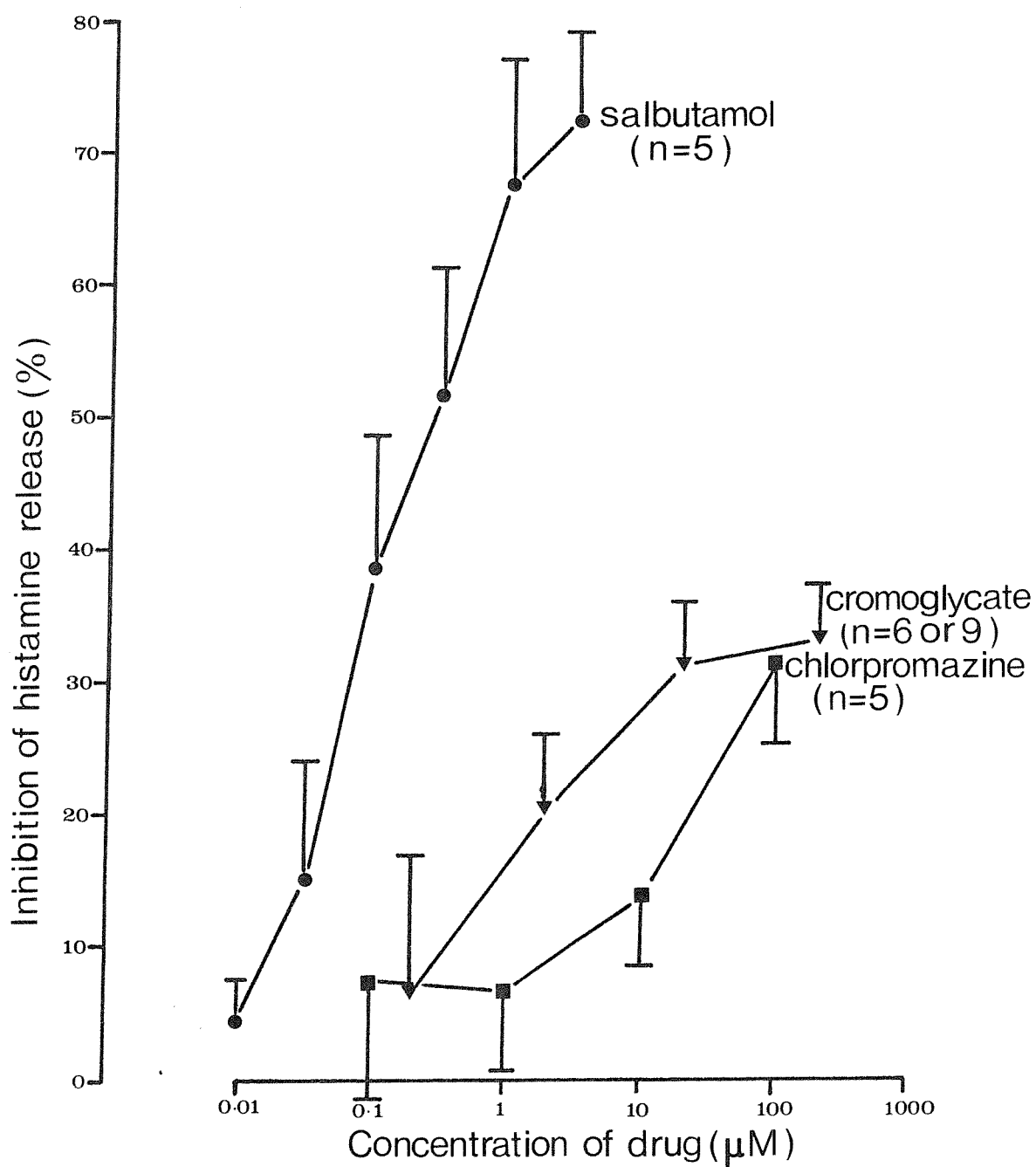


FIG. V.1. Inhibition of histamine release by sodium cromoglycate, salbutamol and chlorpromazine.  $n$  refers to the number of lungs investigated. Inhibition by cromoglycate and salbutamol was significant at all concentrations except the lowest. Inhibition by chlorpromazine was only significant at the highest dose level i.e. 100  $\mu\text{M}$ . Above 100  $\mu\text{M}$ , chlorpromazine enhanced rather than inhibited histamine release (not shown).

by salbutamol was approximately double that achieved by cromoglycate (72.2% compared with 33.0%) which indicated that it was twice as effective.

Chlorpromazine resembled cromoglycate in being a partial inhibitor of histamine release, but appeared to be less potent. A concentration of 100  $\mu$ M was required to significantly inhibit antigen-induced histamine release. This produced 31.3% inhibition. It also increased the spontaneous release of histamine from normal levels of  $2.2 \pm 0.5\%$  of the total histamine content to  $3.2 \pm 0.9\%$  (9 experiments) which suggested that the drug caused a small amount of release. When the higher concentration of 1 mM was investigated in 3 lungs (results not shown), chlorpromazine released  $48.7 \pm 5.3\%$  of the histamine in the tissue.

To assess the relative potencies of the 3 drugs, their  $ID_{30}$  values were compared, ie the dose which caused 30% inhibition of histamine release. These values are shown in Table V.1. Cromoglycate was some 300 times less potent than salbutamol but 5 times more potent than chlorpromazine.

The results of this study demonstrated that the 3 drugs were quite different in the way they inhibited histamine release. Cromoglycate produced only partial inhibition of release even in high concentrations. By comparison, salbutamol was twice as effective and 300 times more potent. Chlorpromazine, on the other hand, was less potent than cromoglycate and its dose-response relationship was biphasic because, in higher concentrations, it actually liberated histamine from the tissue.

## V.2 Variable inhibition by drugs

Just as the response of different lungs to antigen was highly variable (Chapter III), there was much variation in their response to drugs. This is illustrated by results from a survey of many experiments performed with a single, effective concentration of each drug. These are shown in Tables V.2a and V.2b. The values in Table V.2a represent the number and frequency of lungs in which inhibition of antigen-induced histamine release was less than 15%. By this criterion, 25% of the lungs

Drug	Approximate ID <sub>30</sub> ( $\mu$ M)	Relative Potency
Salbutamol	0.06	333
Cromoglycate	20.00	1
Chlorpromazine	100.00	0.2

TABLE V.1. Relative inhibitory potencies of salbutamol, sodium cromoglycate and chlorpromazine in human lung. The approximate ID<sub>30</sub> values were obtained from the dose-response curves shown in Fig. V.1. Relative potencies were calculated by defining the response to cromoglycate as unity.

Drug	n	Poor Inhibition	Frequency (%)
Cromoglycate (200 $\mu$ M)	69	17	25
Salbutamol (0.1 or 0.33 $\mu$ M)	33	8	24
Chlorpromazine (100 $\mu$ M)	28	7	25

Table V.2a. Frequency of poor inhibition by drugs. A poor response was arbitrarily designated as less than 15% inhibition of the antigen-induced histamine release. n refers to the number of different lungs examined.

Drug	n	Mean Inhibition $\pm$ SEM (%)	95% Confidence limits	Coefficient of variation, v (%)
Cromoglycate (200 $\mu$ M)	52	39.8 $\pm$ 2.3	35.2 - 44.4	41.7
Salbutamol (0.1 or 0.33 $\mu$ M)	25	58.7 $\pm$ 4.1	50.2 - 67.1	34.9
Chlorpromazine (100 $\mu$ M)	21	41.5 $\pm$ 3.1	35.0 - 47.9	34.2

Table V.2b. Variable inhibition of histamine release by drugs in different lungs. n refers to the number of different lungs examined; in this survey, results were only included if the inhibition of antigen-induced histamine release was greater than 15%.

did not respond to cromoglycate or chlorpromazine, and 24% were insensitive to salbutamol. As the frequency of poor inhibition was the same for the 3 unrelated drugs, this appeared to be determined by the lung tissue. The values in Table V.2b represent the mean results from lungs in which inhibition of histamine release was greater than 15%. In 52 lungs, cromoglycate produced a mean inhibition of 39.8% with confidence limits of 35.2 and 44.4%. However, the variability associated with the mean value was large, as is indicated by the coefficient of variation of 41.7%. Inhibition by salbutamol and chlorpromazine was similarly diverse ( $v = 34.9\%$  and  $34.2\%$ , respectively). This again suggested that the variation in the effect of each drug in different lungs was due to the tissue and not the drug.

### V.3 Effect of pre-incubation time on the inhibition of histamine release

In this study, each drug was used in a maximally effective concentration, as indicated by the results from the dose-response studies described in section V.1. The concentrations were 200  $\mu\text{M}$  cromoglycate, 1  $\mu\text{M}$  salbutamol and 100  $\mu\text{M}$  chlorpromazine. Drugs were added to lung replicates either 30 sec, 15 min or 19 h before challenge with antigen. Hence the effects of each drug were compared after prolonged as well as acute pre-incubation with the tissue, so that tolerance would be detected if it occurred. In the 19 h treatment, each drug was added at the beginning of sensitisation and was renewed when the lung was washed free of serum, 15 min before challenge. This ensured that the lung was continuously exposed to drug throughout the pre-incubation period. A fourth treatment was included wherein drug was added at the beginning of sensitisation but was not renewed 15 min before challenge.

The results are shown in Table V.3a. When cromoglycate was added 30 sec before challenge, it inhibited antigen-induced histamine release by 36.6%. Adding the drug 15 min before challenge resulted in a slight, but significant loss of inhibitory effect. This was even more marked when the pre-incubation time was extended to 19 h. Under these conditions,

cromoglycate was virtually inactive, producing only 6.3% inhibition of histamine release. The loss of effect in 19 h samples was not due to a short duration of action or metabolism of cromoglycate because the dose of drug was renewed 15 min before challenge. Therefore, it appeared that lung tissue became progressively desensitised the longer it was in contact with the drug. When cromoglycate was washed out after 19 h pre-incubation but not replaced, the effect on antigen-induced histamine release was negligible. Either the drug was completely removed from the tissue by the washing or else the tissue remained desensitised to its effects.

A very different pattern of inhibition was observed with salbutamol. This drug produced 64.7% inhibition of histamine release when added 30 sec before challenge. Its activity was significantly increased by administering the dose 15 min before antigen, but there was no further change in activity after 19 h pre-incubation. Clearly, the tissue showed no sign of tolerance to salbutamol. After salbutamol had been in contact with lung tissue for 19 h, its effects were poorly reversed by washing.

In contrast to the other drugs, chlorpromazine did not inhibit histamine release when added 30 sec before challenge, but its effect increased as the pre-incubation time with the tissue was extended. Thus, after 15 min pre-incubation, release was inhibited by 20.7% and after 19 h contact with lung tissue chlorpromazine produced 75.4% inhibition. This level of inhibition was maintained in the 19 h samples which were washed and then challenged without the drug being renewed.

As the effects of salbutamol and chlorpromazine were not reversed by washing after they had been present throughout the 19 h sensitisation period, it was possible that the drugs interfered with the sensitisation process itself. This possibility was investigated indirectly, by determining if either drug inhibited histamine release from actively sensitised lung tissue under the same conditions; if the persistent inhibition was caused by an interference with passive sensitisation, it should not be observed in tissue in which IgE is already bound. Lung was pre-incubated with drug for 19 h in the absence of allergic serum. It was then washed and challenged with anti-IgE. The results from 1 experiment with each drug

Time of incubation before challenge	Inhibition of histamine release (%)		
	Sodium cromoglycate (200 $\mu$ M)	Salbutamol (1 $\mu$ M)	Chlorpromazine (100 $\mu$ M)
30 sec	36.6 $\pm$ 5.3	64.7 $\pm$ 8.4	3.8 $\pm$ 9.2
15 min	22.6 $\pm$ 8.1*	78.3 $\pm$ 6.6*	20.7 $\pm$ 11.2
19 h <sup>1</sup>	6.3 $\pm$ 4.9**	78.1 $\pm$ 6.6*	75.4 $\pm$ 6.0**
19 h + <sup>2</sup> wash-out	6.9 $\pm$ 6.2**	61.5 $\pm$ 6.9	86.4 $\pm$ 6.8**

Table V.3a. The effect of pre-incubation time on the inhibition of histamine release. Each result is the mean of 4 (chlorpromazine) or 6 (cromoglycate, salbutamol) experiments.

1. The tissue was washed in 100 ml PBS, and the drug renewed 15 min before challenge.

2. The tissue was washed 15 min before challenge but no further drug was added.

\* and \*\* signify  $p < 0.05$  and  $p < 0.01$ , respectively, compared with the inhibition produced after 30 sec pre-incubation.

Drug examined	Histamine release induced by anti-IgE (%)		Inhibition of release (%)
	No drug	19 h pre-incubation with drug + wash-out	
Salbutamol	9.1 $\pm$ 0.7	2.0 $\pm$ 0.8	78.0
Chlorpromazine	18.1 $\pm$ 2.6	4.9 $\pm$ 1.0	72.9

Table V.3b. Effect of 19 h pre-incubation with salbutamol or chlorpromazine on histamine release from actively sensitised human lung. Each value is the mean of 4 replicates.

are shown in Table V.3b. Both salbutamol and chlorpromazine inhibited the immunological secretion of histamine even though neither drug was renewed 15 min before challenge. Hence, both drugs produced persistent inhibition in tissue in which IgE was already bound, so this was due to a direct effect on the lung and not to an interference with the sensitisation process.

To summarise, pre-incubation of cromoglycate with lung tissue lead to a time-related loss of its inhibitory activity, which suggested that the tissue became progressively tolerant to its effects. This did not occur with salbutamol or chlorpromazine, whose inhibitory effects in fact increased with pre-incubation. After prolonged contact with the lung during the 19 h sensitisation period, the effects of salbutamol and chlorpromazine were resistant to washing, but this was not caused by an interference with the sensitisation process.

#### V.4 Effect of pre-incubation time on inhibition of histamine release by ICI 74917 ("Bufrolin")

To determine if tolerance was characteristic of the cromoglycate mode of action in human lung, a compound with similar pharmacological properties to cromoglycate was examined. The compound selected was ICI 74917 since this not only inhibits mast cell secretion in human lung (Church and Gradidge, 1980b), but also demonstrates tolerance and tachyphylaxis in other tissues (Marshall *et al*, 1976). The drug was pre-incubated with lung tissue under the same conditions as those described for cromoglycate in the previous section. A dose of 10  $\mu$ M was chosen for the study, because this had previously been shown to be maximally effective in human lung (Church and Gradidge, 1980b).

The results are shown in Fig. V.2. When ICI 74917 was added 30 sec before challenge, it produced 36.2% inhibition of histamine release. After 15 min pre-incubation the level of inhibition had fallen to 17.3%, but this change was not statistically significant. However, after prolonged pre-incubation with lung for 19 h there was a significant loss of activity, with the drug inhibiting release by only 14.1%. This low level of inhibition was maintained in the 19 h samples which were washed and challenged without replacement of the drug.

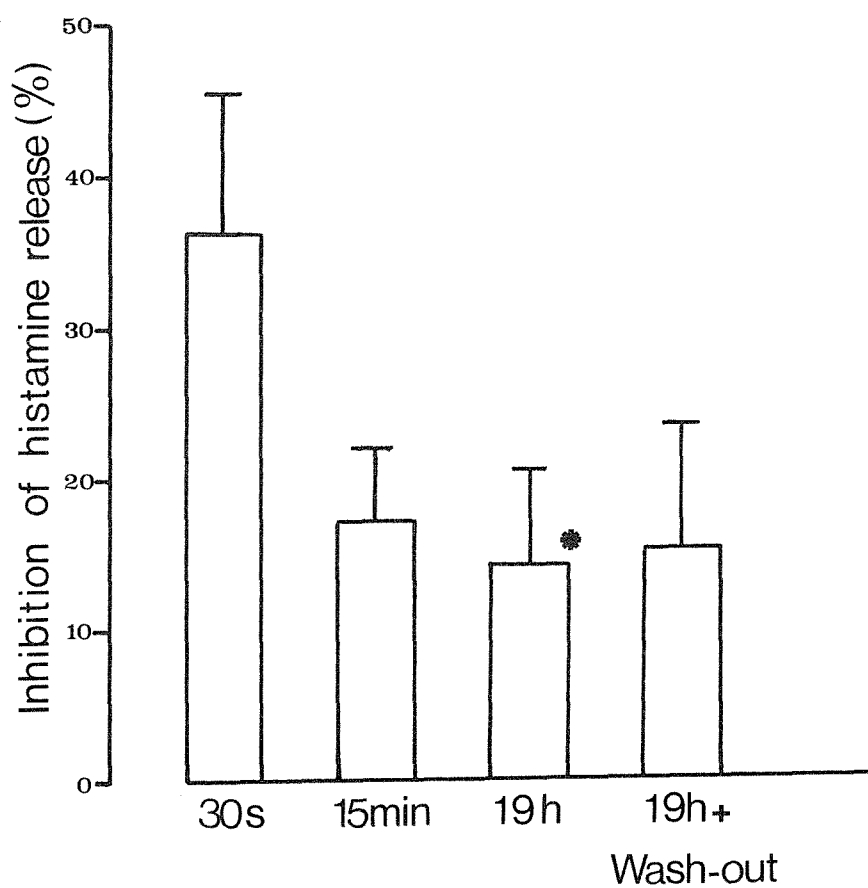


FIG. V.2. Effect of pre-incubation time on the inhibition of histamine release by ICI 74917. Results were obtained in 5 experiments. The asterisk indicates that the inhibition in 19 h samples was significantly less than the inhibition in 30 sec samples.

Hence, the inhibitory activity of ICI 74917 decayed in a similar fashion to cromoglycate, as the pre-incubation time with the tissue was extended. Some activity remained even after the prolonged pre-incubation, whereas cromoglycate was virtually inactive at this stage (Table V.3a). Therefore the tolerance to ICI 74917 was not quite so marked.

#### V.5 Cross-tolerance between cromoglycate and other drugs

Drugs which have a similar mechanism of action to cromoglycate usually exhibit cross-tolerance or cross-tachyphylaxis with the parent compound whereas those which act differently do not (Church, 1978; Cairns, 1979). Therefore, the effects of ICI 74917, salbutamol and chlorpromazine were examined in lung which had been pre-treated with cromoglycate, to determine if tolerance to cromoglycate affected their inhibitory activities.

Lung replicates were pre-incubated with a 200  $\mu$ M dose of cromoglycate for 19 h, thoroughly washed and then challenged in the presence of a second dose, or a dose of 1 of the other drugs. The results are shown in Fig. V.3. Histograms with cross-hatching represent the inhibition of histamine release produced by each drug on its own whereas the plain histograms indicate the drug effect after pre-treatment of the lung with cromoglycate. When added immediately before challenge, cromoglycate produced 38.5% inhibition and ICI 74917 gave 30.5% inhibition of antigen-induced histamine release. The activity of both drugs was significantly reduced by prior exposure of the lung tissue to cromoglycate. Salbutamol inhibited histamine release by 67.8% when added 5 min before challenge. Its activity was slightly but significantly enhanced by the cromoglycate pre-treatment. The same occurred with chlorpromazine, which gave 25.0% inhibition on its own and 36.2% inhibition in pre-treated tissue. The increase in activity of salbutamol and chlorpromazine may have been due to an additive effect with the small amount of carry-over inhibition produced by the cromoglycate pre-treatment (6.7%), which is represented by the solitary histogram in Fig. V.3.

Hence, cross-tolerance was demonstrated between cromoglycate and ICI 74917, indicating that these drugs act via a similar mechanism to inhibit secretion in lung mast cells. In contrast, there was no cross-tolerance between cromoglycate and either salbutamol or chlorpromazine. This suggested that salbutamol and chlorpromazine acted via a different mechanism from cromoglycate.

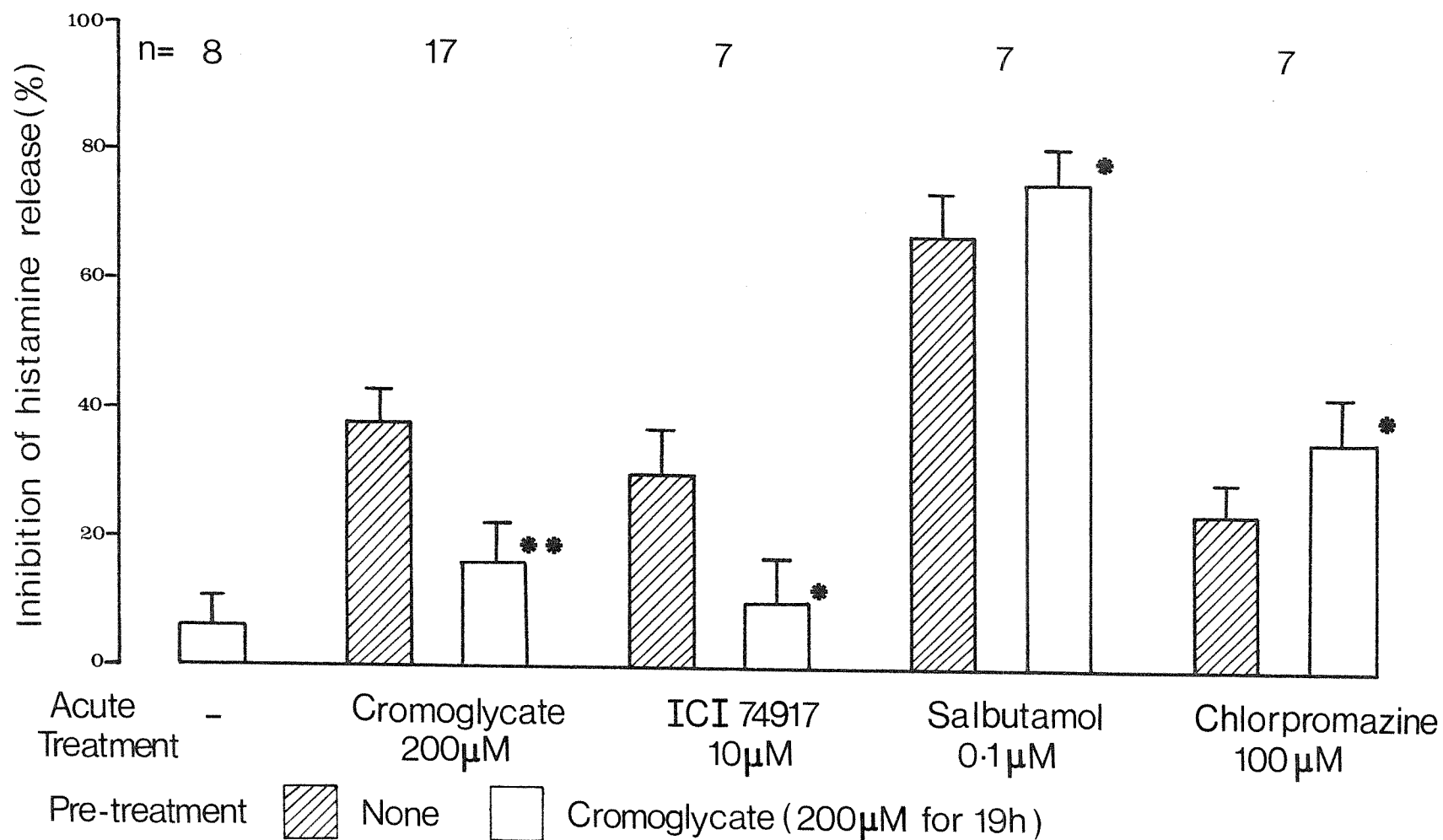


FIG. V.3. Cross-tolerance between cromoglycate and other drugs. In the acute treatment, cromoglycate and ICI 74917 were added 30 sec before antigen; salbutamol and chlorpromazine were added 5 min before antigen. n refers to the number of lungs investigated.

