Histamine Metabolism During Asthma Induced By Inhalation Challenge

Henry Cheuk-ki Poon

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NL-399 (r. 82/08)
HISTAMINE METABOLISM DURING ASTHMA

INDUCED BY

INHALATION CHALLENGE

by

Henry Cheuk-ki Poon

Department of Biochemistry

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
March, 1983

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ABSTRACT

A major objective of this thesis was to re-define the role of histamine (HM) in the mediation of acute human asthma induced by methacholine (MCH). Changes in plasma HM and plasma diamine oxidase (DAO), determined serially together with those occurring in Forced Expiratory Volume in the first second (FEV₁), were taken as an index of similar changes in lung during incremental MCH inhalation by 17 asthmatics and 1 normal volunteer. During 4 self-contained defined control periods not involving MCH inhalation, changes in plasma HM and plasma DAO were similarly determined in 2 asthmatic and 2 normal subjects. Parallel work encompassed effects of MCH on plasma DAO activity in vitro.

HM was determined by enzymatic double isotopic derivative dilution analysis capitalizing on the specificity of the enzyme, histamine-N-methyltransferase (HNMT), with 2 major modifications of existing versions of this principle: (1) HM assays were performed on plasma ultrafiltrates rather than on native plasma for exclusion of factors shown by the writer and others to interfere with HNMT. (2) The latter subsidiary enzyme was harvested from rat brains since conventional enzyme sources (rat kidney, guinea pig brain) affected results for HM in the presence of Cimetidine (CMTD). The modified assay retained its validity in the presence of MCH and Disodium cromoglycate (DSCG) and yielded acceptable results when applied to reconstituted lyophilates prepared by the Mayo Clinic.

DAO was determined at optimum concentrations of substrate (HM) on plasma samples buffered at physiological pH based upon the colour change of O-dianisidine effected by H₂O₂ generated in the oxidative deamination of HM. DSCG and CMTD did not interfere when added in vitro. MCH, however, functioned as a weak substrate in the absence of added HM (apparent
about 1.11 M and 1.14 M at pH 6.0 and 7.4, respectively. At low and high concentrations of HM, MCH enhanced and inhibited DAO activity, respectively.

Inhibition of plasma DAO in vitro as a function of the MCH concentration (0.42-4.2 mMoles/ml) at optimum concentrations of substrate (HM) for pH was separately verified in plasma of 7 asthmatic adults, 10 allergic adults without asthma, 6 normal adults and 1 psychotic boy with multiple allergies. For a given MCH concentration, there was no significant difference in percent activity decrease between groups. At the highest MCH increment, mean inhibition was 34% (pH 6.0) and 17% (pH 7.4); inhibition was more marked in the psychotic boy with multiple allergies (75 and 50%, respectively), suggesting subject variation.

Fluctuations in plasma HM and plasma DAO during four defined periods not involving MCH inhalation were encompassed by their determination every 7 minutes for a total of 91 minutes in 2 asthmatic and 2 normal subjects. Conditions involved resting as well as serial 0.9% NaCl inhalations and/or FEV1 determinations. On an absolute basis, a preponderance of plasma HM values during each condition yielded zero; other values were afforded by short-lived increases to maxima ranging between 4.3 and 19.3 ng/ml. On a relative basis, timed values for each of plasma HM and DAO were not significantly different from the first measurement within the 4 subjects, between the 2 asthmatic and 2 normal volunteers, and between the 4 conditions.

Eighteen subjects inhaled MCH by increments to maximum permissible limits of 224.15 cumulative units (cu); 13 asthmatics were bronchial Responders; 4 asthmatics and 1 normal subject were Non-Responders. In the Responders, the provocative dose of MCH yielding a 20% fall in FEV1 (PD20-
FEV$_1$ ranged from 1.3 to 220 cu. In the case of both plasma HM and plasma DAO, neither mean pre-saline levels nor any timed value obtained during and after the MCH inhalation phase was significantly different from the post-saline control set at 100%. On a relative basis, maxima for plasma HM found at variable stages and grouped separately according to their occurrence during and after inhalation of the highest MCH increment were in each instance significantly higher than the post-saline control. There was no significant difference between plasma HM maxima so defined.

Relative to the post-saline control for both plasma HM and DAO, mean pre-saline values of Non-Responders were not significantly different from those of Responders. Inhalation of the penultimate and maximum permissible MCH increment by Responders and Non-Responders, respectively, yielded a stage common to both groups characterized by the absence of a significant FEV$_1$ fall. Mean relative plasma HM increases by 81 (Responders) and 50% (Non-Responders) above the post-saline control found in blood removed immediately after that stage were not significantly different.

On an absolute basis, unusually high plasma HM levels (> 56.2 ng/ml) were found in 3 of 5 Responders with a high bronchial threshold to MCH (PD$_{20}$ - FEV$_1$ > 78 cu). In the remaining 2 and all of 8 Responders in whom this threshold was lower (PD$_{20}$ - FEV$_1$ < 49 cu), maxima for plasma HM did not exceed 21.6 ng/ml. These findings suggest that the subjects' bronchial sensitivity to MCH did not depend upon the magnitude of HM released from lung. This conclusion would seem to receive support from plasma HM levels as high as 658 ng/ml found in a previous Responder re-challenged with MCH during an acute respiratory illness when he became a Non-Responder.
In only one Responder (PD_{20}-\text{FEV}_1 for MCH, 78 cu) whose absolute values for plasma DAO were higher than those noted in any other subject, did changes in this activity correlate with those in \text{FEV}_1 in a significant manner (P < 0.01); the lowest values for both coincided with the highest (95 ng/ml) of three prominent plasma HM spikes. It remains to be ascertained whether such relationships may point to delayed HM inactivation as a factor implicit in MCH-induced bronchoconstriction.

It is concluded that the changes in plasma DAO and plasma HM occurring during MCH inhalation by increments revealed no clear-cut evidence suggesting that endogenous HM is a major factor mediating MCH-induced bronchoconstriction.
ACKNOWLEDGEMENT

The Clinical Investigation Unit of St. Joseph's Hospital provided space for bronchial challenge tests performed with the assistance of Mrs. L. Moir, Mrs. M. Ruston, Miss F. O'Brien and Mrs. D. Johnson. Work reported in this thesis depended upon the explicit consent of asthmatic patients and other volunteers for serial blood withdrawals. Mr. E. Sorin, Drs. B. Goldberg and B. Gordon granted the use of biomedical laboratory facilities at C.P.R.I.. Dr. G. Gleich from the Mayo Clinic supplied materials for a quality control study. Dr. G. Wells and Mrs. H. Pong of the Statistical and Acturial Sciences Department performed the statistical analyses. Mrs. R. Copper-Dickout did the art work and Mrs. M. Jazey typed this thesis. Financial support was received from London and Middlesex Lung Association, Ontario Thoracic Society Block Term Grant and from St. Joseph's Hospital Pooled Research Fund. The writer extends his thanks to the above individuals and agencies.

Last but not least, he is indebted to Drs. D.R.M. McCourtie and H.L. Haust for their persistent encouragement and guidance.
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NOMENCLATURE

ANOVA  Analysis of variance
ATP  Adenosine triphosphate
cAMP  cyclic adenosine 3', 5'-monophosphate
cGMP  cyclic guanosine 3', 5'-monophosphate
$^{14}$C-MeHm  $^{14}$C-methylhistamine
$^{14}$C-SAM  s-adenosyl-L-methionine (14C-methyl)
cu  cumulative units
CMTD  Cimetidine, TAGAMET (N'-cyano-N-methyl-N' (2(5-methyl-1)-H-imidazol-4-yl) methyl) thio) ethyl) guanidine
DAO  Diamine oxidase, histaminase
DIDDA  double isotopic derivative dilution analysis
DSCG  Disodium cromoglicate, INHAL, Cromolyn sodium
        (disodium salt of 1,3-di(2-carboxy-4-oxochromen-5-sytoxy) propan-2-ol)
EDTA  ethylenediaminetetraacetic acid
FEV1  forced expiratory volume in the first second
HM, Hm  Histamine
HNMT  histamine N-methyltransferase
HRP  horse-radish peroxidase
IgE  Immunoglobulin E
Km  Michaelis-Menten constant
Ki  Inhibitor constant
MCH  Methacholine
PD20-FEV1  provocative dose which yields 20% fall in FEV1
PUF  plasma ultrafiltrate
SRS-A  slow reacting substance of anaphylaxis
U  unit (for plasma DAO, IU = 1 μmole H₂O₂/ml plasma/hr; 37°C)
UF  ultrafiltration
CHAPTER 1: GENERAL REVIEW

1.1 INTRODUCTION: Asthma and Mediators

As early as 1830, bronchial asthma was defined as a "paroxysmal affection of the respiratory organs, characterized by a great difficulty of breathing, tightness across the chest, and a sense of impending suffocation, without fever or local inflammation" (1). By current definition, asthma is due to increased responsiveness of the airways to diverse stimuli (2,3), reflected in typical symptoms such as wheezing, labored breathing with slowing of forced expiration and a tight and dry cough yielding tenacious sputum (4). The common denominator underlying these features is reversible airway obstruction due to bronchoconstriction. Bronchial asthma afflicts and incapacitates a significant segment of the population, including children (5). One important aspect of asthma is its occurrence as an occupational disorder (6,7).

Stimuli implicit in its causation include allergens found in certain grasses, weed and tree pollens, mites, house dust, molds, yeast and animal epithelia (8). Such allergens are known to release chemical mediators from target cells, usually mast cells, via an antigen-antibody reaction involving IgE. Certain drugs such as acetylsalicylic acid (ASA), cold-air, air pollutants, physical exertion and emotional stress may cause asthma in the predisposed (4). The exact mechanism for the bronchospasm from these stimuli has not been defined.
Asthma may be divided into "Intrinsic" and "Extrinsic" forms (9). In intrinsic asthma, no external cause for bronchial narrowing is found aside from infection. This observation does not exclude an immunological mechanism although IgE would not seem to be involved (1). Skin tests to inhaled antigens or food are negative, and a family history of allergies is usually not obtained (9).

Extrinsic or atopic asthma is brought about by an antigen-antibody reaction involving IgE and the above allergens upon their inhalation (10) or, less often, their ingestion. Thus, asthma is likely to be of the "Extrinsic" type when symptoms occur upon exposure to one or more of these allergens. Skin tests to such allergens are then positive, and a family history of allergies is usually present (9). In view of the immediacy of asthmatic symptoms within minutes after exposure to known allergens, extrinsic asthma is a manifestation of the immediate type of hypersensitivity. Exceptions to that rule have recently been documented (11).

In a given person, intrinsic and extrinsic asthma may co-exist ("mixed asthma"). Asthma symptoms occur in certain subjects taking Acetyl-Salicylic acid (4). In still other subjects, exercise remains the only identifiable eliciting factor ("exercise-induced asthma") (12,13,14). Although asthmatic attacks occur in association with emotional stress, there is no clear-cut proof that such stress may by itself produce acute symptoms (10). Finally, asthma symptoms occur in the Carcinoid Syndrome together with facial flushing, abdominal
cramps, malabsorption, telangiectases, and cardiac lesions. These features are associated with the urinary excretion of 5-hydroxy-indoleacetic acid, the breakdown product of serotonin, in excess (15).

Intricately interwoven with the antigen-antibody reaction underlying the immediate type of hypersensitivity is the release of a number of substances collectively called chemical mediators (16).

This reaction involves reagins belonging to the IgE class of immunoglobulins. These serum antibodies activate surface receptors of mast cells when only they unite with polyvalent allergens. It is this union which brings about the release of mediators (17).

Primary chemical mediators of immediate hypersensitivity include histamine, slow reacting substance of anaphylaxis (SRS-A), eosinophil chemotactic factor of anaphylaxis (ECF-A), platelet-activating factor (PAF), basophil kallikrein of anaphylaxis (BKA) and neutrophil chemotactic factor of anaphylaxis (NCFA). Secondary mediators include certain prostaglandins and bradykinin which have the capability of modulating the release and/or action of the primary mediators (18).

