

STUDY OF ANTI-ALLERGIC DRUGS IN RELATION TO PHOSPHODIESTERASE ACTIVITY

THESIS SUBMITTED
UNIVERSITY OF BOMBAY

FOR THE DEGREE OF
MASTER OF PHARMACY
IN
PHARMACOLOGY

SUBMITTED BY
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UNDER THE GUIDANCE OF
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submitted to
THE UNIVERSITY OF BOMBAY**

**for
THE DEGREE OF
MASTER OF PHARMACY
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by

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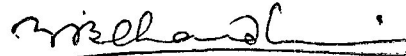
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STATEMENT UNDER UNIVERSITY RULE NO. 0.412 & 0.413

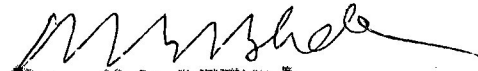
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(Dr. N.B. BHIDE)

Signature of the Research Guide

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INTRODUCTION

Von Pirquet (1906) coined the term allergy and defined it as follows:-

'The vaccinated person behaves towards vaccine lymph, the syphilitic toward the virus of syphilis, the tuberculous patient towards tuberculin, the person injected with serum towards this serum, in a different manner from him who has not yet been in contact with such an agent. Yet he is not insensitive to it. We can only say of him that his power to react has undergone a change. For this concept of a changed reactivity I propose the term "Allergy".'

The altered response may be either due to increased resistance, i.e. immunity, or increased susceptibility, i.e. hypersensitivity. The terms allergy and hypersensitivity have now become synonymous. Hypersensitivity reactions are classified into two major classes, the immediate type and the delayed type. In hypersensitivity reactions, antigens combine with antibodies and cause various syndromes such as systemic anaphylaxis, bronchial asthma, hay fever, serum sickness, Arthus reaction, contact dermatitis etc. In the immediate type symptoms are seen, usually within 24 hours, while in the delayed type it takes much longer. The mechanism of the immediate type of hypersensitivity is better understood

than that of the delayed type. Anaphylactic reactions usually occur in this type of hypersensitivity. Anaphylaxis is an interaction of cell-bound immunoglobulins with a specific antigen or hapten which leads to the release of mediators that cause smooth muscle contraction, vasodilatation and alteration of vascular permeability.

Antibodies of the immunoglobulin E class (IgE) are usually responsible for anaphylactic reactions. However, antibodies of the immunoglobulin G class (IgG) are known to also produce anaphylaxis. Antibodies are formed in response to primary exposure to an antigen. On re-exposure to the same antigen, antigen-antibody complex is formed on the mast cell surface. This induces a number of biochemical processes as a result of which chemical mediators are formed and/or released from the mast cells. These mediators are at least in part responsible for the various symptoms observed. The mediators released are as follows:- (Middleton, 1980)

- 1) Preformed mediators such as;
 - a) Histamine,
 - b) Eosinophil Chemotactic Factor of Anaphylaxis,

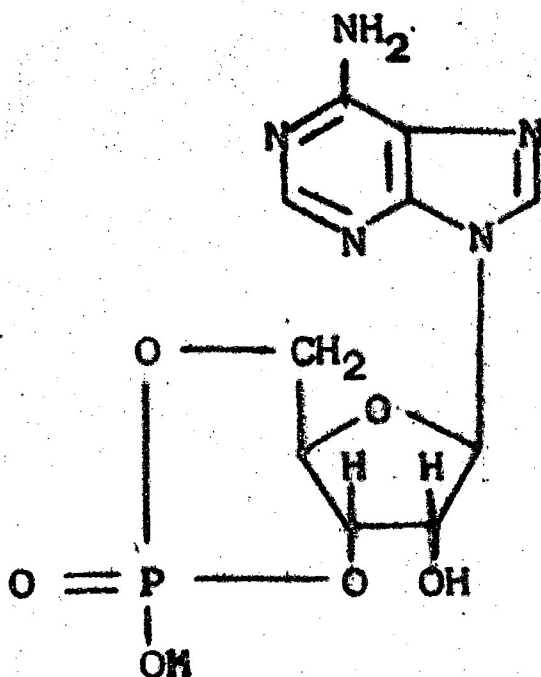
- c) Eosinophil Chemotactic Factor of Oligopeptides,
 - d) Neutrophil Chemotactic Factor,
 - e) Heparin,
 - f) Chymase,
 - g) Lung Kallikrein of Anaphylaxis,
 - h) Superoxide radicals and
 - i) Superoxide dismutase.
- 2) Newly generated mediators such as;
- a) Slow-Reacting Substance of Anaphylaxis (SRS-A),
 - b) Platelet Activating Factor(s),
 - c) Lipid Chemotactic Factor and
 - d) Prostaglandins.

Schild (1936) showed that adrenaline inhibited the anaphylactic release of histamine from guinea-pig lung. This observation was confirmed and extended by Lichtenstein and Margolis (1968) who reported that isoprenaline, adrenaline, methyl xanthines and dibutyric cyclic adenosine monophosphate (cAMP) inhibited allergic release of histamine from human leucocytes and suggested that the effects of all these compounds were mediated by raised intracellular levels of cAMP. Confirmatory findings were also reported by Assem and Schild (1969) with

IgE-mediated release of histamine from human lung and Ishizaka et al. (1971) with the release of SRS-A from monkey lung. It is thus evident that increase in cyclic AMP levels inhibits the anaphylactic mediator release.

cAMP was first reported by Sutherland and Rall (1958). Subsequent work suggested that it has a molecular formula of $C_{10}H_{12}N_5O_6P$ with a molecular weight of 329.2. Its ϵ 260 nm was found to be $15.0 \text{ cm}^2 / \mu \text{ mole}$ (in phosphate buffer 0.1 M, pH 7.0) (Bergmeyer, 1971).

The structure of cAMP is demonstrated to be as follows:-



ADENOSINE-3',5'-MONOPHOSPHORIC ACID,
(Cyclic AMP)

Cyclic AMP is a hydrolysis product of adenosine tri-phosphate (ATP). The hydrolysis in cells is catalysed by the enzyme adenylyl cyclase (E.C. 4.6.1.1). Magnesium ions are required for optimal reaction. Cyclic AMP can be formed and is widely distributed in animal cells. Cyclic AMP, not only mediates the actions of most biogenic amines and polypeptide hormones (Robison *et al.*, 1968; Weiss and Kidman, 1969) but also appears to influence such fundamental processes as cell division (Ryan and Heidrick, 1968), fertilization (Castaneda and Tyler, 1968), embryonic growth and cell differentiation (Zalin and Montague, 1975), blood production (Bottomley *et al.*, 1971; Rodgers *et al.*, 1975; Norris *et al.*, 1974); cardiac contractility and metabolism (Kukovetz and Poch, 1972; Entanani *et al.*, 1969; Mayer *et al.*, 1975) central nervous system function (Weiss and Greenberg, 1975); autonomic nervous system function (Greengard *et al.*, 1972a), vision (Bitensky, 1972); immunological responses (Singh and Dhalla, 1975; Parker *et al.*, 1974), gonadal function (Marsh *et al.*, 1966) and the release of stored intracellular materials such as insulin (Malaisse *et al.*, 1974; Turtle *et al.*, 1967 and Kuo and Kuo, 1975), histamine (Sullivan *et al.*, 1975a and Sullivan *et al.*, 1975b) and lysosomal enzymes (Ignarro, 1975).

The role of cyclic AMP in the regulation of the function of normal cells suggests that any abnormal alteration in its concentration may be responsible for abnormal cell function i.e. diseased state. In the following diseases abnormalities in the formation and/or concentration of cyclic nucleotides have been suggested: asthma, cancer, cardiovascular diseases, Down's syndrome, diabetes mellitus, hyperthyroidism, epilepsy, obesity and psoriasis (Weiss and Hait, 1977).

Intracellular cyclic AMP is degraded by the enzyme phosphodiesterase to yield 5'-AMP. Magnesium ions are once again necessary for the hydrolysis. Phosphodiesterase (PDE) is found in virtually every living cell, its relative activity varying greatly among different tissues (Weiss and Costa, 1968; Butcher and Sutherland, 1962).

As mentioned earlier, increase in cAMP levels have anti-allergic effects. Inhibitors of phosphodiesterase have also been studied for the treatment of cancer, psoriasis, asthma, inflammation, cardiovascular diseases, viral diseases and neuropsychiatric diseases (Weiss and Hait 1977).

In general, phosphodiesterases can be considered to include all the enzymes which hydrolyze the bond between phosphate and one or the other of two molecules possessing alcoholic functional groups which it links (Russell, 1968).

Cromolyn sodium is a potent anti-allergic drug devoid of bronchodilator activity. Cromolyn sodium inhibits cyclic AMP phosphodiesterase (Roy and Warren 1974). Chemically it is bischromone - the disodium salt of 1,3-bis(2-carboxychromon-5-yloxy)-2-hydroxypropane. Since its basic moiety is a chromone, some synthetic chromone derivatives have been evaluated in the present study as regards their anti-allergic activity and their phosphodiesterase inhibitory capacity.

Cromolyn sodium is a prophylactic antiasthmatic agent. Since bronchial asthma is a respiratory tract disease, it was considered useful to study the lung phosphodiesterase, as effects on this enzyme would be more pertinent to disease. Though there are three animal models, guinea-pig, atopic monkey and dog for anti-asthmatic drug screening, the rhesus monkey (*Macaca mulatta*) model of asthma has been shown to most closely simulate human asthma. Hence the enzyme phosphodiesterase was isolated from rhesus monkey lung.

LITERATURE REVIEW

^{As}
 [] quoted in the preceding chapter, Von Pirquet (1906) explicitly stated that allergy included not only hypersensitivity - the altered reactivity, injurious to the host - but also immunity, the alteration beneficial to the host in resisting infection.

Over the years, contrary to Von Pirquet's original intention, the term allergy in common medical, scientific, and lay usage has come to refer only to those immunological reactions that are damaging to the host; hypersensitivity and allergy have now become synonymous.

It is necessary to restrict the term immunity, to those antigen-antibody reactions, in which no injury to the host, but resistance to infection is necessarily implied in the final outcome of the reaction. Immune reactions or immunity are restricted to immunological reactions beneficial or protective to the host organism. Allergic reactions are, then, defined according to common usage as those immunological reactions that are damaging to the tissues or disruptive of the physiology of the host.

Allergic reactions are grossly divided into only two types- the immediate type and the delayed type. The immediate type reactions include all reactions determined

by atopic IgE or IgG₂ types of antibodies found in the circulation and body fluids. They can be transferred from one animal to another through serum. The delayed-type reactions, in general, are not transferable through serum but are transferable through lymphoid cells. It is assumed that none of the presently recognized circulating antibodies is demonstrably responsible for delayed reactions, and the nature of the specific recognition factor(s) for the delayed response is unknown (Ishizaka, 1971).

In immediate type of hypersensitivity, the central event is the process of anaphylaxis. In it, the allergen combines with the cell-bound reaginic antibody on basophils and mast cells, releasing mediators, which in turn have number of pharmacological activities.

The list of mast-cell and basophil-derived mediators includes -

- a) performed mediators such as Histamine, eosinophil chemotactic factor of anaphylaxis, eosinophil chemotactic factor of oligopeptides, neutrophil chemotactic factor, heparin, chymase, lung kallikrein of anaphylaxis, superoxide radical, superoxide dismutase and

b) newly generated mediators such as slow reacting substance of anaphylaxis (SRS-A), platelet-activating factor(s), lipid chemotactic factors, and prostaglandins (Middleton, 1980).

The source, nature, properties, and metabolism of these substances have been reviewed extensively (Austen and Orange, 1975; Austen, 1978; Sullivan and Parker, 1976; Kaliner and Austen, 1974; Coffey and Middleton, 1977; Flaut and Lichtenstein, 1978; Lewis and Austen, 1977).

Histamine is β -imidazolylethylamine and it is synthesized principally by a specific ℓ -histidine decarboxylase. Histamine involved in hypersensitivity reactions of the immediate type, appears to be stored in mast cells (or basophils) as secretory granules, as a complex with heparin and protein. This stored amine undergoes slow turnover in comparison to the histamine of the GI mucosa and the central nervous system.

While still subject to controversy, it is believed that the immunological, calcium-dependent release of histamine involves two processes, perhaps including the dissociation of histamine from the granules and the exocytosis of the subcellular cytoplasmic granules

(Kalinierosak and Diamant, 1978). The heparin-protein complex of mast cell granules has ion-exchange properties, and exposure of the granule matrix to an altered ionic environment results in an exchange of histamine for sodium ions. Once released, histamine contributes to some manifestations of the clinical syndrome of immediate hypersensitivity reactions. Histamine undergoes metabolism by one of two pathways. One, through methylation of the ring structure by imidazole-N-methyl-transferase, yielding methylhistamine, which is then oxidized through monoamine oxidase to methylimidazoleacetic acid. Alternatively, histamine can undergo an oxidative deamination by histaminase or diaminoxidase to imidazole-acetic acid. The products of this metabolism are excreted in the urine.

The mast cell has been implicated as a source of both histamine, and SRS-A (Kalinier and Austen, 1975; Parker, 1979), although other cell types also appear to serve that function. Two experimental systems that generate SRS-A are the sensitized fragmented lung and peripheral blood leucocyte preparations. SRS-A is measured by bioassay on the guinea-pig ileum, and it produces a slow sustained contraction even in the presence of an H_1 -histamine receptor blocker and an

anticholinergic agent. The chemical structure of SRS-A has been only recently elucidated by Lewis *et al.* (1980).

SRS-A was demonstrated to be resistant to proteases and phospholipases but labile to arylsulfatases and oxidation (Kalinin and Austen, 1975; Parker, 1979). Furthermore, it was demonstrated that SRS-A could be derived from arachidonic acid, perhaps through a lipoxygenase pathway, but it appeared to be more polar than, and could be separated from, conventional prostaglandins (Parker, 1979).

Recently, (Fox, 1979), it was suggested that SRS-A may be "identical" to leukotriene C, one of a group of compounds related to the prostaglandins identified by Samuelsson *et al.* of the Karolinska Institute in Sweden. Leukotriene C contains the amino acid cysteine covalently linked to the 20-carbon fatty acid derivative of the prostaglandin precursor, arachidonic acid. Lewis *et al.* (1980) successfully characterized SRS-A from human lung. They reported that human SRS-A is leukotriene C and leukotriene D. Leukotriene D is the major constituent of SRS-A of human lung and accounts for almost all of the biological activity. It is probably formed from leukotriene C, *in vivo* by an enzymatic process of the well known γ -glutamyltransferase type.