SUMMARY

Sodium cromoglycate, salbutamol and chlorpromazine all inhibited mast cell secretion in human lung. Their effects were highly variable from lung to lung and, in some 25% of tissues, they produced very poor inhibition of histamine release. As the frequency of poor inhibition and the extent of the variability in different lungs was very similar for the 3 unrelated drugs, it appeared that the tissue and not the drug was responsible for the variation.

The ways in which the drugs inhibited secretion were quite different. Cromoglycate was a partial inhibitor of antigen-induced histamine release. Even in high concentrations, the drug rarely produced more than 40% inhibition. Tolerance developed to its effects during protracted pre-incubation with the lung tissue. As tolerance also developed to the pharmacologically-related compound ICI 74917, but not to salbutamol or chlorpromazine, it appeared to be specific for cromoglycate-like drugs. This was confirmed when cross-tolerance was demonstrated between cromoglycate and ICI 74917 but not between cromoglycate and either salbutamol or chlorpromazine.

Salbutamol was a much more potent and effective inhibitor of mast cell secretion than cromoglycate. Concentrations in the nanomolar-micromolar range produced a dose-related inhibition of histamine release. The fact that salbutamol did not exhibit tolerance and that its inhibitory effect was maintained in lung which had been desensitised to cromoglycate demonstrated that it was acting via a different mechanism. The effect of the drug marginally increased with pre-incubation and, after salbutamol had been in contact with the lung for 19 h, it persisted in washed tissue. The reasons for this were unclear and required further investigation.

Chlorpromazine was the least potent of the 3 drugs. It only partially inhibited histamine release and, in concentrations greater than 100  $\mu$ M, actually liberated histamine from the tissue. As chlorpromazine did not exhibit tolerance on its own nor cross-tolerance with cromoglycate, it was clearly acting via a different mechanism. Its inhibitory effect increased markedly the longer it was in contact with the lung. As with salbutamol,

the inhibition persisted in tissue which was washed after pre-incubation with the drug for 19 h. Further experiments were required to explain this effect.

Since the 3 drugs appeared to act via different mechanisms, further studies were designed to investigate their individual modes of action.

CHAPTER VI.   STUDIES OF THE MECHANISM OF ACTION  
OF CROMOGLYCATE.

Several reports have shown that cromoglycate inhibits the influx of calcium into stimulated mast cells and this is considered to be one of the main actions of the drug (Johnson and Bach, 1975; Foreman and Garland, 1976; Spataro and Bosmann, 1976). Mast cells in human lung are likely to be very sensitive to this effect, since secretion in these cells is totally abolished by EDTA (Hutchcroft and Orange, 1980) which indicates that it is absolutely dependent on extracellular calcium. Investigations of the mechanisms by which cromoglycate may interfere with calcium transport are described in part A of this chapter.

In part B, studies of the mechanism of tolerance to cromoglycate are described. These studies, performed in human lung, were based on the work of Sung and his colleagues who investigated the mechanism of tolerance in rat lung (Sung et al, 1977a; 1977b).

#### PART A - MECHANISM OF THE INTERFERENCE WITH CALCIUM TRANSPORT

Several mechanisms have been proposed to explain how cromoglycate inhibits calcium influx into mast cells, but none has been substantiated (Kruger and Bloom, 1979). There are 4 ways in which the drug might act: it may

- a) physically stabilise the cell membrane and hence interfere with the opening of calcium channels,
- b) compete with calcium for a membrane channel or receptor site,
- c) bind calcium ions, or
- d) stimulate a membrane or intracellular process which inhibits the transport mechanism.

Some of these possibilities were investigated by examining the effect of the drug both in a mixed suspension of rat serosal cells and in human lung tissue.

VI.1 Stabilisation of the mast cell membrane; effect of cromoglycate on histamine release from mast cells exposed to hypotonic conditions

To determine if cromoglycate physically stabilises the plasmalemma of mast cells, its effect was examined in cells exposed to hypotonic conditions. When any cell is placed in a medium of low tonicity, it accumulates water due to the osmotic gradient which is created. This causes it to swell and eventually burst, releasing all the intracellular contents (Bell, Davidson and Emslie-Smith, 1972). Drugs which physically stabilise membranes increase the resistance of the cell to lysis (Roth and Seeman, 1971). If cromoglycate acts by stabilising the plasmalemma of mast cells, it should prevent the release of histamine when the cells are placed in an hypotonic medium.

The investigation was performed on a mixed suspension of rat serosal cells, which were prepared according to the method described in Chapter II. Its purpose was to examine the effect of cromoglycate in cells exposed to compound 48/80 or a hypotonic saline solution, to determine if concentrations which inhibit 48/80-induced secretion of histamine also inhibit histamine release induced by hypotonic lysis. Therefore, preliminary studies were carried out to establish a concentration of compound 48/80 and a dilution of saline which liberated equivalent amounts of histamine. Cells were incubated with different amounts of 48/80 for 5 min at 37°C, after which they were centrifuged at 4°C to separate them from the supernatant and stop the reaction. Cell aliquots exposed to hypotonic solutions were treated in the same manner. These solutions were prepared by dissolving sodium chloride in Tris buffer, so that the final saline concentration was less than 0.9%.

The results from 1 experiment with cells from the same rat are shown in Fig. VI.1. Compound 48/80 produced a dose-related release of histamine over a narrow range of concentrations. At 150 ng/ml, it liberated 37.2% of the total histamine in mast cells. Release increased with dose, reaching 75-80% with concentrations of the order of 1 µg/ml. Cells incubated with saline solutions of decreasing strengths began to release significant amounts of histamine when the tonicity was reduced to 0.3%. In 0.2% saline, they released 41.4% of their total histamine.

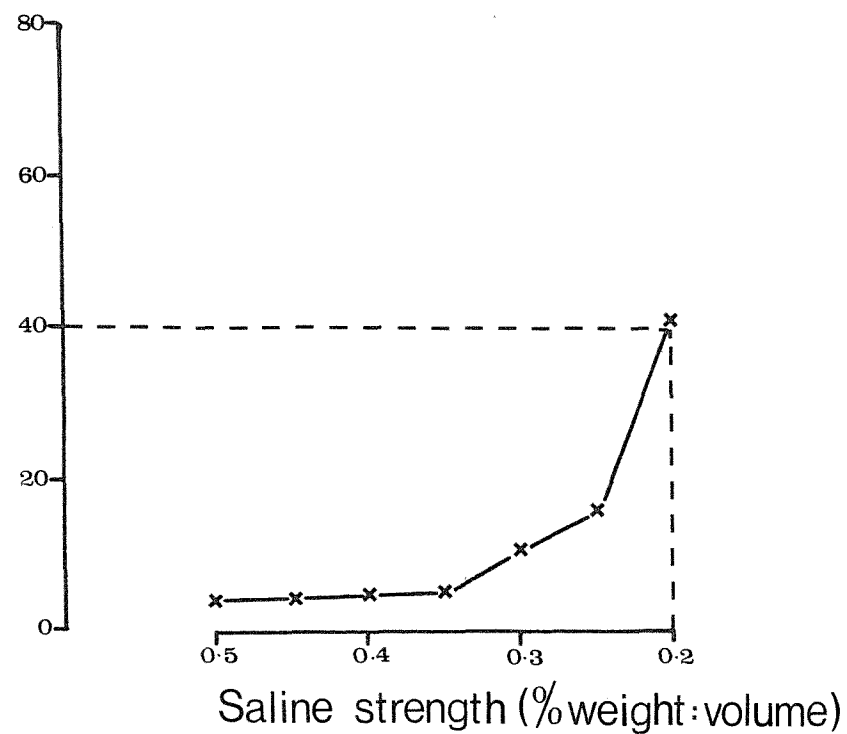
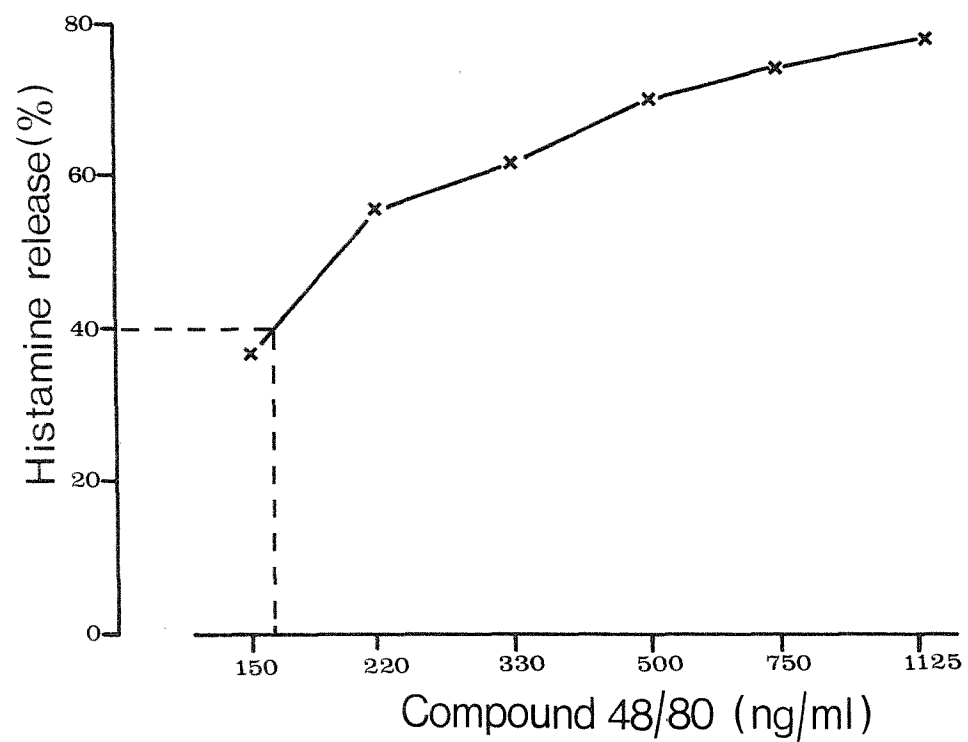


FIG. VI.1. Histamine release from mast cells induced by compound 48/80 or by hypotonic lysis. Each value is the mean from paired observations; the margin of error between replicates was extremely small, so standard deviations have been omitted. Dilutions of saline were prepared in Tris buffer so that the pH remained constant.

Concentrations which produced 40% histamine release were selected for studies with cromoglycate, ie 150 ng/ml compound 48/80 and 0.2% saline. In these studies, cromoglycate was added simultaneously with the histamine-releasing stimulus. Drug concentrations ranging from 50 to 400  $\mu$ M were examined. The results are shown in Fig. VI.2, which illustrates both the amount of histamine liberated in the absence of drug and the inhibitory dose-response curve for cromoglycate against the 2 types of release. The average amount of histamine liberated by compound 48/80 over 4 experiments was 63.1%, which was higher than expected. High levels of release induced by 48/80 are poorly antagonised by cromoglycate (Orr *et al*, 1971) which may be the reason why it produced only 25.7% inhibition of the release at the highest concentration tested (400  $\mu$ M). Nevertheless, there was a significant dose-related inhibition of the response to 48/80 over the whole range of drug concentrations examined. In contrast, none of the doses inhibited histamine release induced by hypotonic lysis. If anything, they caused a slight enhancement, but this was neither significant nor dose-related.

It was conceivable that the conditions for hypotonic lysis had been too severe and had obscured an inhibitory effect of the drug. For example, the membranes of those cells which were lysed may have been ruptured too rapidly for the drug to provide satisfactory protection. Therefore, further experiments were performed in which cells were exposed to a smaller osmotic gradient so that the influx of water and membrane rupture would occur more slowly. Mast cells were incubated with hypotonic solutions for 15 min instead of 5. Over this period, 40% histamine release was obtained in 0.3% saline (Fig. VI.3a). Each concentration of cromoglycate slightly inhibited the release, but the inhibition was neither significant nor dose-related (Fig. VI.3b).

Hence, concentrations of cromoglycate which inhibited the secretion of histamine from mast cells induced by compound 48/80 had no effect on release induced by rupture of the cell membrane under hypotonic conditions. This showed that the drug does not inhibit secretion by physically stabilising the cell membrane. It was ineffective against hypotonic lysis whether the rate of lysis was fast or slow, which denied the possibility that a stabilising effect had been obscured by the membranes being ruptured too rapidly.

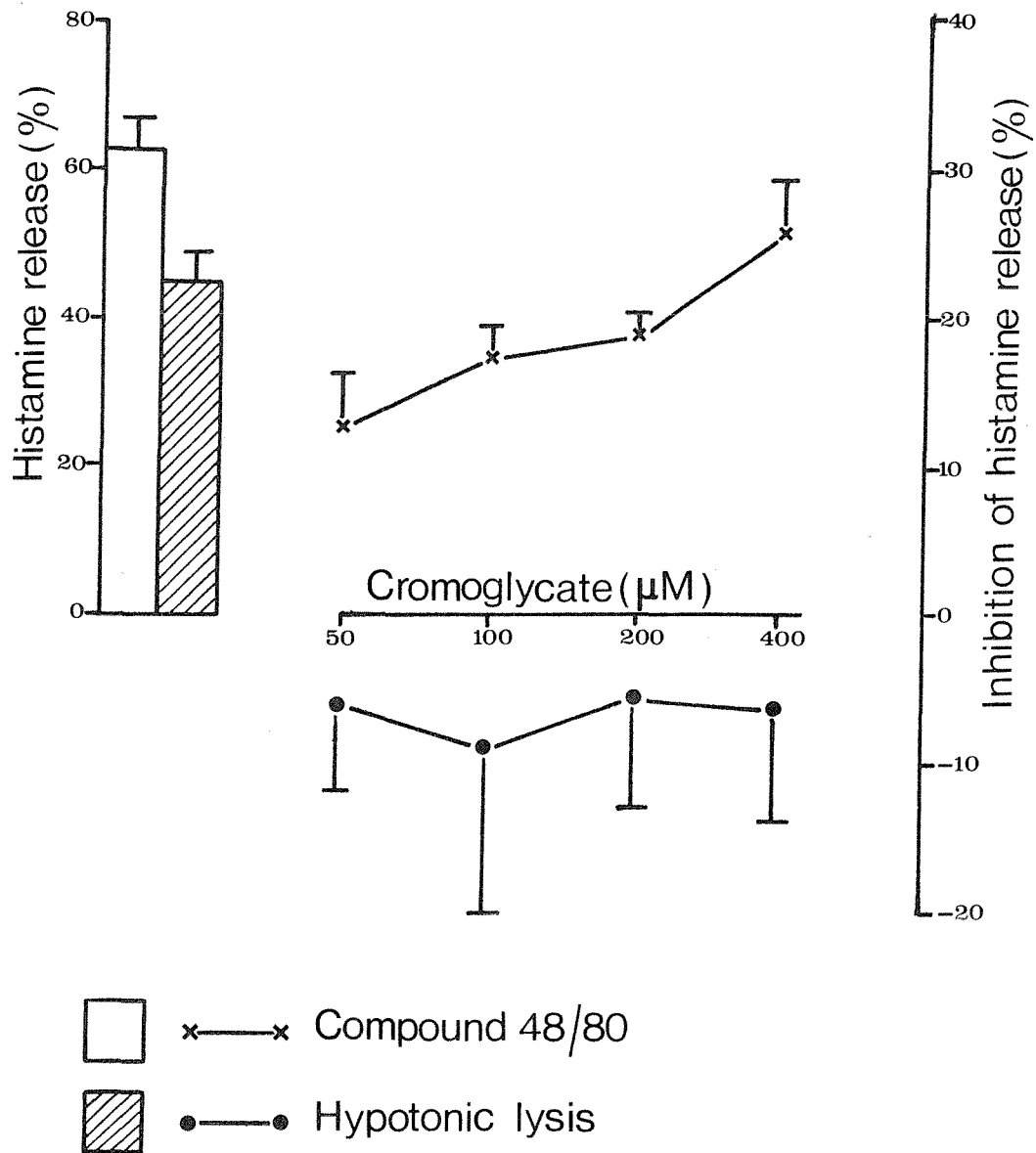


FIG. VI.2. Cromoglycate inhibition of histamine release from rat mast cells. Each value is the mean from 3 (hypotonic lysis) or 4 (compound 48/80) experiments. Inhibition of 48/80-induced release was significant at each dose level ( $p < 0.01$ ).

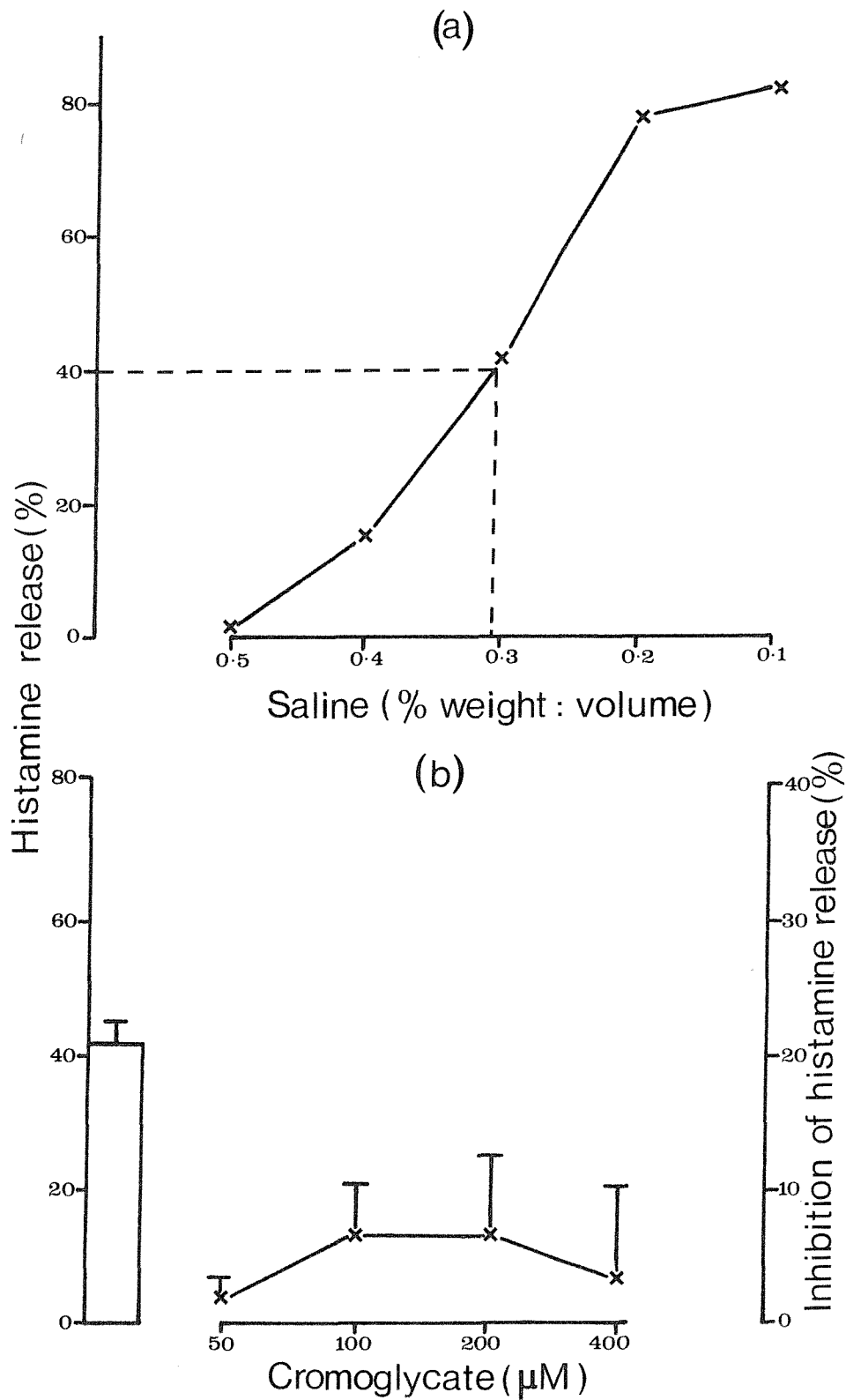


FIG. VI.3. Histamine release from rat mast cells exposed to hypotonic solution for 15 min (a), and its inhibition by cromoglycate (b). Results in (a) are means of paired observations from 1 experiment; standard deviations have been omitted because the error between replicates was extremely small. Results in (b) are mean values from 3 experiments, in which all cells were exposed to 0.3% saline.

VI.2 Inhibition of histamine release by cromoglycate compared with the inhibition by a calcium antagonist and by a calcium-chelating agent

In this study the calcium antagonist, lanthanum, and the specific calcium-chelating agent, EGTA, were investigated for their inhibitory effects on mast cell secretion in human lung. Their activities were compared with that of cromoglycate under similar conditions.

Lanthanum was examined in concentrations ranging from 0.1 - 1000  $\mu\text{M}$ . Solutions of lanthanum chloride were prepared in distilled water rather than in PBS solution, to avoid precipitation of the lanthanum ions before they were added to the lung tissue (Foreman and Mongar, 1972b). Each solution was added as 0.1 ml to sensitised lung replicates 2 min before antigen challenge.

EGTA was difficult to dissolve, particularly in high concentrations, so it was prepared as follows. A 1 mg/ml solution was prepared in calcium-free PBS with warming and stirring for 30 min. This was serially diluted with calcium-free PBS to give EGTA concentrations of 500, 200 and 100  $\mu\text{g/ml}$ . 1.7 ml of each solution was pipetted into test tubes and warmed to 37°C. A 200 mg replicate of sensitised lung was dispensed into each tube, followed by 0.1 ml of a 20 mM calcium chloride solution. All replicates were then challenged with antigen in a final volume of 2 ml.

The results of the study are shown in Fig. VI.4, in comparison with previous results obtained with cromoglycate (section V.1). In concentrations below 10  $\mu\text{M}$ , lanthanum had no effect on antigen-induced histamine release. Between 10  $\mu\text{M}$  and 1 mM, it produced a concentration-related inhibition of release. The 1 mM concentration almost completely blocked the secretory response, producing 91.7% inhibition. The pattern of inhibition with EGTA was almost identical, with complete inhibition of histamine release being obtained by concentrations around 1 mM. As the extracellular calcium concentration used in these studies was 1 mM, this suggested that total inhibition of secretion was achieved when influx of calcium ions into mast cells was blocked on a mole-for-mole basis, either by competitive antagonism (lanthanum) or by chelation (EGTA).