Table I-1 further characterizes both the primary and secondary mediators and summarizes their biological activities in point form.
<table>
<thead>
<tr>
<th></th>
<th><strong>BIOLOGICAL ACTIVITIES</strong></th>
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<tbody>
<tr>
<td><strong>Primary Mediators:</strong></td>
<td></td>
</tr>
<tr>
<td>Histamine</td>
<td>Constricts bronchial smooth muscles, stimulates, irritant receptors, increases venular permeability</td>
</tr>
<tr>
<td>Slow-reacting substance of anaphylaxis</td>
<td>Constricts bronchial smooth muscles, increases venular permeability</td>
</tr>
<tr>
<td>Eosinophil chemotactic factor of anaphylaxis</td>
<td>Selectively attracts and deactivates eosinophils</td>
</tr>
<tr>
<td>Platelet-activating factor</td>
<td>Aggregation and degranulation of platelets</td>
</tr>
<tr>
<td>Basophil kallikrein of anaphylaxis</td>
<td>Bradykinin formation</td>
</tr>
<tr>
<td>Neutrophil chemotactic factor of anaphylaxis</td>
<td>Chemotaxis of neutrophils</td>
</tr>
<tr>
<td><strong>Secondary Mediators:</strong></td>
<td></td>
</tr>
<tr>
<td>Prostaglandins (e.g. PGF$_{2\alpha}$)</td>
<td>Regulate vascular resistance and bronchomotor tone</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>Constricts bronchial smooth muscles, stimulates irritant receptors, increases venular permeability</td>
</tr>
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</table>
1.2 HISTAMINE

Since the writer's work reported in this thesis has focused on the elucidation of a role, if any, of histamine in the mediation of induced human asthma, information available on this amine will be reviewed in subsequent paragraphs in detail.

SYNTHESIS AND DEGRADATION. Histamine of any source is formed by decarboxylation of L-histidine by histidine decarboxylase, an enzyme specific for this reaction requiring pyridoxal phosphate as a co-factor. Preformed histamine undergoes two principle reactions, one involving its methylation to 1-methylhistamine, the other effecting its oxidative deamination yielding imidazole-4-acetaldehyde, ammonia and hydrogen peroxide. 1-methylhistamine is similarly deaminated to 1-methylimidazole-4-acetaldehyde. The two aldehyde forms undergo further oxidation to their respective imidazole-4-acetate. Neither 1-methylhistamine nor any of the degradation products just mentioned reveal the biological and pharmacological effects of histamine proper (19).

TISSUE DISTRIBUTION. Histamine abounds in mast cells of body tissues, in basophils of blood and in the parietal region of the stomach. It also occurs in rabbit platelets. Electron microscopy has shown that histamine of mast cells is present in metachromatic granules. Mast cells abound in perivascular connective tissue. The distribution of histamine in a given tissue would seem to follow its mast cell content rather than its innervation (20).
PHYSIOLOGICAL AND PHARMACOLOGICAL EFFECTS. Effects of endogenous histamine include stimulation of irritant receptors present in lung, constriction of bronchial smooth muscle, and increases in venular permeability. Endogenous histamine furthermore enhances glandular secretions by the nasal mucosa and dilates capillaries while constricting arterioles. Histamine is involved in the physiologic stimulation of gastric acid secretion (21). As a later section of this review reveals, the physiological effects of histamine are mediated by two distinct histamine receptors (21).

Effects of exogenous histamine have been studied following its intravenous infusion as well as its inhalation as an aerosol (22). Infused histamine evokes a sensation of chest tightness and elicits headache, flushing, hypotension and pruritus (23-24). Inhaled histamine may cause bronchoconstriction even in a normal subject. The increased bronchoconstriction exhibited by a preponderance of asthmatics points to bronchial hyperresponsiveness of such patients to this amine. This hyperresponsiveness in turn affords the rationale of including histamine among bronchoprovocative agents commonly used in diagnostic inhalation challenge tests. The resulting acute attack of asthma produced under controlled conditions affords a human model for the study of this disease.

HISTAMINE RELEASE IN VITRO. Histamine release refers to the liberation of this amine from histamine-storing cells such as mast cells
and basophilic leukocytes into the extracellular medium. Defined stimuli are necessary for such liberation. The nature of the stimulus determines whether histamine release will occur as a consequence of cell disruption or cytotoxicity (when other cytoplasmic constituents such as potassium, adenosine triphosphate and lactic dehydrogenase are simultaneously released), or whether it represents a non-cytotoxic secretory process requiring energy. In the following, only non-cytotoxic histamine release will be reviewed because of its involvement in allergic diseases (18, 25).

Mast cells harvested from pleural or peritoneal cavities of rats have been the most convenient source for study of such release. Basophilic leukocytes of man and rabbits, platelets, perfused lungs or lung fragments and slices have afforded alternative models (19-21, 25, 26). Mast cells are extremely rich in basophilic granules which store histamine and, in some species, other biogenic amines such as serotonin and dopamine (26, 27).

Histamine release is an example of stimulus-secretion coupling such that in response to certain degranulating agents or histamine releasers, mast cell granules are extruded from the cell by a specific process of exocytosis (or expoplasmosis). This process consists of the movement of the granules from within the cell to its periphery, followed by fusion of the perigranular membrane with the plasma membrane. Rupture of the fused membrane then permits histamine to diffuse out of the granules, triggered presumably by cation flux in the opposite
direction (28,29). This sequence of events occurs as part of an IgE-mediated–anaphylactic reaction and also in response to selective (non-immunological) histamine-releasers. Such agents include Dextran (30), compound 48/80 (31,32,33), concanavalin A (34,35,36), protamine sulfate (37) and adenosine triphosphate (ATP) (38).

Dependence of exocytosis upon energy emerges from the demonstration of concomitant increases in cellular oxygen consumption, enhancement of glucose metabolism, and increased utilization of ATP. There is also evidence that metabolic stimulation of the mast cell persists once histamine has been released. Presumably, this serves to restore the functional status of the cell through repair of its membrane and replenishment of its amine content (39).

The foregoing generalizations regarding histamine release and associated events find support from the following experimental data.

Incubation of rat peritoneal mast cells with compound 48/80 or \( \text{\textbullet} \) antigen effected histamine release to the extent of 70% of total histamine content, but there was no subsequent reduction in the total number of cells involved in such release (39). Furthermore, there was no leakage of lactate dehydrogenase or other proteins or potassium from such cells (31,40). Moreover, there was no evidence of impairment of the cell membrane (41), nor of a change in its potential (42) or its permeability to vital dyes (41).
The histamine-generating capacity of rat mast cells increased measurably after their exposure to compound 48/80 in vitro (43). Again, this potential increased markedly both in vitro and in vivo in the case of human sensitized leukocytes exposed to antigen (44).

Available evidence suggests that both immunological and non-immunological agents initiate the series of events culminating in histamine release by interaction with specific receptors located on the mast cell (or leukocyte) membrane. Immunoglobulins of the IgE class have themselves the unique affinity for specific receptors located on mast cells as well as on basophils (45). The resulting attachment of IgE to the surface of these cells underlies the phenomenon of passive sensitization (46,47,48). While the nature of these specific receptors awaits clarification (49,50), it is only after passive sensitization that antigens (or allergens) can bridge adjacent surface-bound IgE molecules, thereby triggering histamine release (51,52). IgE receptors do not appear to be the only cell surface site involved in non-cytotoxic histamine release.

The location on rat peritoneal mast cells of receptors specific for compound 48/80 emerges from data showing that covalent binding of compound 48/80 to sepharose beads will attract mast cells to the beads followed by release of histamine and serotonin (53,27). The respective receptors were different from those for IgE (54). The fact that compound 48/80 elicits histamine release from mast cells of the rat but not from mouse or guinea pig mast cells or
human or rabbit basophils, points to species differences as to the presence or absence of specific receptors for this compound on the surface of the cells concerned (55).

Reference was already made to the association of histamine release with cation flux. Evidence suggests that calcium is the principal ion involved. For example, uptake of calcium by mast cells engaged in histamine release takes place whether the enhancer of such release is ATP (58) or phosphatidyl serine (56). Furthermore, substance A 23187, a calcium ionophore, promotes histamine release presumably by ensuring calcium transport across mast cell membranes (57). When calcium is carried in this fashion, histamine release will proceed unimpeded even in the presence of disodium-cromoglycate (58), a well-known inhibitor of such release. Enhancement of anaphylactic histamine release has been effected by both calcium (59) and strontium (60). On the other hand, lanthanum, a trivalent ion, up to a certain concentration inhibits such release (61,62) due, presumably, to its greater affinity for calcium receptor sites (62). Beyond that concentration, histamine release is stimulated by lanthanum even in the absence of antigen (61). Calcium antagonists were found to prevent exercise-induced asthma (63,64) without modification of basal bronchial tone of the asthmatic subjects (63).

The essential role of calcium in the energy-requiring sequence of events implicit in stimulus-secretion coupling culminating in
histamine release would seem to emerge at the following stages:
A diisopropylphosphofluoridate (DFP) - sensitive serine esterase is
originally activated by calcium (= Step I) but is further activated
autocatalytically (= Step II). Energy dependent upon the Embden-
Meyerhoff pathway is required (= Step III) prior to another calcium
requiring step distinct from I and inhibited by EDTA (= Step IV).
This step is forfeited by 2-deoxyglucose. A final step (= Step V),
inhibited by cyclic adenosine monophosphate, is necessary for the
release of both histamine and SRS-A (65).

Recent data (66) on histamine release induced in rat mast cells
by either compound 48/80 or a cationic protein from rabbit neutrophil
lysosomes pin-pointed marked influx of $^{45}$Ca into the stimulated
cells to coincide with the actual release stage. Both histamine release
and influx of $^{45}$Ca were inhibited by 2-deoxyglucose as well as by
2,4-dinitrophenol. Regarding immunological histamine release, it was
recently found that the bridging of IgE receptors on the cell surface
enhanced methylation of phospholipids as a prerequisite for $^{45}$Ca in-
flux prior to actual histamine release (67).

While the foregoing data stress a role for mobilization of extra-
cellular calcium in stimulus-secretion coupling, there is also evi-
dence that intracellular calcium stores are drawn upon whether the
stimulus be dextran (68), antigen (65), or cationic protein from
neutrophil lysosomes (69). It has been suggested that such (membrane-
bound?) calcium may be required to bring about the fusion-fission
response of perigranular and plasma membranes during exocytosis (70).

Addition of antigen to human leukocytes or sensitized rat mast cells does not stimulate histamine release unless calcium is also added. The subsequent addition of calcium will evoke histamine release, but is time-dependent as the cellular susceptibility to calcium fades rapidly. Thus, no histamine will be released when calcium or antigen is added four minutes after the initial antigen exposure (71, 72). This lack of secretory response to repeat exposure to antigen is known as cellular desensitization. Histamine release is not abolished after desensitization to antigens in the presence of the calcium ionophore A23187 (72), suggesting that the ionophore may bypass calcium gates. Cellular desensitization may thus be due to a change in cellular membrane permeability to calcium (72). The validity of this conclusion is hampered if membranes were a priori affected by the calcium-free media used for cellular incubation.

Cellular desensitization extends to repeat exposure of rat mast cells to dextran in the presence of calcium and phosphatidylserine (72). Respective experiments have shown that abolition of histamine release invariably coincided with the time interval at which cellular desensitization was complete. Furthermore, at a lowered temperature, less histamine was released during longer periods. When either theophylline was added or the concentrations of dextran or calcium were lowered, the rate of histamine release was decreased, but its duration was unaffected.
From the foregoing data, it has been concluded that termination of histamine release is a function of cellular desensitization (68, 73, 74) and that the latter process may be a factor controlling the amount of histamine to be released. As already pointed out, desensitization may in turn be the result of the cessation of calcium flux due to altered permeability of mast cell membranes (72).

Functional microtubules emerge as a prerequisite for diverse secretory processes (75, 76, 77) and would seem to be necessary for non-immunological histamine release from human peripheral leukocytes (78, 79) as well as from human lung tissue (80).

While immunologic release of mediators from such tissue and from polypoid nasal mucosa and blood basophils is inhibited by high intracellular levels of cAMP (81, 82, 83) cyclic GMP has the opposite effect (84, 85) and may partially offset the inhibitory action of cAMP (86). Evidence has recently been presented suggesting that in human lung tissue, these opposing effects of the two cyclic nucleotides are mediated by their primary action of modifying the state of microtubular assembly, (87). While colchicine, a known disrupter of secretory reactions by binding to disassembled microtubules, suppressed mediator release, this effect was nullified by 8-bromo-cGMP, suggesting that cGMP stabilized polymerized microtubules. Increases in cAMP levels effected by added 8-bromo-cAMP or isoproterenol (a phosphodiesterase inhibitor) enhanced the suppression of mediator release caused by colchicine, thus pointing to microtubular disassembly as the
mediating event (87).

Cholinergic agents are implicated as promoters of histamine release from rat mast cell preparations. Thus, histamine release was enhanced by carbamyl-choline when compound 48/80 was the primary stimulus (88). When compound 48/80-induced histamine release served as a separate control, various salts of acetylcholine studied alone over a wide concentration range, were ineffective (89). These data conflict with findings demonstrating histamine release from rat mast cells in response to acetylcholine in a dose-dependent fashion (90). Still other findings imply that acetylcholine may promote histamine release from rat mast cells only after active sensitization of the animals (91).