Eosinophils occur in relatively large numbers in pulmonary tissues and mucosal secretions of asthmatics, although their role is not completely understood (Goetzl and Austen, 1975). Investigations demonstrate that neither relatively pure SRS-A nor histamine was chemotactic for these cells. This provided the basis for discovery of eosinophil chemotactic factor of anaphylaxis, another preformed primary mediator. Eosinophil chemotactic factor has a molecular weight of ~ 500 , and is an acidic peptide. Two tetrapeptides with amino acid sequence Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu were recovered from extracts of human lung fragments and were shown to be chemotactic for eosinophils (Kaliner and Austen, 1975; Goetzl and Austen, 1975). Whether these tetrapeptides are, in fact, the eosinophil chemotactic factors remains to be determined conclusively.

Additionally, other pharmacologically potent materials have been shown to be released from previously sensitized lung tissue on immunological challenge. Prostaglandins of E and F classes are rapidly synthesized and released (Kaliner and Austen, 1975; Parker, 1979). Their pharmacological activities are probably mediated by intracellular messengers, the cyclic nucleotides, cyclic guanosine monophosphate (cGMP), and cyclic AMP

and they themselves are considered secondary mediators whose synthesis and release may be governed by histamine or SRS-A .

In addition to the prostaglandins, other chemicals, yet poorly characterized, probably contribute to the symptomatology of allergic asthma. Platelet-activating factor is a lipid released on an immunoglobulin E-antigen interaction on basophils or lung tissue (Kalinin and Austen, 1975). Platelet-activating factor induces aggregation of platelets and the subsequent release of serotonin, which can cause smooth muscle contraction. Serotonin is 3-(β -aminoethyl)-5-hydroxyindole. It is decarboxylated by MAO to 5-hydroxyindoleacetaldehyde. It is further, oxidized to 5-hydroxyindoleacetic acid and in small quantities reduced to 5-hydroxytryptophol by alcohol dehydrogenase. All metabolites are excreted in the urine.

Extensive studies (Austen and Orange, 1975; Austen, 1978; Sullivan and Parker, 1976; Kalinin and Austen, 1974; Coffey and Middleton, 1977; Flaut and Lichtenstein, 1978) of IgE dependent immunological release of chemical mediators from mast cell and basophils have provided a partial picture of the biochemical events involved and,

thus, the various fact for potential pharmacological intervention. Interaction of specific antigen with mast cell-bound IgE antibody presumably results in membrane perturbation associated with extracellular calcium ion transport into the cell. This initial step is accompanied by the activation of a proesterase, which is converted to an active chymotrypsin-like serine esterase, which subsequently engages in further autocatalytic activation. Possibly, calcium-ion influx is essential for initial activation and autocatalytic activation of the proesterase to the esterase. The active esterase decays quite rapidly.

A subsequent energy-dependent step can be inhibited by 2-deoxyglucose. Precisely what function in the mediator release sequence is subserved by the energy requirement is not certain, but it may be related to the function of a "contractile protein". Since dense bands of microfilaments have been observed around mast cell granules, especially during degranulation. Microfilament function is probably involved in mediator release, and the function of microfilaments can be affected by cytochalasins A and B. Studies with these agents indicated that histamine release and SRS-A generation and release can be dissociated. Although

cytochalasins A and B enhance histamine release, they inhibit concomitant formation of SRS-A.

Several studies indicated an important function of microtubules in mediator release from mast cells and basophils. Colchicine, a compound that binds to the microtubular subunit protein, thereby preventing microtubule assembly and function, inhibits histamine release. Heavy water (D_2O), on the other hand, favours microtubule subunit aggregation and facilitates histamine release.

The metabolic regulation of mediator release has been investigated intensively. One aspect of metabolic control of mediator release that is quite well worked out in several *in vitro* systems involves the effects of cAMP, cGMP, and agents that affect their intracellular concentration. Numerous studies (Austen and Orange, 1975; Austen, 1978; Sullivan and Parker, 1975; Kaliner and Austen, 1974; Coffey and Middleton, 1977; Plant and Lichtenstein, 1978) have indicated that agents that increase cAMP levels (e.g. β -agonists, histamine, prostaglandin E and cholera toxin) tend to decrease chemical mediator release, while agents that decrease cAMP levels (e.g. α -adrenergic agonists) tend to increase chemical mediator release. On the other hand, compounds

that increase cGMP levels (e.g. cholinergic agents and, perhaps, α -adrenergic compounds) tend to increase mediator release. Recent evidence appears to establish cGMP as an important mediator of immunological mediator release in human lung (Kalliner, 1977).

Mediator release can be modulated by products of the sympathetic and parasympathetic nervous system and other substances acting through specific receptor systems and affecting the intracellular levels of cAMP and cGMP. Calcium plays a critical role in the control of intracellular cyclic nucleotide concentrations through its ability to inhibit adenylyl cyclase (Robison et al., 1971a), stimulate guanylyl cyclase (Schultz, 1978) and regulate phosphodiesterase regulator protein (Breestorn et al., 1975).

Sutherland and Hall (1958) first reported the presence of cyclic AMP. The cyclic nucleotide that mediates the epinephrine-induced activation of liver phosphorylase, has developed into a broad and novel concept, touching every facet of biological control mechanisms. Adenosine 3',5'-monophosphate (cyclic AMP) and other cyclic nucleotides have now been demonstrated, not only to mediate the effects of most hormones and neurotransmitters, but also to be involved in scores of biochemical and

physiological events (Haynes, 1960; Ball and Sutherland, 1961; Haugeard and Hess, 1965; Sutherland and Robison, 1966; Sutherland et al., 1968; Robison et al., 1968; Exton and Park, 1968; Weiss and Kidman, 1969; Ball et al., 1969; Weiss, 1970; Greengard and Costa, 1970; Weiss and Crayton, 1970a; Benner, 1971; Robison et al., 1971a, b; Hardman et al., 1971; Weiss, 1971a; Hittleman and Burcher, 1972; Greengard et al., 1972b; Ball, 1972; Bitensky and Gorman, 1973; Kahn and Lands, 1973; Drummond et al., 1975; Greengard and Robison, 1973, 1974; Burkard, 1975; Weiss, 1975a; Weiss and Hait, 1977; Weiss and Fortel, 1977; Goldberg and Haddock, 1977).

The evidence that cyclic nucleotides influence the functions of normal cells, suggests that, any abnormal alteration in the concentration of these compounds, may be responsible for abnormal cell function, that is, a diseased state. Indeed, several lines of investigation indicate that cyclic nucleotides may be involved in a variety of clinical conditions ranging from cancer and cardiovascular diseases to mental illness (Levine, 1970; Uzunov and Weiss, 1972a; Murad, 1973; Kahn and Lands, 1973; Schultz and Gratner, 1973; Weiss, 1975a; Weiss and Hait, 1977).

Most of the studies performed during the past several years have dealt with the role of endogenous hormones and neurotransmitters in controlling the intracellular concentration of these cyclic nucleotides. More recently, investigators have turned their attention to the possibility of manipulating the concentrations of these nucleotides by pharmacological means (Breckenridge, 1970; Amer and McKinney, 1973; Amer and Kreighbaum, 1975; Weiss and Fertel, 1977).

If cyclic AMP is involved in so many processes and has so many actions, the question arises, how can drugs that act by changing the concentration of cyclic AMP produce a specific effect? The answer to this question is provided by the evidence that the enzymes responsible for the biosynthesis (nucleotide cyclases) and hydrolysis (cyclic nucleotide phosphodiesterases) of cyclic nucleotides are not simple molecular entities, but, rather, are complex enzyme systems, existing in multiple molecular forms. These enzyme systems are unequally distributed in the different tissues and cell types, and may be differentially activated and inhibited by endogenous and exogenous chemicals. Accordingly, one may be able to develop pharmacological agents, that will selectively change the activity of the form of enzyme

that is present in a certain type of cells. This would result, in a selective alteration of the intracellular concentration of a cyclic nucleotide in that cell and thereby, cause a selective modulation in the function of that cell.

Cyclic nucleotides in immediate hypersensitivity

Lichtenstein and Margolis (1968), reported that isoproterenol, theophylline and dibutyryl cAMP blocked immunoglobulin E (IgE)-mediated histamine release. Hirschhorn et al. (1970) and Smith et al. (1971) showed that increased cyclic AMP levels inhibited lymphocyte proliferation. The inhibition of basophil and lymphocyte function by cyclic AMP was thus confirmed. It leads towards the control of inflammatory and immune cell function, by exogenous drugs and endogenous hormones, which interact with specific receptors to modulate function (Bourne et al., 1974).

Immediate Hypersensitivity

The release of mediators as a result of interaction of allergen with cell-bound IgE antibodies fixed to receptors on basophils and mast cells is the central

event in the pathogenesis of allergic diseases (Lichtenstein, 1972). Sensitized basophils and mast cells are already present at the site of antigen exposure, and accumulation of other cells is not required to produce the hypersensitivity reaction. Thus, following antigen exposure, antigen-antibody complex releases the mediators of 'Immediate hypersensitivity'.

The release of mediators is reduced by cyclic AMP or by several hormones, that interact with cell surface receptors to activate adenylate cyclase (Sourne et al., 1974; Orange et al., 1971). The endogenous hormone-like adenylyl cyclase agonists include, beta-adrenergic agents, prostaglandin E_2 ($PG E_2$), adenosine and histamine. All these agents are potent inhibitors of histamine release.

Histamine itself interacts with a histamine type 2 receptor on human basophils to inhibit histamine release, and this inhibition is blocked by H_2 antagonists such as metiamide or cimetidine (Lichtenstein and Gillespie, 1973). Efforts to delineate an H_2 receptor on mast cells, however, have been unsuccessful (Kalinin, 1978).

Since the effects of epinephrine, histamine and adenosine are blocked by specific antagonists, these

agents (and presumably EGF_2 and prostacyclin) increase cyclic AMP, by acting on receptors linked to adenylate cyclase. The mechanism by which cyclic AMP blocks mediator release is not known. It is suggested that it acts early in the release process, and that, it linked to obligatory inward flux of calcium, and that is related to microtubule function.

Cyclic AMP measurements are mostly made in primate species, either in chopped lung tissue (where mast cell represent a minor component of the tissue) or in mixed leukocyte systems in which the basophils represent less than 1 % of the total cells (Ichisaka et al., 1972).

Adenylate cyclase stimulators, exists *in vivo* and expected to modulate both the allergic response and the subacute and chronic inflammatory processes. In monkey skin, the wheal and flare response to antigen has been inhibited by them. It is also clear that, beta-adrenergic agonists and phosphodiesterase inhibitors are useful in the treatment of allergic conditions. Presumably, their therapeutic effects are due, not only to relaxant effects on vascular and bronchial smooth muscle, but also to their inhibitory effects on mediator release.

However, there are, exceptions when changes in cyclic nucleotide levels do not parallel inhibitor of release. Epinephrine, for example, increases rat mast cell cyclic AMP without affecting histamine release (Sullivan et al., 1975b). A recent experiment with human basophils demonstrates dissociation between cyclic AMP elevation and inhibition of histamine release (Marone et al., 1979). When both lipoxygenase and cyclooxygenase metabolic pathways are blocked by eicosatetraenoic acid (ETYA), histamine release is inhibited. Indomethacin and other non-steroidal anti-inflammatory agents that block the cyclooxygenase pathway induce a dose-dependent enhancement of mediator release.

The effects of changes in the levels of cGMP on immediate hypersensitivity reactions have been less extensively studied. In chopped lung preparations, cholinergic stimulation increases cyclic GMP levels. Cholinergic agents and cyclic GMP derivatives potentiate antigen-induced mediator release (Kaliner et al., 1972a). However, cyclic GMP does not affect the release from rat mast cells, and has minimal or no effect on mediator release from basophils (Gillespie and Lichtenstein 1975).

It is not clear, whether the increase in cyclic GMP levels in lung tissue is due to a direct effect of cholinergic agents on the mast cells or whether, it reflects their actions on other cell types.

It appears to call into question the role of cyclic nucleotides in modulating mediator release (Platshorn and Kaliner 1978; Kaliner, 1978). However, it has to be remembered that the workers were measuring cyclic nucleotides in lung tissue, which contains upto 50 different cell-types, so that it is impossible to ascertain which cell (let alone the intracellular compartment), the cyclic nucleotide changes belong to. However, for reasons as yet unknown, histamine may induce increase in cyclic AMP and cyclic GMP even perhaps in mast cells, in intracellular compartments which are unrelated to the process of mediator release. Alternatively, it may be, as Kaliner speculate that mast cells, unlike basophils, lack histamine receptors.

There is one final piece of evidence which must be considered. Coulson *et al.* (1977) compared the antianaphylactic activity of a variety of compounds, which possess, in varying degree, the power to inhibit degradation of cyclic AMP and cyclic GMP in human lung. They found that as the ability to inhibit hydrolysis of

cyclic GMP increased relative to cyclic AMP, so did antianaphylactic potency increase, possibly because the phosphodiesterases were able to influence intracellular pools of cyclic nucleotide, thereby supporting once again the idea that cyclic AMP plays a key role in reducing mediator release.

Definition of phosphodiesterase (E.C.3.1.4.3)

Phosphodiesterases can be considered to embrace all those enzymes which hydrolyse the bond between phosphate and one or other of the two molecules possessing alcoholic functional groups which it $\square$ links (Russell, 1968).

Discovery and characterization of cyclic nucleotide Phosphodiesterase

In 1957, Rall, Sutherland and Berthet found that, the activity to increase liver phosphorylase formation by a heat-stable substance was rapidly lost during incubation with extracts of heart, liver and brain. This factor, which was subsequently identified as cyclic AMP was shown to be hydrolysed to 5'AMP by an enzyme found in heart (Sutherland and Rall, 1958). Since these studies, this enzyme, cyclic nucleotide phosphodiesterase has been extensively characterised by numerous investigators. The

early work of Drummond and Perrott-Yee (1961) described its distribution in rabbit tissues, its cation dependence and its substrate specificities. This work was confirmed and extended by Butcher and Sutherland (1962). A number of investigators have subsequently studied cyclic nucleotide phosphodiesterase and their results, are discussed in detail in following discussion.

Distribution

Cyclic nucleotide phosphodiesterase activity is found in virtually all living cells, its relative activity varying greatly among different tissues of animals and plants (Weiss and Costa, 1968; Butcher and Sutherland 1962; Weiss and Fertel, 1977; Appleman et al., 1973; Amor and Kreighbaum, 1975). The regional and subcellular distribution of cyclic GMP phosphodiesterase generally parallels that of cyclic AMP phosphodiesterase. Both enzyme activities are found in all subcellular fractions with most of the activity being in the 100,000 x g supernatant fraction (Hait and Weiss, 1977; Weiss and Costa, 1968; Hair, 1966; Campbell and Oliver, 1972; Beavo et al., 1970; Fertel and Weiss, 1974, 1976).