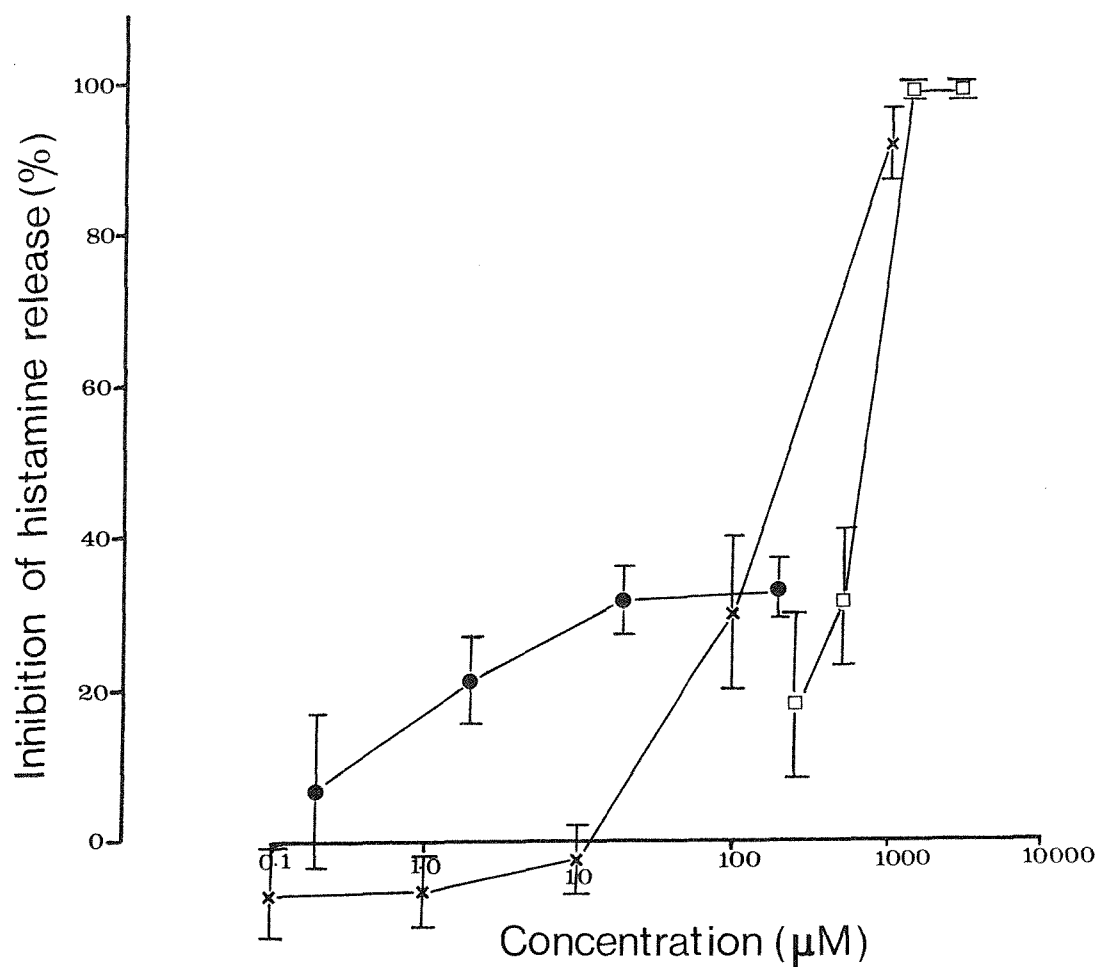


FIG. VI.4. Inhibition of histamine release from human lung by cromoglycate (●—●), compared with inhibition by lanthanum (x—x) and EGTA (□—□). Each result is the mean from several experiments (cromoglycate-9, lanthanum-4, EGTA-3). The molar concentrations of EGTA are only approximate, because they were calculated from its anhydrous molecular weight. Drugs were added either 30 sec or 2 min before antigen challenge.

There were clear differences between the effects of these 2 agents and the effect of cromoglycate. Cromoglycate was active at lower concentrations than either lanthanum or EGTA, but the maximum inhibition of release which it achieved was only 33.0%. Moreover, the slope of the dose-response curve for cromoglycate was quite distinct, all of which indicated that it was acting neither as a calcium antagonist nor by forming a complex with calcium ions.

### VI.3 Effect of extracellular calcium on inhibition by cromoglycate

To determine if the activity of cromoglycate depended on the extracellular concentration of calcium, the effect of the drug was examined under hypocalcaemic and hypercalcaemic conditions and compared with its effect in the presence of the normal concentration of calcium (1 mM).

Sensitised lung was incubated in bulk for 1 h at room temperature in calcium-free PBS solution which contained 100 µg/ml EGTA. The purpose of this was to thoroughly deplete the extracellular calcium in the tissue. At the end of the 1 h period, the tissue was washed thoroughly to remove EGTA, divided into replicates and resuspended in PBS solution minus calcium. The replicates were brought to 37°C and then 200 µM cromoglycate was added. Thirty sec later, calcium was restored by adding 0.1 ml of either 5 mM (hypocalcaemic), 20 mM (normal) or 40 mM (hypercalcaemic) calcium chloride solution. All replicates were challenged with antigen immediately afterwards, in a final volume of 2 ml.

Lanthanum was included in this study as a positive control; one would expect the activity of a calcium antagonist to be strongly influenced by the extracellular concentration of calcium. A dose of 300 µM was used, since results from the dose-response study (section VI.2) predicted that this would produce approximately 50% inhibition of histamine release. Lanthanum was examined under exactly the same conditions as cromoglycate.

When lung was pre-treated with EGTA and challenged without calcium, there was no significant histamine release ( $0.9 \pm 0.4\%$  over 5 experiments). This demonstrated that the chelating agent effectively removed the extracellular calcium from the tissue. The amount of histamine released by

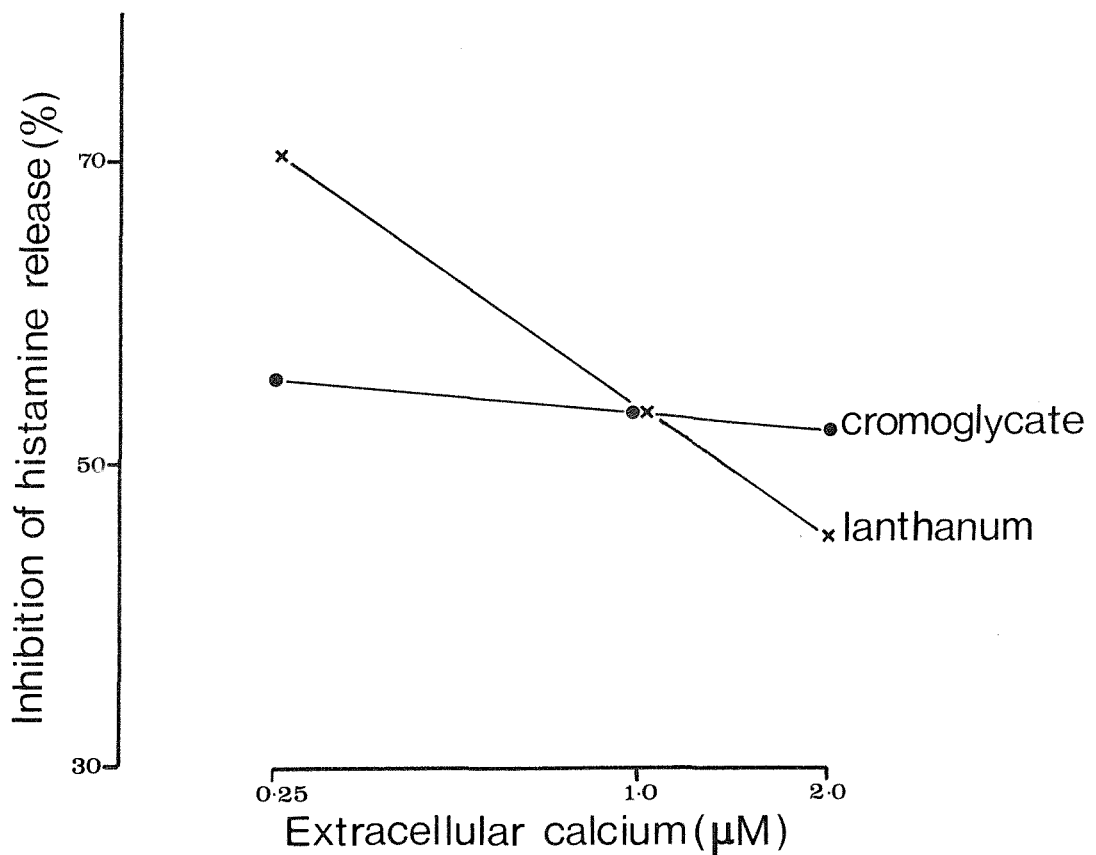
antigen when calcium was restored before challenge, and its inhibition by cromoglycate and lanthanum, is shown in Table VI.1. Over 4 experiments the histamine released in the presence of 0.25 mM calcium was 8.1%, whereas the amount liberated in both 1 mM and 2 mM calcium was approximately 14.0%. In 3 of these experiments, there was no consistent change in the inhibitory effect of cromoglycate as the extracellular calcium concentration was increased. Although the levels of inhibition in the 3 experiments were quite different, the mean values illustrate that cromoglycate produced approximately 50% inhibition whether the reaction was performed in 0.25 mM, 1 mM or 2 mM calcium. Results with lanthanum were completely different. In all 4 experiments, lanthanum was most effective in 0.25 mM calcium but produced less inhibition of histamine release as the extracellular calcium concentration was increased. The mean values revealed that an increase in calcium concentration to 1 mM reduced the inhibition by lanthanum from 70.4 to 53.2%. This was due to the rise in the number of calcium ions per se and not because the response to antigen was more severe in 1 mM calcium, since a further increase in the calcium concentration to 2 mM produced an even greater reduction in lanthanum activity (53.2 to 45.2%) without affecting the levels of antigen-induced histamine release.

The significance of these changes in effect is illustrated in Fig. VI.5. With cromoglycate, there was no correlation between its inhibition of histamine release and the extracellular calcium concentration ( $r = -0.24$ ). In contrast, there was an excellent, negative correlation between the inhibition by lanthanum and the calcium concentration ( $r = -0.92$ ) giving a regression slope of -27.9, ie a 10-fold increase in extracellular calcium produced a 27.9% decline in lanthanum inhibition.

To summarise, inhibition of histamine release by cromoglycate was unaffected by an increase in the concentration of extracellular calcium, whereas inhibition by a calcium antagonist was significantly, albeit not completely, reversed. This confirmed that cromoglycate is not a calcium antagonist and suggested that its action in human lung is totally independent of calcium.

		Calcium concentration (mM)		
		0.25	1.0	2.0
Mean histamine release (%)		8.1 $\pm$ 0.9	14.8 $\pm$ 2.0	14.2 $\pm$ 2.1
Cromoglycate	1)	87.9	77.3	77.4
	2)	21.1	45.1	20.6
	3)	52.0	55.8	46.5
	4)	-	-	-
	MEAN $\pm$ SEM	53.6 $\pm$ 19.3	59.4 $\pm$ 9.4	48.1 $\pm$ 16.4
Lanthanum	1)	82.8	46.4	43.6
	2)	56.7	53.4	41.5
	3)	68.6	55.7	43.7
	4)	73.7	57.4	52.0
	MEAN $\pm$ SEM	70.4 $\pm$ 5.4	53.2 $\pm$ 2.4	45.2 $\pm$ 2.3

Table VI.1. Effect of extracellular calcium on the inhibition of histamine release by cromoglycate and lanthanum. Individual results are shown from 4 experiments; each result is the mean from 3 replicates. The average amount of histamine released by antigen over the 4 experiments is shown at the top of the table.



Drug	Correlation	Slope of regression line $\pm$ SE limits	Significance of slope
cromoglycate	-0.24	$-3.8 \pm 23.8$	NS
lanthanum	-0.92	$-27.9 \pm 5.6$	$<0.01$

FIG. VI.5. Correlation between the inhibition of histamine release by cromoglycate and lanthanum, and the extracellular concentration of calcium. The regression line for each correlation is shown in the graph.

PART B - MECHANISM OF TOLERANCE

In 1977, Sung and co-workers described tachyphylaxis to cromoglycate in passively sensitised rat lung (Sung et al, 1977a; 1977b). They found that pre-incubation of the tissue with 1 dose of the drug reduced the inhibitory effect of a second dose given before antigen challenge. The onset of tachyphylaxis depended on the concentration of the initial dose and the length of pre-treatment. Tachyphylaxis still occurred when calcium was omitted from the pre-incubation medium, and it was only slowly reversed after the drug was removed.

Although these observations refer to tachyphylaxis and not tolerance, they obviously describe the conditions under which the initial dose of cromoglycate induces a state of tolerance. Therefore, similar studies were performed with human lung to determine if tolerance

- a) depends on the dose of cromoglycate and the length of pre-treatment,
- b) occurs in the absence of extracellular calcium, and
- c) is slowly reversed after removal of the drug.

VI.4 Effect of the dose of cromoglycate and the length of pre-treatment on the onset of tolerance

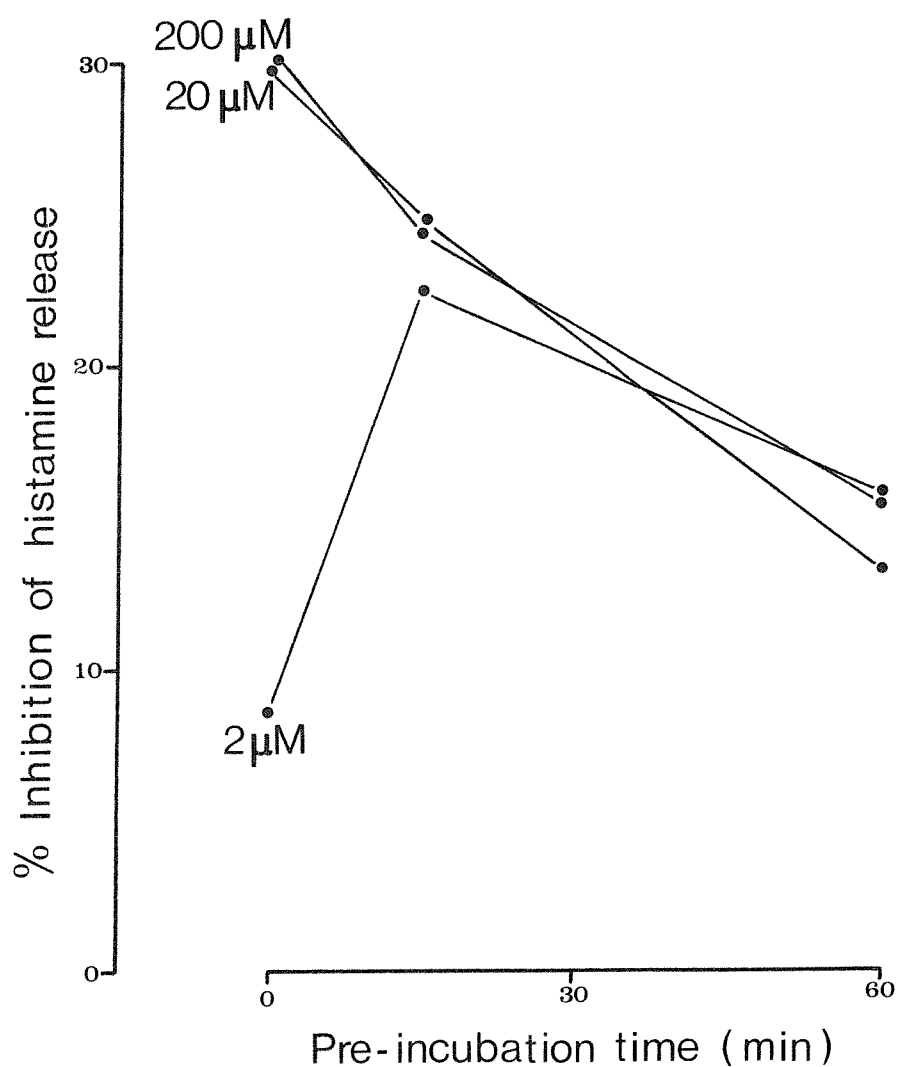
Three concentrations of cromoglycate were selected for this investigation: 20 and 200  $\mu\text{M}$ , which both produced maximal inhibition of histamine release (section V.1), and a less effective dose of 2  $\mu\text{M}$ . Previous studies had shown that some tolerance developed to the 200  $\mu\text{M}$  dose after 15 min pre-incubation with lung tissue but that a longer period of contact was required to cause complete desensitisation (section V.3). In this study, the effect of each dose of drug was examined after 30 sec, 15 min and 60 min pre-incubation.

Lung tissue was sensitised in bulk for 19 h, washed free of serum and divided into replicates. The replicates were incubated at 37°C with drug for the stated period. Then they were all challenged with antigen

simultaneously. Mean results from 9 experiments are shown in the graph in Fig. VI.6. The standard error associated with each mean has been omitted, for clarity. The probability values for the differences between means are shown beneath the graph. When added 30 sec before challenge, the 20 and 200  $\mu\text{M}$  concentrations of cromoglycate both inhibited histamine release by approximately 30%. Their inhibitory effects were reduced in parallel when the pre-incubation time with the tissue was extended. A significant loss of activity was detected after 60 min pre-incubation. Under this condition, inhibition by 200  $\mu\text{M}$  cromoglycate was reduced to 15.5% and inhibition by the 20  $\mu\text{M}$  dose had decreased to 13.2%, which indicated that both doses were approximately 50% less effective.

Pre-incubation of lung with 2  $\mu\text{M}$  cromoglycate produced a different change in activity. When added 30 sec before challenge, this concentration produced only 8.6% inhibition of histamine release, so it was much less effective than the higher doses under the same condition. However, when the pre-incubation time was extended to 15 min, its effect increased significantly to 22.4% inhibition, which was no different from the level of inhibition attained by the higher doses. Thereafter, 2  $\mu\text{M}$  cromoglycate became less active, so that after 60 min pre-incubation it produced only 15.7% inhibition. The decline in activity between 15 and 60 min pre-incubation was not significant but was very similar to that observed with 20 and 200  $\mu\text{M}$  cromoglycate over the same period.

These results showed that the development of tolerance to cromoglycate in human lung depended on the dose of drug administered and on the duration of pre-treatment. Drug doses which were maximally effective after 30 sec pre-incubation became less active as the pre-incubation time was extended. Inhibition by a lower dose of cromoglycate, which was not fully effective when added 30 sec before challenge, initially increased with pre-incubation but thereafter declined. This suggested that tolerance only occurred once the dose of drug had produced its maximum inhibitory effect.



DOSE (μM)	COMPARISON		
	30s v 15min	30s v 60min	15s v 60min
2	<0.05	NS	NS
20	NS	<0.05	NS
200	NS	<0.01	NS

NS denotes a non-significant difference

FIG. VI.6. Dose and time dependency of tolerance to cromoglycate. Each result is the mean from 9 experiments.

### VI.5 Development of tolerance in the absence of calcium

The procedure adopted for this study has been summarised in Fig. VI.7. Chopped lung tissue was divided into 2 groups: lung in group A was used to determine if tolerance occurred in the absence of calcium whereas lung in group B was used to establish the extent of tolerance when calcium was present throughout.

In group A, the chopped tissue was sensitised in bulk for 19 h, washed free of serum and then divided into 2 aliquots. The first was resuspended in calcium-free PBS solution containing 100 µg/ml EGTA and 200 µM cromoglycate. The EGTA was included to chelate any calcium which remained in the extracellular spaces of the tissue. This tissue was left for 1 h at room temperature which was sufficient time for tolerance to develop to cromoglycate (section VI.4). At the end of this period, the aliquot was washed, divided into 200 mg replicates and each replicate was placed in 1.8 ml of calcium-free PBS solution containing 200 µM cromoglycate. Hence, there was continuous contact between the lung tissue and the drug throughout the pre-incubation period. The replicates were then warmed to 37°C and the normal concentration of calcium was restored to each by addition of 0.1 ml of a 20 mM calcium chloride solution. This was immediately followed by challenge with antigen, in a final volume of 2 ml. The second aliquot of sensitised lung in group A was subjected to the same manipulations except that it was not exposed to cromoglycate until 30 sec before challenge. Inhibition of histamine release in these samples represented the activity of the drug on acute administration. Comparison of the effect of cromoglycate in the 2 aliquots of lung from group A indicated the degree of tolerance which had occurred.

In group B, the same procedure was followed except that calcium was present throughout. Hence tolerance to cromoglycate in the absence of calcium (group A) could be compared with the extent of tolerance which occurred in its presence (group B).