In isolated and untreated human adenoidal mast cells, acetylcholine alone sufficed to elicit histamine release in a dose-dependent fashion (91,92). In marked contrast to these observations are findings showing that cholinergic agents do not enhance histamine release from sensitized human basophils (93). Both acetylcholine and carbamyl-choline were found to enhance antigen-induced histamine release from IgE-sensitized human lung (84) and human nasal polyps (82).

Finally, the capacity of acetylcholine to enhance immunological release of both histamine and SRS-A from lung tissue rapidly increased with increasing cGMP levels. Rapid increases in concentrations of the latter nucleotide were also affected by PGE₂ in a dose-related fashion. As opposed to acetylcholine, acetyl-salicylic acid
neither affected control levels of cGMP, nor did it enhance immuno-
logical mediator release (85).

A primary role of cAMP in the modulation of histamine release
emerges from the following data. Histamine release from sensitized
tissues and leukocyte suspensions was found to be inhibited by agents
which either enhanced the formation, or prevented the destruction of
this cyclic nucleotide (85,94,95,96).

Phosphodiesterase inhibitors (such as theophylline), β-adrenergic
agonists (e.g. Isoproterenol), prostaglandins E₁ and E₂, cholera toxin
and histamine proper inhibited histamine release from human leukocytes,
coinciding with increases in cellular cAMP content (97,98). Prosta-
glandin E₁ and β-adrenergic agonists and also theophylline similarly
inhibited histamine release from human and guinea pig lung (81,99) as
well as from peritoneal mast cells of the rat (100,101).

In highly purified rat mast cell preparations, exclusion of cal-
cium from the medium or mere agitation or cellular contact with glass
entailed significant increases in cellular cAMP. Similar effects
were obtained with theophylline in a dose-related fashion. Theophylline
furthermore potentiated effects of epinephrine such that increases in
cAMP elicited by either agent alone were much greater when both agents
were included in the medium. cAMP likewise increased in response to
prostaglandin E₁ and histamine proper (88,102). The epinephrine effect
(of increasing cAMP levels) was preventable by propranolol in a dose-
related fashion. cAMP content of cells decreased immediately,
markedly and progressively in response to compound 48/80, then re-bounded to starting levels.

Histamine release was inhibited by those drugs which counter-acted the depressing effect of compound 48/80 on cellular cAMP content, such as theophylline and prostaglandin E₁. Conversely, histamine release was enhanced beyond the stimulation afforded by compound 48/80 alone and cAMP levels were decreased, in the presence of carbamylcholine, adenine and diazoxide (88).

Notable among the known inhibitors of histamine release is Cromolyn sodium (disodium cromoglycate; DSCG). Not only does this agent inhibit histamine release from rat mast cells after their passive sensitization (103), but also does it prevent their degranulation after sensitization with reaginic antibodies followed by antigen exposure (104). Sensitized guinea pigs pretreated with DSCG revealed significant suppression of histamine release and reduction of total histamine content in their tracheae, lungs and intestines (105). DSCG is furthermore capable of inhibiting non-immunological histamine release from rat mast cells effected by compound 48/80 (106,107). This action of DSCG extends to histamine release induced in rats by dextran in vivo (108,109). These observations credit DSCG with the properties of a "membrane stabilizer" (110) due, presumably, to a primary effect of closing calcium gates in the mast cell membrane (111,112). Indeed, it was already pointed out that DSCG failed to inhibit histamine release when calcium flux was maintained via the
DSCG inhibits cAMP phosphodiesterase in several tissues (113). More recently, DSCG was found to increase cellular cAMP levels in fragmented mouse lung while not affecting cyclic GMP (113).
HISTAMINE RELEASE IN VIVO

The wealth of information pointing to non-cytotoxic immunological and non-immunological histamine release from target cells in vitro has given rise to the question as to the extent to which such release can be validly demonstrated in man in vivo. It has been claimed that measurement of histamine concentrations in venous plasma (as opposed to those in whole blood) may afford a most direct index of histamine release in man under conditions "which are nearest to the physiological situation to be investigated" (sic) (115). It has also been contended that histamine once released, may be metabolized before reaching the venous sampling site (116).

In experimental animals, respective studies are not limited by ethical considerations applying to human experimentation, prompting histamine determinations in both venous and arterial blood and/or plasma.

Thus, in asthmatic dogs inhaling nematode antigen, plasma histamine rose to much higher levels in systemic arterial than in mixed venous blood. From this concentration difference it was concluded that little if any of the inhaled antigen could have been absorbed into the systemic circulation so as to shock the livers, which in this species are uniquely rich in histamine (117,118). The histamine increment in arterial plasma in response to antigen inhalation was therefore ascribed to histamine released from lung (116).
In sensitized dogs inhaling an aerosol of *Ascaris suum* antigen, both arterial and venous plasma histamine rose to their highest values at the end of the inhalation period (7 minutes). However, histamine peaks attained in venous plasma were only some 60% those found in arterial plasma. For a given increment in arterial plasma histamine, the degree of curtailment of pulmonary function varied widely between the animals (119).

In an extension of this work (120) dogs were challenged repeatedly with the same antigen aerosol after intervals of 90, 180 and 360 minutes. Although arterial plasma histamine rises after the second (90 min) challenge were blunted, histamine rose once more significantly after the third (180 min) challenge. The highest increases in arterial plasma histamine levels occurred after repeat challenge at 360 minutes. Histamine in venous plasma was not measured. With successive challenges, pulmonary function was progressively more curtailed. The rises in arterial plasma histamine were equated with histamine released from lung. The blunted responses observed after repeat challenge at 90 minutes were thought to reflect incomplete replenishment of histamine stores in degranulated mast cells at that interval. However, increases in the rate of local histamine degradation were not excluded (120).

In subsequent work (121) inhalation of *Ascaris suum* antigen by allergic dogs was shown to entail rises in arterial plasma histamine whether the aerosol was delivered to the upper or peripheral airways.
Degrees of airway obstruction were comparable irrespective of topographic differences in antigen delivery. It was concluded that reflex bronchoconstriction may have accounted for the similarity in results and that the degree of bronchoconstriction achieved with antigen inhalation may depend upon the product of the local histamine concentration and the area over which histamine release extends (121).

In yet another extension of this work (122) only 6 out of 10 dogs inhaling acetylcholine responded with modest increases in arterial plasma histamine. Since airway obstruction was marked in all 10 animals, it was concluded that local histamine release, if any, was not responsible for this effect. Repeat inhalation of acetylcholine after 90 minutes entailed a new histamine rise in arterial plasma in those animals which had responded with such a rise before. Differences in mucosal mast cell content, in mast cell histamine content, or in mast cell sensitivity to acetylcholine were envisaged to account for the fact that arterial plasma histamine did not increase in all animals exposed to this inhalant. Repeat inhalation of histamine proper by the dogs was accompanied each time by increases in arterial plasma histamine. With successive inhalations, airway obstruction became more marked, suggesting that bronchial sensitivity increased progressively (122).

Data on histamine concentrations in venous blood in man under various clinical conditions are conflicting. One report implies that there is no difference in these concentrations between allergic
and non-allergic subjects (123). In another study, increased blood concentrations of histamine were found in asthmatic patients, but basophilic leukocytes were likewise increased (124,125), thus militating against the lung as a major source. In still other work, blood histamine increases in asthmatics were found to be confined to the acute attack whether spontaneous (124,126) or induced by exercise (127,128) or inhalation challenge with antigen or methacholine (129). Recently, it was reported that in patients with atopic and non-atopic asthma, blood histamine levels were higher during an acute attack than during symptom-free periods and were even higher in status asthmaticus (130).

Comparable levels of histamine in whole blood were found in patients with severe asthma, patients with other pulmonary diseases, and normal subjects. However, histamine measured concomitantly in plasma was higher in the asthma patients as well as those with other pulmonary diseases than in the normal control group (131), thus militating against peripheral leukocytes as a source of the higher histamine levels in the former two groups. When, in turn, the asthma patients were given corticosteroids, histamine in whole blood decreased to barely detectable levels, while histamine in plasma yielded values comparable to those found in the group of normal subjects (131).

In an extension of this work (132) histamine determinations in arterial and venous whole blood and plasma were complemented by counts of peripheral eosinophils and basophils in a group of patients with
acute asthma and one representative of patients treated with steroids. Patients with diverse acute non-respiratory ailments formed a separate group. Data were compared to results obtained on normal subjects. From this work, no significant arteriovenous differences emerged in any of the variables measured for any of the three patient groups, thus militating against net loss of arterial histamine in the peripheral circulation. However, all of arterial and venous blood histamine levels and eosinophils and basophils in asthma patients not receiving steroids significantly exceeded those of patients with acute non-respiratory ailments. Significant increases in venous plasma histamine levels of patients with acute illness other than asthma, relative to values found in normal subjects, suggested that acute illness per se may have a bearing on plasma histamine levels. Administration of hydrocortisone and bronchodilators to asthma patients entailed a marked fall in whole blood histamine as well as in peripheral eosinophils and basophils (132).

Histamine concentrations in venous plasma of asthmatic subjects correlated with naturally occurring asthmatic episodes, such that the highest values (>1.25 ng/ml) were found in all of 20 patients with severe attacks. Only 3 of 20 patients who were in partial remission and only 3 of 20 symptom-free patients revealed plasma histamine values greater than 1 ng/ml. Values were below 1 ng/ml in all of 50 normal subjects (133).

In five of seven patients with bronchial asthma, significant
elevations in plasma histamine were found to be associated with antigen-induced bronchospasm. These elevations peaked about 5 minutes following inhalation of pre-determined single threshold doses of skin-test positive allergens and persisted for periods of up to 30 minutes. The highest elevations in plasma histamine were found in patients showing the largest decline in FEV$_1$. No rises in plasma histamine occurred when the same patients inhaled pre-determined single threshold doses of methacholine (134).

In 6 antigen-sensitive subjects, inhalation of ragweed or grass antigen by increments to a broncho-provocative threshold followed by serial histamine determination in venous plasma entailed elevations of this amine within 2 minutes after this threshold had been attained. Peaks ranged between 18 and 80 ng/ml. Comparable inhalations of antigen by 4 non-atopic subjects neither elicited bronchospasm, nor entailed rises in plasma histamine, even when antigen concentrations were increased ten-fold. Bronchospasm accompanied by sustained plasma histamine increases ascertainable within 1 minute was produced by inhalation of 2.5 to 10 mg of methacholine by 3 atopic and 3 non-atopic subjects. In two atopic and 5 non-atopic subjects not responding to the foregoing methacholine doses with bronchospasm, no plasma histamine occurred even with higher doses of methacholine (135).

In 7 asthmatics intolerant to acetyl salicylic acid (Aspirin), controlled challenges with this agent given orally entailed significant decreases in FEV$_1$ accompanied by increases in venous plasma histamine.
No decreases in FEV₁ or changes in plasma histamine occurred in any of these patients with Maalox or sodium salicylate given under comparable conditions. Acetyl salicylic acid given to 10 non-asthmatic control subjects likewise had no effect on these two variables. Two of 10 asthmatics with purported tolerance of acetyl salicylic acid responded to this agent with increases in a priori elevated plasma histamine concentrations although FEV₁ remained unaffected. It was concluded that these 2 patients were examples of hitherto unsuspected Aspirin intolerance, and that histamine is one of the mediators of bronchospasm in Aspirin-induced asthma (136).

In an elegant study designed to define factors accounting for the well-known occurrence of wheezing in asthmatics predominantly at night and in the early morning (137,138,159), histamine in venous plasma was one of the variables measured around the clock (140). Subjects included 5 asthmatic patients displaying such a typically circadian rhythm of symptoms, with skin tests positive to common allergens. Five normal subjects were also studied. Plasma histamine in the asthmatics rose markedly at 04:00 hours in the early morning when peak expiratory flow had markedly decreased and plasma epinephrine and cAMP had reached their lowest values. Plasma cortisol was somewhat out of phase such that the lowest values measured at midnight, preceding the fall in expiratory flow. In the 5 normal subjects, plasma epinephrine likewise reached its lowest values at 04:00 hours; although plasma histamine by that time had risen, respective values were not significantly different from those measured at 16:00 hours, and the increment
was significantly less than that observed in the asthma group (140). In an extension of this work, epinephrine was infused into the 5 asthma patients at two dose levels, viz. 0.01 µg and 0.075 µg/kg/min., respectively. The lower epinephrine dose entailed a reduction in plasma histamine levels at 09:00, 16:00 and 04:00 hours whereas with the higher dose, plasma histamine was increased at each of these intervals so as to exceed the 09:00 and 04:00 hour values measured in the patients before any infusion. It was concluded that the circadian fall in plasma epinephrine observed at 04:00 hours was responsible for a similar fall in cAMP in the uninfused patients, thus causing nocturnal asthma via release of histamine (and other mediators) from their sensitized mast cells. While a similar fall in plasma epinephrine extended to the 5 normal subjects, a concomitant significant rise in plasma histamine did not occur presumably because mast cells were not sensitized. Epinephrine may, thus, influence bronchomotor tone in asthmatics by inhibiting mediator release from sensitized mast cells in vivo. This conclusion is supported by in vitro data (84), and by the reduction in plasma histamine achieved in these patients by infusion of the lower dose of epinephrine. The rise in plasma histamine occurring in these patients with the higher dose of infused epinephrine may point to an α-adrenergic-mediated increase in mediator release, for which separate evidence has been presented (84). Bronchodilation in the face of such an increase may alternatively be due to a direct effect of epinephrine on bronchomotor tone, outweighing its indirect effect on sensitized mast cells (140).
In attempts to clarify the role of histamine as a mediator of bronchospasm, barriers to human experimentation have only recently been overcome by measuring histamine in arterial plasma.