Although, phosphodiesterase is largely soluble enzyme particulate form of enzyme also exists. The particulate enzyme may be of greater physiological importance since it contains a high affinity form of the enzyme (Appelman *et al.*, 1973; Amor and Kreighbaum, 1975; Clark *et al.*, 1973). A soluble fraction also contains a high affinity form of the enzyme (Usunov *et al.*, 1974; Brooker *et al.*, 1968; Lagarde and Colobert, 1972; Weiss and Strada, 1972). It is more likely that both the low and high K_m forms of phosphodiesterase play a role in regulating the intracellular concentration of cyclic nucleotides, the low K_m form of the enzyme being responsible for the basal levels and the high K_m form controlling the cyclic nucleotide concentrations following hormonal stimulation of the nucleotide cyclases (Weiss and Strada, 1972).

Substrate Specificity

The cyclic nucleotide phosphodiesterases are not completely substrate specific since some of the purified phosphodiesterase enzymes can hydrolyze cyclic AMP and cyclic GMP as well as a number of other cyclic nucleotides. However, the different isozymes of phosphodiesterase do have different relative affinities for the various cyclic

nucleotides (Rosen, 1970b; Thompson and Appelman, 1971b; Song and Cheung, 1971; Beavo et al., 1971; Sung et al., 1972; Miller et al., 1973; Amer and Nyol, 1973; Russell et al., 1973; Brostrom and Wolff, 1976).

Kinetic Properties

Phosphodiesterase exhibits anomalous kinetic behavior, suggesting either the existence of a single enzyme form with the property of negative co-operativity (Russell et al., 1972) or the existence of at least two forms of phosphodiesterase, one having a low affinity (high K_m) for cyclic AMP and the other having a high affinity (low K_m) (Usunov et al., 1974; Weiss and Strada, 1972; Thompson and Appelman 1971a; Loten and Snoyd, 1978; Weiss et al., 1972; Schonhofer et al., 1972; Weiss and Strada, 1973; Strada et al., 1974). Evidence suggests that both possibilities are true. On one hand, it has been shown that there are different molecular forms of phosphodiesterase and on the other hand, it has been shown that even highly purified isoenzymes of phosphodiesterase exhibit negative or positive co-operativity, suggesting that there are allosteric sites on the enzyme that influence the catalytic site (Beavo et al., 1971; Russell et al., 1972; Hait and Weiss, 1977; Franks and MacManus, 1971).

pH Dependence

The pH optima for phosphodiesterase is between 7.5 and 9.0 (Drummond and Perrott-Yee, 1961; Hair, 1966; Menahan et al., 1969; Weiss et al., 1972; Schroeder and Rickenberg, 1973; Cheung, 1971a; Szabo and Burke, 1972; Rosen, 1970a; Song and Cheung, 1971; Huang and Kemp, 1971; Goren and Rosen, 1972a; Ritten et al., 1973).

Ion-dependence

Phosphodiesterase has an absolute requirement for Mg^{++} and under certain conditions, calcium has been shown to be required for enzyme activity (Butcher and Sutherland, 1962; Kakiuchi et al., 1971, 1972, 1973; Cheung, 1971a; Teo and Wang, 1973).

Multiple molecular forms

The existence of multiple molecular forms of phosphodiesterase has been demonstrated in many tissues by numerous investigators using a variety of techniques (Uzunov et al., 1974; Bertel and Weiss, 1974; Clark and Perkins, 1971; Strada et al., 1974; Thompson and Appleman, 1971; Goren et al., 1971; Monn and Christensen, 1971;

Ugunov and Weiss, 1972b; Klotz et al., 1972; Schroder and Bickenberg, 1973; Pichard et al., 1973; Pledger et al., 1974; Strada and Pledger, 1975; Wells et al., 1975; Singer et al., 1976).

The pattern and ratio of these phosphodiesterase isozymes vary with the specific tissue (Ugunov et al., 1974; Thompson and Appleman, 1971; Monn and Christiansen, 1971; Ugunov and Weiss, 1972b; Singer et al., 1976) and cell type (Ugunov et al., 1974, 1973).

Endogenous activator

While purifying phosphodiesterase from bovine brain Cheung (1970a and b) noticed that the phosphodiesterase activity decreased as the enzyme became more pure, suggesting that a factor that activates phosphodiesterase was being removed during the purification process (Cheung et al., 1975). This activator has now been isolated and has been studied (Brostrom et al., 1976; Kakiuchi et al., 1970; Goren and Easen, 1971; Teo and Wang, 1973; Wolf and Brostrom, 1974; Wickson et al., 1975; Levine and Weiss, 1976). The activator is a heat-stable, calcium-dependent protein (Ugunov and Weiss, 1972b; Wolf and Brostrom, 1974; Kakiuchi and Yamasaki, 1970; Wang et al., 1975; Lin et al., 1975), with a molecular weight between 11,000 and 40,000

(Cheung 1971; Teshima and Kakinuchi, 1974). The mechanism by which the activator increases phosphodiesterase activity is quite complex. This endogenous activator is of particular interest because it is extremely potent and highly selective. Less than 1 μ g of activator is capable of increasing the activity of one of the molecular forms of phosphodiesterase more than ten-fold while the activity of other isozymes of phosphodiesterase isolated from the same tissue remains essentially unaltered (Weiss *et al.*, 1974). Moreover, this activable form of phosphodiesterase is not present in all tissues (Hait and Weiss, 1976, 1977; Fortel and Weiss, 1976) and where it is found, it is present in different amounts relative to the other phosphodiesterase isozymes (Uzunov and Weiss, 1972b; Greenberg and Weiss, 1976).

Inhibitors of phosphodiesterase

The effect of any inhibitor on a given enzyme system is usually expressed, either as, the dissociation constant of the inhibitor-enzyme complex (K_i) or as the concentration of the inhibitor causing 50 % inhibition of enzyme activity at a given substrate concentration (I_{50}). These two factors are equal only under certain kinetic conditions (Cheung and Prusoff, 1973). Since there is a

differential distribution of phosphodiesterase isoenzymes, according to tissue and cell type (Uzunov et al., 1974) and since these isoenzymes have a differential response to inhibitors (Weiss et al., 1974) a comparison of the effects of phosphodiesterase inhibitors based on studies using different phosphodiesterase sources is difficult. Moreover, many investigators have studied the effects of phosphodiesterase inhibitors in the presence of high concentrations of substrate. Under these assay conditions the total phosphodiesterase activity will reflect both the high K_m and low K_m enzyme.

The following classes have been studied for their ability to inhibit phosphodiesterase (Weiss and Fertel, 1977) :-

- 1) Xanthine analogs
- 2) Papaverine and its analogs
- 3) Cyclic nucleotides and their analogs
- 4) Imidazolidine derivatives
- 5) Pyrazole and pyridine derivatives
- 6) Substituted imidazopyrazines
- 7) Substituted pyrroles, triazines and pyridazines
- 8) Substituted quinoxalines

- 9) Imidasoline derivatives
- 10) Semicarbazones
- 11) Phenethylamines
- 12) Ergot alkaloids and lysergic acid derivatives
- 13) Reserpine and reserpine analogs
- 14) Phenothiazines
- 15) Benzodiazepines
- 16) Dibenzazepines
- 17) Sulfonylureas
- 18) Steroids
- 19) Cardiac glycosides
- 20) α -Tocopherol and its analogs
- 21) Disodium cromoglycate
- 22) Tri-iodothyronine and thyroxine
- 23) Eritadenine
- 24) Ascorbic and dehydroascorbic acid
- 25) Acid mucopolysaccharides
- 26) Flavoxate
- 27) Benzopyran derivatives
- 28) Miscellaneous pharmacological agents
 - a) Diuretics
 - b) Metabolic inhibitors
 - c) Alkylating agents
 - d) Bitters
 - e) Anti-inflammatory agents

Mechanism of Inhibition of phosphodiesterase

From the data available upto date, drugs may inhibit phosphodiesterase by several distinct mechanisms as follows:-

- 1) by competitive blockade of the active enzyme site, thereby preventing access of substrate to the site (e.g. theophylline, cyclic GMP and low concentrations of papaverine)
- 2) by acting as non-competitive antagonists of the substrate (high concentrations of papaverine and inhibitors which are structurally unrelated to the substrate)
- 3) by interfering with the activation of phosphodiesterase (e.g. trifluoperazine and related phenothiazines, calcium binding compounds).

Since the use of cromolyn sodium as an anti-allergic agent, chromone derivatives were synthesised and studied for their anti-allergic activity (Ellis and Shaw, 1972; Ellis and Barker, 1972; Barker *et al.*, 1973; Wright and Johnson, 1973; Kohara *et al.*, 1977; Pantick *et al.*, 1976; Devlin *et al.*, 1977, 1978).

Assay of Phosphodiesterase

Since the discovery, a considerable amount of work has been done to characterize the cyclic nucleotide phosphodiesterase (E.C. 3.1.4.17) enzymes and clarify the significance of their activities under physiological conditions. Several methods are currently available for monitoring phosphodiesterase activity. The most sensitive of these methods is the radio-isotopic assay method described by Thompson and Appleman (1971). Cyclic AMP is hydrolyzed by phosphodiesterase to 5'-Adenosine monophosphate, which is in its turn, further cleaved to adenosine and phosphate group by addition of snake venom. The radiolabelled amount of adenosine is measured.

Another sensitive method of measuring phosphodiesterase activity depends on the ability of 5'-AMP to inhibit fructose-bis-phosphatase activity (Hans et al., 1977).

The other methods are less sensitive. The most widely utilized method is measurement of orthophosphate released from the product 5'-AMP by snake venom. The inorganic phosphate can be either measured by the method of Fiske and Subbarow (1925) or Garbanga et al. (1980), by colourimetry. Several more spectrophotometric methods have been published depending on

- a) measurement of 5'-inosine monophosphate (5'-IMP) produced from 5'-AMP by incubation with 5'-AMP deaminase (Drummond and Perrott-Yee, 1961)
- b) measurement of proton released during hydrolysis of cAMP with the incorporation of phenol red (Friele et al., 1979)
- c) conversion of 5'-AMP by myokinase to ATP which is then measured by coupling to the pyruvate kinase and lactate dehydrogenase catalyzed reactions (Carter et al., 1979)
- d) measurement of inosine, by cleaving the product 5'-AMP by adenosine deaminase (Bergmeyer, 1974)
- e) measurement of p-nitrophenol using bis-p-nitrophenyl phosphate as substrate (mainly used for snake venom phosphodiesterase) (Bergmeyer, 1974)
- f) using Luciferin-luciferase technique (Weiss et al., 1972). In this technique, phosphodiesterase is the rate limiting step in a sequence of reactions starting with conversion of cyclic AMP to 5'-AMP and ending with the formation of ATP. ATP is then quantitated by firefly luciferin-luciferase technique. The light generated is measured by luminescence bismeter or liquid scintillation spectrophotometer.

One of the non-spectrophotometric methods consists of direct measurement of protons released by titrimetric method (Cheung, 1969).

Comparison of sensitivity of phenol red assay with other methods of measuring phosphodiesterase activity.

<u>Assay Method</u>	<u>Sensitivity (n mol/min)</u>	<u>Reference</u>
1) Radiochemical: discontinuous	0.001	Thompson and Appelgren, 1971
2) Inhibition of fructose- bis-phosphatase by 5'-AMP: discontinuous	0.050	Hans et al., 1977
3) Spectrophotometric determination of phenol red titration: continuous	0.075	Frielle et al., 1979
4) Coupling of 5'-AMP production with myokinase, pyruvate kinase, and lactate dehydrogenase reactions: discontinuous	1.0	Adams, 1963
5) Coupling with 5'-AMP deaminase and spectrophotometric determination of 5'-IMP: continuous	1.0	Drummond and Perrott-Yee 1961
6) Titrimetric determination of 5'-AMP production: continuous	2.0	Cheung, 1969
7) Hydrolysis of 5'-AMP with 20.0 5'-nucleotidase and measurement of molybdate orthophosphate: discontinuous		Eutcher and Sutherland, 1962

Anti-allergic studies

The experimental animal models are based on the "mediator concept" of allergic disorders. The rationale for this approach of screening lies almost totally on the known pharmacology of the prototype prophylactic anti-allergic agent cromolyn sodium and its well-established clinical activity.

Cromolyn sodium is not orally active and is, therefore administered as a powdered aerosol. The drug is efficacious in only a portion of the asthmatic patients for reasons that are not at present understood. Consequently, a compound that is orally active or that can protect a broader group or preferably all asthmatics would be a significant advance in therapy of asthma.

In vitro models based on the above rationale

- a) Isolated mast cells (Sullivan et al., 1975a),
- b) Blood leukocytes (basophils) (Lichtenstein and Gillespie, 1973),
- c) Fragmented lung tissue (Sheard et al., 1967; Chakrin et al., 1974; Orange et al., 1971; Krell and Chakrin, 1978).

Isolated mast cells - A commonly used technique for obtaining a relatively "pure" population of mast cells involves the use of rat peritoneal fluid. In brief, cells are removed from the peritoneal cavity of passively sensitized rats by a simple washing with a physiological salt solution and centrifugation of the washings. Cells are then suspended in an appropriate physiological medium and challenged with antigen to induce histamine release. Preincubation with anti-allergic agents reduces the amount of histamine release on challenge.

Similarly, blood leukocytes (basophils) which also contain histamine and may be prepared from actively sensitized animals or allergic human donors, suspended in an appropriate medium and challenged with the appropriate antigen to release the mediators of anaphylaxis. These types of systems are relatively simple and economic and they provide an essentially homogeneous cell population to study anti-allergic agents.

The next model in higher order of complexity in the testing sequence is the *in vitro* fragmented lung model prepared from various species including rats, guinea-pigs, dogs, rhesus monkeys and humans (Sheard et al., 1967; Chakrin et al., 1974; Orange et al., 1971; Krell and Chakrin, 1978). Lungs obtained from normal or actively

sensitized animals or humans are chopped into small fragments, passively sensitized with reaginic antibody (for those obtained from normal animals), placed in physiological medium and then challenged with the appropriate antigen. Many chemical substances can be released on immunological challenge from fragmented lung tissue, and some appear to be species specific. The sheer number of substances released as well as the rather sophisticated technology required to assay each substance, precludes the routine analysis of more than two or three substances. Consequently, many laboratories involved in the search for novel anti-allergic agents often measure either histamine or SRS-A or both. The selection of these two mediators is based on the observation that much of the body's histamine is sequestered within mast cells, the cells believed to play a major role in pathophysiological process. Thus, inhibition of histamine release by anti-allergic agents provides evidence that the molecule is altering the mast cell release process and hopefully, that the inhibition will extend to all substances emanating from this cell.