The results of the study, performed on 2 different lungs are shown in Table VI.2. In the first lung, cromoglycate produced the same inhibition of histamine release in tissue which had been left without calcium

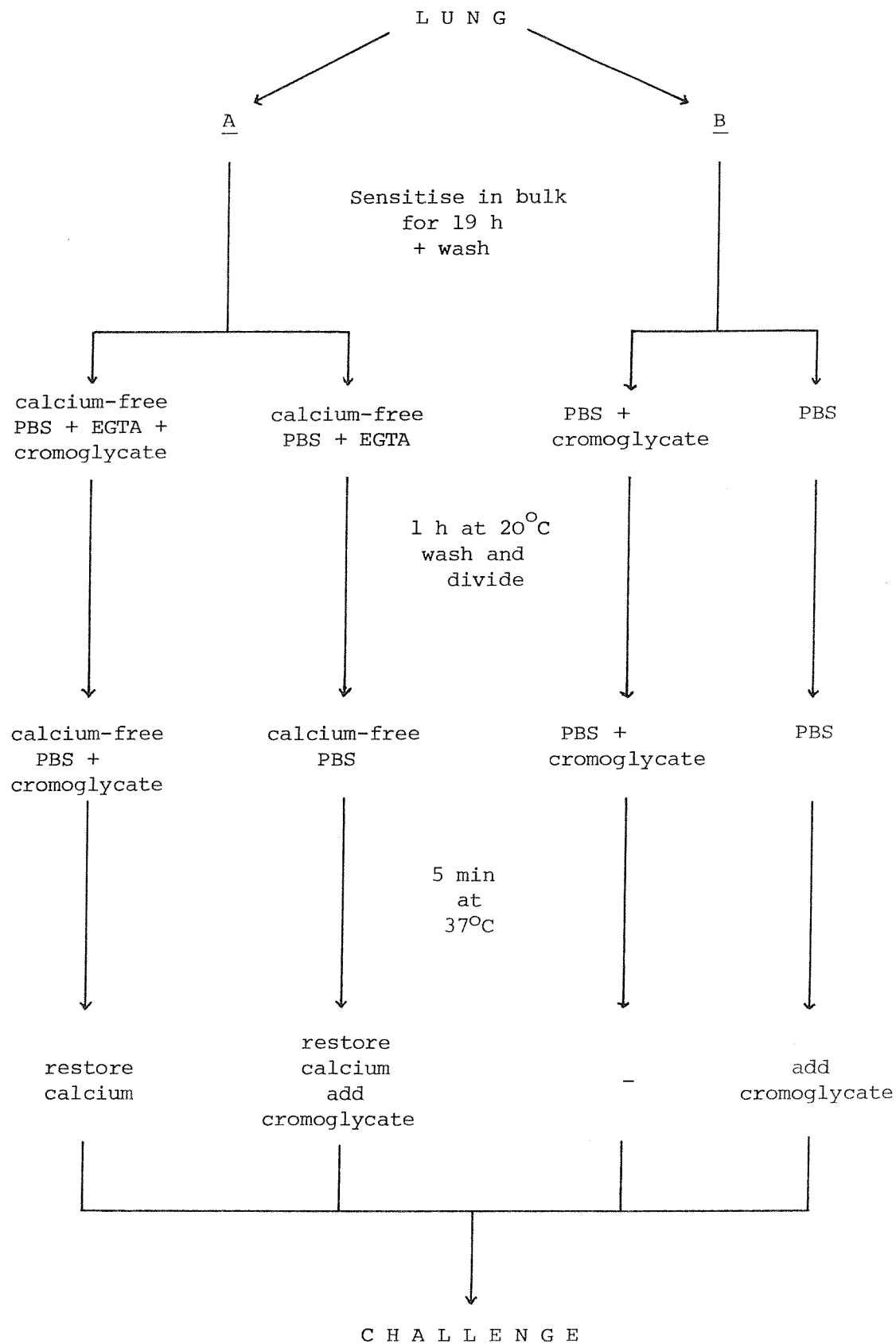


Fig. VI.7. Scheme for comparing the development of tolerance to cromoglycate in the absence (A) and presence (B) of calcium.

for 1 h prior to challenge as in tissue which had been maintained in calcium-containing buffer. When added 30 sec before challenge, it gave 49.6% inhibition in group A samples and 42.5% in group B. The inhibition was reduced to 18.0% and 17.5%, respectively, when the drug was pre-incubated with the tissue for 1 h. Hence, the net loss of inhibition was similar in both groups. This was reproduced in the second experiment, although cromoglycate was only marginally effective in the second lung, the net loss of inhibition was approximately 20%, regardless of whether calcium was omitted from, or included in, the pre-incubation medium.

These results showed that the development of tolerance to cromoglycate was independent of extracellular calcium.

#### VI.6 Recovery from tolerance after removal of the drug

This study was performed on the same lung tissues as the previous investigation. Recovery from tolerance was assessed as follows. Lung was sensitised in bulk, washed and resuspended in PBS solution containing 200 $\mu$ M cromoglycate. After pre-incubation at room temperature for 1 h, the lung was thoroughly washed and transferred in bulk to fresh buffer solution without drug. The tissue was then left for 30 min, washed again and divided into replicates. The replicates were brought to 37°C and challenged in the presence of a fresh, 200 $\mu$ M dose of cromoglycate, added 30 sec before antigen. Hence, there was a period of 30-40 min between the end of the first pre-incubation with cromoglycate and renewal of the dose before challenge, in which the lung was not incubated with drug. Inhibition of histamine release by cromoglycate under these conditions was compared with its inhibition in lung which had been pre-incubated in PBS solution alone. This was to determine if leaving the pre-treated tissue for 30 min in cromoglycate-free buffer restored its sensitivity to the drug.

The results from 2 lungs are shown in Table VI.3, together with results which indicate the tolerance created by continuous pre-incubation with cromoglycate (from section VI.5). After continuous pre-incubation with the first lung for 1 h, cromoglycate produced only 17.5% inhibition of histamine release, compared with 42.5% when it was added 30 sec before challenge. When the lung was pre-treated with the drug and then transferred to drug-free buffer for 30 min, there was no response to a fresh

	Inhibition of histamine release (%)		Net loss of inhibition (30 s - 1 h)
	Pre-incubation time 30 s	1 h	
A) No calcium during 1 h pre-incubation	49.6	18.0	31.6
B) Calcium present throughout	42.5	17.5	25.0
A) No calcium during 1 h pre-incubation	28.2	6.9	21.2
B) Calcium present throughout	15.0	-8.2	23.1

Table VI.2. Development of cromoglycate tolerance in the absence of calcium. Results from 2 experiments are shown. Each value is the mean of 4 replicates.

Inhibition of histamine release (%)				
	Cromoglycate added 30 sec before challenge	Continuous pre-incubation with cromoglycate for 1 h	Cromoglycate added 30 sec before challenge	Pre-incubation with cromoglycate for 1 h, 30 min wash, then drug renewed
LUNG 1	42.5	17.5	45.4	-1.6
LUNG 2	15.0	-8.2	49.9	32.5

Table VI.3. Maintained tolerance to cromoglycate in washed lung tissue. Results from 2 experiments are shown. Each value is the mean of 4 replicates.

dose added before challenge; this produced -1.6% inhibition of histamine release compared with 45.4% in tissue which had not been pre-treated. The tachyphylaxis to the second dose demonstrated that the lung was still desensitised.

Results from the second lung are less easily compared because the response of the 2 sets of samples to acute administration of cromoglycate was quite different (ie 15% and 49.9% inhibition). Nevertheless, tachyphylaxis still occurred in the pre-treated tissue which was left for 30 min before cromoglycate was renewed. In these samples, the fresh dose of drug produced 32.5% inhibition of histamine release compared with 49.9% in samples which had received no pre-treatment.

The results suggested that, once tolerance had developed to the first dose of cromoglycate, lung mast cells did not recover their sensitivity during the 30 min period without drug. However, it was possible that the initial dose was not completely washed from the tissue so that tolerance persisted because the drug was still present. To test how efficiently cromoglycate could be removed by washing, lung was incubated with the drug for a short period (5 min), washed thoroughly and then challenged with antigen. Inhibition of histamine release under these conditions was compared with the inhibition produced by the drug in tissue which was not washed. The results from a single experiment are shown in Table VI.4. Washing the tissue reduced the inhibition by cromoglycate from 60.9% to 2.1%, ie it abolished the effect of the drug. This showed that after a short period of contact with lung tissue the drug could be completely removed by washing, suggesting that it was only loosely bound. Therefore, unless it became more strongly bound with increasing pre-incubation, cromoglycate should also have been washed out of tissue with which it had been in contact for 1 h. If so, the persistence of tolerance in washed lung could not have been caused by a continued presence of drug.

		Histamine release (%)		Inhibition of release (%)
		antigen	cromoglycate + antigen	
a)	No wash	19.7 $\pm$ 1.7	7.7 $\pm$ 0.7	60.9
b)	2 min wash	19.4 $\pm$ 1.3	19.0 $\pm$ 1.4	2.1

Table VI.4. Reversal of the acute effect of cromoglycate by washing. Results are shown from a single experiment. Mean values for the antigen-induced histamine release were obtained from 6 replicates, whereas the mean release in the presence of cromoglycate was obtained from 4. Sensitised lung was pre-incubated in bulk with 200  $\mu$ M cromoglycate for 5 min at 37°C. It was then either  
a) divided into replicates and immediately challenged with antigen, or  
b) thoroughly washed and transferred to 250 ml of fresh buffer without drug at 37°C. After 2 min continuous stirring, the tissue was washed again, divided into replicates and challenged with antigen.

SUMMARY

Studies described in Part A of this chapter were designed to investigate the mechanism by which sodium cromoglycate blocks the influx of calcium into stimulated mast cells. The 4 possibilities which were considered are that the drug

- a) physically stabilises the cell membrane and hence interferes with the opening of calcium channels,
- b) competes with calcium for a membrane channel or receptor site,
- c) binds calcium ions, or
- d) stimulates a membrane or intracellular process which inhibits the transport mechanism.

Studies in rat mast cells demonstrated that concentrations of cromoglycate which inhibited secretion did not protect the cells from lysis when they were placed in a medium of low tonicity. This indicates that the drug acts more selectively than by causing a physical stabilisation of the mast cell membrane. Studies in human lung tissue revealed clear differences between the effect of cromoglycate and the effect of a calcium antagonist or a calcium-chelating agent. Therefore, it appears that cromoglycate acts neither by competing with calcium for a membrane channel or receptor, nor by chelating the free or bound ion. Further studies showed that it produced similar inhibition of secretion in lung mast cells regardless of the concentration of free calcium in the incubation medium. In contrast, the effect of a calcium antagonist was highly dependent on the concentration of free calcium. This confirmed that cromoglycate is not a calcium antagonist and indicated that its action is totally independent of calcium. Of the 4 possibilities considered, the most likely mode of action of the drug is stimulation of a membrane or intracellular process which inhibits calcium transport.

The investigations described in Part B were concerned with the mechanism of tolerance to cromoglycate. The tolerance which developed to the drug in human lung appeared to be very similar to that observed in rat

lung (Sung et al, 1977a; 1977b) except that it was slower in onset:-

- a) Tolerance occurred much more readily with high, maximally-effective concentrations of cromoglycate than with lower, sub-liminal doses. This implied that it was produced by hyperstimulation, eg of a receptor or cellular process.
- b) Even in the presence of high concentrations of cromoglycate, the onset of tolerance was slow; significant tolerance was seen after pre-incubation of the lung with drug for 1 h, but this was only partial. Complete desensitisation only occurred after a longer period of pre-treatment (section V.3). This demonstrated that the biochemical or pharmacological change responsible for the loss of tissue sensitivity occurred gradually rather than rapidly.
- c) Tolerance still occurred when calcium ions were omitted from the pre-incubation medium, so the mechanism appeared to be independent of calcium.
- d) There was no significant recovery from tolerance during a 30-40 min period after the drug was removed. Assuming that cromoglycate was not still bound to the tissue, this indicates that either the sensitivity of the tissue became irreversibly impaired or that the reversal of tolerance was a slow process. The latter explanation seems more likely because, in rat lung, the tissue does eventually recover its sensitivity to cromoglycate but this takes at least 2 h to occur (Sung et al, 1977b).

These results are discussed in Chapter IX, in the light of current concepts concerning cromoglycate tolerance.

CHAPTER VII. STUDIES OF THE MECHANISM OF  
ACTION OF SALBUTAMOL

Studies described in Chapter V demonstrated that the  $\beta$ -adrenergic agonist, salbutamol, is a potent inhibitor of mast cell secretion in human lung. Assem and Schild (1969) found the same with a series of  $\beta$  agonists and also showed that propranolol antagonises their effect, which indicates that it is mediated via a  $\beta$  receptor. However, the tolerance which characterises the action of  $\beta$  agonists in other tissues (Mukherjee *et al*, 1975) did not occur when lung was pre-incubated with salbutamol either for a short (15 min) or long (19-20 h) period. This suggested either that the  $\beta$  receptor which modulates mast cell secretion in human lung is atypical or that inhibition by salbutamol, particularly on prolonged pre-incubation, is not mediated by a  $\beta$  receptor. Therefore a series of studies was performed

- a) to confirm that the acute effect of salbutamol was caused by stimulation of  $\beta$  receptors in the lung, and
- b) to determine if the chronic effect of the drug was also receptor-mediated.

#### VII.1 Effect of propranolol on the inhibition of histamine release produced by short-term incubation of lung tissue with salbutamol

In this study, inhibition of histamine release by salbutamol was examined in the presence of the  $\beta$  receptor antagonist, propranolol. Replicates of sensitised lung were pre-incubated with 1  $\mu$ M propranolol for 2½ min at 37°C. Then 0.1  $\mu$ M salbutamol was added and 5 min later the samples were challenged with antigen. Inhibition of histamine release in these samples was compared with the inhibition produced by each drug on its own.

The results are shown in Table VII.1. Over 7 experiments, propranolol showed no interference with antigen-induced histamine release. Salbutamol inhibited release by 33.9%, but this was reduced to 3.2% by prior incubation of the tissue with propranolol. Hence, in the presence of a  $\beta$  receptor antagonist, salbutamol was ineffective.

	Propranolol alone	Salbutamol alone	Propranolol/ salbutamol
n	7	5	5
Inhibition of histamine release (%)	0.2 $\pm$ 5.6	33.9 $\pm$ 9.1	**3.2 $\pm$ 4.6

Table VII.1. Effect of propranolol (1  $\mu$ M) on the inhibition of histamine release from human lung by salbutamol (0.1  $\mu$ M).

The asterisks indicate a significant difference between the inhibition by salbutamol alone and its effect in the presence of propranolol. n refers to the number of experiments performed on different lungs. The concentration of salbutamol used in this study was selected from the linear part of the dose-response curve (Fig. V.1, section V.1) ie. the region where the inhibition of histamine release was linearly related to the dose of drug.

### VII.2 Stereoselective blockade by propranolol

To confirm that propranolol antagonised the effect of salbutamol by blocking  $\beta$  receptors in the lung, a study was performed using different stereoisomers of the drug. Drug binding to  $\beta$  receptors is stereospecific such that the (-) isomer of  $\beta$  agonists and antagonists is always more potent than the (+) form (Nickerson and Collier, 1975). Therefore, if propranolol was acting on  $\beta$  receptors in the lung, one would expect (-) propranolol to be more potent at antagonising the response to salbutamol than (+) propranolol. Experiments were performed to determine if this was so. Unfortunately, (-) propranolol was not available so the racemate was used, instead.

Propranolol was added to replicates of sensitised lung in concentrations ranging from 100 pM to 1  $\mu$ M. After 2½ min incubation at 37°C, salbutamol (0.33  $\mu$ M) was added and 5 min later the samples were challenged with antigen. Antagonism of the response to salbutamol is shown in Fig. VII.1. The racemate of propranolol produced dose-related antagonism in the concentration range 1 nM to 100 nM, with the 100 nM dose completely blocking the response to salbutamol (95.6% antagonism). (+) propranolol also produced dose-related antagonism but it was clearly less potent than the racemate. The approximate concentrations of the 2 forms which gave 50% antagonism were 5 nM and 330 nM, respectively, which indicated that the racemate was 66 times more potent. Assuming that the racemate comprised a 1:1 mixture of the (-) and (+) forms, then the (-) isomer was some 130 times more potent than the (+) isomer.

The relative potencies of (-) and (+) propranolol in this study equate well with their potencies as  $\beta$  receptor antagonists in other tissues (Nickerson and Collier, 1975; Kalsner, 1980). This provided strong evidence that their antagonism of the response to salbutamol was caused by blockade of  $\beta$  receptors.

### VII.3 Effect of propranolol on the inhibition produced by salbutamol after prolonged pre-incubation with lung tissue

In this study, salbutamol was added to lung tissue at the beginning of sensitisation and was renewed 19 h later when the tissue was washed free of serum prior to antigen challenge. Propranolol was added either

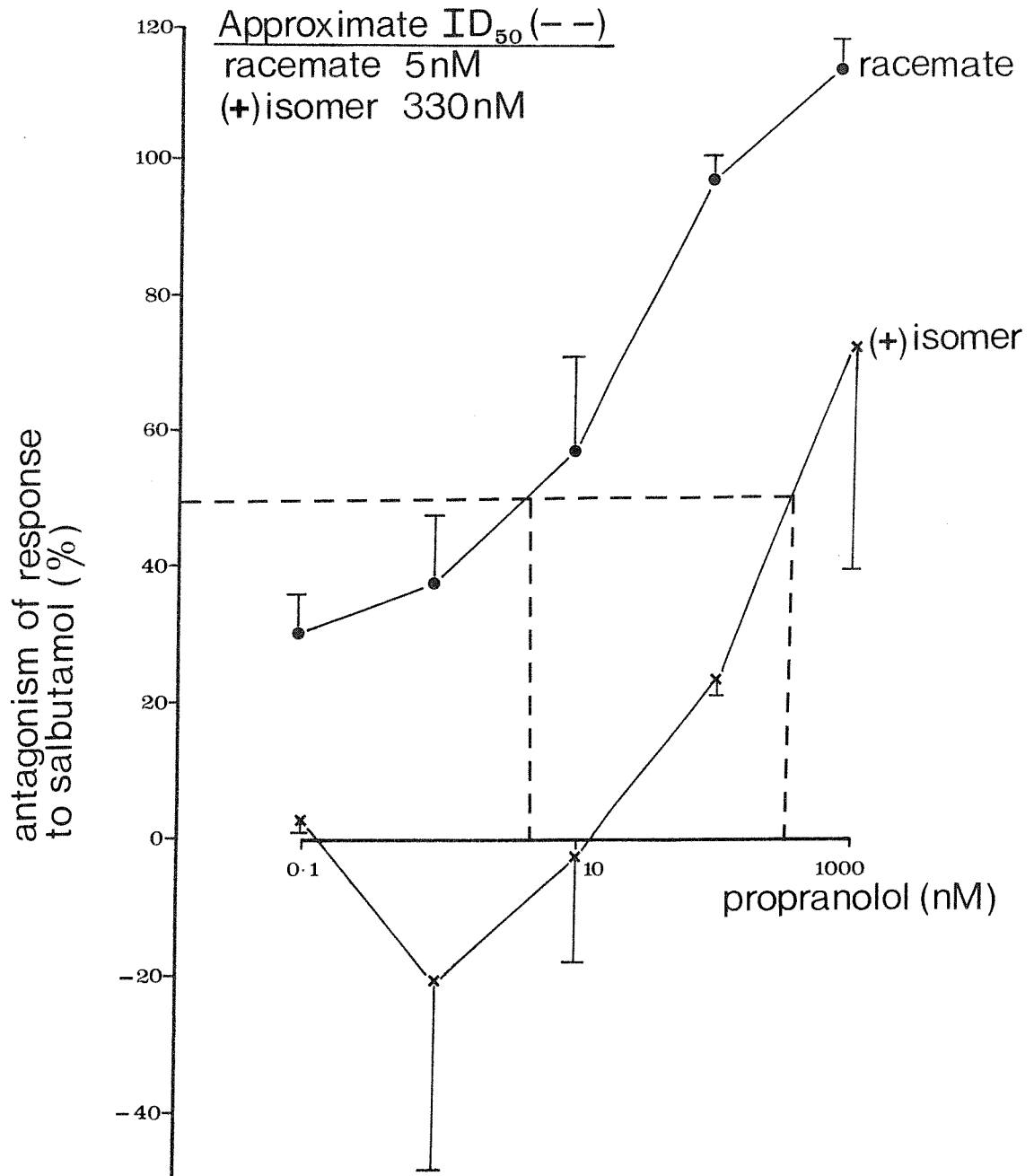


FIG. VII.1. Antagonism of the response to salbutamol in human lung by the racemate and (+) isomer of propranolol. Mean results from 2 experiments are shown. In the first experiment, salbutamol produced 74.4% inhibition of histamine release but gave only 23.6% inhibition in the second. Therefore, for ease of interpretation, results with propranolol have been expressed as the percentage antagonism of the response to salbutamol.

before the first dose of salbutamol and maintained throughout the pre-incubation period, or it was introduced only when the dose of salbutamol was renewed.

Results from experiments on 2 lungs are shown in Fig. VII.2. Prolonged pre-incubation with salbutamol produced 88.2% inhibition of antigen-induced histamine release in the first lung and 26.1% inhibition in the second (histogram a). In both experiments, the inhibition was prevented when propranolol was present throughout the 19-20 h pre-incubation period (histogram b). This demonstrated that the anti-allergic effect produced by salbutamol after prolonged contact with lung tissue was caused by stimulation of  $\beta$  receptors. When propranolol was introduced as the dose of salbutamol was renewed, it only partially blocked the inhibition of histamine release (histogram c). The remaining inhibitory effect under these circumstances must have been caused by the initial dose of the agonist, because propranolol completely antagonised the effect of acutely-administered salbutamol (section VII. 1). This suggested that, after prolonged pre-incubation with the lung, salbutamol either remained bound to the tissue or it produced an effect which could not be blocked by propranolol.

#### VII.4 Recovery from the acute and chronic effects of salbutamol

Although the effects of salbutamol after acute and prolonged pre-incubation with lung tissue were both caused by stimulation of  $\beta$  receptors, only the acute effect was reversed when the tissue was washed. This is illustrated by the results of 2 separate studies which are shown in Table VII.2 and Fig. VII.3. When lung tissue was pre-incubated with salbutamol for 5 min, thoroughly washed and then challenged with antigen, there was very little inhibition of histamine release. In 2 experiments, washing reduced the inhibition by salbutamol from 72.1% to 21.6% and from 51.6% to -2.8%, respectively (Table VII.2). This demonstrated that, after acute administration, the drug could be easily washed out of the tissue which indicated that it was only loosely bound. However, if lung was pre-incubated with salbutamol for 19 h, the inhibition of histamine release was not abrogated by washing. In 2 experiments, inhibition by the drug was maintained even after the tissue had been placed in drug-free buffer for periods of up to 1 h before it was challenged (Fig. VII.3).