One such study was designed to trace a relationship, if any, between arterial plasma histamine concentrations and the degree of lung dysfunction prevailing in 17 asymptomatic asthmatics prior to controlled exercise. Post-exertional airway changes and histamine concentrations in arterial plasma were also studied. Baseline levels of the amine were highest in subjects with a priori curtailed pulmonary function. These subjects responded to exercise with severe obstruction in the main of peripheral airways. Post-exertional obstruction in subjects whose pulmonary function was less curtailed (coinciding with the lower histamine levels in arterial plasma) was less severe and involved predominantly the larger airways. In no instance was there a change in arterial histamine levels after exercise. It was concluded that these levels may serve as a marker of the state of inflammation of airways (141).

In another investigation involving measurement of histamine in arterial plasma, 8 patients with documented exercise-induced asthma and 2 normal subjects inhaled saline followed by controlled exercise. After a lapse of 140 minutes, each subject then inhaled terbutaline sulphate prior to repeat exercise. In 5 of the asthmatics, increases in
arterial plasma histamine associated with bronchoconstriction occurred when exercise was preceded by saline inhalation. Bronchoconstriction subsided with repeat exercise after terbutaline inhalation, but arterial plasma histamine rose further. This additional increase in arterial plasma histamine was ascribed to local histamine accumulation (rather than histamine release) within lung interstitium due to changes in the pulmonary microcirculation effected by the bronchoconstriction associated with exercise prior to terbutaline. Failure of arterial plasma histamine to rise in 3 out of the 8 asthmatics was ascribed to its inactivation by diamine oxidase prior to entering the pulmonary microcirculation. Considering that the rate of such inactivation may vary, measurement of histamine in systemic arterial plasma might then not necessarily afford a valid index of pulmonary histamine release (142).
HISTAMINE RECEPTORS

The physiological effects of histamine are mediated by histamine receptors. At present, two such receptors are definitely recognized, called H1 and H2, respectively. This distinction is based upon the following criteria.

Receptors of the H1 type are uniquely activated by 2-methylhistamine as well as by 2-pyridyl-ethylamine, but are blocked by mepyramine and similar antihistaminic agents (145). These blocking functions of antihistamines antagonize effects of histamine such as constriction of smooth muscle in diverse organs.

Receptors of the H2 type are selectively stimulated by 4-methylhistamine (144,145) dimaprit and imipramine (146). They are blocked by burimamide, an agent developed by Black et al. (144) in 1972. H2 receptors are similarly blocked by metiamide and cimetidine (146). H2 blockers typically antagonize effects of histamine such as stimulation of acid secretion in the stomach (144,147,148), increases in heart rate (147,149), and inhibition of contraction of rat uterus (144,148). According to Grechishkin et al. (150), H1 and H2 receptors are further distinguishable based upon the lipophilic (H1) or hydrophilic (H2) character of their adjacent zones, hydrogen bonds being responsible for histamine binding in either instance.

While airway constriction, capillary dilatation and increased
capillary permeability have been traditionally ascribed to histamine released during allergic reactions, the recognition of H2 receptors has made it possible to ascertain other and more subtle effects of this amine. Thus, by virtue of a negative feedback mechanism, histamine may not only modulate its own release during an allergic reaction (151,152,153) but also that of other mediators such as SRS-A (146,154), thromboxanes (155) and prostaglandins (156). In certain animals at least, such modulatory functions of histamine have been demonstrated, and have been shown to operate via histamine H2-receptors (157).

In dogs, pulmonary responsiveness to inhaled histamine was significantly potentiated by H2 receptor blockade with cimetidine. This enhancing effect of cimetidine upon histamine-induced bronchoconstriction was more marked in animals showing the lesser initial responsiveness to histamine. Explanations given for this intriguing finding envisaged a dual effect of the histamine inhaled, culminating in bronchoconstriction via stimulation of H1 receptors, and in airway relaxation via stimulation of H2 receptors. Blockade of the latter receptors by cimetidine would, then, no longer oppose H1-mediated bronchoconstriction, thus accounting for enhanced airway responsiveness to the histamine inhaled (158).

Studies on the presence and importance of H2 receptors in human airways have yielded conflicting results. In normal and asthmatic subjects, histamine-induced bronchoconstriction remained unaffected by cimetidine, thus casting doubt upon the importance of H2 receptors.
in the airways of man (159). However, in other work involving asthmatic subjects inhaling histamine, the threshold dose of this amine necessary for a critical fall in FEV₁ was increased after prior administration of chlorpheniramine (a typical H₁ blocker), but was decreased when cimetidine was given. No change in this threshold occurred when both agents were administered simultaneously (160). These data not only point to the presence of both H₁ and H₂ receptors in human airways, but also show that as in the case of dogs, cimetidine may potentiate the bronchoconstrictive effect of inhaled histamine.

Conflicting data obtained on human subjects as to the effect, if any, of cimetidine on histamine-induced bronchoconstriction may be reconciled by data suggesting that asthmatic subjects may differ as to a critical balance between H₁ and H₂ receptors such that some forms of asthma may be due to frank H₂ receptor deficiency (161,162). This possibility is supported by data obtained on non-asthmatic non-allergic subjects: Cimetidine prevented the bronchoconstrictive effect of inhaled histamine in those most sensitive to this amine, but enhanced this effect in less sensitive subjects. Cimetidine failed to affect airways of individuals not responding to histamine at all. Any histamine-induced airway responses in these subjects were largely abolished by chlorpheniramine (a H₁ blocker). These data suggest that there may be an abundance of H₁ and a paucity of H₂ receptors in lungs of subjects most sensitive to histamine; conversely, less H₁ and more H₂ receptors may be present in less sensitive subjects; finally, in
those unresponsive to histamine, the number of both H1 and H2 receptors may be significantly curtailed (163).

Not in conflict with the foregoing thesis are data suggesting that cimetidine, hitherto thought to be a typical H2 antagonist, may in addition have H1 blocking properties (163, 164).

It has recently been found that histamine-mediated significant increases in lymph flow and lymph proteins in the skin of dogs can be abolished by blocking either H1 or H2 receptors, suggesting that both receptors may play a role in microvascular permeability (165).

The presence of an additional receptor(s) in eosinophils has been inferred from data showing that neither H1 nor H2 receptor antagonists (pyrilamine and metiamide, respectively) affected the chemoattractant activity of histamine proper (166). Antagonists of either receptor also failed to block the inhibition exerted by histamine on epinephrine-induced platelet aggregation (167).
HISTAMINE INACTIVATION

It was already indicated that two pathways are open for histamine inactivation. Catalyzed by the highly specific enzyme, histamine-N-methyltransferase, histamine may be methylated to yield 1-methyl-4-(B-aminoethyl) imidazole ("methylhistamine"), s-adenosyl-methionine being the methyl donor. Methylhistamine is subject to further deamination by monoamine oxidase, yielding 1-methyl-imidazole-4-acetic acid ("methyl imidazole acetic acid") (21).

In the alternative pathway, histamine is deaminated by diamine oxidase, yielding imidazole-4-acetaldehyde, ammonia and hydrogen peroxide. The term, Diamine Oxidase (DAO), collectively refers to a group of enzymes, effecting oxidative deamination of a number of other aromatic and aliphatic diamines. Although the histamine deaminating properties of these enzymes have given rise to the designation "Histaminase", none of these activities would seem to exhibit substrate specificity for histamine alone. Imidazole-4-acetaldehyde is further oxidized to imidazole-4-acetate which is usually conjugated with ribose prior to its excretion as 1-ribosyl-imidazole-4-acetic acid (21).

While the latter step is unique in that no other known metabolite would seem to undergo conjugation with ribose, it has recently been found that therapeutic doses of aspirin may block formation of ribosyl imidazole acetic acid in man and other species (21). It remains to be ascertained whether there is any relationship between this blocking
action of aspirin and its properties of inducing asthma in the pre-
disposed.

In a study of the tissue disposition of $^{14}$C-histamine in the whole
mouse, it was found that the initial disappearance of radioactivity
attributable to histamine proper was exceedingly rapid. $^{14}$C-
methylhistamine, appearing within minutes, reached peak concentrations
at 30 minutes. Minor amounts of both radioactive compounds were still
detectable at 48 hours. Total radioactivity declined sluggishly, still
fat exceeding the sum of $^{14}$C-histamine and $^{14}$C-methylhistamine at 16
hours. These data afforded a basis for studying histamine methylation
in the whole mouse in vivo as affected by drugs 10 minutes after admin-
istration of $^{14}$C-histamine. Aminoguanidine markedly diminished the dis-
appearance rate of $^{14}$C-histamine proper, and significantly enhanced
the appearance of radioactivity in methylhistamine, suggesting a diver-
sion of histamine metabolism into the methylation pathway in response
to the known inhibition exerted by aminoguanidine on DAO (168).

The disposition of a subcutaneous dose of $^{14}$C-histamine at speci-
fied time intervals in individual tissues of the rat (in which hista-
mine methylation was found to be negligible) was also studied. Total
radioactivity exceeded the concentration of radioactive histamine in
all tissues at 1 and 24 hours and was greatest in heart and lowest in
brain. At the 1 hour interval, lung afforded 30% of radioactivity in
histamine, some 40% in imidazole acetic acid and some 30% in its ribo-
side. At 24 hours, radioactivity found in the riboside far exceeded
that found in the free acid in lung and other tissues (168).

The relative importance of the two pathways of histamine inactivation has also been studied from the angle of the cellular and subcellular localization of the enzymes involved. Earlier data yielding evidence of a mitigating effect of eosinophils and extracts of eosinophils on the intradermal responses to histamine pointed to the presence in eosinophils of a histamine degrading enzyme (169).

By specific thin-layer radiochromatic assays, this enzyme was recently shown to be "histaminase" (DAO), its presence extending to human neutrophils, but not to circulating mononuclear cells. Conversely, histamine methyltransferase was detected in monocytes, but was not present in granulocytes, eosinophils, lymphocytes or platelets. The leukocyte histaminase enzyme resembled human placental histaminase functionally and chemically, being mainly localized in a 27,000 g granule-rich fraction of eosinophil and neutrophil homogenates. Histamine methyltransferase was confined to the 100,000 g cell sap supernatant fraction of monocytes (170).

Low levels of DAO activity usually found in normal human plasma rise dramatically during pregnancy (171), in patients suffering from medullary carcinoma of the thyroid (172) and as a consequence of heparin administration (173,174). High levels of DAO activity in human plasma during pregnancy are manifest during the first trimester and persist into the third trimester. The source of this
excess plasmatic activity is the maternal component of the placenta (175). Earlier observations of an association of low values or rapid decreases in maternal plasma DAO activity with high risk pregnancies (171) were not verified in subsequent work (176). Hence, maternal plasma levels are no current criterion to judge the intactness of the feto-placental unit.

High levels of DAO activity in plasma of patients with medullary carcinoma of the thyroid coincide with similarly high levels of such activity in the tumor proper (172). Marked clinical improvement occurred in one of several such patients receiving aminoguanidine (172), the specific inhibitor of DAO activity.

Heparin administration entails drastic rises in plasma DAO activity in both experimental animals (174) and man (173). In animals, this rise is preceded by activity increases in mesenteric and thoracic duct lymph (174). In man, a concomitant decrease in gastric secretion upon heparin administration, suggesting rapid catabolism of histamine, is preventable by prior administration of aminoguanidine (177). Other conditions associated with increased DAO activity in human plasma include penicillin shock (178) as well as bronchial (179) and uterine cancer (180).

In patients receiving heparin for haemodialysis treatment, plasma DAO activity and total amines were measured at hourly intervals. A pronounced transitory fall in a priori elevated activity levels
occurred at the 4th hour of treatment, coinciding with a marked
but similarly transient increase in plasma total amines. These
changes occurred whether or not haemodialysis was performed in the
fasting state (181). The reciprocity of these changes suggests a
cause and effect relationship such that the decrease in plasma DAO
activity accounted for the increase in plasma total amines observed
(181).