In vivo models

Rat passive cutaneous anaphylaxis (Ovary and Bier 1952) is the most widely used test for primary laboratory experimental system. Its principal advantages are that it is simple, rapid, economical and semiquantitative. Furthermore, the potential oral efficacy of compounds may be assessed at an early state in the pharmacological testing sequence. Major disadvantages are that it provides only circumstantial evidence as to the mechanism of action of the compounds and that efficacy is established in cutaneous tissue rather than in designated therapeutic site, pulmonary and tracheobronchial tissue.

Rat passive peritoneal anaphylaxis (Kalinin et al., 1972b)

Rat passive peritoneal anaphylaxis is mostly used when quantitation of the mediator release is desired.

Rat passive paw anaphylaxis (Martel and Ellicius, 1977)

This is an extremely simple technique. It depends upon the measurement of edema produced in rat hind-paw by antigen-antibody reaction. Here again, the permeability factors play a dominant role. Further, not individual mediators but the combined effect of all mediators is evaluated.

Various *in vivo* models of "allergic asthma" have been developed in several species to gain information on the potential efficacy of compounds in preventing antigen-induced bronchospasm. Since these models are considerably more complex than ICA or PPA, compound evaluation in these methods generally occupies the terminal step in the pharmacological testing sequence. Models have been developed in small animals such as the rat and guinea-pig, utilising either active or passive sensitization, most frequently with ovalbumin or dinitrophenol-ovalbumin conjugates (Stotland and Share, 1974; Framar et al., 1975; Armitage et al., 1952). Antigen may be presented parenterally for precipitation of systemic anaphylaxis or by an aerosol to induce pulmonary anaphylaxis. Pretreatment with an efficacious anti-allergic agent would be expected to reduce the severity of the response to antigen. The major advantages of these models are that the principal mediators of pulmonary anaphylaxis have been determined in both species (Stotland and Share, 1974; Framar et al., 1975; Armitage et al., 1952) and that the sensitized animals are easily and economically available in large numbers.

A major disadvantage of these models is that until recently, they have not allowed for the use of sophisticated technology for measurement of pulmonary mechanics

due to the small size of animals (Diamond and O'Donnell, 1977). Determination of the effects of antigen or drug-antigen combinations usually involve an indirect assessment of lung compliance rather than a true determination of resistance to air flow in large and small airways.

In large animals, principally dogs and rhesus monkeys with a spontaneously occurring hypersensitivity to an antigen prepared from *Ascaris suum* roundworms are used (Chakrin and Krell, 1978). An alternative to these actively sensitized models is passive sensitization of the airways of nonhypersensitive dogs and monkeys with high titer reaginic serum obtained from hypersensitive donors (Patterson and Kelly, 1974). Major disadvantages of large animal models include :

- 1) Animals with spontaneous positive bronchial antigen hypersensitivity, particularly rhesus monkeys are relatively scarce.
- 2) Antigen challenge can be no more frequent than every 2 weeks. Shorter intervals lead to highly erratic pathophysiological pulmonary responses. Thus, large numbers of animals are required to evaluate reasonable numbers of experimental compounds.

- 3) Pulmonary responses may be extremely variable, even in the same animal.
- 4) Large animal models are costly to develop and maintain routinely.
- 5) No clear insight has yet been gained regarding the biochemical pharmacology underlying the pulmonary pathophysiology. Therefore, unexpected results are difficult to understand.
- 6) Various pharmacological mechanisms other than inhibition of mediator release can account for a reduction in the response. For example, end-organ antagonism of preincent mediators and smooth muscle bronchodilator activity both reduce response severity.
- 7) Apparently, humans uniquely suffer from asthma. No case of allergic asthma in a non-human species has been reported. Therefore, while there is presently no alternative, these laboratory models may ultimately prove irrelevant to the clinical syndrome.

MATERIALS AND METHODS

Ammonium sulphate A.R.	Sarabhai Chemical Co., Baroda
Sodium chloride G.R.	---
Magnesium chloride, 6H ₂ O GR	---
Copper sulphate, 5H ₂ O GR	---
Pot.Sod. Tartarate, 6 H ₂ O	---
Sod.bi-carbonate	---
Sod.dihydrogen phosphate	---
Glucose	---
Pot.Chloride	---
Tris (hydroxymethyl) aminomethane	Sigma Chemical Co., St.Louis, U.S.A.
Bovine Serum Albumin (Cohn fraction V, Nitrogen content 15.3 %)	---
Adenosine 3',5'-cyclic phosphate (cAMP), M.W. 329.22	Sisco Research Laboratories, Bombay
Phenol Red G.R.	Lehn-chemie, Germany
Heparin Inj. I.P. (25000 I.U. in 5 ml)	---
Activated Charcoal	HDE, Glaxo Laboratories, Bombay
Sod. Carbonate AR	---
Theophylline	---
Folin-Ciocalteu reagent	---
Cal.Chloride, dihydrate GR	---
Atropine	---
Bordetella Pertussis	Haffkine Bio-Pharmaceutical Corporation Ltd., Bombay
Cromolyn Sodium	Fisons Pharmaceuticals Ltd., England
β -mercaptoethanol	B.Merck, Darmstadt, Germany
Anaesthetic ether	Industrial Solvents and Chemicals Pvt. Ltd., Bombay

Isolation of the enzyme phosphodiesterase

Preparation of reagents

Reagents:

1) Activated Charcoal,

2) 25 mM Tris-hydrochloride buffer -- 3.027 gm of Tris-hydrochloride in 1000 ml gives a 25 mM solution.

Prior to use, the activated charcoal (100 mesh) was treated with 0.1 N HCl by successive sedimentations, then washed repeatedly with water and neutralized by resuspending in several volumes of 25 mM Tris-HCl buffer, pH 7.5. The resuspension was done at least three times. The sediment was again washed with distilled water (pH 8.0). Then it was filtered through Whatman No.1 filter and dried at 90°C^{to} constant weight and stored at room temperature till use.

3) Tris-Hydrochloride buffer (50 mM) -- This was prepared by dissolving 6.055 gms of Tris-hydrochloride (M.W. 121.1) in 1000 ml of distilled water.

4) β -mercaptoethanol (3.75 mM) -- 292.91 mg of β -mercaptoethanol (M.W. 78.13) dissolved in 1000 ml of distilled water yielded 3.75 mM solution.

Procedure

Green et al. (1977) have described the method for the isolation of phosphodiesterase from human lung. Newman et al. (1974) have worked on rhesus monkey lung cAMP dependent phosphodiesterase. Hence a modified method of Newman et al. was used for the isolation of the enzyme.

Isolation

The process is mainly divided into

- 1) Homogenisation.
- 2) Ammonium Sulphate purification.
- 3) Charcoal treatment.

1) Homogenization :

Lungs from Rhesus monkey (*Macaca mulatta*, weight 1.5 to 2.0 Kg) were removed surgically and immediately frozen until used. The tissue was thawed and trimmed of excess fat and connective tissue. The tissue was homogenised using Cenco homogenizer in 3 $\times$ volumes of Tris hydrochloride buffer 50 mM, containing

β -mercaptoethanol 3.75 mM (pH 6.0). The crude homogenate was centrifuged on ^{Beckman} J-2W centrifuge at 100,000 $\times$ g for 30 minutes at 0°C.

2) Ammonium Sulphate ^{purification} :

The supernatant fraction of the above spin was stirred with ammonium sulphate in a concentration of 351 gms/litre for 60 minutes. The temperature was maintained at 2°C. After this, the solution was centrifuged at 100,000 x g for 30 minutes at 0°C. The pellet was redissolved in minimum quantity of Tris-hydrochloride buffer (50 mM containing 3.75 mM β -mercaptoethanol (pH 6.0). The temperature of the buffer was maintained at 4°C. This fraction was dialysed as described below against Tris-hydrochloride buffer (50 mM, pH 6.0). After 6 hours the fluid was changed and dialysed for 6 hours more.

Dialysis :

The enzyme solution was dialysed through dialysis tubing (Arthur H. Thomas Co., U.S.A.).

The appropriate dialysis tubing (No. 3787-B47, cut off value 3,500) was boiled in distilled water repeatedly to remove the incorporated salts and glycerol from the tubing. The tubing was then tied at one end and the enzyme solution to be dialysed was filled in it. The tubing was closed from the other end and placed in a beaker containing the dialysis buffer. The dialysis buffer

was continuously agitated with a magnetic stirrer at 4°C. After appropriate time the dialysis buffer was changed and the process continued as before. At the end of the dialysis the tubing was taken out and the enzyme was collected.

3) Charcoal treatment :

For removal of protein-bound adenine nucleotide charcoal treatment was carried out.

To the dialysed enzyme solution activated charcoal was added in a concentration of 20 mg/ml. The mixture was agitated intermittently for 15 minutes at 0°C. The charcoal was removed by centrifugation on Janetaki K-24 model at 500 x g for 40 minutes.

The supernatant of this spin was dialysed against distilled water (pH 7.7) for 6 hours. After this the enzyme was redialysed against distilled water pH 8.0 for 6 hours.

After dialysis the enzyme was stored in 1 ml aliquots at -20°C.

Enzyme parameters of Phosphodiesterase

Enzyme doubling :

To check whether any reaction is due specifically to an enzyme action or not, a parameter of the activity which is often studied is the relationship between the concentration of the enzyme and the reaction velocity.

To study this parameter various concentrations of the enzyme were added. The enzyme activity was then determined as described later in each case.

Optimum pH :

A characteristic parameter of an enzyme activity is its optimum pH i.e. the pH at which the enzyme activity is maximum. This parameter of the phosphodiesterase was also studied.

In this experiment, assay mixtures having various pH values ranging from 7.0 to 8.8 were prepared. The enzyme solutions were also made between the same pH range as that of assay mixtures, using dialysis against distilled water of the appropriate pH.

The enzymatic activity was measured and plotted against the pH. Since the reaction is, one of hydrolysis, it is dependant upon the pH of the environment. The non-enzymatic hydrolysis of the substrate was also simultaneously studied.

The pH, at which the enzyme activity was maximum with minimal non-enzymatic hydrolysis, was taken as the optimum pH for the enzyme activity.

Kinetics of the enzyme reaction - The Michelis-Menten constant:

The Michelis-Menten constant, or the K_m value for an enzyme activity is perhaps one of the most important parameters in the identification of the enzyme activity. The K_m value is that concentration of the substrate at which the reaction rate is maximum.

The determination consists of measuring the reaction velocities at various substrate concentrations. By plotting the reciprocal of the reaction velocity against, that of the substrate concentration, or the Lineweaver-Burke plot, the value of the Michelis-Menten constant can be calculated.

In this determination various substrate concentrations (50 μM to 500 μM) were used.

The enzyme activities were determined as described in all the above cases and the plot of the reciprocal of the reaction velocity against that of the substrate concentration was drawn. The negative intercept on the X axis is the reciprocal of the Michelis-Menten constant.

Protein Estimation by Folin Phenol Reagent (Lowry et al., 1951) :

Principle :

The method consists of measuring the colour produced by the reaction of peptide bonds with alkaline copper tartarate and between phenolic groups on proteins and the Folin-Ciocalteu reagent. The colour produced has a broad absorbance maximum around 660 nm, the wavelength at which this colour is measured.

Reagents :

1) Folin-Ciocalteu Reagent (EDH) - This was diluted 1:2 with water before use.

2) Alkaline copper tartarate solution - This was prepared fresh by mixing solutions A and B in the ratio of 1:50.

Solution A : A solution of 0.5 % hydrated copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and 1 % sodium potassium tartrate.

Solution B : A 2 gm % solution of sodium carbonate in 0.1 N sodium hydroxide.

3) Standard Protein Solution - This was prepared as a 10 mg % solution of Bovine serum albumin (Cohn Fraction V; Sigma) containing 15.3 % nitrogen by weight.

Procedure :

One ml of protein solution was treated with 5.0 ml of alkaline copper tartrate solution and shaken vigorously. After exactly 15 minutes 0.5 ml of the Folin-Ciocalteu reagent was added after dilution and the mixture shaken thoroughly. Thirty minutes later, the optical density of the mixture was measured at 660 nm, on a Spectronic 20 (Bausch and Lomb) colorimeter.

A standard curve was prepared by developing the colour in protein solutions containing graded concentrations

of protein (10 μ g to 100 μ g). Optical density was then plotted against the protein concentration, to get a standard graph.

The protein concentration determined as above was used to express all values as a function of the protein content of the enzyme, PDE.

Assay of Enzyme Activities

The enzyme assay was carried out on the crude and the partially purified fractions by the method of Frielle *et al.* (1979).

Principle :

This assay is based on the fact that the hydrolysis of cyclic nucleotides to the corresponding 5'-phosphate ester by phosphodiesterase makes available, an additional titrable proton of the 5'-phosphate group. By incorporating Phenol Red in the assay mixture, the rate of the proton production can be continuously measured by monitoring the decrease in absorbance of the basic chromophore of Phenol Red at 560 nm.

The primary advantages of the spectrophotometric assay used for assaying ADK inhibition by this method are:

- a) it provides initial velocity measurements, and thus is ideally suited for studying the kinetic properties of partially purified preparations of enzyme and,
- b) it does not require the tedious and time consuming purification of commercially available substrate which is often required when radioisotopic assays are used.

Materials

Reagents :

Preparation of stock solution - A 2.0 mM aqueous stock solution of phenol red was made and stored at 4°C for limited periods (1-2 weeks). The molar absorptivity of each new stock solution was determined by the titration of a 10 μ M solution of the indicator to pH 9.8 and measuring the absorbance at the 560 nm, absorption maximum. The value obtained was approximately $5.3 \times 10^4 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

2 mM Phenol Red solution : 35.438 mg of Phenol Red was dissolved in 50 ml to give 2.0 mM solution.

40 mM Sodium Chloride solution : 233.80 mg NaCl dissolved in 100 ml of Phenol Red (125 μ M) solution.

5 mM Magnesium Chloride solution : 101.6 mg of $MgCl_2 \cdot 6H_2O$ was dissolved in 100 ml of 125 μ M Phenol Red.

1.9 mM cAMP solution : 62.551 mg of cAMP dissolved in 100 ml of 125 μ M Phenol Red solution.

10 mM Theophylline solution : 18.02 mg of Theophylline dissolved in 10 ml of distilled water.

Glass distilled water was boiled for 10 to 20 minutes to make it Carbon-dioxide free, and then used.

pH of all solutions was adjusted to 8.0.