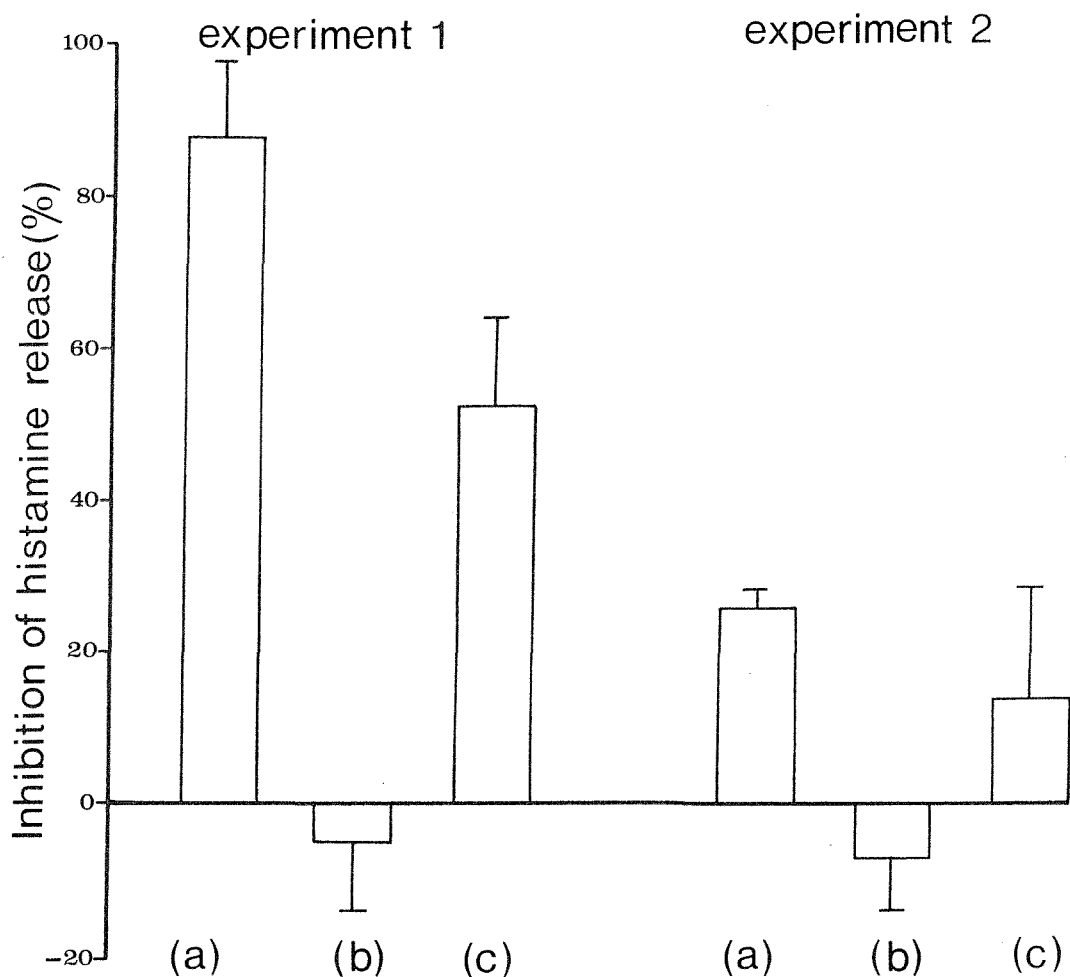


FIG. VII.2. Inhibition of histamine release produced by salbutamol after 19-20 hours pre-incubation with lung tissue, and its antagonism by propranolol. Each result is the mean from 4 replicates. Salbutamol was used at a concentration of  $1 \mu\text{M}$ , since this was the dose investigated in the studies of pre-incubation described in section V.3.

- a) lung tissue was continuously pre-incubated with salbutamol for 19-20 h.
- b) propranolol ( $1 \mu\text{M}$ ) was present throughout the 19-20 h. (prolonged pre-incubation with propranolol did not affect antigen-induced histamine release).
- c) propranolol ( $1 \mu\text{M}$ ) was introduced before the dose of salbutamol was renewed, 5 min before challenge with antigen.

Experiment	Inhibition of histamine release (%)	
	No wash	Wash
1	72.1 $\pm$ 2.9	21.6 $\pm$ 14.9
2	51.6 $\pm$ 8.6	-2.8 $\pm$ 10.0

Table VII.2. Inhibition of histamine release in tissue which was washed after 5 min pre-incubation with salbutamol. Each result is the mean of 3 replicates. Sensitised lung was pre-incubated in bulk at 37°C with 0.33  $\mu$ M salbutamol. After 5 min it was thoroughly washed and transferred to 250 ml of fresh buffer without drug, at 37°C. The solution was continuously stirred for 2 min, then poured through a piece of thin gauze to collect the lung. The lung was divided into replicates and immediately challenged with antigen. Control samples were pre-incubated with salbutamol but challenged without being washed.

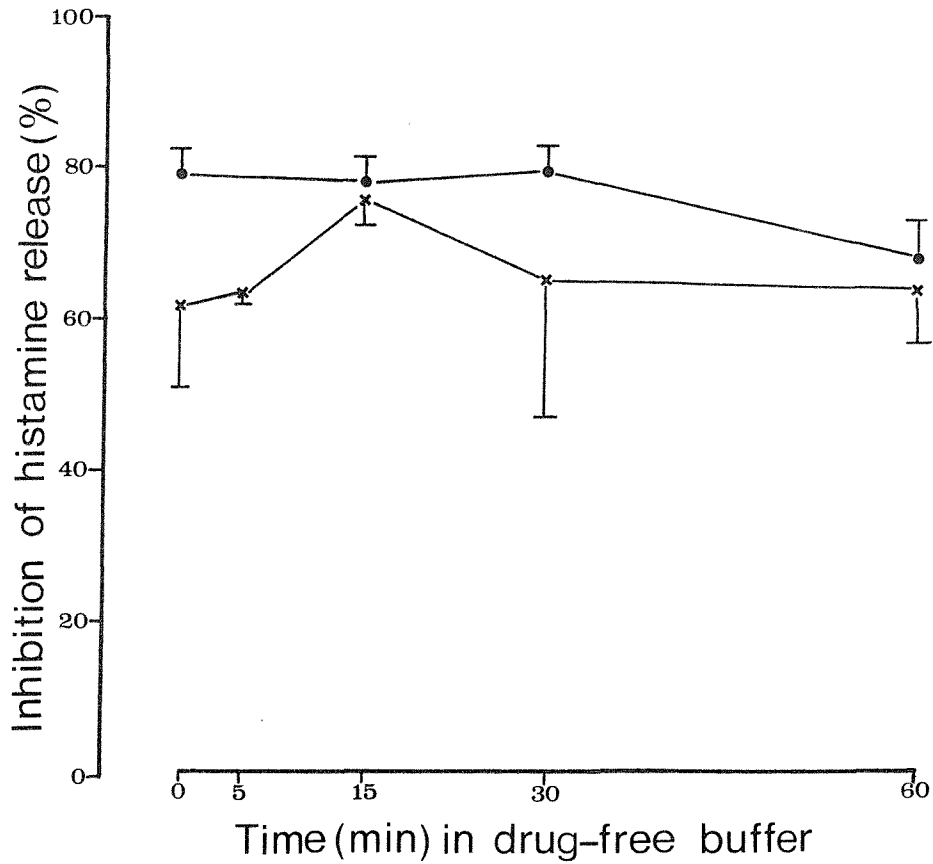


FIG. VII.3. Inhibition of histamine release in lung tissue which was washed and placed in drug-free buffer for increasing periods after it had been pre-incubated with salbutamol for 19 hours. Individual results from 2 experiments are shown, each result is the mean from 3 replicates. Lung was pre-incubated in bulk with  $1 \mu\text{M}$  salbutamol for 19 hours, washed thoroughly and divided into replicates. The replicates were placed in fresh buffer without drug for increasing periods, at the end of which they were washed again and challenged with antigen.

Clearly, some change occurred during the prolonged pre-incubation with salbutamol. Either the drug became more strongly bound so that it was not removed by washing, or it produced an irreversible change in the tissue which depressed secretion in mast cells.

#### SUMMARY

There are 2 conclusions from this series of studies:-

- 1) On acute administration, salbutamol inhibited mast cell secretion in the lung by stimulation of  $\beta$  receptors.

The evidence for this was that the  $\beta$  receptor antagonist, propranolol, totally blocked the anti-allergic effect of salbutamol. It did so in a stereoselective manner which indicated that it was specifically binding to  $\beta$  receptors.

- 2) On chronic administration, salbutamol also produced its effect through stimulation of  $\beta$  receptors but there was a change in the nature of the inhibition produced.

The inhibition of immunological histamine release produced by salbutamol after 19-20 h contact with the lung tissue was abolished if propranolol was present throughout the pre-incubation period. This demonstrated that the chronic effect of the agonist must have developed through its action on  $\beta$  receptors. However, 2 observations indicated that a change occurred during the prolonged treatment with salbutamol. Firstly, propranolol did not fully antagonise the effect of a fresh dose of salbutamol added to tissue which had been pre-treated with the agonist for 19 h, whereas it completely blocked the effect of salbutamol in lung which had not been pre-treated. The second observation came from experiments in which lung tissue was washed between pre-incubation with the drug and challenge with antigen; immunological histamine release was still inhibited in tissue which had been pre-treated with the drug for 19 h but it was not inhibited in tissue which had been pre-treated for only 5 min. These observations can be interpreted in 2 ways: either

- a) salbutamol became more strongly bound after prolonged pre-incubation with the lung so that it was not removed by washing, or
- b) continuous stimulation of  $\beta$  receptors by the drug for 19 h produced an irreversible change in the tissue, and this was responsible for the suppressed secretion in mast cells.

CHAPTER VIII.   STUDIES OF THE MECHANISM OF  
ACTION OF CHLORPROMAZINE

Chlorpromazine has many pharmacological actions including potent local anaesthetic activity (Byck, 1975). There is evidence that this property contributes to its anti-allergic effect. Like chlorpromazine, several local anaesthetics inhibit secretion in rat mast cells (Kazimierczak, Peret and Maśliński, 1976; Johnson and Miller, 1979). Moreover, chlorpromazine exhibits very similar characteristics of inhibition to lignocaine in these cells (Johnson and Miller, 1979). In the following section, evidence is presented to show that there are also similarities between the effects of these 2 drugs on mast cell secretion in human lung, suggesting that at least part of the action of chlorpromazine is due to its local anaesthetic property. Also described in this section are studies designed to find out how this property influences the secretory process.

#### VIII.1 Dose-response comparison of lignocaine and chlorpromazine

Studies described in chapter V demonstrated that chlorpromazine exerts a dual effect on antigen-induced histamine release, ie it inhibits release at low concentrations but becomes less inhibitory as the dose is increased because it starts to liberate histamine. Lignocaine was examined at various dose levels to determine if it exhibited similar characteristics. Solutions of lignocaine were prepared in PBS, in concentrations ranging from 3.3  $\mu\text{M}$  to 1 mM. The drug was added to sensitised lung tissue 5 min before antigen challenge, to simulate conditions under which chlorpromazine was investigated (section V.1).

The results are shown in Fig. VIII.1, in comparison with results from studies with chlorpromazine. At concentrations below 100  $\mu\text{M}$ , lignocaine had no effect on antigen-induced histamine release. The 100  $\mu\text{M}$  dose produced a slight enhancement of release but this was not significant. The higher concentrations of 300  $\mu\text{M}$  and 1 mM significantly inhibited release, producing 18.5 and 34.5% inhibition, respectively. This suggested that the inhibitory effect of lignocaine was dose-related between 100  $\mu\text{M}$  and 1 mM.

Lignocaine was approximately 10 times less potent than chlorpromazine: the concentrations of the 2 drugs which produced 30% inhibition were 1 mM and 100  $\mu\text{M}$ , respectively. Not only did these concentrations give equivalent

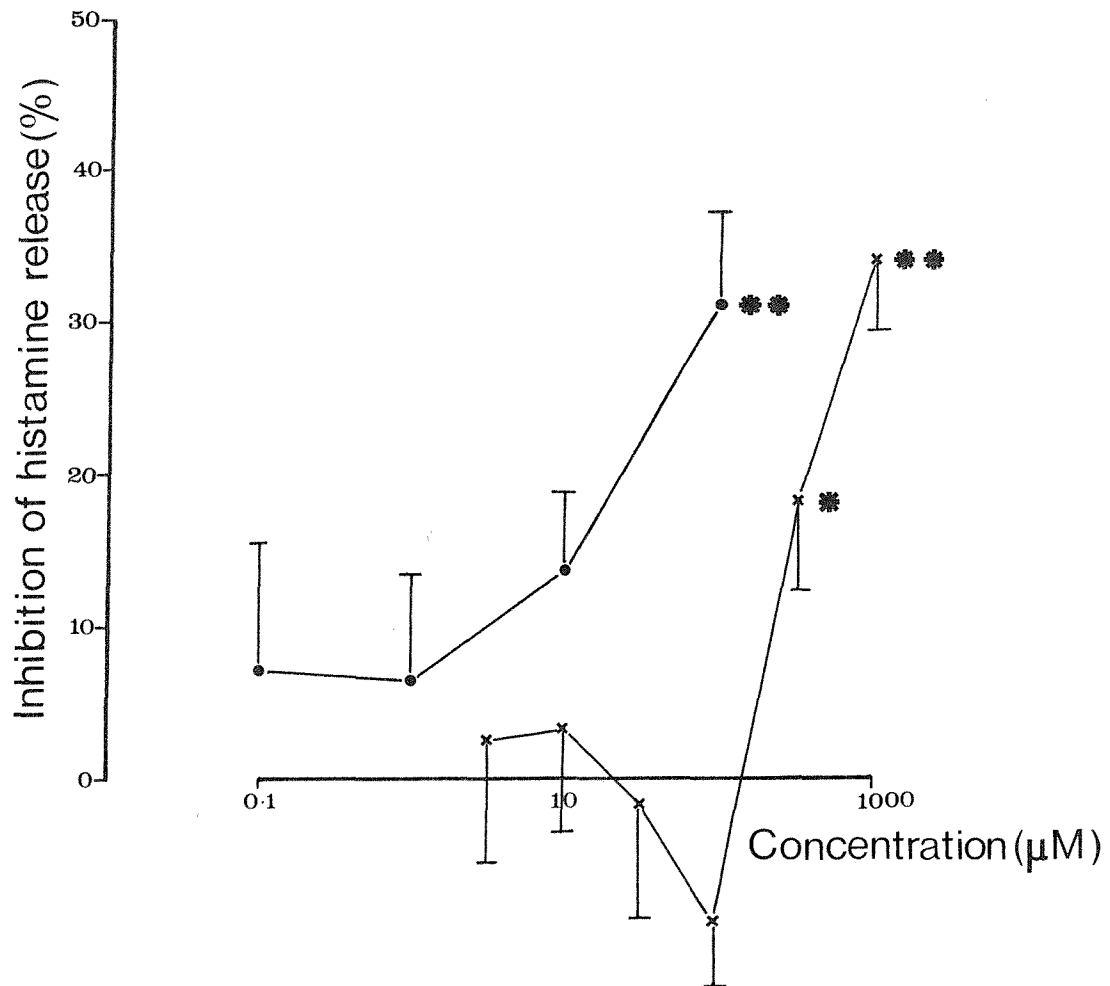


FIG. VIII.1. Inhibition of histamine release by chlorpromazine (●—●) compared with the inhibition by lignocaine (x—x). Each result is the mean from 4 or 5 experiments. Drugs were added 5 min before antigen challenge.

inhibition, they also increased the spontaneous release of histamine from the tissue (Table VIII.1). In the presence of 1 mM lignocaine, spontaneous release rose from 0.7 to 1.1% of the total histamine contained in the tissue, whereas in the presence of 100  $\mu$ M chlorpromazine it increased from 2.2 to 3.2%. Whilst neither increase in release was statistically significant, this implied that both drugs began to liberate histamine from mast cells at these dose levels.

These results showed that lignocaine produced a similar antagonism of antigen-induced histamine release to chlorpromazine, though it was less potent. They also suggested that, like chlorpromazine, lignocaine started to liberate histamine from mast cells at higher concentrations.

#### VIII.2 Effect of extending the period of pre-incubation on the inhibition of histamine release by lignocaine and chlorpromazine

Studies described in chapter V demonstrated that the inhibitory effect of chlorpromazine was increased by extending the period of pre-incubation with lung tissue before antigen challenge. Therefore a study was performed to determine if the activity of lignocaine was similarly increased. Maximally effective concentrations of lignocaine and chlorpromazine were chosen for this study, ie 1 mM and 100  $\mu$ M, respectively. Each drug was pre-incubated at room temperature with replicates of sensitised lung for periods of up to 1 h before challenge. Five min before antigen was added the replicates were warmed to 37°C. All replicates were challenged simultaneously.

Individual results from 3 experiments are shown in Fig. VIII.2. In 2 experiments, the change in activity of lignocaine was qualitatively very similar to the change in activity of chlorpromazine. In the first of these, there was a small increase in the inhibition of antigen-induced histamine release by both drugs as the pre-incubation time with the tissue was extended from 5 min to 1 h. In the second, their inhibitory effects initially increased with pre-incubation but then declined. There was some divergence in the pattern of inhibition in the third experiment: both drugs slightly increased in effect between 5 and 30 min pre-incubation but thereafter lignocaine continued to become more inhibitory whereas the inhibition by chlorpromazine declined.

	n	Spontaneous release of histamine (%)	
		no drug	+ drug
Lignocaine	4	0.7 $\pm$ 0.4	1.1 $\pm$ 0.4
Chlorpromazine	9	2.2 $\pm$ 0.5	3.2 $\pm$ 0.9

Table VIII.1. Effect of lignocaine (1 mM) and chlorpromazine (100  $\mu$ M) on spontaneous release of histamine from human lung. n refers to the number of different lungs investigated.

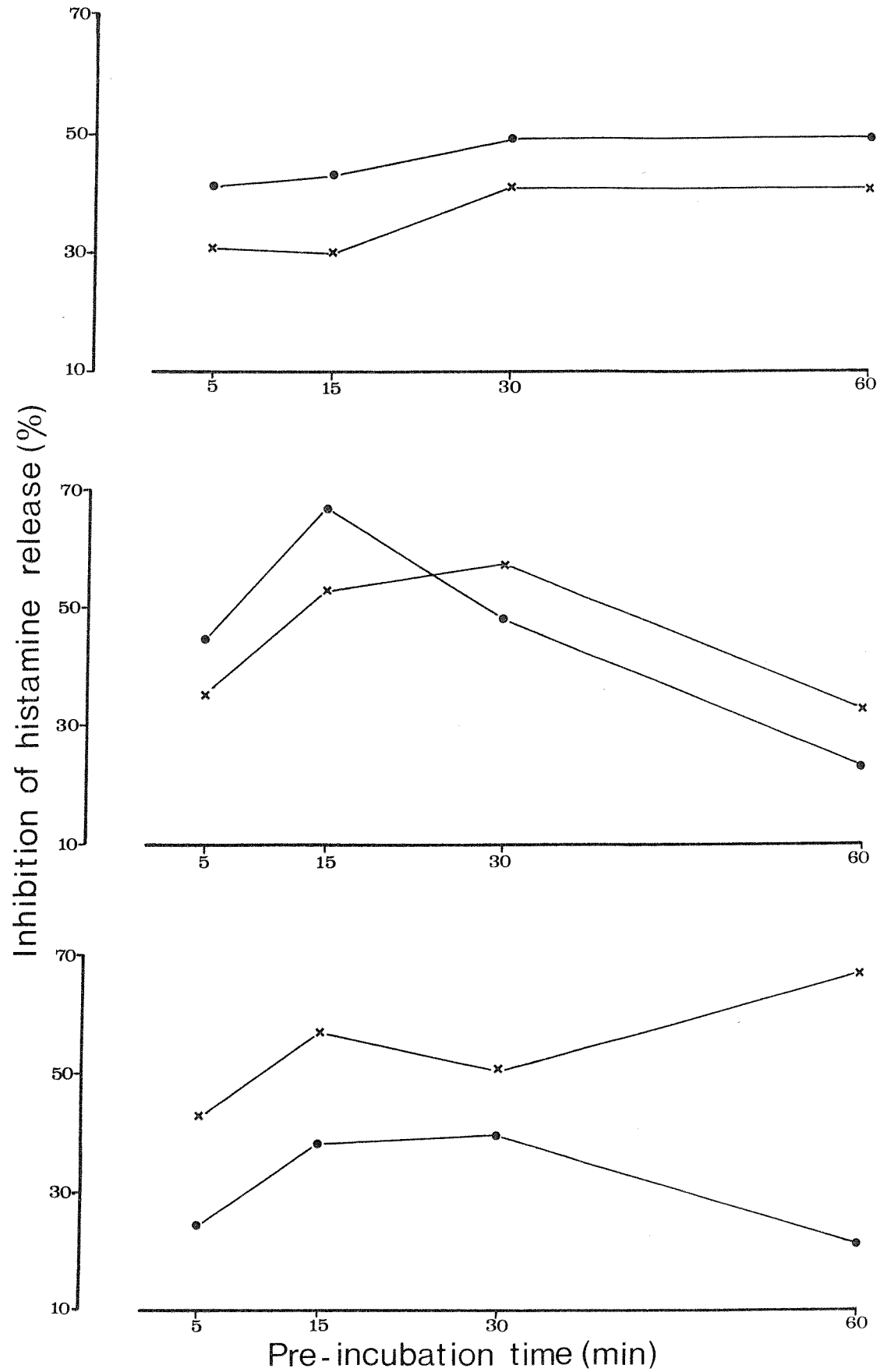


FIG. VIII.2. Qualitative changes in the inhibition of histamine release by lignocaine (x—x) and chlorpromazine (●—●) caused by extending the period of pre-incubation with lung tissue. Each result is the mean from 3 replicates.

The decrease in inhibitory effect after the longer periods of pre-treatment, seen in 1 experiment with lignocaine and in 2 with chlorpromazine, was probably due to the drugs liberating histamine from the tissue. This was indicated by the rise in spontaneous histamine release which occurred after 60 min pre-incubation with each drug (Table VIII.2). In each of the 3 experiments with lignocaine spontaneous release was increased, although the mean increase from 1.1 to 1.7% was not statistically significant. Sixty min pre-incubation of lung tissue with chlorpromazine significantly increased the release from 1.4 to 4.5%.

Hence, the changes in activity of lignocaine and chlorpromazine which occurred when the pre-incubation period was extended were qualitatively very similar. Although the pattern of change was different in each experiment, the results suggested that both drugs were more inhibitory when added 15-30 min before challenge instead of 5, but that they began to liberate histamine if the pre-incubation time was extended to 1 h.

As there were similarities between the effect of chlorpromazine on antigen-induced histamine release and the effect of a local anaesthetic, it appeared that the anti-allergic activity of chlorpromazine was related to its local anaesthetic property. This lead to studies of how the property might influence secretion in mast cells.

### VIII.3 Stabilisation of the mast cell membrane; effect of chlorpromazine on rat mast cells exposed to hypotonic conditions

Chlorpromazine, local anaesthetics and lipid-soluble drugs in general, all dissolve in membranes and cause them to expand. This creates a conformational change in the membrane which obstructs the passage of ions and also allows the drug access to membrane enzymes (Seeman, 1972). Experiments were performed to determine if the anti-allergic effect of chlorpromazine was mediated by an action on the plasmalemma of mast cells.

The study was based on a report by Roth and Seeman (1971). These authors demonstrated that concentrations of lipid-soluble drugs which were anaesthetic in neuronal tissues protected erythrocytes from lysis when the cells were placed in a medium of low tonicity. Both effects were caused

		Spontaneous release of histamine (%)	
		no drug	+ drug
Lignocaine	1)	0.4	0.9
	2)	1.9	2.4
	3)	1.0	1.8
	MEAN $\pm$ SEM	1.1 $\pm$ 0.4	1.7 $\pm$ 0.4
Chlorpromazine	1)	2.3	4.4
	2)	0.6	2.4
	3)	1.4	6.6
	MEAN $\pm$ SEM	1.4 $\pm$ 0.5	*4.5 $\pm$ 1.2

Table VIII.2. Effect of 60 min pre-incubation with lignocaine (1 mM) or chlorpromazine (100  $\mu$ M) on spontaneous release of histamine from human lung. Individual results from 3 experiments with each drug are shown; each result is the mean of 3 replicates.