Hydralazine is another effective inhibitor of DAO activity both
in vitro and in vivo, thus explaining its antihypertensive properties,
in part at least; on the basis of delayed histamine inactivation
(182).

Increases in tissue levels of histamine and potentiation of some
of its pharmacological effects in response to DAO inhibitors account
for the conclusion that DAO is in large part responsible for the
termination of the biological effects of histamine (178). This con-
clusion requires reconciliation with evidence showing that in man,
histamine inactivation by DAO and ribosylation of imidazole acetic
acid actually represent a minor pathway of histamine catabolism (21).

Data obtained in sheep indicated that the sensitivity of this
species to exogenous histamine (given orally) was enhanced markedly,
accompanied by increases in urinary levels of endogenous histamine,
when aminoguanidine was administered (183).

When histamine was given subcutaneously either alone or together
with estrogen or SC-11800 (a progestational agent) to premenopausal and post-menopausal non-pregnant women, no increases in plasma DAO activity occurred. Moreover, exogenous histamine did not prevent the typical decline in plasma DAO activity occurring in women post-partum (184). Increases in DAO activity accompanied by increases in endogenous circulating histamine occurred in rabbits and rats as typical consequences of anaphylactic shock (185,186,187). However, no further increases in DAO activity could be elicited by exogenous histamine (188) or by increments in the dose of antigen administered although with such increments the plasma histamine level rose further (189). These data lend support to the thesis that in man and animals, ongoing histamine release rather than an increased level of circulating histamine may function as a substrate inducer of DAO activity.

However, in both rats and guinea pigs, DAO activity in plasma, intestine and lung rose within 5 minutes of thermal injury effected by hot water immersion even after prior depletion of the animals' histamine stores by polymyxin B (190).

In attempts to define the importance of the two pathways of histamine inactivation (methylation versus oxidative deamination) in human asthma, histamine metabolites have been measured in urine under diverse conditions.

In asthmatic and non-asthmatic children subsisting on histamine-restricted diets, no difference was found in excretion patterns of
histamine proper, 1-methyl-imidazole-4-acetic acid, and methyl-
histamine (191).

In patients with intrinsic asthma, the urinary excretion of both methylhistamine and methyl-4-imidazole acetic acid was higher during an acute attack than during remissions. Corticosteroid administration during remissions had no effect on histamine excretion or that of its metabolites (192).

In similar patients and normal subjects, urinary excretion of total radioactivity attributable to $^{14}$C-histamine was comparable and complete within 24 hours whether the amine was instilled intrabronchially or given intravenously, suggesting that histamine rapidly penetrated the bronchial mucosa. $^{14}$C-Histamine given intrabronchially revealed a higher methylation rate than $^{14}$C-histamine given intravenously (193). This may be in line with in vitro data showing that human lung tissue has a considerable methylating capacity (194).

In patients with extrinsic asthma subjected to bronchial challenges by allergen inhalation, urinary excretion of both histamine and methylhistamine was higher in those responding with a minimum decrease in FEV$_1$ by 20%. The majority of such patients also revealed increased excretion of methylimidazole acetic acid. Inhibition of histamine release in vivo was inferred from smaller increases in urinary histamine and methylhistamine after prior administration of Disodium cromoglycate (195).
In recognition of difficulties encountered in histamine determinations, plasma "Histaminase" (DAO) activity was measured together with circulating eosinophils in asthmatic and normal subjects (196). Both histaminase activity and total numbers of eosinophils were higher in asthmatics in whom both parameters correlated significantly. Upon prednisolone administration (2 x 5 mg/day for 3 days), histaminase activity and eosinophils dropped markedly in both groups. Since plasma histamine proper was not measured concomitantly, these studies afforded no clue as to whether the high levels of histaminase activity in the asthmatics reflected an "increased need for histamine catalysis" (sic) (196).
HISTAMINE DETERMINATION

In the foregoing, data on changes in histamine levels studied under diverse conditions were reviewed without scrutiny of the methodology involved. A survey of methods used for histamine determinations is given below. Histamine was first determined chemically by colorimetry after coupling it to a diazotized aromatic amine or to dinitrofluorobenzene (197). Alternatively, histamine was measured by isotopic dilution by coupling it with labeled pipsylchloride followed by addition of unlabelled dipipsyl-histamine and recrystallization to constant specific activity (198,199).

More recent methods capitalize on the greater sensitivity and specificity of fluorometric estimations (200-204) and, in the case of enzymatic isotopic procedures, on the specificity of the enzyme, histamine N-methyl transferase (205-211). None of the two last-named analytical approaches have completely superseded the classical procedure of determining histamine by bioassay, especially in circumstances in which comparisons with other smooth muscle contractants (e.g. SRS-A) are warranted (212).

Separation procedures have included ion exchange chromatography (213) and thin layer chromatography (214). Only very recently has histamine been determined in biological tissues (215,215-217) and fluids (265,216) and in food industries (214,218) by high pressure liquid chromatography (HPLC) (215,217) and reversed phase HPLC (218).
A modification of the fluorometric determination of histamine in whole blood with O-phthalaldehyde by the method of Anton and Sayre (219) (itself a modified version of the procedure originally proposed by Shore et al. (200)), was used in earlier work reported by the writer (129). Elaborate purification steps including both column chromatography and solvent extraction introduced by Loréz et al. since (220) are claimed to ensure more valid determination of histamine in plasma by fluorometry. These steps are time consuming, limiting their suitability for serial or large-scale analyses.

Biological (221,222) and fluorometric (223-226) assays hitherto yielded values for plasma histamine in normal subjects ranging between 3 and 8 ng/ml (221-226). However, with the preparatory steps aimed at rigid separation of histamine from interfering substances mentioned above, mean levels of plasma histamine in such subjects were below 1 ng/ml (range 0.1 - 1.4 ng/ml) (220,227,228). It has, therefore, been entertained that a preponderance of absolute values for histamine in plasma reported in the literature must be artifacts (229).

Enzymatic isotopic histamine assays based upon the specificity of histamine-N-methyltransferase (HNMT) have been claimed to be superior to fluorometry due to their direct applicability, in theory at least, to biological materials, thereby opening new avenues for research in histamine metabolism (205,206). As originally proposed by Snyder et al. (230), $^{14}$C-methyl-histamine is formed in these assays from histamine and S-adenosyl-methionine-$^{14}$C-methyl in the presence of
this enzyme. This principle, now widely used in several modifications which include single (231,252) and double (205,206,233,234) isotope derivative dilution analyses, has revealed its own pitfalls. A major disturbing finding is that in most analytical runs, values for histamine in plasma were lower than those given by enzyme blanks (206, 235). Furthermore, inhibition of the added crucial enzyme HNMT by human plasma proper was found by Beaven et al. (205) when the usual plasma volume assayed, 100 μl or less, was increased to 200 μl. On the assumption that proteinaceous substances accounted for such inhibition, Nisam et al. (207) resorted to a boiling step followed by high-speed centrifugation for their removal. This boiling step may in addition destroy any endogenous S-adenosyl methionine, as originally suggested by Snyder et al. (230). The writer found this step to be impractical for reasons given in the experimental section of this thesis (cf. page 66).

HNMT whether prepared from guinea pig brain or rat kidney has been found to be inhibited by its own substrate, histamine, at concentrations above 10^{-5}M and also by its product, N-methylhistamine, at concentrations above 10^{-3}M (209). In addition, HNMT from either source has been shown to be inhibited by a number of H1 and H2 receptor agonists and antagonists and by certain antimalarials (209,236,239). These findings have forfeited the validity of the enzymatic isotopic assay for histamine when applied to urine of patients receiving these drugs. In these patients, urinary amounts of these drugs apparently sufficed to inhibit HNMT completely (209,236,237).
An interfering enzyme activity, present in hitherto conventional HNMT preparations from guinea pig or rat brains (205, 206, 230, 233, 240) was found to limit the sensitivity of the assay due to the production of measurable amounts of chloroform-extractable radioactive material (205). HNMT preparations from an alternative source, viz., rat kidney, were only recently shown to be devoid of this interference, as judged by lower values for assay blanks (209).

Finally, it was recently found that any absolute values for this amine in biological specimens reported in the literature must be regarded with caution whether based upon the enzymatic isotopic or fluorometric assay (241). This conclusion emerged from widely discrepant results, with unacceptably large coefficients of variance, obtained with one and/or the other of the two analytical approaches by 21 voluntary participants in a large-scale quality control survey (241). This outcome should underscore the difficulty of attempts at meaningful comparisons of data in a review of the literature on histamine metabolism:
1.3 **BRONCHIAL INHALATION CHALLENGES**

Bronchial provocation tests have been mentioned earlier whenever they were part of the experimental design underlying data on histamine metabolism already quoted. Since such tests are an intricate part of work to be reported in this thesis, the present section reviews the rationale and limitations of such tests from a clinical viewpoint.

At present, the most common agents used for inhalation challenge procedures include antigen, methacholine (MCH) and histamine.

Inhalation of antigen aerosols has permitted identification of the specific allergens(s) giving rise to asthmatic symptoms although the large number of antigens makes selection difficult (242). Antigen inhalation has further resolved discrepancies between history and skin tests reactions in a given patient and in situations in which excessive or absent skin tests reactivity was at variance with clinical impression (242). Antigen inhalation by a proven asthmatic may be of relevance for subsequent intervention by immunotherapy or specific avoidance and may be indicated in the recognition and evaluation of hitherto unsuspected allergens (243).

The diagnostic usefulness of bronchial provocation with methacholine rests upon the observation that the majority of asthmatic subjects are extremely sensitive to the bronchoconstrictive effects of
cholinergic agents (244, 245). Thus, a bronchoconstrictive response to methacholine has been attested to asthma in patients who by history should have reversible airway obstruction, but in whom a typical attack was never witnessed by the clinician and/or did not materialize with antigen inhalation challenge. Other areas of usefulness have included differentiation of a cough attributable to asthma from one of another etiology; consideration of an obstructive lesion in a wheezing patient with a negative methacholine response; screening of job applicants for high risk operations, e.g., red wood lumber workers; screening of suspected asthmatics wishing to join the armed forces or certain police agencies (246). However, the methacholine test is not infallible; evidence points to the possibility of false positives in non-asthmatic subjects with rhinitis or chronic bronchitis, and of false negatives in some patients with convincing histories of asthma (247). Moreover, corticosteroids have been reported to increase bronchial sensitivity to methacholine in some patients (248, 249), and not to affect it in others (246).

The asthmatic subject is similarly sensitive to the bronchoconstrictive effects of inhaled histamine even after having been free from symptoms for many years (244, 246, 250). Indications for histamine inhalation challenge are thus similar to those mentioned above for methacholine. Evidence suggests that the relative response to the inhalation of histamine and methacholine is a better criterion for differentiation between asthmatics and non-asthmatics than the response
to antigen inhalation (251-253).

However, differences in responses to these two agents by individual asthmatics have been reported (246). In a large-scale study, patients capable of sustaining high doses of methacholine did not suffer from a severe form of asthma whereas those tolerating high doses of histamine tended to have the reagin-mediated form of the disease, suggesting that inhalation of the two agents may be useful in the delineation of subgroups of asthmatics (246).

Recognized limitations and inconsistencies in bronchial inhalation challenge testing involve problems inherent to the inhalants or their administration as well as problems arising from the measurement of the response. As an example of the former, non-specific bronchoconstrictive reactions may occur with antigen inhalation as a result of irritant or toxic impurities (254,255) stressing a need for rigidly purified allergens and their standardization for clinical use.

With existing delivery systems, there has been uncertainty as to whether the desired dose of the inhalant reaches the responsive airway (s). At the present time, the accepted and only method of measuring response is by pulmonary function criteria. Factors known to affect pulmonary function testing include the observer error, lack of consistency of the subject's effort, the known effect of the subject's repetitive effort in serial measurements, and his suggestibility (256).

In cognizance of some of these drawbacks, criteria for procedures
and materials for standardization of bronchial inhalation challenges were delineated by a panel of investigators from the Asthma and Allergic Disease Control Centers (AADC) and the National Institutes of Health, Bethesda, Md. (257). This panel suggests the change in the Forced Expiratory Volume in the First Second (FEV₁) as an adequate criterion for measuring response to inhalation of antigen, methacholine, and histamine by increments, in each instance considering its minimum fall by 20% as a positive response (257). Lesser percent decreases have been suggested by other workers as a criterion for such a response (251,258,259), thus in some instances foregoing comparison of data.
1.4. **SCOPE OF THESIS**

From the foregoing literature review, conflicting evidence emerges for a role of histamine in the mediation of acute human asthma in vivo. Therefore, the objective of this thesis was to re-define this role based upon relationships between changes in Forced Expiratory Volume in the first second (FEV₁), plasma histamine (HM) and plasma Diamine oxidase (DAO) occurring in human subjects during asthma induced by inhalation of methacholine (MCH).