Procedure

The assay mixture consisted of 40 mM NaCl, 5 mM $MgCl_2$, 125 μ M Phenol Red and cAMP at a concentration of 1.9 mM. The pH was adjusted to 8.0. The total reaction volume was 1.0 ml. The reaction was initiated by the addition of phosphodiesterase (67.5 μ g). Theophylline was used as a known standard inhibitor of the enzyme. The drugs to be tested were added in the reaction mixture before the addition of PDE. Blanks were run simultaneously. All reactions were carried out at 22-24°C. The pH of the standard drug and test-drugs was adjusted to 8.0. The continuous titration was recorded by measuring the decrease in absorbance at 560 nm. All assays were performed in triplicate.

Methodology of anti-allergic studies:

Preparation of IgE antibodies

Female albino rats (Wistar strain) weighing between 100-200 g were injected with egg albumin (10 mg/mg) intra-muscularly and Bordetella Pertussis organisms (2×10^{10}) intraperitoneally. After 12 days the animals were anaesthetised using anaesthetic ether. The blood was collected by cardiac puncture. The serum was collected from individual

rats and pooled. The presence of IgE antibodies was tested by the method described by Ovary and Bier (1952) which is as follows:

Testing of IgE antibodies

Passive cutaneous anaphylaxis reactions were produced by the technique of Ovary and Bier (1952). The backs of the albino rats (Wistar) were shaved with an electric hair clipper, care being taken to avoid irritation. Usually, three intradermal injections of 0.1 ml antiserum were made on each side of dorsal skin, about 1.5 cm from the midline, with a sharp 26-gauge short bevel hypodermic needle. Twenty-four hours later, the animals were injected intravenously with 1 ml of Evans Blue (25 mg/kg) in saline (0.85 % NaCl) containing antigen (25 mg/kg). Twenty-five to 30 minutes after antigen injection, the animals were killed, the skin was reflected and the lesion diameter was measured on the inner surface of the skin with a transparent scale. The presence of IgE antibody was established by destruction on heating (56°C for 4 h) and by persistence of the PCA reaction upto at least nine days.

Rat passive peritoneal anaphylaxis

Rat anti-egg albumin serum was diluted 1:2 with 0.15 M saline (0.85 % NaCl). One ml of this diluted serum was injected into the peritoneal cavity of male rats weighing 150 to 200 gms. After a latent period of two hours the animals were challenged intraperitoneally with 2 mg of egg-albumin in 5 ml of heparinized (50 µg/ml) tyrode solution. The animals were sacrificed after 5 minutes, the abdomen was opened and the peritoneal fluid was collected.

The peritoneal fluid was centrifuged at 1000 g for 5 minutes. The supernatant was assayed for histamine content on atropinized isolated guinea-pig ileum.

Testing of drugs

Drugs tested for phosphodiesterase activity were screened for anti-allergic activity by using rat passive peritoneal anaphylaxis. All drugs in the volume of 1 ml were injected intraperitoneally (10 mg/ml) one minute prior to the administration of antigen.

The drugs were tested for their anti-histaminic activity if any, on atropinized guinea-pig ileum.

Assay of Histamine release

A guinea-pig (*Cavia porcellus*) was killed by a blow on the head and the throat was cut. The abdomen was opened and the ileum lifted out. A piece of the ileum, about 5 cm long, was removed from the region about 6-8 cm away from the caecal end, and placed in a dish containing Tyrode solution (Na^+ , 149.2 mM; K^+ , 2.7 mM; Ca^{++} 3.6 mM; Mg^{++} 2.1 mM; Cl^- 145.3 mM; H_2PO_4 0.4 mM; HCO_3 11.9 mM; Glucose 5 mM) previously warmed to 37°C.

Atropine (10 µg/ml) was added to the Tyrode solution. The ileum was then gently flushed with warm Tyrode (37°C) and the mesentery cut. A piece about 3 cm long was then cut off and tied with threads antidiagonally and mounted in an organ bath containing Tyrode solution through which air was bubbled continuously. The preparation was allowed to equilibrate for 30 minutes, with changes in solution after every 10 minutes, before the start of the experiment.

Responses were recorded to standard concentrations of Histamine (10-60 ng/ml) using a frontal writing lever (Magnification $\times 10$; load = 0.5 g) on smoked kymographic paper. Responses of peritoneal fluid content were also taken similarly.

RESULTS

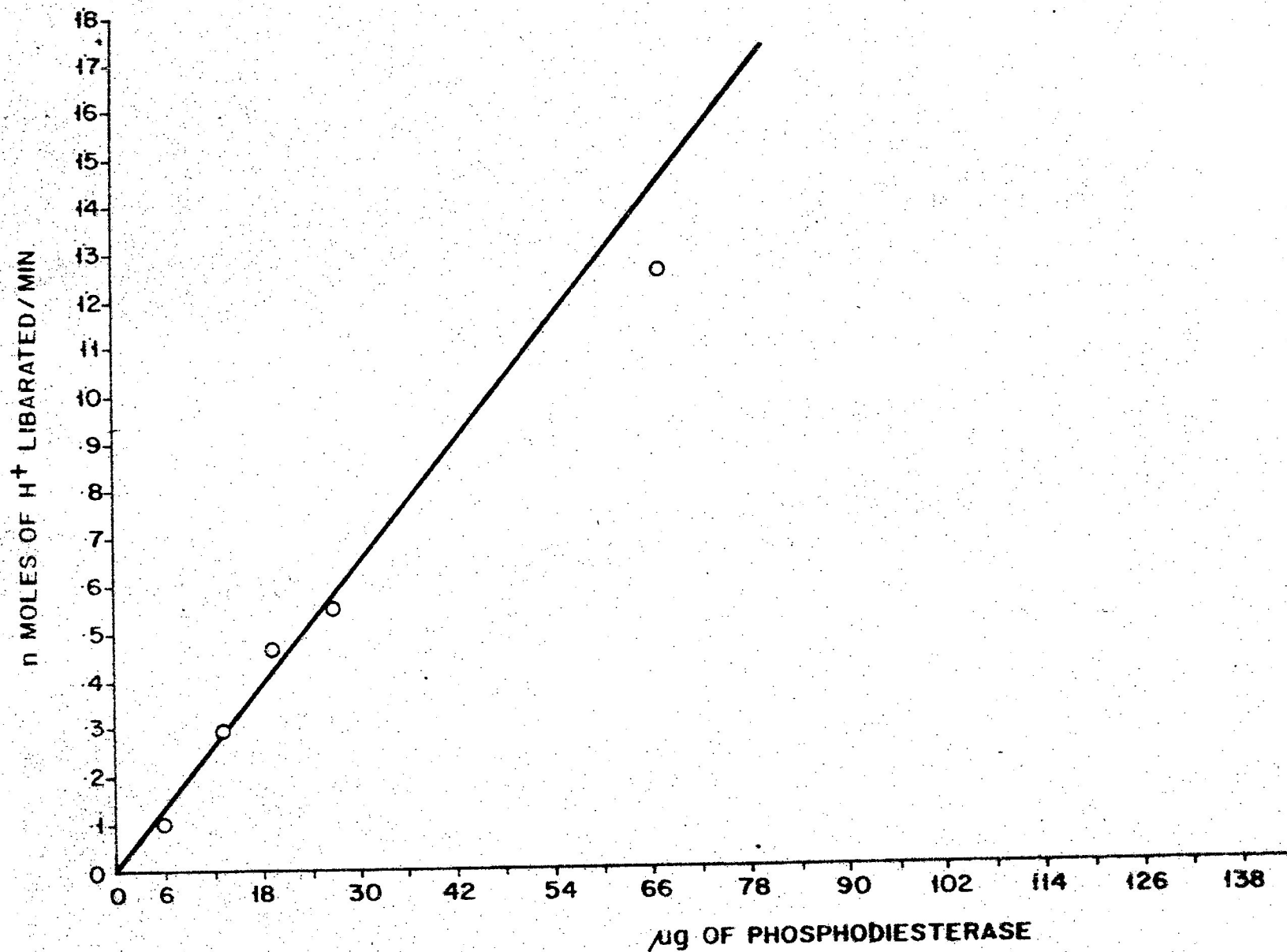
The protein content as estimated by Lowry's method, of the supernatant fraction after first spin at 100,000 g was 7.7 mg/ml. While that of the final sample was 2.25 mg/ml. The specific activity of 100,000 g spin was 7.79 n moles of cAMP hydrolysed/min/mg of protein, while that of final enzyme preparation was 14.817 n moles of cAMP hydrolysed/min/mg of protein.

The hydrolysis of cyclic AMP by the enzyme phosphodiesterase was found to be linear for 45 minutes. The effect of various enzyme concentrations on the hydrolysis is shown in (Graph No. 1). It is evident from it that the rate of hydrolysis proportionately increases with the increase in enzyme concentration.

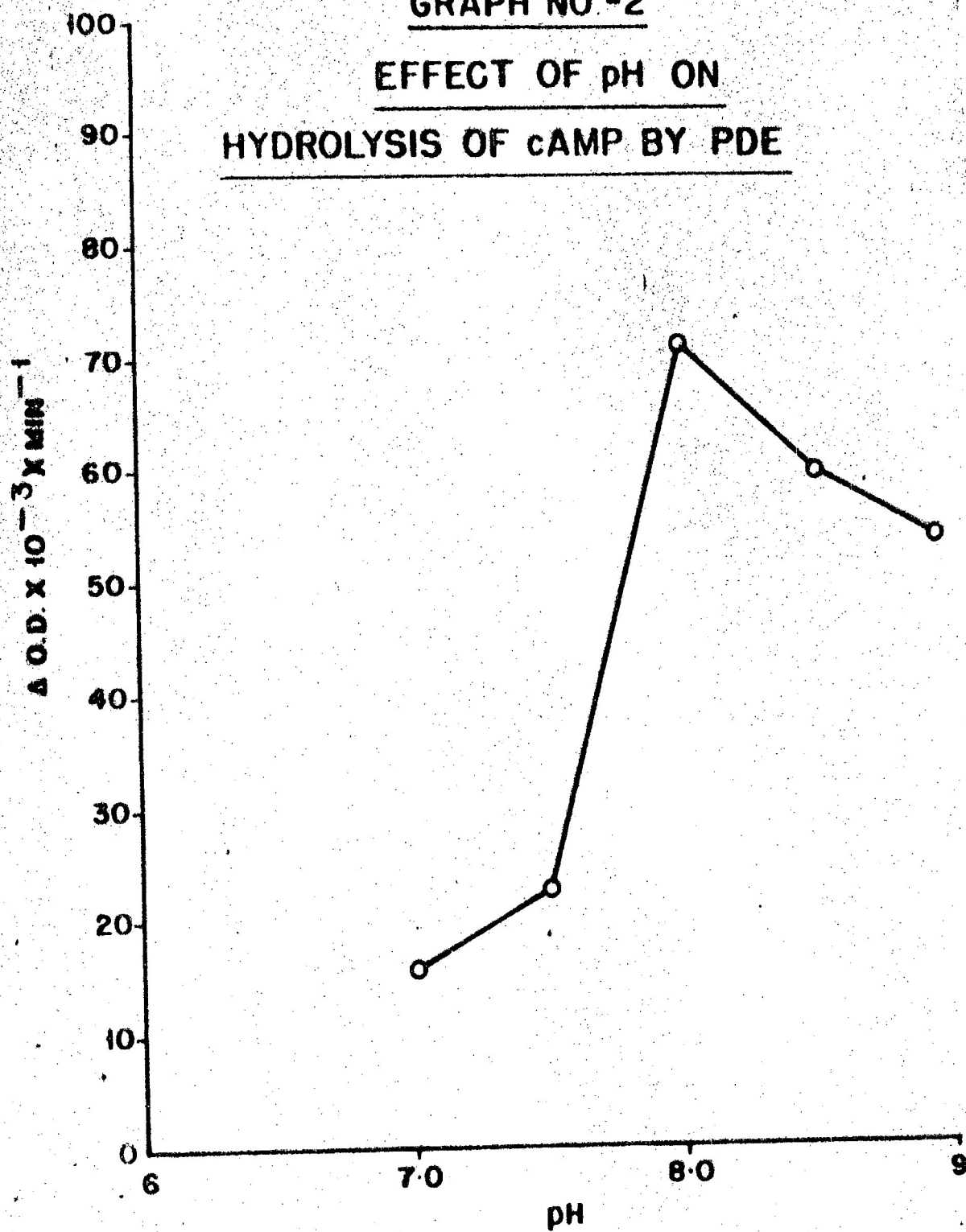
The effect of changes in pH on the assay system is shown (Graph No. 2). The pH range selected was between 7.0 to 8.8. It is clear from the nature of the graph that rate of hydrolysis of cAMP increases with increase in pH till pH 8.0 and then decreases. Hence optimum pH of the enzyme is 8.0.