\* denotes a significant increase in the release.

by the drugs integrating with the plasma membrane (Seeman, 1972). Therefore, chlorpromazine was examined in mast cells exposed to either a secretory stimulus or hypotonic conditions, to determine if concentrations which inhibit the secretion of histamine also inhibit histamine release induced by hypotonic lysis. The study was performed on an unpurified suspension of rat mast cells and compound 48/80 was chosen as the stimulus for secretion. Preliminary experiments designed to establish concentrations of chlorpromazine which inhibited the response to compound 48/80 showed that the drug was effective over a very narrow dose range. The results of 2 such experiments are shown in Fig. VIII.3a. The drug inhibited histamine release in doses ranging from 2 to 6.6  $\mu\text{M}$ . At concentrations greater than 6.6  $\mu\text{M}$  in the first experiment and above 4.4  $\mu\text{M}$  in the second, the drug liberated histamine in a dose-related fashion. As 15  $\mu\text{M}$  chlorpromazine produced 80% histamine release, there was clearly very little difference between concentrations which were inhibitory and those which liberated histamine.

On the basis of these preliminary experiments, the concentration range chosen for the comparative study of its effect against 48/80- and hypotonically-induced histamine release was 0.83 to 15  $\mu\text{M}$ . The drug was examined in 0.66 increments in dose so that small changes in effect would be detected. Mean results from 5 experiments are shown in Fig. VIII.3b. The histamine released in response to compound 48/80 and hypotonic conditions was very similar: between 50 and 60% of the total contained by mast cells. Chlorpromazine inhibited both types of release in an almost identical, biphasic fashion. It produced dose-related inhibition of 48/80-induced release over the range 1.9 to 6.7  $\mu\text{M}$ . It was slightly more potent against release induced by hypotonic lysis, producing dose-related inhibition between 0.83 and 2.9  $\mu\text{M}$ . Maximum inhibition of both types of release was approximately 30% and the slopes of the inhibitory part of the 2 dose-response curves appeared very similar. In the concentration range 6.7 to 15  $\mu\text{M}$ , there was a dose-related loss of inhibitory effect, which was caused by the drug liberating histamine from the cells (Fig. VIII.3a).

Hence, concentrations of chlorpromazine which inhibited the secretion of histamine from mast cells also inhibited histamine release induced by hypotonic lysis of the cells. The similar slopes and maxima of the 2

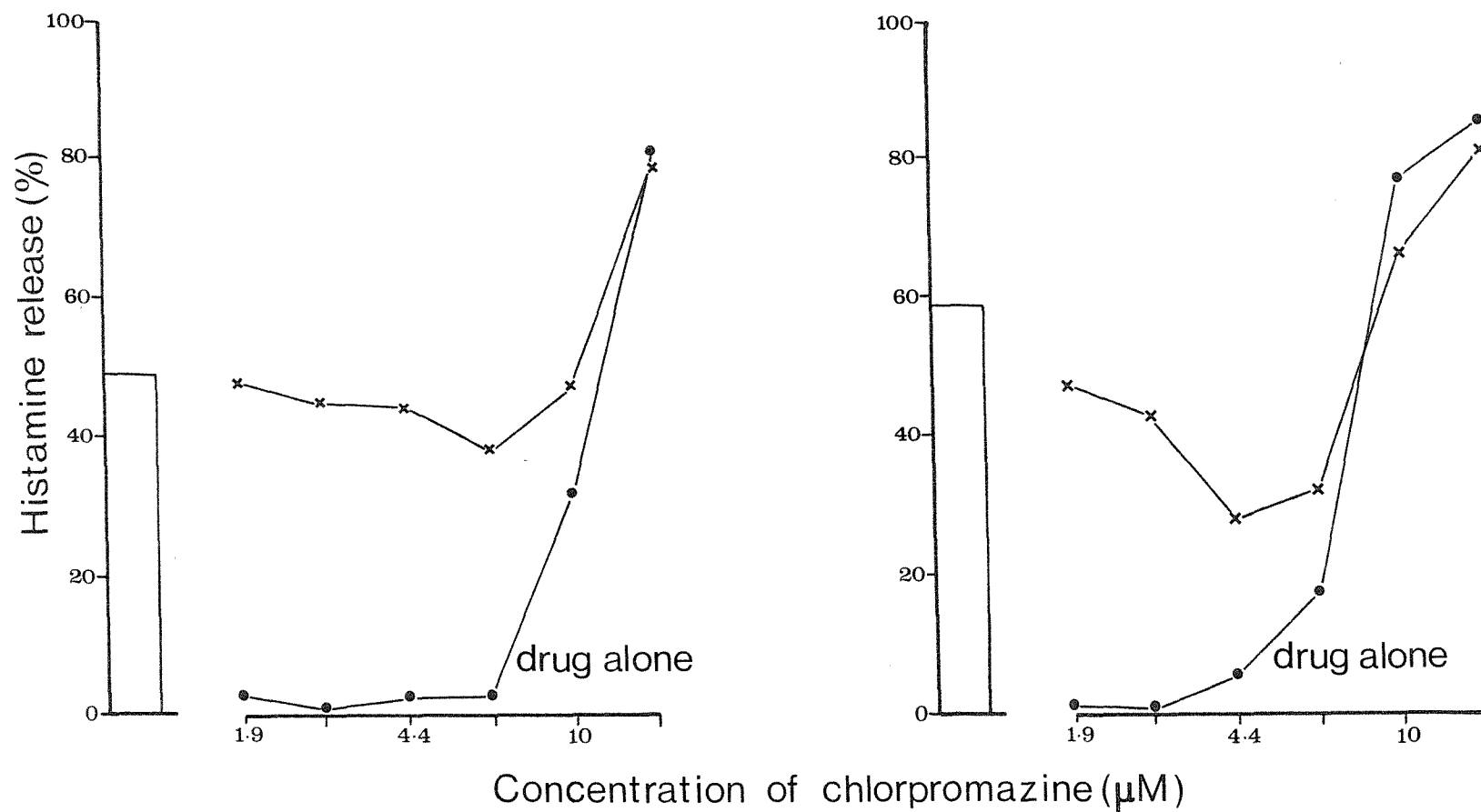


FIG. VIII.3a. Effect of chlorpromazine on histamine release from rat mast cells induced by compound 48/80. Results from 2 experiments are shown; each result is the mean from 2 replicates. Standard deviations have been omitted because the agreement between replicates was extremely good. The histograms represent the release in the presence of 48/80 alone (200 ng/ml). Chlorpromazine was added 5 min before challenge.

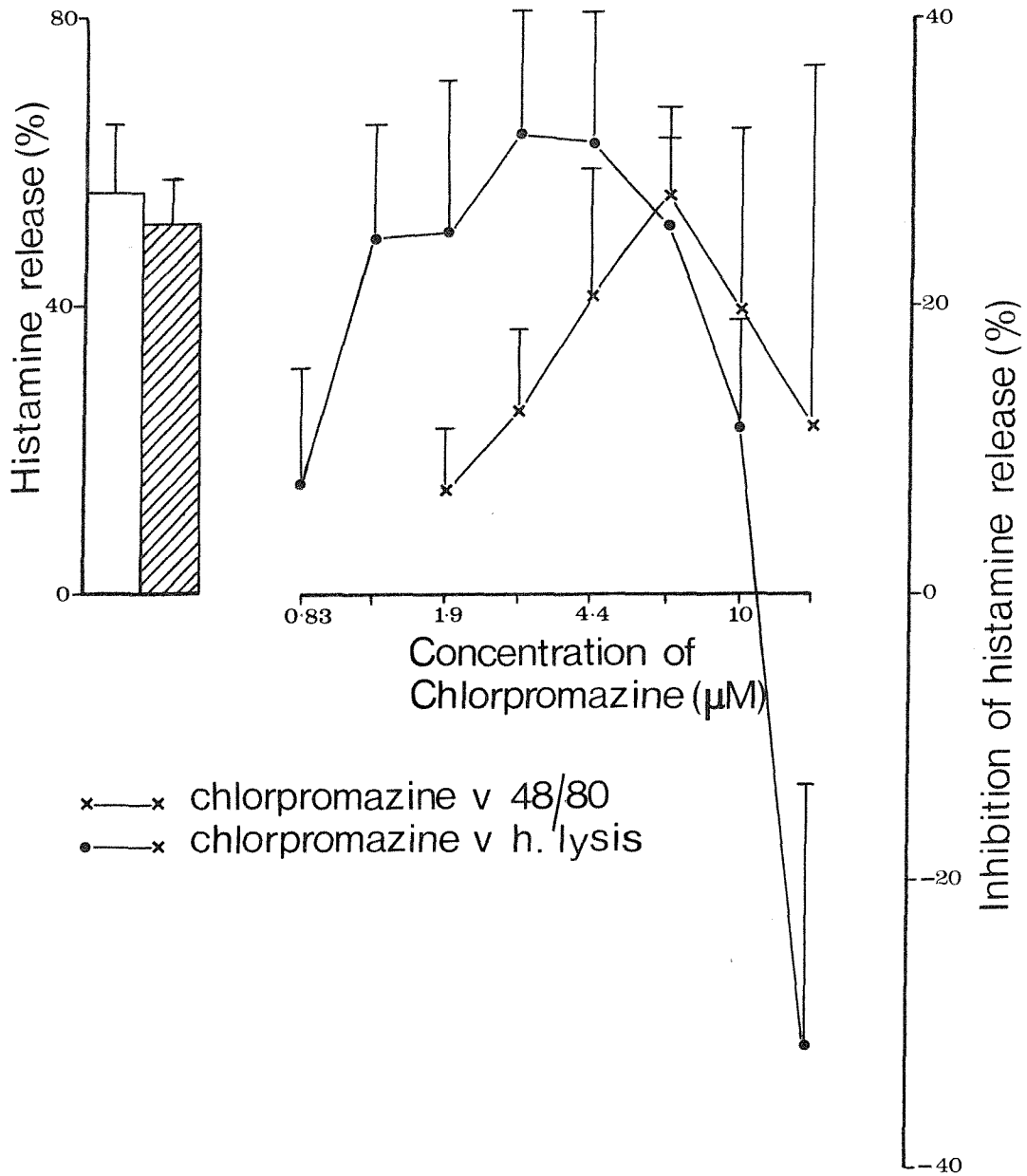


FIG. VIII.3b. Histamine release from rat mast cells induced by compound 48/80 (x—x) and hypotonic lysis (●—●) and its antagonism by chlorpromazine. Cells were incubated for 5 min with 150 ng/ml compound 48/80 (in PBS solution) or with 0.2% saline (in Tris buffer). These conditions were selected on the basis of studies described in section VI.1, which indicated that they resulted in similar levels of histamine release. Chlorpromazine was added 5 min before challenge.

dose-response curves indicated that it inhibited both types of release by a similar mechanism. Therefore, the anti-allergic effect of chlorpromazine appeared to be totally mediated by its physical alteration of the mast cell membrane.

#### VIII.4 Effect of extracellular calcium on inhibition of histamine release by chlorpromazine

By binding to and physically stabilising the membrane of a cell, local anaesthetics may have 1 of 2 effects: firstly, they depress the movement of ions across the membrane and, secondly, they interfere with the function of membrane-associated enzymes (Seeman, 1972). In particular, they inhibit calcium movements by competing with the ion for binding to phospholipids (Hebborn, 1972). There is evidence that this occurs in mast cells: both lignocaine and chlorpromazine inhibit the influx of calcium in rat mast cells stimulated by the calcium ionophore, A23187 (Johnson and Miller, 1979). Moreover, inhibition of histamine release from these cells by lignocaine can be antagonised by increasing the extracellular concentration of calcium (Kazimierczak *et al*, 1976). Therefore, experiments were performed to find out if chlorpromazine competes with calcium for sites on lung mast cells.

Firstly, the effect of the drug was examined in the presence of increasing concentrations of free calcium. Sensitised lung was pre-treated with EGTA in calcium-free buffer to remove extracellular calcium from the tissue (section VI.3). The lung was then washed, divided into replicates and each replicate was resuspended in calcium-free buffer. The replicates were brought to 37°C and 100 µM chlorpromazine was added. Five min later, calcium ions were introduced in the form of calcium chloride, to give concentrations of 0.25, 1 and 2 mM calcium. This was immediately followed by challenge with antigen.

Mean results from 4 experiments are shown in Fig. VIII.4, in comparison with results obtained in the same tissues with the calcium antagonist, lanthanum. In 0.25 mM calcium, chlorpromazine produced 55.3% inhibition of antigen-induced histamine release. The inhibition was reduced as the concentration of calcium was increased: an 8-fold increase in calcium to

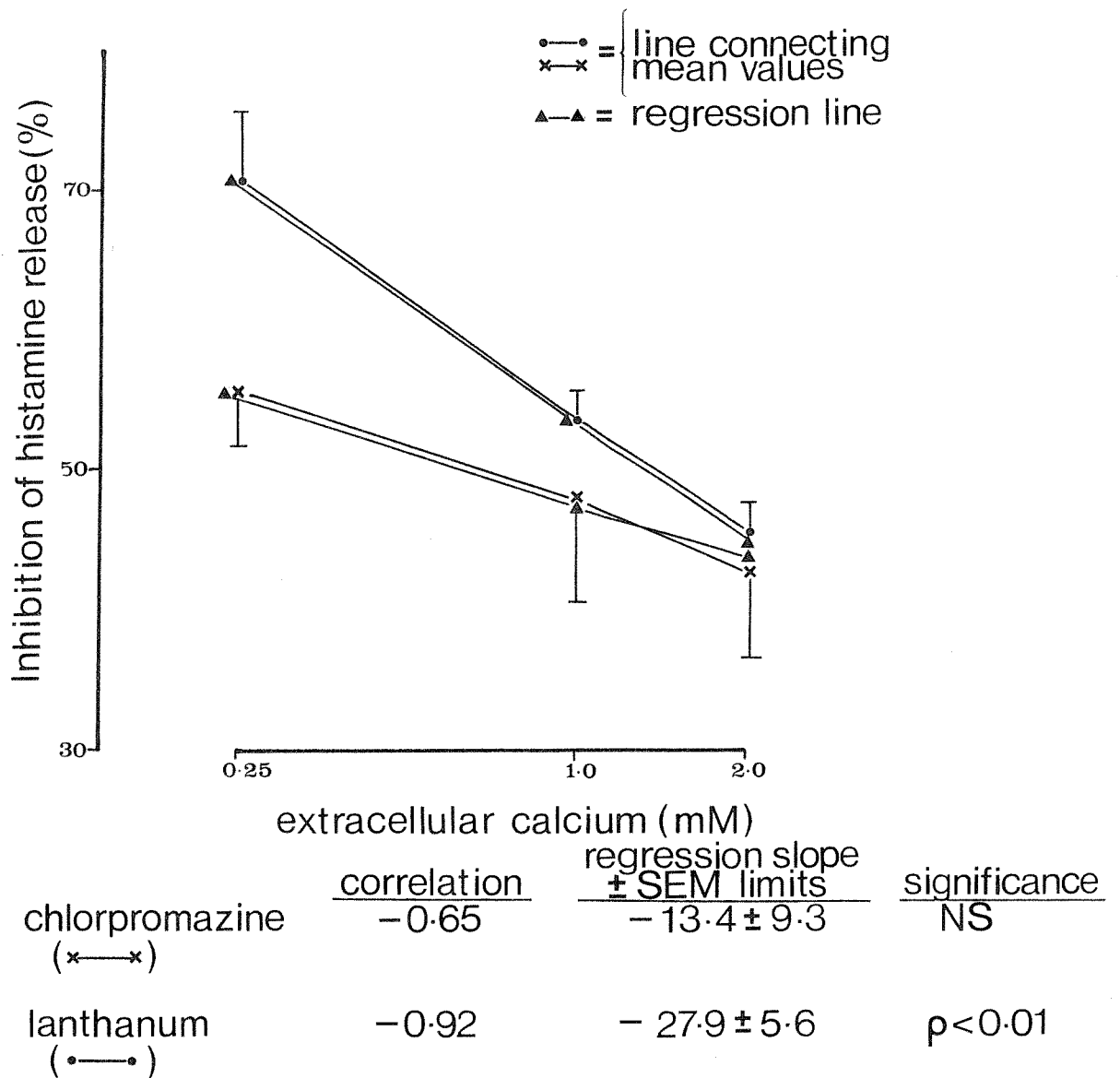


FIG. VIII.4. Effect of extracellular calcium on the inhibition of histamine release from human lung by chlorpromazine (100  $\mu$ M) and lanthanum (300  $\mu$ M). Mean results from 4 experiments are shown in the graph. Values beneath the graph refer to the linear regression of each relationship. Drugs were added 5 min (chlorpromazine) or 30 sec (lanthanum) before antigen challenge.

2 mM produced a 12.4% decline in inhibition, ie chlorpromazine became 22.4% less effective. However, the effect of chlorpromazine was less dependent upon calcium than the effect of lanthanum. This is reflected by the relatively poor correlation between inhibition of histamine release by chlorpromazine and the calcium concentration ( $r = -0.65$ ), and by the smaller regression slope for this relationship (-13.4) which was not statistically significant.

These results demonstrated that inhibition by chlorpromazine was partly overcome by calcium but, relative to the inhibition by lanthanum, it was poorly antagonised. This suggested either that chlorpromazine was more strongly bound than lanthanum and hence was more difficult to displace, or that only a minor part of its action involved competition with calcium for a membrane binding site. Results from another study supported the second hypothesis. In this study, inhibition of histamine release by chlorpromazine was assessed when calcium was present during the 5 min pre-incubation with lung before antigen challenge, and compared with the inhibition produced when calcium was absent during this period. The results are shown in Table VIII.3. The presence of calcium slightly reduced the development of inhibition by chlorpromazine: the drug produced a mean inhibition of 37.0%, compared with 51.9% after it had been pre-incubated with lung tissue in the absence of calcium. However, the difference between the two mean values was not significant, suggesting that the inhibitory effect of chlorpromazine developed similarly whether calcium was present or not.

The results of the 2 studies showed that the anti-allergic effect of chlorpromazine in human lung was mostly independent of calcium. The implications of this are briefly discussed at the end of the chapter.

#### VIII.5 Differences between the effects of chlorpromazine and lignocaine in human lung

Although the anti-allergic activity of chlorpromazine in human lung was related to its local anaesthetic property (sections VIII.1, VIII.2) results from some studies suggested that its action involved additional effects. These studies showed that there were differences between the

Experiment	Inhibition of histamine release (%)	
	(a)	(b)
1	49.9	61.1
2	63.6	61.7
3	13.9	17.7
4	20.7	67.1
MEAN $\pm$ SEM	37.0 $\pm$ 11.8	51.9 $\pm$ 11.5

Table VIII.3. Development of chlorpromazine inhibition in the presence (a) and absence (b) of calcium. Individual results from 4 experiments are shown; each result is the mean of 4 replicates.

a) chlorpromazine was pre-incubated with lung tissue for 5 min in the presence of calcium.

b) chlorpromazine was pre-incubated with lung tissue for 5 min in the absence of calcium. Prior to chlorpromazine addition, the tissue had been pre-treated with EGTA to remove the extracellular calcium (section VI.3). Calcium was restored to these samples immediately before antigen challenge.

There was no significant difference between the inhibition of histamine release under the two conditions.

inhibitory effects of chlorpromazine and lignocaine on antigen-induced histamine release. For example, the effect of lignocaine was reversed by washing whereas the effect of chlorpromazine was not. This is illustrated in Fig. VIII.5, which represents the inhibition of histamine release when sensitised lung was exposed to either drug for 5 min at 37°C, thoroughly washed with buffer solution, then challenged with antigen in fresh buffer without drug. The washing reduced the inhibition by lignocaine from 51.1% to -15.0%, ie it abolished the effect of the drug. In contrast, washing did not diminish the inhibition by chlorpromazine.

The other finding which suggested a difference between the actions of the 2 drugs came from a study of their combined effect on antigen-induced histamine release. In this study, replicates of sensitised lung were pre-incubated with 1 mM lignocaine for 15 min at 37°C, so that the dose of drug would produce its maximum inhibitory effect (section VIII.2). Over 3 experiments, lignocaine gave 43.2% inhibition of antigen-induced histamine release (Table VIII.4). When 100 µM chlorpromazine was added 5 min before challenge to lung samples which had been pre-treated with lignocaine, the inhibition of release was significantly increased to 64.3%. The extra inhibition was easily accounted for by the effect of chlorpromazine alone (37.5% inhibition).

Both the additivity between the effects of lignocaine and chlorpromazine and their differences in response to washing could be explained by a stronger binding of chlorpromazine in the lung tissue. However, it is equally conceivable that chlorpromazine exerted an additional effect which was unrelated to its local anaesthetic property.