As a preliminary to this object, the validity of the principle of enzymic double isotopic derivative dilution analysis chosen for serial determinations of plasma HM was scrutinized, highlighting a necessity of performing these determinations on plasma ultrafiltrates for exclusion of interfering substances (Chapter 2).

A similar scrutiny of the assay for plasma DAO activity (which measured the H₂O₂ generated in the oxidative deamination of HM) culminated in the characterization of hitherto unsuspected concentration-dependent effects of MCH of enhancing or inhibiting this activity in vitro (Chapter 3).

In self-contained control tests, changes in plasma HM and plasma DAO occurring in normal and asthmatic subjects were determined serially during four distinct periods not involving inhalation of bronchoconstrictive agent (Chapter 4).
Changes in these two variables and in FEV₁ were encompassed in volunteers during controlled attempts at inducing asthma with MCH inhaled by increments to a defined permissible limit. Based upon bronchial sensitivity to this agent, subjects were retrospectively divided into two groups (Responders versus Non-Responders) for within group and between group comparisons of results (Chapter 4).

A discussion (Chapter 5) of the data presented is followed by a summary and conclusion (Chapter 6).

An appendix to this thesis compares and discusses changes in plasma HM and DAO occurring in a limited number of subjects who re-inhaled methacholine after pre-medications with Disodium cromoglycate (DSCG) and Cimetidine (CMTD).
CHAPTER 2: DETERMINATION OF HISTAMINE IN ULTRAFILTRATES OF HUMAN PLASMA

BY DOUBLE ISOTOPIC DERIVATIVE DILUTION ANALYSIS (DIDDA)

2.1 INTRODUCTION

The review of methods used for determination of histamine in biological materials culminated in the recognition that even the double isotope enzymatic assay capitalizing on the specificity of the enzyme, HNMT, is not free from drawbacks. These drawbacks may be re-iterated as follows:

(1) Inhibition of purified HNMT by human plasma was found by Beaven et al. (205) when the usual plasma volume assayed, 100 μl or less, was increased to 200 μl.

(2) In a preponderance of analytical runs, values computed for histamine in plasma were lower than those given by enzyme blanks (206).

(3) Purified HNMT prepared from recommended sources (guinea pig brain or rat kidney) was found to be inhibited by substrate (histamine) and product (N-methylhistamine) alike when either exceeded certain critical concentrations (209).

(4) HNMT was also found to be inhibited by the H2-blocker Cimetidine (CMTD) (209), thus forfeiting the validity of the assay for histamine during bronchial provocations of subjects pre-medicated with this agent.
(5) Widely divergent results for histamine were obtained in a published large-scale quality control study.

The greater sensitivity of the enzymatic double isotopic derivative dilution principle relative to fluorometry made it ideally suited for detection of changes in histamine concentrations within the low range of values reported for human plasma (220, 227, 228), provided its above-mentioned drawbacks could be overcome.

This section describes the modifications necessary to eliminate some of these drawbacks, culminating in the introduction of an ultrafiltration step and the harvesting of HNMT from rat brains in lieu of the conventional sources already stated.

The principle of Enzymatic Double Isotopic Derivative Dilution Analysis for histamine under scrutiny will henceforth be referred to as DIDDA.

STATISTICAL METHODS

A programmable electronic calculator (Texas Instruments, Model SR-51-II) was used for computation of means and standard deviations, coefficient of variance and linear regression analyses.
2.2 MATERIALS

REAGENTS: Spectrally purified chloroform, scintillated tolune, perchloric acid, hydrochloric acid, sodium hydroxide, sodium phosphate monobasic, sodium phosphate dibasic, and crystalline non-labeled histamine dihydrochloride purchased from Fisher Scientific Company (Toronto, Ontario) were of analytical grade.

S-adenosyl-L-methionine ($^{14}$C-methyl), $^{14}$C-SAM; 46-47 mCi/mmol (200 nCi/ml in H$_2$SO$_4$). 2',5-diphenyloxazole (PPO) of scintillation grade and Triton X-100 (alkyl phenoxyl polyethoxy ethanol) were obtained from New England Nuclear Corporation (Dorval, Quebec).

$^{3}$H-histamine dihydrochloride, 15.6 Ci/mmol (1 mCi/ml ethanol) (Amersham/Searle Corporation, Oakville, Ontario) was diluted with glass distilled water to a concentration of 1 μCi/ml. The diluted material was partitioned into multiple aliquots, which were stored at -60°C. Twenty microlitres of diluted radioactive histamine solution containing 20 nCi 3H-histamine were used as internal standards to monitor histamine recovery.

Stock solutions containing 1.0 mg of non-labeled histamine (free base) per millilitre of 0.1N HCl were similarly partitioned and kept frozen at -60°C. Dilutions of histamine stock solutions providing 1.25, 5.00 and 10.00 nanograms histamine/100 μl H$_2$O were prepared immediately prior to each analytical run.
Crystalline non-labeled 1-methylhistamine (1-methyl-4(8-amin-ethyl)imidazole) dihydrochloride of "A" grade (Calbiochem-Behring Corp., La Jolla, Calif., U.S.A.) was dissolved in 0.4N perchloric acid to a concentration of 100 μg/200 μl and stored at -60°C.
HNMT: PARTIAL PURIFICATION

HNMT was partially purified from rat brains by a modification of the method of Brown et al. (230,260) as summarized in Fig. 2-1. All manipulations were carried out at 4°C. In brief, 100 brains obtained from male Sprague-Dawley rats weighing between 200 and 300 g (Pel Freeze Biologicals, Rogers, Arkansas, U.S.A.) were homogenized in 0.25M sucrose using a Polytron model PCU-1 (Brinkmann, Rexdale, Ontario). The homogenate was centrifuged at 30,000 g for 40 minutes. To the supernatant, ammonium sulfate was added to a final concentration of 45%, followed by agitation. After centrifugation at 30,000 g for 30 minutes, ammonium sulfate was added to the supernatant to a final concentration of 70%. The pellet obtained after agitation and centrifugation at 30,000 g for 30 minutes was dispersed in 0.1M sodium phosphate buffer, pH 7.4. The dispersed material was dialyzed in three stages for a total of 24 hours against 30 volumes of 0.01M sodium phosphate buffer (pH 7.4) per stage. The dialysate was centrifuged at 5,000 g for 20 minutes. The supernatant was partitioned into several aliquots which were immediately frozen at -60°C, followed by their lyophilization at -20°C for 40 hours. The lyophilates of HNMT were stored at -20°C.

Protein content was monitored by the dye-binding assay principle available in kit form from Bio-Rad Laboratories (Mississauga, Ontario, Technical Bulletin 1051).

HNMT activity was monitored by the reciprocal (232) of the DITIDA principle for histamine presently under scrutiny.
Homogenize rat brains in 9 volumes of 0.25M Sucrose \[\ldots\ldots\] Fraction (A)

- Pellet (discard)
  - Supernatant
    - + (NH₄)₂SO₄ to 45\% (.258g/ml)
      - stir for 30 mins.
      - allow to set for 30 mins.
      - centrifuge @ 30,000 g for 30 mins.

- Pellet (discard)
  - Supernatant \[\ldots\ldots\] Fraction (B)
    - + (NH₄)₂SO₄ to 70\% (.156g/ml)
      - stir for 30 mins.
      - allow to set for 30 mins.
      - centrifuge @ 30,000 g for 30 mins.

- Supernatant (discard)
  - PELLET
    - + 0.1M sodium phosphate buffer, pH 7.4 \[\ldots\ldots\] Fraction (C)
      - dialyse against 0.01M sodium phosphate buffer pH 7.4
  - DIALYSATE
    - centrifuge @ 5,000 g for 20 mins.

- Pellet (discard)
  - Supernatant
    - freeze @ -60°C for 1 hr.
    - lyophilize @ -20°C for 40 hrs.

LYOPHILATE OF HNMT
Store @ -20°C

FIG. 2-1: Flow Sheet - Purification of rat brain Histamine N-methyltransferase
Table 2-1 is illustrative of the gain in specific activity and recovery of HNMT when this enzyme is partially purified from rat brain, as summarized in Fig. 2-1. Omission of respective data from similar work dealing with the harvesting of HNMT from rat brains (233) precluded direct comparisons. The 5.8 fold increase in specific activity emerging from the data shown in Table 2-1 is comparable to the gain achieved when HNMT is partially purified from a conventional source (guinea pig brains) (230).

In agreement with data for HNMT partially purified from the latter source (209,261) HNMT partially purified from rat brains, showed a pH optimum of 7.9 (Fig. 2-2) as well as inhibition by substrate concentration (Histamine) greater than $2 \times 10^{-5}$M (Fig. 2-3). Inhibition of HNMT beyond that substrate concentration extended to physiological pH (Fig. 2-3).

The $K_m^{app}$ for the partially purified rat brain enzyme, $7.1 \pm 0.5 \times 10^{-5}$M at pH 7.9 (Fig. 2-4), is in reasonable agreement with the respective value reported for HNMT partially purified from guinea pig brain (209,261).

As described in detail later (cf. page 75), the activity of HNMT partially purified from rat brains was unaffected by lactose, DSCG, CMTD and MCH when used for histamine determinations in plasma by DIDDA.
<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Total Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (U/mg protein)</th>
<th>Total Units (U)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Whole homogenate</td>
<td>338</td>
<td>81</td>
<td>27,378</td>
<td>0.11</td>
<td>3115</td>
<td>100</td>
</tr>
<tr>
<td>(B) Supernatant of 45% ammonium sulphate</td>
<td>449</td>
<td>12</td>
<td>5,388</td>
<td>0.27</td>
<td>1434</td>
<td>46</td>
</tr>
<tr>
<td>(C) Pellet of 70% ammonium sulphate, dissolved in 0.1M buffer</td>
<td>221</td>
<td>6</td>
<td>1,266***</td>
<td>0.64</td>
<td>805</td>
<td>26</td>
</tr>
</tbody>
</table>

* as defined on flowsheet (Fig. 2-1)
** one unit (U) of HNMT = 1 nmole of $^{14}$C-methylhistamine/mg protein/hr @ 37°C

$= 213,310$ DPM/mg protein/hr @ 37°C

*** >95% (w/w) of lyophilate
Fig. 2-2: Relationship between pH and CPM = $^{14}$C-Methylhistamine at two concentrations of Histamine
Fig. 2-3: Effect of substrate (histamine) concentration on rat brain Histamine N-methyltransferase activity at pH 7.9 (optimum) and 7.4
2.3 Method

For determinations of HM, the procedure of Beaven et al. (205) was followed. In brief, 100 μl of native plasma were combined with 346 μl of 0.1M sodium phosphate buffer, pH 7.9; 1 mg of HNMT; 4 μl of $^{14}$C-SAM and 20 μl of $^3$H-histamine dihydrochloride (DHC) to a final volume of 470 μl in siliconized glass culture tubes provided with teflon-lined screw caps. After brief centrifugation followed by agitation, the mixture was incubated at 37°C for 90 mins in a Magni-Whirl constant temperature water bath. The reaction yielding radioactive methylhistamine as the critical product was then stopped in an ice bath.

Methylhistamine, 200 μg provided in 200 μl of a 0.4N solution of HClO₄ was then added as a carrier, followed by addition of 200 μl of 10N NaOH. Chloroform, 4.0 ml was next added. After vigorous shaking for 10 minutes for extraction of radioactive and carrier methylhistamine, tubes were centrifuged at 500 g for 10 minutes followed by aspiration of the aqueous phase. The chloroform phase was then washed with 1.0 ml of 3.5N NaOH, followed by centrifugation at 500 g for 10 minutes.

Of the washed chloroform solution containing methylhistamine, 3.0 ml was pipetted into a scintillation counting vial and permitted to dry in air. The residue was dissolved in 10.0 ml scintillation fluid which was comprised of 12.02 g of PPO dissolved in 2.04 L of toluene and 1.0 L of Triton X-100.
An assay reagent blank and three standard assay mixtures containing histamine in known amounts were included in each analytical run.

Radioactivity was counted in a Beckman Scintillation System model LS-150 at a counting efficiency of 36.0 ± 1.6 (S.D.) % for \(^3\)H and 78.1 ± 1.1 % for \(^14\)C based upon 30 and 38 analytical runs, respectively.