The inhibition pattern by theophylline, a potent phosphodiesterase inhibitor was studied (Graph No. 3). A 0.1 mM solution of theophylline inhibited 29.47 % and

GRAPH NO.-1
EFFECT OF PDE CONCENTRATION ON cAMP HYDROLYSIS
EFFECT OF ENZYME CONCENTRATION

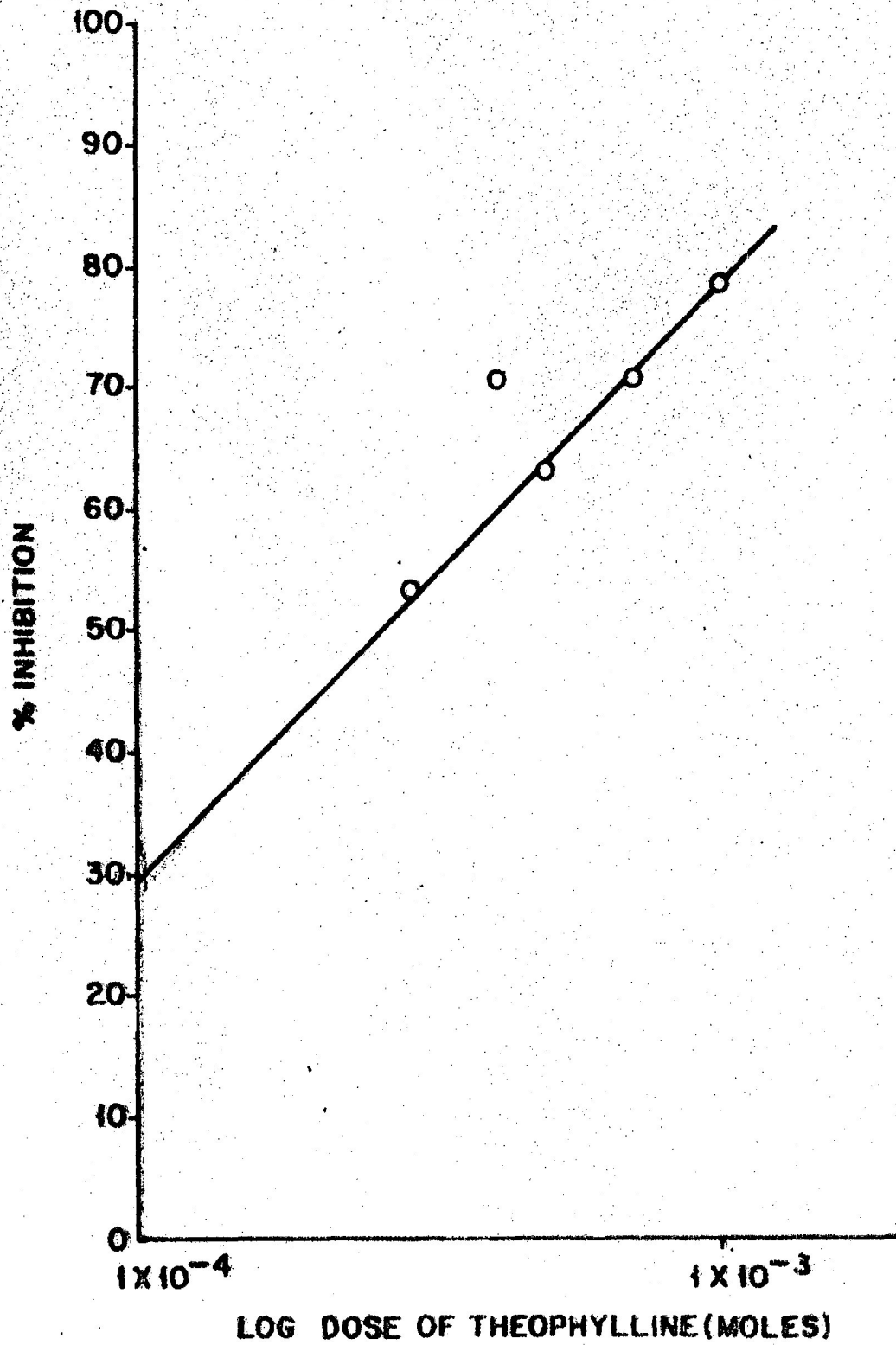


GRAPH NO -2
EFFECT OF pH ON
HYDROLYSIS OF cAMP BY PDE



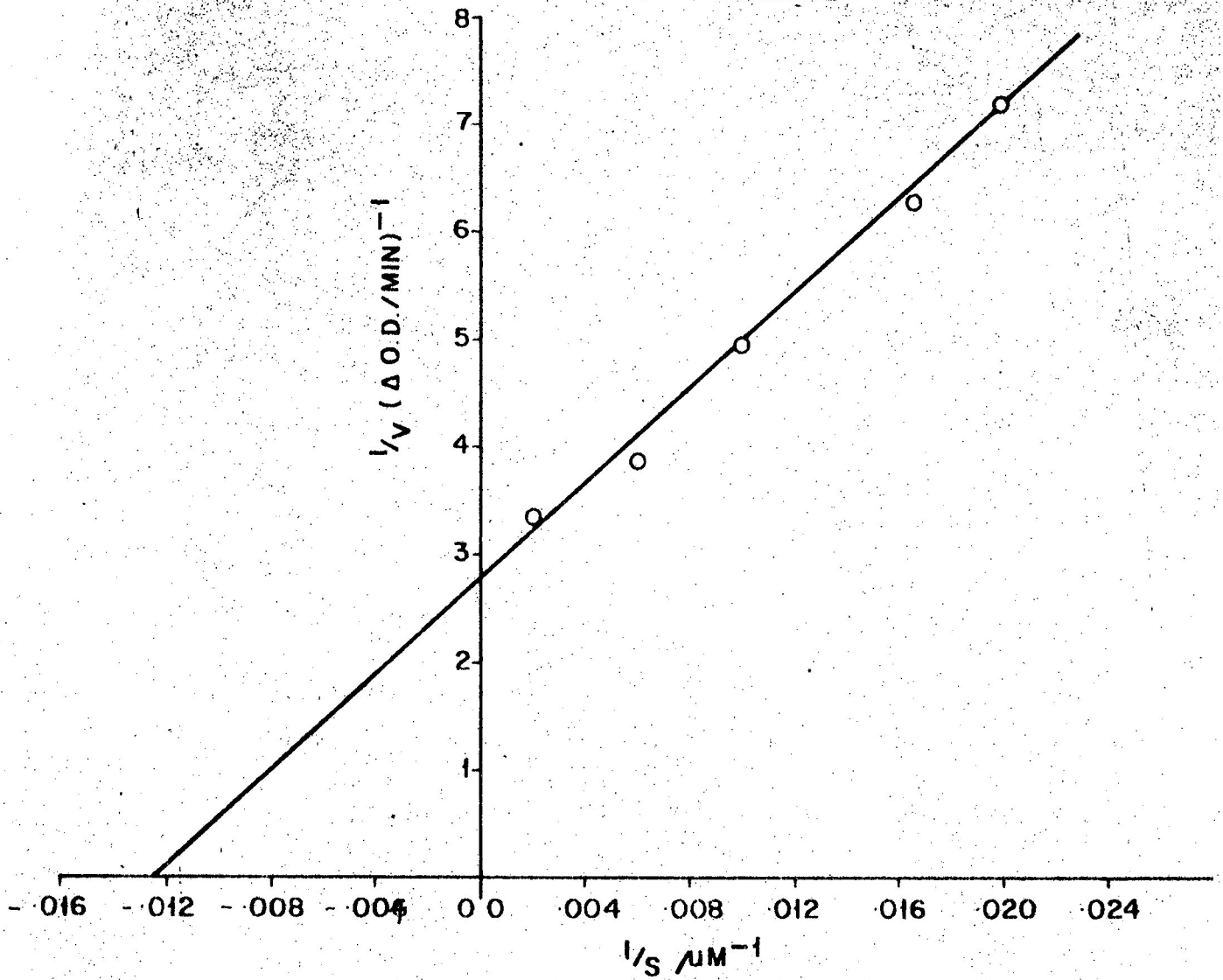
GRAPH NO.-3

% INHIBITION OF PDE BY THEOPHYLLINE



GRAPH NO-4

LINEWEAVER-BURKE PLOT FOR PDE FROM
RHESUS MONKEY LUNG



0.5 mM 63.11 %, the hydrolysis. The highest concentration (1×10^{-3} M) of theophylline produced 78.85 % inhibition of the hydrolysis of cAMP.

The Michaelis-Menten constant (K_m) for the hydrolysis of cyclic AMP by rhesus monkey lung phosphodiesterase was observed to be 80 μ M. The value was calculated by using the Lineweaver-Durke Plot (Graph No. 4).

DRUGS

The following table indicates the effect of test compounds on the enzyme phosphodiesterase obtained from rhesus monkey lung and their anti-allergic activity, using rat passive peritoneal anaphylaxis. None of the compounds had any anti-histaminic activity per se.

	% inhibition of PDE		% inhibition of PPA
	0.1 mM	0.5 mM	
1) Sod. salt of 2-carboxylic chromone	30.36		98.6
2) Sod. salt of 3-hydroxy chromone		60.68	22.14
3) Sod. salt of 6-methyl, 3-hydroxy chromone		43.68	43.05

	% inhibition of PDE		% inhibition of PFA
	0.1 mM	0.5 mM	
4) 2-Hydroxy-1H-naphtho [2,1-b]pyran-1-one		59.15	-28.29
5) 3-propargyloxy- chromone	21.19		-33.21
6) 6-methyl chromone	37.38		6.15
7) 8-methyl chromone	30.605		24.6
8) 7-methoxy, 2-styryl isoflavone	39.698		45.51
9) 5,7-dimethoxy, 2-styryl isoflavone	70.43	71.133	41.82
10) 2,8-dimethyl, 4,6-dioxo-3,5,7,10H- [1,2-b:5,4-b'] dipyran	42.30		54.12
11) 4,6-dioxo-2,3,5,7,8, 10H-benzo [1,2-b:5, 4-b']dipyran	82.61		31.98
12) 2,4 diacetyl Resorcinol	63.245		49.2
13) 2(β-naphthoxy)dimethyl fumarate	44.11		81.98

	% inhibition of PDE		% inhibition of PFA
	0.1 mM	0.5 mM	
14) 3,7-dialdehyde, 4,6-dioxo-2,5,8, 10H-benzo[1,2-b:5, 4-b']dipyran	46.72		45.51
15) p-4-methoxy-N [4'-oxo-4H(1) benzopyran-3'- -formylidene] aniline		18.561	103.32
16) p-4-methoxy-N[4'-oxo- 6-methyl-4H(1) benzopyran-3'-formyl- lidene]aniline		35.69	100.86
17) 3-[2-hydroxy-5-methyl-56.69 phenyl]-3-oxo-1- [1-piperidyl] prop-2-ene	56.57		18.53
18) 3-[2-hydroxy-phenyl]- -3-oxo-1-[1-piperidyl] prop-2-ene	7.42		24.6
19) 2-(3',4'-dihydroxy- phenyl)oxymethyl-8- 7-dihydroxy-4-oxo- 4H(1)benzofuro (3,2-b)pyrone	100	100	100

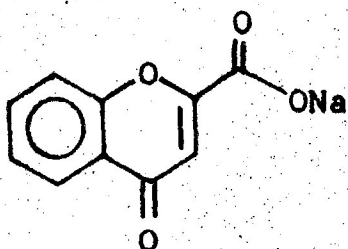
60 % inhibition or more in phosphodiesterase studies was considered active.

70 % inhibition in anti-allergic experiment was considered to be active.

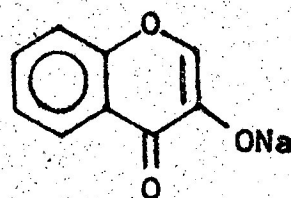
* sign indicates the increase in Histamine content

All percentage inhibition figures have been calculated with reference to the activity 2-(3',4'-dihydroxy-phenyl)oxymethyl-8-7-dihydroxy-4-oxo-4H(1)benzofuro (3,2-b)pyrone, which is considered 100%.

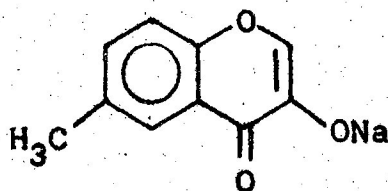
DRUGS TESTED FOR PDE-INHIBITION AND
ANTIALLERGIC ACTIVITY.



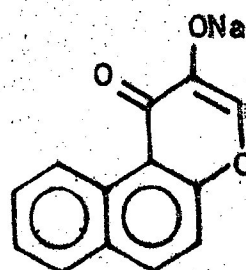
7 MCCS/GSN
Sod. salt of 2-carboxylic
chromone.



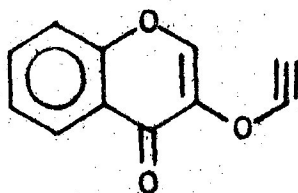
GSN-B-1
Sod. salt of 3-hydroxy
chromone



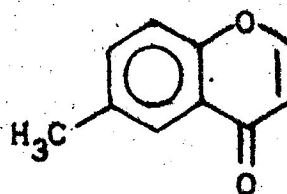
GSN-B-2
Sod. salt of 6-methyl,
3-hydroxy chromone.



GSN-B-4
2-Hydroxy-1H-naphtho(2,1-b)-
Pyran-1-one.

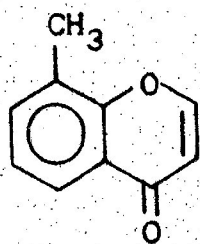


GSN-B-5
3-Propargyloxy chromone



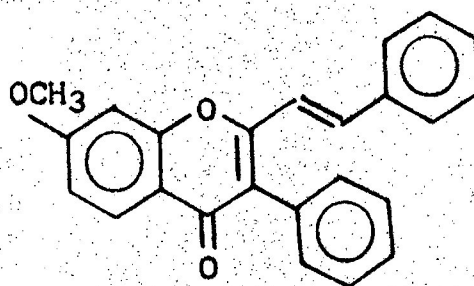
GSN-B-11
6-Methyl chromone

CONTD...



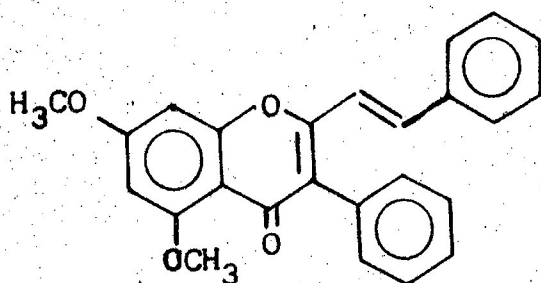
GNS-B-12

8-Methyl chromone



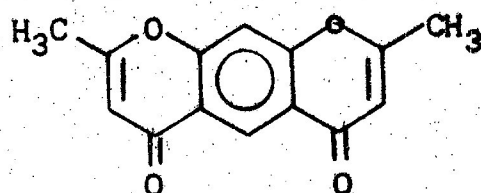
GNS-B-14

7-Methoxy,2-styryl isoflavone.



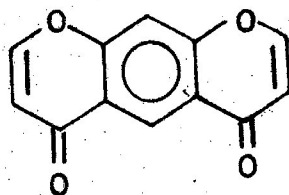
GNS-B-17

5,7-Dimethoxy,2-styryl-
isoflavone



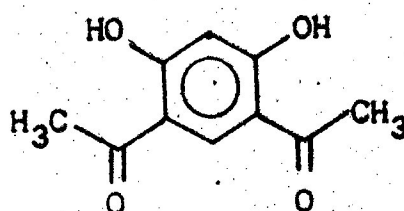
GNS-BV-35

2,8-Dimethyl-4-6-dioxo-
3,5,7,10-H-benzo-(1,2-b ;
5,4-b)- dipyrans.



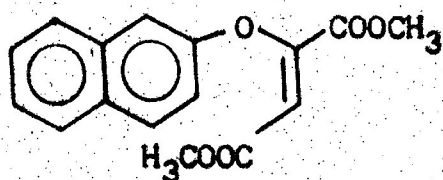
GNS-BV-36

4,6-Dioxo-2,3,5,7,8,10-H-
benzo-(1,2-b: 5,4-b:)-
dipyrans.