#### SUMMARY

When chlorpromazine and lignocaine were added to passively sensitised human lung in a range of concentrations, they inhibited antigen-induced histamine release in a similar fashion. There was a 10-fold difference between the potencies of the 2 drugs. This equates well with their relative potencies in rat mast cells (Johnson and Miller, 1979). Both drugs were capable of liberating histamine from the lung tissue if they were used in high enough concentrations or if the pre-incubation time with

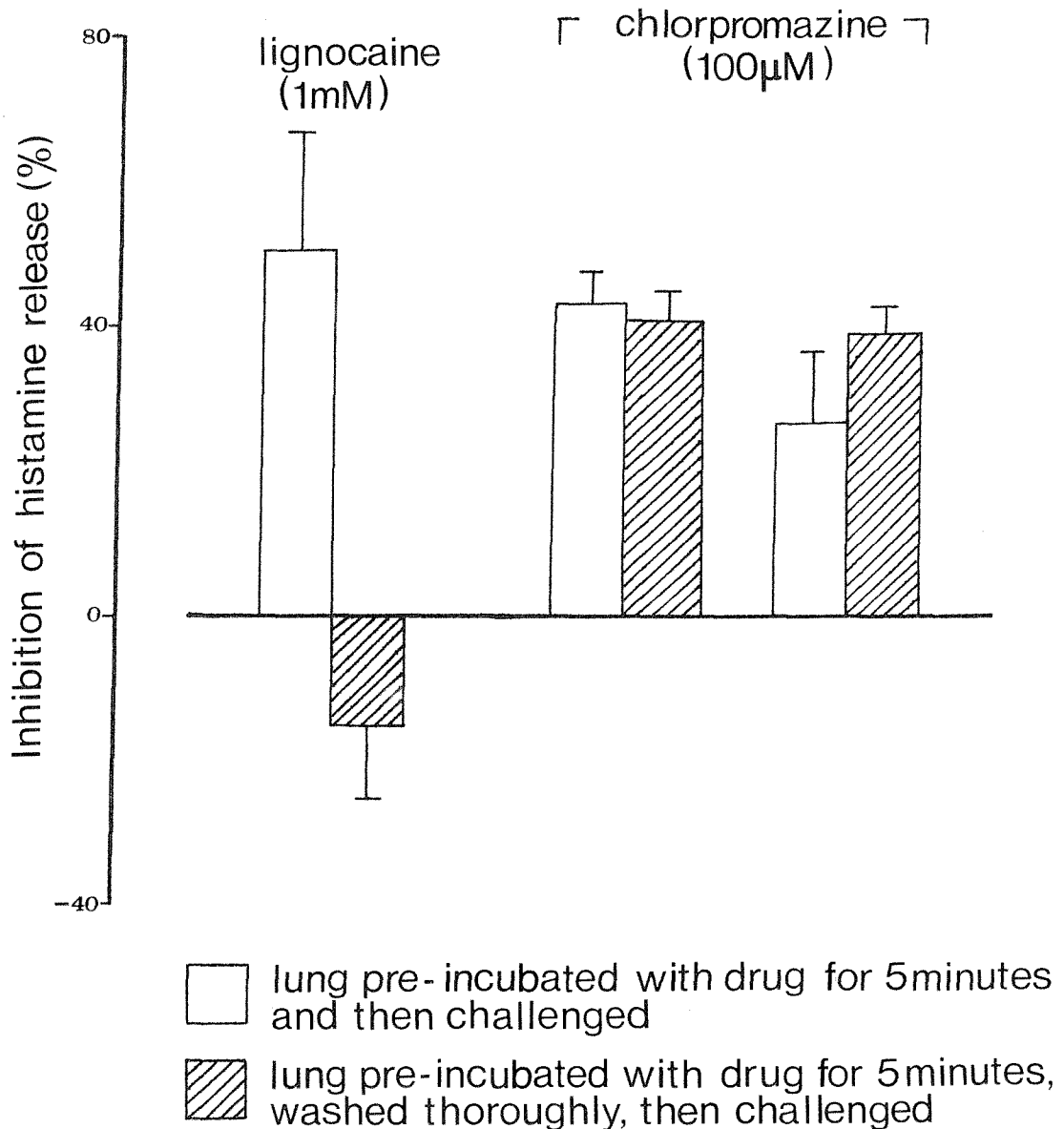


FIG. VIII.5. Inhibition of histamine release in lung tissue which was washed after pre-incubation with lignocaine or chlorpromazine. Results from 1 experiment with lignocaine and 2 experiments with chlorpromazine are shown; each histogram represents the mean  $\pm$  standard deviation from 3 replicates. The tissue was washed as follows: after 5 min pre-incubation with drug, in bulk, it was rinsed in 50 ml of warm PBS solution and transferred to 250 ml of fresh buffer. The solution was continuously stirred for 2 min, then poured through a piece of thin gauze to collect the lung. The lung was divided into 200 mg replicates and then challenged.

Experiment	Inhibition of histamine release (%)		
	Lignocaine alone	Chlorpromazine alone	Lignocaine + Chlorpromazine
1	48.7	61.0	79.4
2	39.9	17.0	59.4
3	41.0	34.5	54.1
MEAN $\pm$ SEM	43.2 $\pm$ 2.8	37.5 $\pm$ 12.8	*64.3 $\pm$ 7.7

Table VIII.4. Additive inhibition between lignocaine (1 mM) and chlorpromazine (100  $\mu$ M). Individual results are shown from 3 experiments; each result is the mean of 3 replicates. The asterisk indicates a significant difference between the effect of lignocaine alone and its combined effect with chlorpromazine.

the tissue was extended. The similarities between their dose-response characteristics and changes in effect on pre-incubation indicated that they acted via a similar mechanism. This implied that the anti-allergic activity of chlorpromazine was related to its local anaesthetic property. Experiments were performed to determine how this property might influence mast cell secretion:-

- a) Firstly, the effect of the drug on the cell membrane was investigated. Chlorpromazine was found to protect rat mast cells from hypotonic lysis which suggested that it was bound and integrated into the plasma membrane (Seeman, 1972). The concentrations of drug which prevented hypotonic lysis also inhibited secretion induced by compound 48/80. Thus, binding of the drug to the cell membrane appeared to explain its interference with secretion.
- b) Secondly, studies were performed to determine if chlorpromazine antagonised secretion by competitively inhibiting the binding of calcium. Experiments with human lung showed that, whereas the anti-allergic effect of a calcium antagonist was reversed as the free calcium concentration was increased, for the most part the effect of chlorpromazine was not. This tends to suggest that the anti-allergic action of the drug was not caused by competition with calcium. Alternatively, it may be that the drug did act by interfering with calcium binding but since its affinity for the binding site was much greater than that of calcium it could not be displaced. As calcium readily displaces chlorpromazine from membranes in other tissues (Seeman, 1972), the former explanation seems more likely to be correct.

There were differences between the effects of chlorpromazine and lignocaine in human lung. These may have been caused either by stronger binding of chlorpromazine in the lung tissue or by the drug exerting additional effects to its local anaesthetic action. Further experiments were required to discriminate between these possibilities.

CHAPTER IX.   GENERAL DISCUSSION

The pathophysiological events which lead to allergic bronchial asthma involve an interaction between inhaled antigens and immunoglobulins, mainly IgE, fixed to the surface of lung mast cells. The resultant secretion of granules from each cell generates a variety of active components which can induce airways obstruction (Austen and Orange, 1975). These mechanisms can be represented in vitro by passively sensitising fragments of human lung with allergic serum and then challenging them with specific antigens. By measuring the release of histamine one can assess the extent of granular secretion (Sheard et al, 1967). The purpose of the project was to use this model to investigate the ways in which secretion in lung mast cells may be antagonised by drugs and, from this, gain a better understanding of the pharmacological mechanisms which may be exploited in the treatment of allergic asthma.

## A. ANAPHYLAXIS IN THE LUNG

### Antibodies which mediate histamine release

Sensitisation of mast cells is said to involve the specific binding of at least 2 antibody classes, IgE and IgG, to the plasma membrane (Ishizaka, Ishizaka and Hornbrook, 1966a,b; Block, 1967), cross-linkage of which results in mediator secretion (Ishizaka and Ishizaka, 1969; Bach *et al*, 1971a,b). When lung fragments were passively sensitised with serum from an allergic donor, histamine release was induced either by the antigen to which the donor was sensitive or by an antiserum directed against human IgE, but not by an antiserum raised against human IgG. This was true whether the serum used for sensitisation was from donors allergic to grass pollen, or from donors sensitive to the house dust mite, Dermatophagoides pteronyssinus. The donors with house dust mite allergy were specifically selected because their sera contained anaphylactic IgG antibodies (Godfrey, personal communication).

These results confirm the finding of Augustin (1973) that in human lung, as in human basophils and rat mast cells (Ishizaka and Ishizaka, 1975), IgE is the major antibody which mediates histamine release. The failure to demonstrate an IgG-mediated release of histamine may have been because the experimental conditions were inadequate for sensitisation with anaphylactic IgG. This type of antibody requires a short latent period for sensitisation and its binding to mast cells is weak (Bach *et al*, 1971b; Parish, 1970; 1971). However, even when lung fragments were sensitised over a shortened period of 2 h and washing of the tissue prior to challenge was kept to a minimum, anti-IgG did not evoke histamine release. Thus, no evidence was found for an involvement of IgG in the activation of lung mast cells.

### Time course and extent of histamine release

When lung fragments were incubated with antigen or anti-IgE, release of mast cell-derived histamine took 15-30 min to reach a maximum. This is a much longer time course of release than that which occurs in isolated cells; histamine release from mast cells isolated from human lung reaches

completion within 3 min (Caulfield et al, 1980). This suggests that, in chopped fragments of lung, time is required for antigen and anti-IgE to penetrate the tissue and activate mast cells which are deeply embedded, and for the histamine released by the stimuli to equilibrate with the supernatant fluid. This may explain why the time course of histamine release was biphasic, ie there was a rapid liberation of histamine during the first 5 min incubation with antigen or anti-IgE, followed by a slower release between 10 and 30 min. The initial rapid release was probably due to secretion of granules from surface-lying mast cells, whilst the second phase of release probably occurred after the secretory stimulus had reached and activated the cells which were deeply embedded.

Even after an optimal period of incubation with antigen, only a small percentage of the total histamine contained by the tissue was liberated. A survey of antigen-induced histamine release over 80 experiments revealed that the average release was only 16.3%. In similar experiments, Augustin (1973) and Stanworth (1973) also obtained low levels of release. It is unlikely that this is due to the physical problem of poor tissue penetration by antigen because mast cells isolated from human lung also release only a minor proportion of their histamine; on incubating cells from 4 lungs with ragweed pollen, Caulfield et al (1980) found that histamine release did not exceed 20%.

If the low percentage of histamine release is not due to penetration problems, it may be explained in 3 ways. Firstly, it is conceivable that only a small proportion of lung histamine is contained by mast cells, the remainder being held by cell-types which do not expel their contents on exposure to antigen. This possibility can be rejected because there is evidence that more than 90% of the histamine contained in human lung is mast cell-associated (Paterson, Wasserman, Said and Austen, 1976). Secondly, there may be 2 completely different populations of lung mast cells, one which responds to antigen and another which does not. In this case, histamine release would reflect complete degranulation by a small proportion of the total number of cells. Thirdly, most, if not all, of the mast cells may be capable of responding to antigen but to varying extents. Hence, histamine release would reflect partial secretion of

granules from the majority of cells rather than complete degranulation by a few. Evidence from electron micrographs of human lung mast cells indicate that this third alternative is the most likely. Micrographs taken after the cells are exposed to antigen reveal many cells in various stages of degranulation, even when histamine release has been completed (Caulfield *et al*, 1980). In rat mast cells, there appears to be a feedback mechanism which limits the extent to which each cell is activated (Foreman *et al*, 1977; Wasserman, 1977). Perhaps feedback control mechanisms are well-developed in human lung mast cells and, in consequence, the majority of cells only undergo partial degranulation.

#### Variability of the anaphylactic reaction

Throughout the present studies there was great variation in the amount of histamine released from the lung tissue, even though the conditions for passive sensitisation and antigen challenge were kept constant. This was particularly noticeable in lung samples from different donors but was also seen in replicates from the same lung. At least 3 other investigators have found the same (Bukhari, 1967; Stanworth, 1973; Coleman, 1980).

One factor which must have been a major cause of the intra-experimental variation in histamine release was the variable size of the lung fragments. Fragment size would govern the accessibility of sensitising sera and specific antigens to the interstitial mast cells and thereby determine the extent of the anaphylactic reaction. Although every effort was made to chop fragments to a standard size, it was not possible to keep them all identical.

Inter-experimental variation in histamine release was probably determined by differences in mast cell reactivity. It is likely that mast cells in different lungs have wide-ranging affinities for IgE antibodies and therefore vary in their capacities for passive sensitisation. Furthermore, they probably respond quite differently to an antigenic stimulus. In particular, their capacities for passive sensitisation must have been influenced by IgE antibodies from the lung donor. The presence of these antibodies was apparent from the demonstration that anti-IgE provoked histamine release

from lung which had not been exposed to allergic serum, a finding previously reported by Kay and Austen (1971). By occupying receptor sites for IgE on mast cells, the donor antibodies would have interfered with the uptake of antigen-specific IgE from the allergic serum. The degree of sensitisation with donor IgE varied from lung to lung, so its interference with passive sensitisation will also have varied.

A further factor which probably influenced both the intra- and inter-experimental variation in release was the diverse nature of the lung fragments. Lung used in the present studies came almost entirely from patients suffering with lung carcinoma. The parenchyma of individual lungs was often inconsistent, with some areas being macroscopically quite different from others. Parenchyma of different lungs ranged from being grossly emphysematous to being very solid and poorly aerated. It is likely that the permeability of these tissues to sensitising sera and specific antigens was extremely varied and that, in consequence, anaphylactic reactions induced in the tissues were diverse.

The variability of passive anaphylaxis in human lung imposed certain limitations on its value as a model for investigating the anti-allergic effects of drugs. For example, in approximately 1 in 10 of the lungs investigated, antigen-induced histamine release was less than 5%, ie too low to allow a meaningful investigation of drug action. The percentage variation in antigen-induced release from replicates of the same lung was sometimes as high as 30%. In consequence, each drug had to produce a marked change in the release for its effect to be detected.

#### B. EFFECTS OF ANTI-ALLERGIC DRUGS

Sodium cromoglycate, salbutamol and chlorpromazine all inhibited mast cell secretion in the lung. They were quite distinct in the inhibition they produced, differing both in potency and in efficacy. Several investigators have reported that the activity of cromoglycate in human lung fragments is highly variable (eg, Morr, 1978; Butchers, Fullarton, Skidmore, Thomson, Vardey and Wheeldon, 1979). This was found to be true in the present studies. In lung samples from different donors, the inhibitory

effect of cromoglycate was diverse: over 52 experiments, the coefficient of variation for the inhibition produced by a 200  $\mu\text{M}$  dose of the drug was 41.7%. However, cromoglycate was not unique in this respect. The inhibitory activities of single, effective doses of salbutamol and chlorpromazine were similarly diverse (coefficients of variation = 34.9% and 34.2%, respectively). Hence, there was general variation in the inhibition of mast cell secretion by drugs rather than specific variation with one particular compound.

As the variation in inhibition was so similar for the 3 unrelated agents, it was most probably governed by the test system. Factors such as variable distribution of the drugs throughout the lung tissue, variable responsiveness of mast cells to the drugs and the variable anaphylactic reaction against which the drugs were tested were probably the main causes.

Since the inter-lung variation in the activities of cromoglycate, salbutamol and chlorpromazine was high, only major effects of these drugs were reproducible from experiment to experiment. Furthermore, in order to detect an increase or decrease in their activities, such changes had to be gross rather than discrete.

#### Dose-response characteristics of sodium cromoglycate

When sodium cromoglycate was examined in a range of concentrations (0.2 - 200  $\mu\text{M}$ ) it was found to produce a variable dose-response pattern. In most tissues, its inhibition of antigen-induced histamine release was linearly related to dose but in some there was a clear loss of inhibitory effect at higher concentrations. Contrary to the findings of a previous report (Church and Gradidge, 1978), there was no evidence whatsoever that this loss of effect was due to potentiation of an IgG mechanism of histamine release. The drug appeared to act solely by interfering with IgE-mediated secretion.

Generally, high concentrations (2 - 200  $\mu\text{M}$ ) of cromoglycate were required to inhibit mast cell secretion. This could have been due to poor distribution of the drug throughout the tissue but, as similar concentrations are required to inhibit secretion in isolated rat mast cells (Orr *et al*,

1971; Taylor et al, 1974), it seems more likely that cromoglycate was simply not very potent. Even in maximally effective concentrations (20 - 200  $\mu$ M), cromoglycate produced only 30-35% inhibition of histamine release. Hence, at best, the drug was only a partial antagonist of the release mechanism. These findings confirm those of others who have studied the effect of the drug in human lung (eg, Sharpe, Ross and Spicer, 1978; Butchers et al, 1979).

#### Effect of pre-incubation time on the activity of cromoglycate

Rat mast cells, both in vitro and in vivo, quickly become desensitised to cromoglycate when they are pre-incubated with the drug. Depending on the model investigated, complete desensitisation takes from between 10 and 30 min to occur (Kusner et al, 1973; Thomson and Evans, 1973; Sung et al, 1977a). In the present studies with human lung tissue, there was no significant decrease in cromoglycate activity when the pre-incubation time was extended from 30 sec to 15 min. This showed that the rapid loss of inhibitory effect seen in rat models of anaphylaxis does not occur in passively sensitised human lung. A recent report supports this finding (Butchers et al, 1979). However, there was a loss of effect when lung tissue was pre-incubated with the drug for a period of 1 h or longer. After 19-20 h pre-incubation, cromoglycate produced negligible inhibition of histamine release. To ensure continuous exposure to the drug under this condition, a second dose of cromoglycate had to be added to the sensitised samples after they had been washed, about 15 min prior to challenge. Therefore, the loss of inhibitory activity could not be explained by inactivation or a short duration of action of the drug. Hence, it appeared that mast cells in the tissue became tolerant to its effect.

Tolerance produced by prolonged pre-incubation with cromoglycate only affected the activity of cromoglycate-like drugs. This was revealed by the demonstration that ICI 74917, a compound with similar pharmacological properties to cromoglycate (Marshall et al, 1976), was inactive in tissue which had been desensitised to the parent compound whilst 2 other agents unrelated to cromoglycate, ie salbutamol and chlorpromazine, remained fully effective under this condition. Clearly, cromoglycate induced a specific sub-sensitivity in the lung mast cells rather than a general reduction in their response to drugs.

Mechanism of action of cromoglycate

## a) Interference with calcium entry into mast cells.

Cromoglycate inhibits the uptake of calcium ions by stimulated mast cells and this effect is considered by many to be responsible for its anti-secretory action (Johnson and Bach, 1975; Foreman and Garland, 1976; Spataro and Bosmann, 1976). The mechanism by which it does this is unknown. The possibility was considered that cromoglycate stabilises the plasma membrane of mast cells in an anaesthetic-like manner. Anaesthetic drugs physically integrate with the plasma membrane of various types of cell and this is one of the mechanisms by which they interfere with ionic fluxes (Seeman, 1972). By virtue of this membrane effect, they also increase the resistance of cells to lysis when the cells are exposed to hypotonic conditions (Roth and Seeman, 1971). Cromoglycate produced no such effect on mast cells; concentrations of the drug which inhibited secretion did not protect the cells from hypotonic lysis. This suggests that the drug has no anaesthetic-like action on the plasma membrane.

Some investigators have proposed that cromoglycate reduces calcium availability, by forming a complex with the free or bound ion (Cox, 1974; Spitzer, 1979). This possibility was investigated by comparing its anti-allergic effect with that of the calcium chelator, EGTA. Cromoglycate was active in much lower concentrations than EGTA but was not nearly so effective. The vast dissimilarity between the effects of the 2 agents suggested that they acted via different mechanisms. Hence, although cromoglycate does form a complex with calcium under certain conditions (Spitzer, 1979), this does not seem to explain its anti-allergic action.

It is possible that cromoglycate inhibits calcium influx by directly competing with the ion for a membrane channel or receptor. However, differences between the anti-allergic effect of the drug and the effect of lanthanum indicate that this is not so. Lanthanum is an agent which blocks the movement of calcium across membranes (Miledi, 1971). Although there is some dispute about its exact mechanism of action (Rahwan, Piascik and Witiak, 1979), it is suggested that it competitively inhibits calcium

binding (Lettvin, Pickard, McCulloch and Pitts, 1964). This certainly seems to explain its anti-allergic action; inhibition of histamine release from human lung by lanthanum was reversed as the concentration of free calcium was increased, suggesting that it competed with calcium for a binding site on the lung mast cells. Pearce and White (1981) have recently shown that lanthanum acts similarly on mast cells from rats. In human lung, cromoglycate differed from lanthanum both in potency and in efficacy. Moreover, its anti-allergic effect remained unaltered when the concentration of free calcium was raised. These findings indicate that cromoglycate is not a calcium antagonist.

Since the findings of these studies were basically negative, it is impossible to make firm conclusions from them about the mechanism of cromoglycate inhibition. However, if the drug acts neither by stabilising the plasma membrane of mast cells in an anaesthetic-like manner, nor by interacting or competing with calcium ions, it seems likely that it blocks calcium influx by an indirect mechanism. Ennis and co-workers are of the same opinion. From studies of rat mast cells, they have shown that cromoglycate inhibits secretion even when calcium is omitted from the incubation medium. They propose that cromoglycate activates intracellular pumping mechanisms which extrude calcium from the cytosol and that inhibition of calcium influx is secondary to this (Ennis, Atkinson and Pearce, 1980; Ennis, Truneh, White and Pearce, 1981). Certainly, cromoglycate stimulates the phosphorylation of a specific protein in the mast cell which is involved in recovery of the cell after it has been activated (Sieghart *et al*, 1978; Theoharides *et al*, 1980). Perhaps this protein forms part of the intracellular pumping mechanism.

The possibility should not be excluded that the inhibitory effect of cromoglycate on mast cell secretion depends on actions other than interference with calcium movements. For example, through phosphorylation of a regulator protein within the mast cell (Theoharides *et al*, 1980), the drug may affect the changes in membrane permeability, the aggregation of microtubules or the contraction of microfilaments which are essential to stimulus-secretion coupling (Greengard, 1978; Holgate *et al*, 1981a).

## b) Mechanism of tolerance to cromoglycate

In rat lung, mast cell tolerance to cromoglycate exhibits the following characteristics: i) it is rapid in onset, complete desensitisation of the cells occurring within 10 min of exposure to cromoglycate, ii) it is dose-dependent, ie it occurs more readily with high doses of the drug, iii) it is independent of calcium and iv) once it has developed, recovery from tolerance is slow, with only partial sensitivity being restored even 2 h after the drug is removed (Sung et al, 1977a,b). In human lung, mast cell tolerance was found to exhibit the same characteristics except that the time taken for tolerance to develop was much longer (complete desensitisation occurred only after several hours of pre-incubation with the drug). Tolerance occurred more readily with 20 and 200  $\mu$ M cromoglycate than with the lower dose of 2  $\mu$ M, it occurred whether or not calcium was present in the incubation medium and tolerance was still detectable 30 min after the drug had been washed out of the tissue. These findings indicate that the mechanism of tolerance to cromoglycate in humans and rats is the same.

On the basis of data obtained in studies with rat mast cells, several hypotheses have been submitted to explain how tolerance to cromoglycate occurs. It is suggested that tolerance involves loss or modification of receptor sites on, or in, the mast cell (Marshall et al, 1976; Sung et al, 1977b), depletion of a labile factor which is normally released by the drug (Kusner et al, 1973; Thomson and Evans, 1973) or intracellular activation of a phosphodiesterase isoenzyme which is insensitive to cromoglycate (Taylor et al, 1974). As tolerance in human lung mast cells develops more slowly than it does in cells from rats, it may be that receptors for cromoglycate on the human cells are less susceptible to modification. Alternatively, perhaps the intracellular levels of the labile factor which the drug is said to release (Kusner et al, 1973; Thomson and Evans, 1973) are much higher in the human cells and are therefore less readily depleted, or perhaps the activation of cromoglycate-insensitive phosphodiesterases is a much more gradual process.

Antagonism of mast cell secretion by salbutamol

The  $\beta$  adrenergic agonist salbutamol was a highly potent and effective inhibitor of secretion in lung mast cells. It produced 60-70% inhibition of antigen-induced histamine release in concentrations as low as 1  $\mu$ M.

Assem and Schild (1969) have demonstrated that the effect of adrenergic agonists on mast cell secretion in human lung is blocked by the  $\beta$  adrenoceptor antagonist propranolol. Results obtained in the present studies confirm this finding. Inhibition of histamine release by salbutamol was completely abolished if propranolol was first added to the lung fragments. That propranolol exerted its effect by binding to  $\beta$  receptors and not by some other mechanism was verified by the demonstration that its antagonistic effect was stereospecific ( - the relative potencies of (-) and (+) propranolol at antagonising the anti-secretory effect of salbutamol were found to equate well with their potencies as  $\beta$  receptor antagonists in other tissues, as described by Nickerson and Collier, 1975, and Kalsner, 1980). These results show that  $\beta$  agonist drugs, such as salbutamol, inhibit mast cell secretion in human lung by stimulation of a  $\beta$  receptor. Evidence from a recent study indicates that the receptor is predominantly of the  $\beta_2$  sub-type (Butchers, Skidmore, Vardey and Wheeldon, 1980).