Counts per minute (CPM) of \(^14\)C due to methylhistamine were corrected for those given by the reagent blank prior to application of the following equation for calculation of histamine:

\[
(I) \quad \frac{\text{CPM}_{\text{H}}^{14}}{\text{CPM}_{\text{H}}^{\text{recovered}}} \times \frac{\text{CPM}_{\text{H}}^{3}}{\text{CPM}_{\text{H}}^{\text{added}}} = \text{CPM}_{\text{H}}^{14} \text{ corrected for 100% recovery}
\]

The histamine content in plasma was then determined from the regression line obtained by applying the above equation to the standard assay mixtures.

Typical examples of such regression lines, illustrating linearity between known amounts of histamine (ranging from 78 picograms to 50 nanograms/470 μl assay mixture) and \(^14\)C due to methylhistamine, are shown in Fig. 2-5.

Table 2-2 shows the variation in \(^14\)C counts given by blanks and known amounts of histamine (corrected for 100% recovery of \(^3\)H-histamine) over a period of 7 months.
Fig. 2-5: Relationship between $^{14}$C-Methylhistamine and concentration of histamine

*To convert HM concentration in ng/ml to nMoles/L; divide by a factor of 0.11.*
TABLE 2-2: Variation in $^{14}$C Counts Given by Assay Blanks and Known Amounts of Histamine

<table>
<thead>
<tr>
<th>Histamine (ng/assay mix.)</th>
<th>$^{14}$C-methylhistamine* (CPM/3.0 ml CHCl$_3$)</th>
<th>Coefficient of Variance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>635 ± 61**</td>
<td>9.6</td>
</tr>
<tr>
<td>1.25</td>
<td>1216 ± 86</td>
<td>7.1</td>
</tr>
<tr>
<td>5.00</td>
<td>2894 ± 114</td>
<td>3.9</td>
</tr>
<tr>
<td>10.00</td>
<td>4580 ± 435</td>
<td>9.5</td>
</tr>
</tbody>
</table>

* Corrected to 100% recovery
** Mean ± S.D. (N=9)
While the data shown so far illustrate the validity of DIDDA when applied to standard concentrations of histamine dissolved in buffer, its applicability to human plasma was hampered by the limitations already stated by Beaven et al. (205). These limitations implied that HNMT was inhibited by human plasma when the usual plasma volume assayed, 100 μl or less, was increased to 200 μl.

Attempts were therefore made by the writer to determine the minimum plasma volume exerting such inhibition; any smaller plasma volume should then be suitable for valid determination of its histamine content. Accordingly, volumes of a given plasma ranging from 25 to 200 μl were incorporated in the assay mixture at the expense of buffer.

Fig. 2-6 representative of 18 distinct plasma sources, invariably shows an inverse relationship between plasma volume and counts due to 14C-methylhistamine; even at the lower echelon of the range of plasma volumes analyzed, inhibition exerted by plasma on HNMT was apparent. Fig. 2-6 thus illustrates that the validity of DIDDA for histamine in human plasma is hampered beyond the limitations already stated by Beaven et al. (205).

On the assumption that proteinaceous substances present in plasma exerted the inhibition on HNMT, Beaven et al. (205) suggested boiling of plasma followed by high-speed centrifugation for their removal as an option prior to analysis. A similar maneuver was applied by Nisam et al. (207) as a routine. Snyder et al. (230) have drawn attention
to the possibility of partial destruction of histamine by boiling. With large amounts of plasma, the writer found no difference in counts given by $^1\text{C}$-methylhistamine between boiled and non-boiled samples, thus militating against interference by endogenous s-adenosyl methionine (230) with the analytical principle under scrutiny. Boiling followed by high-speed centrifugation of 1.0 ml of native plasma yielded supernatants of unpredictable volumes which in most instances were too small for duplicate or even single histamine analyses. Since plasma histamine was furthermore to be determined serially by DIDDA in human subjects undergoing bronchial inhalation challenge, removal of larger amounts of blood, thereby increasing the amounts of plasma available for boiling followed by high-speed centrifugation, was ill advised.

Stabilization of the sulfhydryl groups of HNMT by incorporation into the assay mixture of reduced glutathione, dithiothreitol or zinc sulfate did not overcome the inhibition exerted on HNMT by plasma demonstrated in Fig. 2-6.

**INTRODUCTION OF PLASMA ULTRAFILTRATION STEP.** Ultrafiltration of plasma followed by application of DIDDA for histamine to plasma ultrafiltrates suggested a solution to the latter problem.

Membrane cones retaining molecules above 25,000 MW were used ("CENTRIFLO" type CF25, Amicon Canada Ltd., Oakville, Ontario). In keeping with the manufacturer's instructions, these re-usable cones
Fig. 2-6: Relative change in corrected counts of $^{14}$C in methyl-histamine with volume of plasma subjected to DIDDA.
were first soaked in distilled water for at least one hour, followed
by their insertion into the manufacturer's conical support and centri-
fugation to remove excess water.

In preliminary experiments, $^3$H-histamine was added to pooled human
plasma. After establishing its radioactivity as CPM/50 μl, volumes
ranging from 0.25 to 1.0 ml were placed onto the cones, followed by
centrifugation at 1,000 g for 30 minutes at room temperature. Radio-
activity was then re-determined on 50 μl aliquots of plasma ultrafil-
trates so obtained. Similar aliquots of ultrafiltrate were subjected
to the DIONDA principle for histamine under scrutiny.

Table 2-3 shows that irrespective of the differences in plasma
volumes subjected to ultrafiltration, counts due to $^3$H in 50 μl of ultra-
filtrates were comparable to those measured in identical volume of native
plasma, thus attesting to the efficiency of the ultrafiltration process.

Furthermore, after application of DIONDA to ultrafiltrates, counts
reflecting $^3$H in the double-labeled methylhistamine formed and extracted
into chloroform were recovered to a remarkably constant percentage
($52.04 ± 1.21\%$ (SD)), again despite the differences in original volumes
of native plasma filtered.

Fig. 2-7A reveals linear regression lines of comparable slopes for
known amounts of $^3$H-histamine present in native plasma (1.0 ml) and
counts given by 50 μl plasma aliquots before and after ultrafiltration.
In addition, Fig. 2-7A establishes a direct relationship between the
TABLE 2-3: Recovery of $^3$H-histamine from Plasma Ultrafiltrates Before and After DIDDA

<table>
<thead>
<tr>
<th>Volume native plasma filtered (ml)</th>
<th>Before DIDDA $^3$H-histamine/50 µl ultrafiltrate</th>
<th>After DIDDA Double-labeled MeHm/50 µl ultrafiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPM</td>
<td>% Recovery</td>
<td>CPM ($^3$H)</td>
</tr>
<tr>
<td>-----</td>
<td>------------</td>
<td>-------------</td>
</tr>
<tr>
<td>.025</td>
<td>697,056 ± 1056</td>
<td>112.4</td>
</tr>
<tr>
<td>.50</td>
<td>679,510 ± 16758</td>
<td>109.5</td>
</tr>
<tr>
<td>.75</td>
<td>652,982 ± 6130</td>
<td>105.2</td>
</tr>
<tr>
<td>1.00</td>
<td>653,914 ± 10090</td>
<td>105.4</td>
</tr>
</tbody>
</table>

* Corrected for 100% recovery; based upon N=8, the concentration of histamine in pooled plasma, calculated according to equation 1 (cf. p.62), was 28.00 ± 1.15 ng/50 µl (mean ± S.D.).
Fig. 2-7A: Relationship between known amounts of $^3$H-histamine (Hm) present in native plasma, counts given by 50 ul aliquots before and after plasma ultrafiltration, and counts given by $^{14}$C of the double-labeled methylhistamine formed in 50 ul ultrafiltrates after DIDDA.

Fig. 2-7B: Application of DIDA to aqueous solutions containing unlabeled Hm in known concentrations before and after their ultrafiltration: Relationship between concentration of Hm and counts due to $^{14}$C-methylhistamine.
counts of tritiated histamine present in native plasma and counts given by $^{14}$C of the double-labeled methylhistamine formed in 50 µl aliquots of plasma ultrafiltrate subjected to DIDDA.

Analysis by DIDDA of identical volumes of standard solutions containing unlabeled histamine in widely varying amounts before and after their ultrafiltration, yielded comparable-linear regression lines between concentration of histamine and $^{14}$C of double-labeled methylhistamine (Fig. 2-7B).

Fig. 2-8 is representative of varying volumes of distinct plasma sources subjected to DIDDA before (Left half; native plasma) and after (right half) ultrafiltration. Data for native plasma not only show an inverse relationship between plasma volume and counts given by $^{14}$C in the methylhistamine formed, but also confirm observations by others (206) to the effect that regardless of the volume analyzed, a preponderance of corrected counts is below those of enzyme blanks. By contrast, a direct relationship between corrected counts and volume, starting from much higher values, emerges for plasma ultrafiltrates; only in two instances which were confined to the lowest volume (50 µl) of ultrafiltrate, were the corrected counts slightly below blank values.

Fig. 2-8 thus demonstrates that inhibitors of HNMT present in native plasma are effectively retained by ultrafiltration so as to change an inverse relationship between $^{14}$C counts of methylhistamine
Fig. 2-8: Analysis of varying volumes of distinct plasma samples for Histamine by DIDDA: Relationship between volume analyzed and counts due to $^{14}$C-methylhistamine before and after plasma ultrafiltration.
and volume analyzed by DIDDA to a direct relationship when plasma ultrafiltrates are used.

Through the courtesy of Dr. G. J. Gleich—(Allergic Diseases Research Laboratory, Mayo Medical School and Mayo Clinic and Foundation)—the writer was in a position to analyse by DIDDA coded lyophilates containing known amounts of histamine dihydrochloride. These lyophilates were prepared in his laboratory for the large-scale voluntary quality control study involving 22 participating laboratories referred to earlier (cf. page 43 and ref. 241). In keeping with the instructions of Gleich and Hull (241), lyophilates were reconstituted by the writer with 2.4 ml deionized water to provide the concentrations of histamine listed in Table 2-4.

This table shows that with the modifications under scrutiny (HNMT prepared from rat brains; ultrafiltration of samples), the over-all accuracy was 95.87 ± 17.88 (SD) % of known values* when histamine was determined by DIDDA by the writer, with an over-all C.V. (N=5) between two analytical runs of 13.31 ± 13.80 (SD) %. The coefficient of variance for the 15 duplicate determinations was 4.75 ± 3.85 (SD) %.

Separate data of the reproducibility of values obtained on ultrafiltrates of unknown plasma samples by DIDDA using HNMT prepared from rat

* Data are presented with the explicit understanding that the lyophilates were received from Dr. Gleich some 2 years after completion and publication (241) of the quality control study for which they were prepared; for this reason, their histamine content was known to the writer beforehand.
TABLE 2-4: Histamine in coded lyophilizes: Known versus determined values after reconstitution with 2.4 ml deionized water

<table>
<thead>
<tr>
<th>Code</th>
<th>Histamine added (ng/ml)</th>
<th>July 21, 1981 (ng/ml)</th>
<th>July 31, 1981 (ng/ml)</th>
<th>Mean ± S.D. (ng/ml)</th>
<th>% of known value</th>
<th>C.V. #(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21Z</td>
<td>4.00</td>
<td>2.80 ± 0.01*</td>
<td>4.29 ± 0.53</td>
<td>3.55 ± 1.05</td>
<td>88.75 ± 18.50</td>
<td>29.58</td>
</tr>
<tr>
<td>92A</td>
<td>12.80</td>
<td>15.07 ± 0.59</td>
<td>14.94 ± 1.44</td>
<td>15.01 ± 0.09</td>
<td>117.18 ± 0.70</td>
<td>0.60</td>
</tr>
<tr>
<td>45X</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4FP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>295SP</td>
<td>4.80</td>
<td>4.74 ± 0.31</td>
<td>5.25 ± 0.17</td>
<td>4.00 ± 1.05</td>
<td>85.33 ± 21.95</td>
<td>26.34</td>
</tr>
<tr>
<td>2KP</td>
<td>11.20</td>
<td>10.96 ± 0.33</td>
<td>12.40 ± 0.52</td>
<td>11.68 ± 1.02</td>
<td>104.28 ± 9.10</td>
<td>8.73</td>
</tr>
<tr>
<td>42DP</td>
<td>16.00</td>
<td>13.67 ± 0.20</td>
<td>13.93 ± 0.11</td>
<td>13.80 ± 0.18</td>
<td>86.25 ± 1.12</td>
<td>1.30</td>
</tr>
</tbody>
</table>

# The coefficient of variance for all analytical runs was 13.31 ± 13.80 (SD)% (N=5).
b Buffered saline samples.
* Mean ± range between sample duplicates.
p Plasma samples.
brains are shown in Table 2-5.

The combined evaluation of data shown in Table 2-4 and 2-5 yields a coefficient of variance of $13.29 \pm 12.72$ (SD) % between analytical runs, attesting to the accuracy of the procedure.