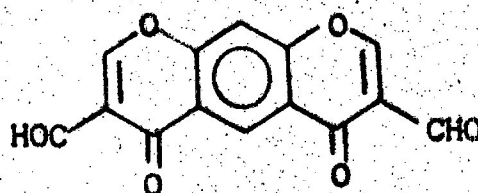


GNS-BV-37

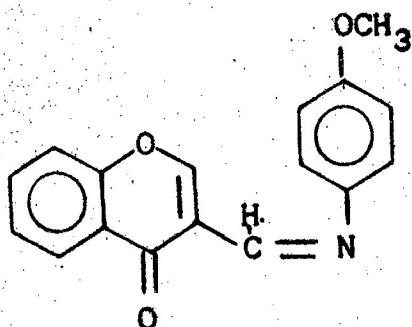
3,4-Diacetyl resorcinol



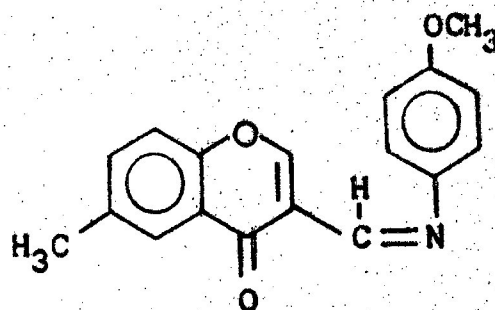
GSN-BV-38
2-(β -Naphthoxy)-dimethyl-
Fumarate.



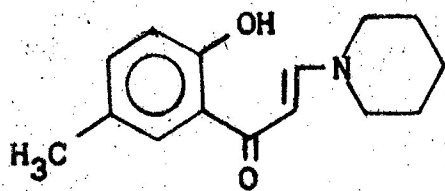
GSN-BV-39
3,7-Dialdehyde-4,6-dioxo-
2,5,8,10-H-Benzo(1,2-b; 4,5-b)-
Dipyrane



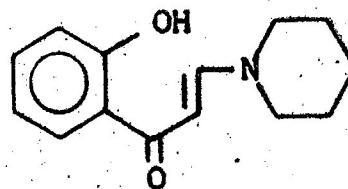
GSN-C-2
p-4-Methoxy-N-(4'-oxo-4H(1)-
benzopyran-3'-formylidene)-
Aniline.



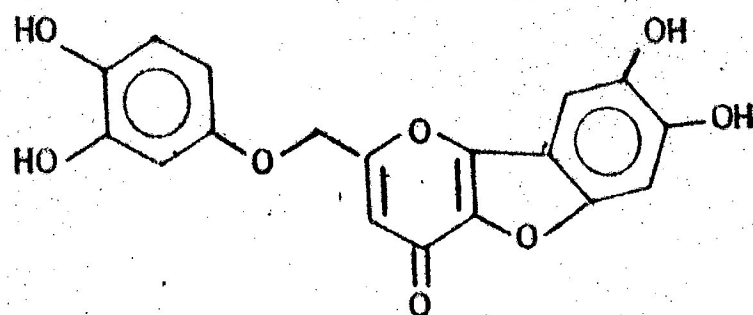
GSN-C-4
p-4-Methoxy-N-[4'-oxo-6-
methyl-4H(1)benzopyran-3'-
formylidene]-Aniline.



GSN-C-5
3-[2-Hydroxy-5-methylphenyl]-
3-oxo-1-[1-piperidyl]-prop-2-ene.



GSN-C-7
3-(2-Hydroxy-phenyl)-3-
oxo-1-(1-piperidyl)-
prop-2-ene.



(KR/NVS/E)

2-(3',4'-Dihydroxyphenyl)-oxymethyl-8,7-dihydroxy-
4-oxo-4H(1)-benzofuro(3,2-b)-pyrone.

For phosphodiesterase inhibition, theophylline (1,3-dimethyl xanthine, M.W. 180.2) was used as a standard drug, while Cromolyn sodium [disodium salt of 1,3-bis(2-carboxychromon-5-yloxy)-2-hydroxy propane] was considered as the reference compound for anti-allergic activity. Compound 2-(3',4' dihydroxy phenyl)oxymethyl-8-7-dihydroxy-4-oxo-4H(1) Benzofuro (3,2-b) pyrone, has shown significant activity. All results listed in the table are expressed with reference to the activity of the same. Cromolyn sodium showed 8 % anti-allergic activity, when used at a dose of 10 mg/kg 4,6-dioxo-2,3,5,7,8,10H, benzo [1,2-b:5,4-b'] dipyran, 5,7-dimethoxy 2-styryl isoflavone, 2,4-diacetyl resorcinol, Sodium salt of 3-hydroxy chromone, and 2-Hydroxy-1H-naphtho[2,1-b]pyran-1-one have promising phosphodiesterase inhibitory property. 3-[2-hydroxy-phenyl]-3-oxo-1-[1-piperidyl]prop-2-ene have lowest phosphodiesterase inhibitory action.

In anti-allergic activity, p-4-methoxy-N[4'-oxo-4H(1)benzopyran-3'-formylidene]aniline, its methyl derivative at position 6 of benzopyran moiety, sodium salt of 2-carboxy chromone and 2-(β -naphthoxy) dimethyl fumarate show promising anti-allergic activity. Sodium salt of

3-hydroxy chromone, 2-Hydroxy-1H-naphtho(2,1-b)pyran-1-one and 3-propargyloxy-chromone are not only devoid of anti-allergic activity, but increased histamine release from mast cells.

From the results of inhibition of rhesus monkey lung cyclic AMP phosphodiesterase and inhibition of immunologically released histamine release from rat mast cells, the compounds can be divided into two major groups, considering the highest activity.

Group one, consists of compounds having good activity in both systems 2, (3',4'-dihydroxyphenyl) oxyethyl-8,7-dihydroxy-4-oxo-4H(1)benzofuro (3,2-b) pyrone, 4,6-dioxo-2,3,5,7,8,10H-benzo(1,2-b:5,4-b') dipyrans and 5,7-dimethoxy, 2-styryl isoflavone.

Group second, consists of all other compounds which do not have significant inhibitory activity in both the systems.

DISCUSSION AND SUMMARY

From the results, the order of the drugs having anti-allergic activity and phosphodiesterase inhibitory property is as follows:

2-(3',4'-dihydroxyphenyl)oxymethyl-6,7-dihydroxy-4-oxo-4H(1)benzofuro(3,2-b)pyrene; 4,6-dioxo-4H,6H-benzo [1,2-b:5,4-b']dipyran; 5,7-diethoxy, 2-styryl isoflavone; 2,4 diacetyl resorcinol; sodium salt of 3-hydroxy chromone; sodium salt of 2-hydroxy-1H-naphtho [2,1-b]pyran-1-one.

3-[2-hydroxy-phenyl]-3-oxo-1-[1-piperidyl]

prop-2-ene has shown the lowest phosphodiesterase inhibitory property and low anti-allergic activity. Introduction of methyl group at position 5 of the phenyl ring of this compound increased PDE-inhibitory property but decreased anti-allergic activity. Cyclisation of 2-hydroxy group with adjacent carbon atom yielding the compound 6-methyl chromone, decreased both. Shift of methyl group from position 6 to 8 causes further decrease in PDE-inhibition, but slight increase in anti-allergic activity. Removal of methyl group with introduction of carboxy group at 2 position produces no change in PDE-inhibitory action, but promising anti-allergic activity. Removal of carboxy group with incorporation of hydroxy group at 3 position increases PDE-inhibitory property to a significant extent, but

produces no increase in anti-allergic activity. On the contrary, it enhances histamine release from immunologically stimulated mast cells. Etherification of this compounds decreased PDE-inhibition, causing maximum release from mast cells. Introduction of methyl group at 6 position to 3-hydroxy chromone increased PDE-inhibitory property to a greater extent than the increase due to etherification, but to a lesser extent than the unsubstituted derivative, the anti-allergic activity being moderate.

Substituting hydroxy group by methoxybenzylidene moiety to yield, p-methoxy-8[4'-oxo-6-methyl-4H(1) benzopyran-3'-formylidene]aniline, does not changes PDE-inhibition, but increases significantly anti-allergic activity. Removal of methyl group of position 6, decreases slightly PDE-inhibition, but increases anti-allergic activity slightly.

Substitution in chromone nucleus at 2,3 and 7 position, yielding 7-methoxy,2-styryl isoflavone, which has moderate activity in both systems. Incorporation of an additional methoxy group at position 5 increased both PDE-inhibitory activity as well as anti-allergic activity.

Considering the γ pyrone part of the chromone moiety as an important pharmacophore, another γ -pyrone has been fused within the nucleus to get 4,6-dioxo-2,3,5,7,8,10H benzo $\left[1,2\text{-}b';5,4\text{-}b'\right]$ dipyrans, which has excellent PDE-inhibition with promising anti-allergic activity. Further addition of methyl groups at 2 and 8 position respectively causes slight decrease in PDE-inhibition and slight increase in anti-allergic activity. Incorporation of aldehyde groups instead of methyl groups at positions 3 and 7, yields compounds that have similar PDE-inhibitory activity, and anti-allergic activity intermediate to that of the unsubstituted parent compound and of the methyl derivative.

From the results it is evident that 2-(3',4'-dihydroxyphenyl)oxymethyl-8,7-dihydroxy-4-oxo-4H(1) benzofuro (3,2-b) pyrone; 5,7-dimethoxy 2 styryl isoflavone; 4,6-dioxo-4H, 6-H-benzo $\left[1,2\text{-}b';5,4\text{-}b'\right]$ dipyrans have both significant phosphodiesterase inhibitory property as well as anti-allergic activity.

Possible reasons for the failure
of the correlation

For the study of the inhibition of rhesus monkey lung phosphodiesterase, the enzyme isolated was cytoplasmic in location, i.e., an intracellular enzyme is used. ^{In} This system the drug comes directly in contact with the enzyme, while in the *in vivo* system it has to pass membrane barriers. In the *in vivo* system, solubility, lipophilicity, systemic absorption, and various other factors play an important role. Most of the chromone-derivatives are insoluble in aqueous ^{medium}, hence the possibility of the drug not coming in contact with the intracellular enzyme can not be ruled out. Due to high lipophilicity, there is a chance of a high percentage of the drugs binding to lipo-proteins, forming insoluble complexes. In case of soluble drugs, the systemic absorption may be high. Due to this, the drug may undergo various modifications such as conjugation, or biotransformation so as to make the drug inactive after absorption. Davoren and Sutherland (1963) reported that cyclic AMP is secreted by cells and this process may involve an active transport. The effect of test-drugs on this may have an important role in deciding its *in vivo* activity.

The effect of these drugs on calcium ions will also be a critical factor in determining their effect on ADP in *vivo*. The relationship between the phosphodiesterase activator and calcium ion had been firmly established now (Kakinuchi and Yamasaki, 1970; Kakinuchi et al., 1973; Teo and Wang, 1973; Teshima and Kakinuchi, 1974; Wolf and Brostrom, 1974; Wickson et al., 1975). Recently, a heat-stable, calcium-dependent protein, originally shown to increase phosphodiesterase activity (Cheung, 1970a, b) has been demonstrated to activate adenylate cyclase as well (Brostrom et al., 1975; Cheung et al., 1975).

Pharmacological agents can differentially inhibit the various isozymes of phosphodiesterase (Usunov et al., 1974; Weiss et al., 1974; Weiss, 1975b; Fertel and Weiss, 1976). The inhibition of isozymes of phosphodiesterase may have a significant role in *in vivo* studies. There are distinct phosphodiesterase isozymes associated with a particulate cell fraction (Thompson et al., 1973; Russell et al., 1972) and it is possible that these isozymes are also differentially responsive ^{to} drugs affecting phosphodiesterase.

A role for cyclic AMP in physiological and biochemical events has now been well established. Several of the actions of cyclic GMP appear to be opposite of those of cyclic AMP. These observations are akin to the so-called yin-yang hypothesis (Goldberg *et al.*, 1975). Hence the effects of test-drugs on the enzyme guanylate cyclase (E.C.4.6.1.2), responsible for synthesis of cGMP and cGMP phosphodiesterase causing hydrolysis of cGMP will also throw additional light on the anti-allergic activity of these drugs.

Besides acting on the nucleotide cyclases or phosphodiesterases, test-drugs could have also altered the effects of cyclic AMP by preventing the interaction of cyclic AMP with its receptor. Cyclic AMP appears to exert most of its effects by activating protein kinase (Langan, 1973). Since protein kinase may exist in several different molecular forms (Reisann *et al.*, 1971; Rubin *et al.*, 1972; Labrie *et al.*, 1971) it may be possible that the test-drugs have differential inhibition patterns, as shown for the phosphodiesterases, and hence they may be exerting varying influence on mediator release.

In the evaluation of anti-allergic activity histamine release during the anaphylaxis^{is} studied. The selection of histamine is based on the observation that much of the

body's histamine is sequestered within mast cells, the cells believed to play a major role in the pathophysiological process. Thus inhibition of histamine release by anti-allergic drugs provides evidence that the molecule is altering the mast cell release process and, hopefully, that the inhibition will extend to all substances emanating from this cell. Histamine secretion from mast cells may be inhibited by elevated intracellular levels of cyclic AMP and by several anti-allergic drugs (Berridge, 1975; Garland *et al.*, 1980; Pepys *et al.*, 1979; Ennis *et al.*, 1981). These compounds are claimed to act directly on the calcium-gating mechanism activated by the anaphylactic reaction, preventing influx of Ca^{++} from the external environment and so blocking exocytosis (Foreman *et al.*, 1977a, b; Foreman *et al.*, 1975; Fowtrel and Gomperts, 1977). The test-compounds might modulate the calcium signal by activating membrane pumps to extrude the cation from the cytosol or promote sequestration of the ion into internal stores, as does cyclic AMP. The anti-allergic compounds have been shown to prevent the accumulation of radiolabelled calcium by stimulated mast cells (Foreman *et al.*, 1977a,b). This effect was attributed to a decreased influx of the ion, but is equally well explained by an increased efflux

of the cation. Accumulation studies alone will not distinguish between these possibilities (Berridge, 1975). Alternatively, the drugs might have effects not immediately related to calcium homeostasis and might promote disaggregation of microtubules (Gillespie and Lichtenstein, 1972) involved in the phosphorylation of proteins in the plasma or perigranular membranes and so prevent the fusional changes involved in exocytosis (Sullivan and Parker, 1976; Theoharides et al., 1980) or have an as yet ill defined stabilizing effect on the mast cell membrane.

Besides calcium ions, the biochemical requirements for the immunological release of mediators consist of an intact glycolytic pathway and the activation of a serine esterase from its precursor state (Kaliner and Austen, 1975). The effect of test-drug on these might have played an important role in inhibition of histamine release from mast cells.

The reaction of mast cell membrane bound antibody with antigen leads to sphingolipid clustering, and this clustering is prevented by anti-allergic drugs of chromone type. Since it has been suggested that these drugs act by preventing Ca^{++} influx into the cell, it seems possible that the glycosphingolipid clusters may

be associated with the calcium gate (Curtain *et al.*, 1981). Hence, degree of inhibition of sphingolipid clustering by test-drugs will also reflect on the anti-allergic activity.