The results do not necessarily indicate that salbutamol acted directly on mast cells. It is possible that, through stimulation of  $\beta$  receptors on another cell-type, it caused the release of some form of inhibitory substance and this was responsible for the suppression of mast cell secretion. Such a mechanism of action for  $\beta$  agonists has already been proposed by Taylor and Sheldon (1977), on the basis of evidence they obtained in studies with isoprenaline in rats. However, results from recent experiments with purified human lung mast cells argue against this hypothesis; these experiments show that, in a suspension of 99% pure mast cells, salbutamol and other  $\beta$  agonists still inhibit secretion, indicating that they act directly on the mast cell itself (Dr. H.H. Newball - personal communication).

Binding of adrenergic agonists to  $\beta$  receptors causes each receptor to couple with, and activate, the plasma membrane enzyme adenylyl cyclase. The activated enzyme then catalyses the cytoplasmic conversion of ATP to cyclic AMP (Swillens and Dumont, 1980). It is suggested from studies with human lung fragments that this is the mechanism underlying the inhibitory effect of salbutamol and other  $\beta$  agonists on mast cells. Stimulation of lung  $\beta$  receptors with salbutamol, isoprenaline or adrenaline increases the tissue levels of cyclic AMP (Orange, Kaliner, Laraia and Austen, 1971; Davis, Conolly and Greenacre, 1980). Moreover, the concentrations of isoprenaline and adrenaline required to produce this effect are those which inhibit secretion (Orange, Kaliner, Laraia and Austen, 1971). Perhaps,  $\beta$  agonists enhance the turnover of a specific pool, or pools, of cyclic AMP within the mast cell which, through activation of protein kinases and phosphorylation of regulator proteins (Greengard, 1978), causes the inhibition of secretion.

In studies of the change in activity of salbutamol with pre-incubation, it was found that the drug became more effective when added to lung fragments 15 min instead of 30 sec before antigen challenge. Butchers and co-workers, who studied the change in activity of the drug over 20 min pre-incubation, also found that its activity increased (Butchers *et al*, 1979). This finding can be explained in 2 ways. Firstly, the drug may have been slow to distribute throughout the tissue and therefore it would have required a certain length of time to achieve its maximum inhibitory concentration in the extracellular fluid. Secondly, there may have been a rate-limiting step at the cellular level which prevented salbutamol from immediately producing its maximum effect; for example, time may have been required either for salbutamol to become fully bound to  $\beta$  receptors on mast cells or for adenylyl cyclase in the plasma membrane to become fully activated and cause maximum hydrolysis of ATP. Certainly, in other cells, adenylyl cyclase activation appears to be rate-limiting. For example, in turkey erythrocytes, the enzyme does not become fully activated until 10-15 min after adrenergic agonists have been added, yet binding of the agonists to  $\beta$  receptors in the erythrocyte membrane is extremely rapid (Sevilla, Steer and Levitzki, 1976).

A time-related desensitisation to the effects of  $\beta$  agonists has been demonstrated in many types of cell, eg guinea pig macrophages (Remold-O'Donnell, 1974), frog erythrocytes (Mukherjee et al, 1975) and human bronchial smooth muscle cells (Davis and Conolly, 1980). The desensitisation, or tolerance, is slow in onset reaching a maximum, for example, in frog erythrocytes in 24 h (Mukherjee et al, 1975). In the present studies with human lung fragments, there was no sign of tolerance to salbutamol in mast cells. After 19 h of pre-incubation with the fragments, there was no reduction in the inhibitory effect of the drug on histamine release. However, the nature of salbutamol inhibition under this condition differed from that seen after acute pre-incubation in that the inhibition could not be reversed by washing, or prevented by the acute addition of propranolol. The activity of salbutamol after prolonged pre-incubation was not due to a non-specific or toxic effect because, when propranolol was present throughout the 19 h period of pre-treatment, salbutamol inhibition was completely blocked.

Although the reasons underlying the failure to induce tolerance to salbutamol were not investigated further, the following possibilities are considered:-

- i) Firstly, the receptor which mediates the anti-secretory effect of  $\beta$  agonists in human lung may have a more pronounced conformational stability than  $\beta$  receptors in other tissues.  $\beta$  agonist-induced subsensitivity is thought to be caused by uncoupling of  $\beta$  receptors from adenyl cyclase and by internalisation of the agonist-receptor complex by the cell (Harden, Cotton, Waldo, Lutton and Perkins, 1980). Perhaps the  $\beta$  receptors which modulate lung mast cell secretion are less susceptible to uncoupling or internalisation than  $\beta$  receptors on other cells.
- ii) Secondly, long term exposure of lung mast cells to salbutamol may cause a prolonged rise in the intracellular levels of an inhibitory pool of cyclic AMP. Maintained levels of this nucleotide in the cell cytoplasm may continue to inhibit the process of secretion, even if  $\beta$  receptors on the membrane surface become uncoupled or internalised.

iii) Thirdly, lengthy exposure of the mast cells to salbutamol may produce a prolonged rise in the intracellular pool of cyclic AMP which is involved in the activation of secretion (Lewis *et al*, 1979). As a result, the protein kinase which propagates the effect of this cyclic AMP pool (Holgate *et al*, 1980b) might become activated and depleted, long before the mast cells are exposed to antigen. If, for example, resynthesis of the kinase is a slow process, then insufficient amounts of free enzyme would be available to expedite the immunological activation of secretion.

iv) Fourthly, continuous stimulation of lung  $\beta$  receptors for 19 h may produce an irreversible change in the tissue which suppresses secretion in mast cells. For example,  $\beta$  adrenergic agonists are known to affect the turnover of several membrane phospholipids. In particular, they trigger an intensive decomposition of phosphatidyl choline (Nemecz and Farkas, 1980). Perhaps prolonged exposure of lung mast cells to salbutamol produces a change in the phospholipid composition of the plasma membrane and this interferes with the role of the phospholipids in stimulus-secretion coupling, ie to prepare a suitable membrane environment for calcium influx and granule exocytosis (Ishizaka *et al*, 1981; Sullivan, 1981).

Lack of tolerance to  $\beta$  adrenergic agonists does not seem to be confined to mast cells in human lung. In a recent study of the effect of intradermally-administered terbutaline on mast cell degranulation in human skin, Grönneberg and Strandberg (1981) found that this  $\beta$  agonist inhibited allergen-induced degranulation even when it was injected 8 h prior to challenge. This suggests that mast cells in human skin, like human lung mast cells, do not develop tolerance to  $\beta$  adrenergic drugs.

#### Antagonism of mast cell secretion by chlorpromazine

In agreement with a report by Church and Gradidge (1980a) the effect of chlorpromazine on mast cells in human lung was found to be biphasic. In low doses ( $< 100 \mu\text{M}$ ), the drug inhibited antigen-induced secretion of histamine whereas at relatively higher concentrations ( $> 100 \mu\text{M}$ ) it provoked histamine release. The release was probably caused by a cytotoxic effect

on the mast cell membrane: in rat mast cells, concentrations of the drug which liberate histamine also cause release of the cytoplasmic enzyme lactate dehydrogenase (Frisk-Holmberg, 1972); the same drug concentrations cause invagination and rupture of the plasma membrane of erythrocytes (Seeman, 1966).

As an inhibitor of secretion, chlorpromazine was not very potent, though this may have been partly because of its distribution throughout the tissue. Chlorpromazine is highly lipophilic (Seeman, 1972) and binds avidly to proteins (Rosen and Tham, 1980), so it is likely that it became bound to many types of cell in the lung. Hence, the concentration to which each mast cell was exposed was probably much less than that added to the incubation medium. In consequence, amounts of drug far in excess of those required to inhibit secretion in individual cells would have to have been added for it to prevent histamine release. Certainly, the concentrations of chlorpromazine necessary for inhibiting release from human lung fragments (c. 100  $\mu\text{M}$ ) were much greater than those which were inhibitory in isolated rat serosal cells (1.9 - 6.7  $\mu\text{M}$ ).

On acute pre-incubation with lung tissue, chlorpromazine became more effective at inhibiting antigen-induced histamine release. After prolonged pre-incubation with lung for 19 h, it produced even greater inhibition but this was not necessarily due to further increase in its activity. Chlorpromazine started to release histamine from mast cells once it had been in contact with the tissue for 1 h. Hence, when it was present for 19 h, it must have liberated some of the stored histamine and this would have been removed when the tissue was washed free of serum, 15 min prior to antigen challenge. Consequently, the inhibition produced by the drug in 19 h samples was probably due to a combination of antagonism of secretion and of its having depleted the histamine available for release.

The inhibitory effect of chlorpromazine in human lung was mimicked by the local anaesthetic lignocaine, as it is in rat mast cells (Johnson and Miller, 1979). This suggested that the local anaesthetic property of the drug (Byck, 1975) contributed to its antagonistic action. Local

anaesthetics exert their effects by binding to membranes (Hebborn, 1972), so experiments were performed to determine if chlorpromazine became bound to the plasma membrane of mast cells. The demonstration that the drug increased the resistance of isolated mast cells to lysis when they were exposed to hypotonic solutions verified that it did become bound; Seeman and colleagues have shown that anaesthetic drugs inhibit hypotonically-induced lysis by binding and integrating with the cell membrane (Roth and Seeman, 1971; Seeman, 1972). Since the concentrations of chlorpromazine which protected mast cells from lysis were the same as those which inhibited secretion, it was clear that binding of the drug to the plasma membrane was responsible for its anti-secretory effect.

Through binding to the plasma membrane of other cells, chlorpromazine and local anaesthetics in general have 2 main actions: firstly, they competitively inhibit the binding of calcium to membrane phospholipids (Kwant and Seeman, 1969; Lüllmann, Plösch and Ziegler, 1980) and, secondly, they interfere with the functioning of membrane enzymes (Seeman, 1972). Both of these actions may have contributed to the antagonistic effect of chlorpromazine in mast cells. By interfering with calcium binding, chlorpromazine would have upset the calcium-dependent trigger process which activates secretion (Foreman et al, 1977; Ennis et al, 1980). By inhibiting the function of certain enzymes it may have disrupted the sequence of reactions which leads to triggering of the cell and to exocytosis of granules. For example, chlorpromazine is a highly potent inhibitor of calmodulin-dependent enzymes; it binds to calmodulin and thereby inhibits its function as an enzyme activator (Weiss and Levin, 1978). At least 2 enzymes which are involved in mast cell triggering, ie adenylyl cyclase (Holgate et al, 1980a) and calcium-magnesium dependent adenosine triphosphatase (Batchelor et al, 1979) have been shown in other tissues to be calmodulin-dependent (Klee et al, 1980). Moreover, chlorpromazine interferes with enzymes involved with glycerolipid turnover (Sturton and Brindley, 1977). In this way, it may have upset the methylation of membrane phospholipids which is thought to promote calcium influx and to generate fusogenic phospholipids for the process of granule exocytosis (Ishizaka et al, 1980).

Since inhibition of histamine release by chlorpromazine was poorly antagonised by calcium, it appeared that competition with calcium played only a minor part in its action. This assumes that the affinities of chlorpromazine and calcium for binding sites on lung mast cells were similar (if the affinity of chlorpromazine was much greater than that of calcium, it is possible that the anti-secretory effect of the drug remained unaltered because calcium could not displace it). The assumption seems justified by evidence from studies of the binding of chlorpromazine and calcium to the plasma membrane of human erythrocytes. Calcium displaces chlorpromazine from the surface of these cells as readily as chlorpromazine displaces calcium (Kwant and Seeman, 1969; Seeman, 1972), indicating that the 2 agents have similar membrane binding affinities.

Some results in the present studies implied that the inhibitory effect of chlorpromazine on lung mast cells involved not only its local anaesthetic action but also some other action of a different nature. For example, inhibition of histamine release by chlorpromazine was not reversed by washing the lung tissue prior to antigen challenge whereas inhibition by the local anaesthetic lignocaine was reversed. Moreover, combined treatment of lung tissue with chlorpromazine and lignocaine produced additive inhibition of histamine release, indicating that chlorpromazine might be acting at a different site from lignocaine. However, these findings may be equally well explained by a stronger or more extensive binding of chlorpromazine in the lung tissue. Being a more lipophilic drug than lignocaine, chlorpromazine is more soluble in cell membranes (Seeman, 1972; Johnson and Miller, 1979), so it is quite probable that it was more extensively bound. As a result, it would have been less easily washed from the tissue and this might explain why its inhibitory effect was irreversible. Moreover, it would have penetrated the plasma membrane of mast cells much better than lignocaine and perhaps interfered with enzyme pathways which lignocaine could not effect. Experiments required to discriminate between these possibilities were beyond the scope of this thesis.

Comparison of the effects of cromoglycate, salbutamol and chlorpromazine

From the points already discussed, it seems that cromoglycate, salbutamol and chlorpromazine inhibited mast cell secretion in human lung by completely different mechanisms. Direct evidence for this can be obtained by comparing their inhibitory profiles (Table IX.1). The characteristic which distinguished the action of cromoglycate was that it exhibited tolerance on pre-incubation. Salbutamol and chlorpromazine showed no sign of tolerance and they remained fully effective in lung desensitised to cromoglycate. The absence of cross-tolerance, in particular, proved that the mechanisms of action of salbutamol and chlorpromazine were different from that of cromoglycate.

Salbutamol was distinctive because it was considerably more potent and effective than either of the other 2 drugs. Chlorpromazine, on the other hand, was the only agent whose inhibitory effect was irreversible by washing and which was capable of liberating histamine from the tissue.

	Sodium cromoglycate	Salbutamol	Chlorpromazine
relative potency	1	300	0.2
maximum inhibition	33.0%	72.2%	31.3%
reversibility	✓	✓	×
histamine release	×	×	✓
tolerance	✓	×	×

Table IX.1. Drug profiles in human lung.

The relative potencies and values for maximum inhibition were taken from data presented in Chapter V (section V.1).

C. IMPLICATIONS FOR THE DRUG TREATMENT OF ASTHMA

As conditions in vitro can never entirely mimic those found in vivo, it is unwise to make firm predictions about the clinical relevance of the data. This is particularly so because the human lung model simulates only one of the many processes which contribute to allergic asthma, ie immunological release of granule-associated mediators from lung mast cells. Other events, such as generation of secondary mediators from other cell-types and mediator-induced changes in bronchial smooth muscle tone (Kaliner, 1980) are not represented in the model. Even in simulating mast cell degranulation in asthma, the model has certain limitations. First and foremost, experiments are performed, not on asthmatic lung, but on lung tissue obtained from patients suffering with lung carcinoma. Secondly, sensitisation of the tissue mast cells towards specific antigens is brought about passively rather than actively, ie the cells are sensitised by immunoglobulins from an allergic serum rather than by immunoglobulins from the lung donor. This may alter the cells' reactivity; Schmutzler and co-workers have shown that, compared with actively sensitised cells, passively sensitised mast cells are functionally less responsive (Schmutzler, Poblete-Freundt, Rauch and Shoenfeld, 1979). Thirdly, in order to release sufficient amounts of histamine which can be measured, it is necessary to induce a much more severe reaction in vitro than would normally be expected in vivo. Orr (1977) has suggested that such reactions in vivo would bring an asthmatic near to status.

Even though the predictive value of the technique is limited, the implications of the results in asthma are worthy of consideration because the results were all obtained in human lung tissue. The finding that sodium cromoglycate failed to suppress histamine release in 25% of lungs suggests that mast cells in the lungs of many subjects are insensitive to its effects. This may explain why there is a high proportion of asthmatics who do not respond to cromoglycate therapy (Brogden et al, 1974a). A similar degree of insensitivity was found with salbutamol and chlorpromazine. However, those lungs which did not respond to cromoglycate very often did respond to one, or both, of the other two agents. Hence, a patient whose asthma is refractory to cromoglycate may well benefit from treatment with another type of anti-allergic drug.

In lung fragments, cromoglycate was a partial antagonist of mast cell secretion producing a maximum inhibition of antigen-induced histamine release of only 30-35%. It seems unlikely that, in vivo, this would be sufficient to prevent symptoms of asthma developing. These findings may be reconciled in two ways. Firstly, it is possible that the drug is more active in vivo than the results predict. Studies with rat mast cells have shown that there is an inverse relationship between cromoglycate efficacy and the strength of the anaphylactic reaction (Orr et al, 1971; Krüger and Bloom, 1979); anaphylaxis in the lungs of asthmatics is considered to be much less severe than that induced in sensitised lung fragments (Orr, 1977), so in vivo cromoglycate may exert greater activity. Secondly, it is conceivable that other actions of the drug contribute to its prophylactic effect. Breslin and co-workers have shown that cromoglycate prevents bronchoconstriction induced by cold air hyperventilation (Breslin, McFadden and Ingram, 1980), a reaction in which there is no increase in circulating mast cell-derived mediators (Deal, Wasserman, Soter, Ingram and McFadden, 1980). They propose that the drug has a direct action on bronchial smooth muscle, or that it inhibits reflex-induced bronchoconstriction as it does in dogs (Jackson and Richards, 1977). Orr (1977) has suggested that cromoglycate interferes with the activation of complement, though he has no direct evidence for this.

Whether tolerance to cromoglycate, seen after one hour's pre-incubation of lung fragments with a 20 or 200  $\mu\text{M}$  dose of drug, occurs clinically is unknown. After being inhaled by asthmatics or normal subjects, cromoglycate is cleared quite slowly from the lung and blood plasma (approximate time for 50% clearance: lung = 1 h, plasma = 2 h - Moss, Jones, Ritchie and Cox, 1971; Brown, Hodder and Neale, 1981) so it is certainly in the vicinity of mast cells for a sufficient period to produce tolerance. However, concentrations of the drug required to cause desensitisation of the cells are unlikely to be achieved. A large percentage of each inhaled dose of 20 mg does not reach the lungs because it is swallowed or deposited in the mouth and throat. The remainder becomes distributed throughout the bronchi (Moss et al, 1971). Hence, it seems unlikely that the concentration of cromoglycate in the extracellular fluid of the lung would rise as high as 20 - 200  $\mu\text{M}$ , ie to levels which produce tolerance. Therefore, the likelihood

that the phenomenon occurs in vivo is small. Nevertheless, the mere fact that cromoglycate is capable of producing tolerance in lung mast cells is disadvantageous to its use as a prophylactic drug.

Salbutamol inhibited immunological histamine release from lung fragments in low concentrations, so it is likely to be a potent antagonist of lung mast cell secretion in vivo. The results imply that it would be more potent and more effective at preventing anaphylactic reactions in the lungs of asthmatic patients than sodium cromoglycate. The results also indicate that, unlike the action of cromoglycate, the effect of salbutamol would not diminish with time due to tolerance. Hence a prophylactic role for salbutamol and related  $\beta$  adrenergic agonists in asthma should be given serious consideration. Prophylactic administration of these drugs may be considered unwise because tolerance might develop to their bronchodilator action. In studies with normal subjects, Holgate et al (1977) have shown that the airways response to salbutamol decreases with repeated drug administration. However, Harvey and Tattersfield (1979) were unable to reproduce this in asthmatic subjects. Moreover, in a recent survey of the clinical trials performed with  $\beta$  stimulant drugs, Paterson and colleagues concluded that tolerance to their bronchodilator action was rare (Paterson, Woolcock and Shenfield, 1980). Consequently, the premise that these drugs should not be given prophylactically in asthma seems to be unfounded.

Whilst it has been suggested that phenothiazine-type drugs should be developed as prophylactic agents for the treatment of allergies (Lichtenstein and Gillespie, 1975), it is unlikely that they would be as beneficial in asthma as cromoglycate-like compounds or  $\beta$  adrenergic agonists. The phenothiazine, chlorpromazine, only inhibited secretion in human lung mast cells in high concentrations. In vivo, these would almost certainly have side-effects, eg on the central nervous system (Byck, 1975). The capacity of the drug to liberate mediators from mast cells could even worsen a patient's asthma. Therefore, chlorpromazine and other phenothiazines are unlikely to be of therapeutic value as anti-asthmatic drugs.

#### 4. CONCLUSION

Passive anaphylaxis in human lung in vitro is one of several techniques available by which one can study the mechanism of action of anti-allergic drugs in asthma. Its value as a screening model lies unquestionably in the fact that all results are obtained in human lung (albeit not asthmatic lung) and not in lung tissue from an animal species. However, the technique suffers from several practical limitations. Firstly, despite standardisation of the experimental conditions for passive sensitisation and antigen challenge, some lungs release only a very small proportion of their mast cell-derived histamine. In these tissues, it is impossible to accurately assess the inhibitory effects of drugs. Secondly, within each lung, there is often great variation in the amount of histamine released from different tissue replicates. When this occurs, the errors associated with determinations of histamine release are large and, in consequence, drugs have to produce a marked change in the release for their effects to be detected. Thirdly, the inhibitory activity of drugs in different lungs is diverse so only their major effects are reproducible from experiment to experiment. Because of these limitations, i) many experiments have to be performed to ensure that a particular response to drug is significant and ii) it is only possible to examine gross drug effects.

Studies in which their inhibitory effects were compared and contrasted indicate that sodium cromoglycate, salbutamol and chlorpromazine inhibit secretion in lung mast cells by completely different mechanisms. The exact mode of action of cromoglycate remains unknown but seems not to involve membrane stabilisation, or competition or interaction with calcium. Salbutamol exerts its effect through stimulation of  $\beta$  receptors in the lung whilst chlorpromazine acts by binding to, and interfering with the functioning of, the mast cell plasma membrane. The action of cromoglycate suffers from the disadvantage that long periods of contact with the lung can cause desensitisation. The action of chlorpromazine is unsatisfactory because the drug is only effective in high concentrations and it can cause mediator release. The anti-secretory effect of salbutamol does not suffer from these disadvantages. Furthermore, salbutamol is much more potent and

effective at inhibiting secretion than either of the other 2 agents. It appears that stimulation of  $\beta$  receptors in the lung offers the best prospect for in vivo activity.

To investigate the effects of these drugs further, studies need to be performed in which their pharmacological actions are linked with specific biochemical changes within lung mast cells. It is impossible to perform this task with chopped lung tissue; a purified suspension of mast cells is required. Methods do exist whereby human lung mast cells can be isolated and purified (Paterson et al, 1976; Newball, manuscript in preparation) but these involve extensive enzymatic digestion of the tissue which may radically alter the reactivity of the cells. Our goal should be to develop a method for purification of tissue-bound mast cells which exposes them as little as possible to destructive enzymes.

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