Though Coulson *et al.* (1977) have shown the inter-relationship of cyclic nucleotides and anaphylactic reactions, some other investigators have studied phosphodiesterase inhibition for studying mechanism of drug action (Tateson and Trist, 1976; Bagli *et al.*, 1979; Georgiev *et al.*, 1979; Miller *et al.*, 1980; Jarvest and Love, 1980; Glenon *et al.*, 1981a,b).

SUMMARY

As discussed earlier, phosphodiesterase inhibition may be studied to evaluate the anti-allergic activity of synthetic drugs. It will also focus light on the mechanism of action of the drugs. From our studies sodium salt of 2-carboxylic cromone and 2-(3',4'-dihydroxyphenyl)oxymethyl-8,7-di hydroxy-4-oxo-4H(1)benzofuro (3,2-b)pyrone have shown promising anti-allergic activity as tested by rat passive peritoneal anaphylaxis. The first compound has no promising activity as a phosphodiesterase inhibitor. The later showed excellent activity. Therefore, further work on this compound as an anti-allergic drug is desirable.

Chromones in general showed phosphodiesterase inhibitory activity suggesting further work on this series of compounds.

Model derivatives of 5,7 dimethoxy, 2 styryl isoflavone and 4,6-dioxo-4H, 6H-benzo[1,2-b:5,4-b']dipyran may be synthesized and tested. It is interesting to note that the later compound is a fused Benzodipyran. Further work on this type of compounds may be rewarding.

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SYNOPSIS

S Y N O P S I S

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Name of Guiding Teacher : Dr. Bhide, M.B.
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Subject : STUDY OF ANTIALLERGIC DRUGS IN
RELATION TO PHOSPHODIESTERASE
ACTIVITY.

Anaphylaxis is an interaction of the cell bound immunoglobulins IgE with the specific antigen or hapten which lead to the release of mediators of immediate hypersensitivity to cause vasodilation, smooth muscle contraction and alteration of vascular permeability. ¹

A vast number of chemical substances are known to be released during experimental anaphylaxis, important among these are histamine, serotonin, prostaglandin ² SRS-A, bradykinin and permeability factors.

One of the earliest attempts showed that adrenaline inhibited the anaphylactic release of histamine from guinea-pig lung.³ This observation was confirmed and extended by Lichtenstein and Margolis,⁴ who reported that isoprenaline, adrenaline, methyl xanthines and dibutyric cyclic AMP inhibited allergic release of histamine.

It has become clear that stimulatory effects of catecholamines on adenylyl cyclase in many tissues are mediated by typical β -adrenergic receptors.⁵ It is now accepted that β -stimulants inhibit histamine release by elevating the intra-cellular levels of cAMP.⁶

The enzyme, phosphodiesterase plays the role of breaking down cyclic AMP to its inactive metabolites.



In general, phosphodiesterases can be considered to embrace all these enzymes which hydrolyse the bond between phosphate and one or the other of two molecules possessing alcoholic functional groups which it links.⁷

Theoretically any inhibition of the enzyme phosphodiesterase would lead to an increase in intracellular cAMP concentrations and thus inhibit a histamine release.

This mechanism holds good for a large number of drugs, which inhibit phosphodiesterase.¹

The present approach towards the development of anti-asthmatic agents is therefore directed towards development of

- a) drugs which stimulate adenylyl cyclase;
- b) drugs which inhibit phosphodiesterase.

Studies on intracellular cAMP concentrations and effects of adrenergic drugs suggested a close parallelism in intracellular cAMP concentration and effects of the drugs. It was suggested by Sutherland and his co-workers that adrenergic agonist might act by stimulating the adenylyl cyclase enzyme and the effects are mediated through Beta-receptors.⁵ Working on this basis, a number of laboratories are actively pursuing the first type of drugs and a wide variety of drugs are being presently tested for their effects on β -receptors.¹

Since the elucidation of mechanism of hydrolysis of cAMP, systemic exploitation of phosphodiesterase inhibitors for potential antianaphylactic agents has been going on. Therefore an attempt was made to screen certain new compounds for their phosphodiesterase inhibition in relation to their anti-anaphylactic activity.

Phosphodiesterase studies :

The enzyme phosphodiesterase is found virtually in all living cells, its relative activity varying greatly among different tissues.⁸ Different tissues and cell types have their own unique pattern of the multiple forms of phosphodiesterase.⁹ Bronchial^a asthma being a disease of the respiratory tract it was essential to study the lung phosphodiesterase as effects on this enzyme would be more pertinent to the disease. The rhesus monkey model of asthma has been shown to most closely, simulate human asthma.¹⁰ Hence the enzyme phosphodiesterase was isolated from rhesus monkey lung using modified method of David Green et al.¹¹ as follows :

Lungs from Rhesus monkey (*Macaca mulatta*) were obtained surgically and immediately frozen until used. The tissue was thawed, trimmed of excess fat and connective tissues and homogenised in 3X Tris-buffer (50mM) containing β -mercaptoethanol (3.75 mM) having pH 6.0. The crude homogenate was centrifuged at 1,00,000 g for 30 minutes. The supernatant was stirred with ammonium sulphate (351 g/l) for 60 mins. at 4°C. The phosphodiesterase fraction was recovered by centrifugation, the pellet was redissolved and dialysed against Tris buffer (12 hrs.). To the dialysed enzyme charcoal treatment was given.¹²

The enzyme was redialysed against double distilled water, pH 8.0 (12 hrs.). The aliquots was divided in small tubes and kept at -20°C . All handling during enzyme preparation was done at 4°C . Protein concentrations were determined according to Lowry¹³.

All characteristics of monkey lung phosphodiesterase were studied (Time dependence, K_m , $\square$, pH.).

The continuous spectrophotometric assay method was used for assaying an enzyme, phosphodiesterase.¹⁴ All reactions were carried out at $22-24^{\circ}\text{C}$ and in a total volume of 1 ml. Drugs used for testing were added in solution form or in finely uniform mixture (insoluble drugs admixed in distilled water only. Theophylline was used as standard inhibitor of the enzyme. All drugs were tested either at 0.1 mM or 0.5 mM concentration.

Anti-allergic studies :

The drugs were tested for their anti-allergic activity using passive peritoneal anaphylaxis in rats.¹⁵

Preparation of rat antisera : Rats weighing between 100-200 g were injected with egg-albumin (10 mg/kg intramuscular) and *B. pertussis* organism (2×10^{10} organism). After 12 days the animals were anaesthetised and the blood was collected. Serum was stored at -20°C .

Rat anti-egg albumin serum was diluted 1:2 with 0.15 M saline. One ml of this diluted serum was injected into the peritoneal cavity of male rats (150-200 g). After a latent period of 2 hours the animals were challenged intraperitoneally with 2 mg of egg-albumin in 5 ml of heparinized (50 μ g/ml) Tyrode solution. The animals ^{were} sacrificed after 5 minutes, abdomen were opened, the peritoneal fluid collected and centrifuged at 1000 g for 5 mins. The supernatant was assayed for histamine content on atropinized guinea-pig ileum. Test drug in the value of 1 ml were injected intraperitoneally one minute prior to the administration of antigen.

RESULTS

The protein concentration of monkey lung phosphodiesterase was found to be 2.25 mg/ml. The approx. K_m was observed to be 80 μ M. Theophylline at 1 mM conc. inhibited 78.85 % of phosphodiesterase, while 0.5 mM and 0.1 mM theophylline inhibited 63.11 % and 29.47 % of monkey lung phosphodiesterase. The optimum pH was observed to be 8.0. The DSCG inhibited 89 % of the histamine release from mast cells. Compound GSN-2-17 and GSN-EV-36, GSN-B-1 and GSN-4 have shown significant phosphodiesterase inhibitory activity, but less significant anti-anaphylactic

activity. Compound GSH-C₇ have shown lowest phosphodiesterase inhibitory activity, and weak anti-anaphylactic inhibitory activity. Compound 7 MCCS/GSH, GSH-C-2 and GSH-C-4 though showed poor phosphodiesterase inhibition, the anti-anaphylactic activity was very significant. Compound GSH-B-5 showed weak phosphodiesterase inhibition and lowest anti-anaphylactic activity. Compound KR/NVS/E has shown significant activity in both systems.

DISCUSSION

Levels of intracellular nucleotides play an important role in the functions of wide variety of cells. The cyclic AMP has been identified as the controlling factor of the secretory activity of mast cells. Studies on enzymes affecting the levels of this nucleotide have yielded important clues towards the mechanism of action of various drugs. A number of presently used anti-asthmatic agents have demonstrated significant effects on both the adenylyl cyclase and the phosphodiesterase. We hereby attempted to correlate phosphodiesterase inhibitory activity of new drugs with ^{their} anti-anaphylactic activity.

The results obtained from our studies with monkey lung phosphodiesterase demonstrate that though in some cases a good correlation is found between enzyme inhibition

and anti-anaphylactic activity (KR/NVS/E and GSH-B-17) in other cases it was not found to be so.

We have divided the drugs studied into two groups, mainly 1) in which a correlation is seen (KR/NVS/E and GSH-B-17); 2) in which the drugs affect one system but not the other. The two systems studied by us are entirely different from each other. An intra-cellular enzyme has been used in one system after solubilization. Factors such as drug transport across the cell membrane and effects on other enzymes do not affect the phosphodiesterase studies. These could, however, play important role in rat passive peritoneal anaphylaxis.

The mechanism of actions of known drugs lead us to accept that phosphodiesterase inhibition is not the only mechanism by which inhibition of histamine release is achieved. Drugs can also act by stimulating adenyl cyclase¹⁶ or by stabilising cell membrane.¹⁷ Hence, not all anti-anaphylactic agents can be detected by this system.

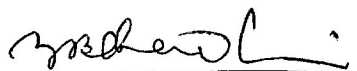
In conclusion, we have demonstrated the activity of a wide range of compounds on the phosphodiesterase enzyme obtained from monkey lungs. This test is a very useful tool for studying the mechanism of action of drugs which inhibit immunogenic histamine release.

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(D.B. Chauchari)

Signature of the Candidate

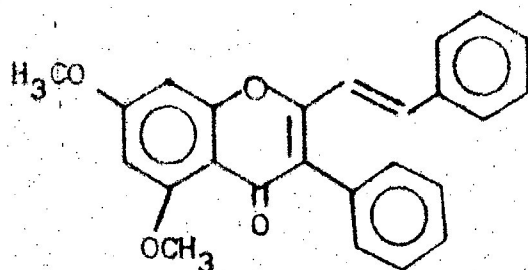


(Dr. M. B. Bhida)

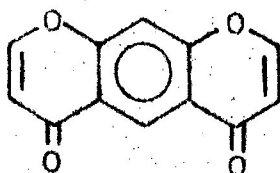
Signature of the Research Guide

APPENDIX - I

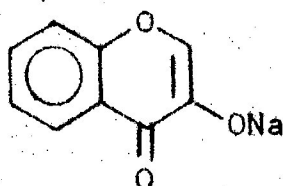
No.	Compounds	% Inhibition PDE w.r.t. Compd. E		% Inhibition of PPA w.r.t.
		0.1 mM	0.5 mM	
1.	7 MCCS/GSN	30.361		98.4
2.	KR/NVS/E	100	100	100
3.	GSN-B-1		60.68	-22.14
4.	GSN-B-2		43.68	43.05
5.	GSN-B-4		59.15	-28.29
6.	GSN-B-5	21.19		-33.21
7.	GSN-B-11	37.38		- 6.15
8.	GSN-B-12	30.605		24.6
9.	GSN-B-14	39.698		45.51
10.	GSN-B-17	70.43	71.133	41.82
11.	GSN-BV-35	42.30		54.12
12.	GSN-BV-36	82.61		31.98
13.	GSN-BV-37	63.245		49.2
14.	GSN-BV-38	44.11		81.98
15.	GSN-BV-39	46.72		45.51
16.	GSN-C-2		18.561	103.32
17.	GSN-C-4		35.69	100.86
18.	GSN-C-5	56.684	56.57	18.53
19.	GSN-C-7	7.42		24.6

APPENDIX - IIGSN-B-17

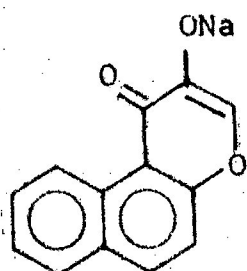
5,7-Dimethoxy,2-styryl-
isoflavone.

GSN-BV-36

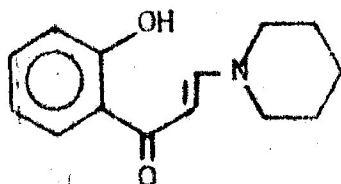
4,6-dioxo-2,3,5,7,8,10-H-benzo-
(1,2-b:5,4-b)-dipyrans.

GSN-B-1

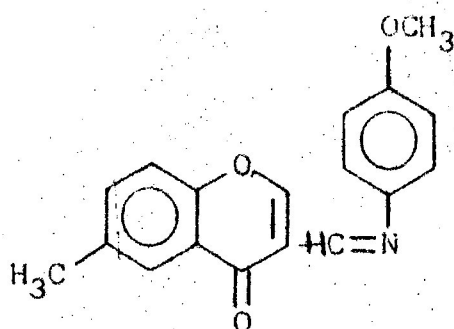
Sodium salt of 3-hydroxy-
chromone-2,3-ene.

GSN-B-4

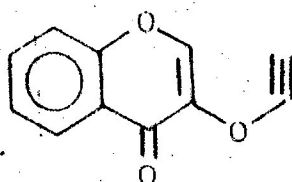
2-Hydroxy-1H-Naphtho-
(2,1-b)Pyran-1-one.

GSN-C-7

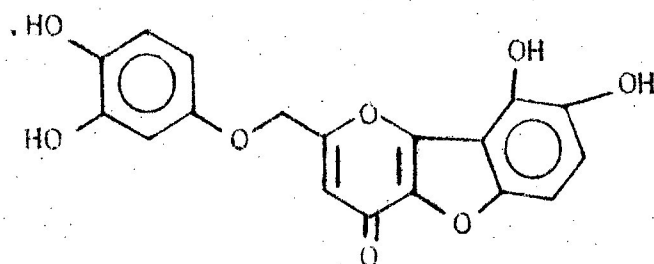
3-(2-Hydroxyphenyl)-3-oxo-1-
(1-piperidyl)-prop-2-ene.

GSN-C-4

p-4-Methoxy-N-(4'-oxo-6-methyl-4H(1)-benzopyran-3'-formylidene)-aniline.

GSN-B-5

3-Proparglyoxy-chromone.

KR/NVS/E

2-(3',4'-Dihydroxyphenyl)oxymethyl-8,7-dihydroxy-4-oxo-4-H(1)-benzofuro(3,2-b)-pyrone.