VALIDITY OF DIDDA APPLIED TO PLASMA ULTRAFILTRATES IN PRESENCE OF POTENTIALLY INTERFERING AGENTS. Using HNMT routinely prepared from rat brain, as described earlier (cf. p.54), the validity of DIDDA was tested in the presence in standard assay mixtures of DSGC, lactose (its inhalation vehicle) and CMTD in widely varying concentrations. Table 2-6 shows that the net counts given by 10 ng of histamine in the presence of these agents were comparable to those given in their absence.

Similarly comparable results for histamine by DIDDA were obtained when lactose, DSGC, CMTD, and also MCH were added in known amounts to plasma ultrafiltrates (Table 2-7) and when native plasma was preincubated with these agents for 1/2 hr prior to its ultrafiltration (Table 2-8).

The effect of varying the amounts of methacholine (MCH) added to known amounts of histamine present in standard mixtures was separately studied. Table 2-9 shows that for a given histamine concentration, corrected counts for $^{14}C$-methylhistamine in the presence of methacholine were comparable to those obtained in its absence.
TABLE 2-5: Reproducibility of values obtained for histamine by DIDDA of ultrafiltrates of unknown plasma

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>ng/ml</th>
<th>Mean ± SD</th>
<th>C.V. (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB</td>
<td>2.96</td>
<td>2.72 [12]**</td>
<td>2.84 ± 0.17</td>
</tr>
<tr>
<td>9HLH11</td>
<td>521.60</td>
<td>310.06 [7]</td>
<td>414.85 ± 149.58</td>
</tr>
<tr>
<td>9HLH13</td>
<td>658.29</td>
<td>515.66 [7]</td>
<td>586.98 ± 71.32</td>
</tr>
</tbody>
</table>

* C.V. for entire series (N=5): 13.27 ± 13.19 (SD)%
** [ ] = number of days between analytical runs
TABLE 2-6: Effects of Lactose, DSCG and CMTD on HNMT in 470 μl Assay mixtures containing 10 ng histamine.

<table>
<thead>
<tr>
<th>CONCENTRATION</th>
<th>Net CPM = $^{14}$C-Methylhistamine/ 3 ml Chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIL</td>
<td>1395 ± 4*</td>
</tr>
<tr>
<td>Lactose:</td>
<td></td>
</tr>
<tr>
<td>6.2 x 10^{-8}M</td>
<td>1533 ± 48</td>
</tr>
<tr>
<td>6.2 x 10^{-7}M</td>
<td>1466 ± 24</td>
</tr>
<tr>
<td>6.2 x 10^{-6}M</td>
<td>1519 ± 88</td>
</tr>
<tr>
<td>6.2 x 10^{-5}M</td>
<td>1426 ± 36</td>
</tr>
<tr>
<td>DSCG:</td>
<td></td>
</tr>
<tr>
<td>4.2 x 10^{-8}M</td>
<td>1414 ± 14</td>
</tr>
<tr>
<td>4.2 x 10^{-7}M</td>
<td>1402 ± 15</td>
</tr>
<tr>
<td>4.2 x 10^{-6}M</td>
<td>1357 ± 11</td>
</tr>
<tr>
<td>4.2 x 10^{-5}M</td>
<td>1551 ± 42</td>
</tr>
<tr>
<td>CMTD:</td>
<td></td>
</tr>
<tr>
<td>8.4 x 10^{-7}M</td>
<td>1475 ± 2</td>
</tr>
<tr>
<td>8.4 x 10^{-6}M</td>
<td>1661 ± 8</td>
</tr>
<tr>
<td>8.4 x 10^{-5}M</td>
<td>1432 ± 167</td>
</tr>
<tr>
<td>8.4 x 10^{-4}M</td>
<td>1358 ± 13</td>
</tr>
</tbody>
</table>

*Mean ± range after correction for reagent blanks yielding 431 ± 79 CPM*
TABLE 2-7: Effects of Lactose, DSCG, CMTD and MCH on HNMT in 470 µl assay mixture containing 100 µl of plasma ultrafiltrate (PUF)

<table>
<thead>
<tr>
<th>Addition to PUF (Moles/Litre)</th>
<th>(^{14}\text{C} = \text{methylhistamine (CPM/3.0 ml CHCl}_3\text{)})</th>
<th>Histamine (ng/ml PUF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIL</td>
<td>10022 ± 82*</td>
<td>42.0 ± 5.5</td>
</tr>
<tr>
<td>Lactose: (6.2 \times 10^{-5})</td>
<td>10924 ± 757</td>
<td>45.7 ± 5.2</td>
</tr>
<tr>
<td>DSCG: (4.2 \times 10^{-5})</td>
<td>9939 ± 226</td>
<td>41.6 ± 1.0</td>
</tr>
<tr>
<td>CMTD: (8.4 \times 10^{-4})</td>
<td>11657 ± 575</td>
<td>48.8 ± 2.4</td>
</tr>
<tr>
<td>MCH: (6.7 \times 10^{-4})</td>
<td>11517 ± 136</td>
<td>48.2 ± 0.6</td>
</tr>
</tbody>
</table>

* Mean ± range after correction for reagent blanks yielding 2312 ± 23 CPM
TABLE 2-8: Pre-incubation of native plasma with Lactose, DSCG, CMTD and MCH: Effect on DHNDA for histamine applied to plasma ultrafiltrates.

<table>
<thead>
<tr>
<th>Addition to native plasma (Moles/Litre)</th>
<th>Determination of Histamine</th>
<th>14C methylhistamine (CPM/3.0 ml CHCl3)</th>
<th>Histamine (ng/ml PUF*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIL</td>
<td></td>
<td>752 ± 15**</td>
<td>15.56 ± 0.31</td>
</tr>
<tr>
<td>Lactose: 6.2 x 10^-5</td>
<td></td>
<td>761 ± 16</td>
<td>15.89 ± 0.33</td>
</tr>
<tr>
<td>DSCG: 4.2 x 10^-5</td>
<td></td>
<td>711 ± 22</td>
<td>14.04 ± 0.43</td>
</tr>
<tr>
<td>CMTD: 8.4 x 10^-4</td>
<td></td>
<td>733 ± 12</td>
<td>14.85 ± 0.24</td>
</tr>
<tr>
<td>MCH: 6.7 x 10^-4 **</td>
<td></td>
<td>745 ± 19</td>
<td>15.30 ± 0.38</td>
</tr>
</tbody>
</table>

* PUF = plasma ultrafiltrate
** Mean ± range after correction for reagent blanks yielding 532 ± 59 CPM
TABLE 2-9: Determination of histamine by DIDDA: Effect of MCH added to standard concentrations of histamine on formation of $^{14}$C-methylhistamine

<table>
<thead>
<tr>
<th>Histamine (ng/470μl)</th>
<th>METHACHOLINE ADDED</th>
<th>CPM = $^{14}$C-MeHm/3.0 ml CHCl₃ *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>(NIL)</td>
<td>250 ± 13 (100)</td>
</tr>
<tr>
<td>5.00</td>
<td>(6.7 x 10⁻⁶M)</td>
<td>242 ± 19 (96.8)**</td>
</tr>
<tr>
<td>10.00</td>
<td>(6.7 x 10⁻⁵M)</td>
<td>259 ± 13 (103.6)</td>
</tr>
<tr>
<td>5.00</td>
<td>(6.7 x 10⁻⁴M)</td>
<td>254 ± 19 (101.6)</td>
</tr>
</tbody>
</table>

*CPM = $^{14}$C-MeHm/3.0 ml CHCl₃*

*After correction for closely agreeing reagent blanks yielding 648 ± 19 CPM (mean ± S.D.)*

**For a given histamine concentration, values in parentheses express counts as a percentage of those given by mixtures devoid of methacholine**
2.4 SUMMARY

(1) A principle of double isotope derivative dilution analysis for histamine capitalizing on the specificity of the enzyme histamine-N-methyltransferase was scrutinized for its suitability for serial determinations of histamine in plasma of human subjects undergoing bronchial provocations with methacholine before and after pre-medication with Disodium cromoglycate and Cimetidine.

(2) A known drawback of this procedure, consistent with an inverse relationship between values for histamine and plasma volume analyzed, was eliminated by its routine application to plasma ultrafiltrates.

(3) Known inhibition exerted by Cimetidine on histamine-N-methyltransferase prepared from conventional sources (guinea pig brains and rat kidneys), yielding spuriously low results for histamine, was not observed when this enzyme was harvested from rat brains.

(4) Values obtained for histamine in plasma ultrafiltrates were not affected by Disodium cromoglycate, lactose (its inhalation vehicle) or methacholine whether added to plasma prior to or after its ultrafiltration.

(5) It appears that ultrafiltration of plasma is a critical preliminary to plasma histamine determinations by the method under scrutiny presumably by retaining proteinaceous inhibitors of HNMT; rat brains emerge as a similarly critical source of this enzyme
if such determinations are to be valid in the presence of Cimetidine.
2.5 CONCLUSION

From the data presented, two advantages not offered by Beaven's original procedure for determination of histamine by DIDDA would seem to emerge as major prerequisites for valid analyses:

(1) Direct linear relationships between $^{14}$C-methylhistamine and volume analyzed are obtained when this principle is applied to plasma ultrafiltrates.

(2) HNMT prepared from rat brains rather than from guinea pig brain and rat kidney is not inhibited by CMTD. Therefore, this enzyme source would seem to be critical for valid determination of histamine by DIDDA in the presence of this H2 blocker*.

Furthermore, HNMT prepared from rat brains was not inhibited by DSCG and MCH, it remaining to be ascertained whether these compounds would affect HNMT prepared from conventional sources (guinea pig brain and rat kidney) so as to affect the DIDDA principle for histamine.

In view of the foregoing advantages, serial determinations of plasma histamine by DIDDA in human subjects undergoing the bronchial provocations described in Chapter 4 were routinely performed in duplicate on 100 µl aliquots of plasma ultrafiltrates using HNMT prepared from rat brain.

* This advantage should outweigh the fact that a batch preparation of HNMT from 100 rat brains permits only about 1300 histamine assays to be performed. To accomplish the same number of assays requires partial purification of HNMT from 40 guinea pig brains or, alternatively, from less than one rat kidney (209).
The coefficient of variance (C.V.) for duplicate determinations of \(^{14}\text{C}\)-methylhistamine formed in standard assay mixtures containing between 1.25 and 10.0 ng of histamine was 3.46 ± 1.12\% (mean ± SD) (N=60). The C.V. for reagent blanks was 6.39 ± 4.54\% (N=20). The respective value for plasma ultrafiltrates analyzed in duplicate was 3.95 ± 1.40\% (N=756).

Recoveries of 20 nCi of 2,5-\(^{3}\text{H}\)-histamine dihydrochloride (added in a 20 \(\mu\)l volume yielding 8663 ± 1961 (SD) CPM) as double-labeled methylhistamine from histamine standard mixtures, blanks and plasma ultrafiltrates were 57.9 ± 7.4 (N=60), 55.4 ± 16.1\% (N=20), and 55.6 ± 11.5\% (N=756), respectively, after corrected for 100\% recovery.

During each analytical run, 100 \(\mu\)l aliquots of plasma ultrafiltrates of a given experimental series to be analyzed were combined to yield a pool comprised of amounts of histamine proportionate to those measured in individual ultrafiltrates. By separate analysis of 41 such pools for histamine content, the sum of these amounts was reproduced to the extent of 102.96 ± 10.10\% (SD).
CHAPTER 3. DETERMINATION OF DIAMINE OXIDASE ACTIVITY IN HUMAN PLASMA

3.1 INTRODUCTION

Criteria used for quantitating Diamine Oxidase (DAO) activity include: the amount of oxygen consumed (262-264); the amount of ammonia liberated (262); the amount of imidazole acetaldehyde produced (265); the rate of substrate disappearance as determined by bioassay (264, 266, 267); fluorometry (268), or residual radioactivity in tritiated substrate (269). The rate of oxidation of indigo sulphonate (270, 271) or of O-dianisidine (272, 273) by the hydrogen peroxide formed in the oxidative deamination of added histamine has also been used.

The latter principle, warranting the inclusion of horse-radish peroxidase (HRP) in the assay system as a catalyst, was chosen by the writer. The sequence of reactions culminating in the oxidation of added O-dianisidine by hydrogen peroxide is shown in Fig. 3-1.
Histamine

\[ \text{DAO} \quad \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{HN} + \text{CH}_2\text{CHO} + \text{H}_2\text{O} + \text{NH}_3 \]

\[ 2\text{H}_2\text{O}_2 + \text{O}_2 \rightarrow \text{HN} + \text{CH}_2\text{CHO} + \text{H}_2\text{O}_2 + \text{NH}_3 \]

\[ \text{O-Dianisidine} \quad 3,3'\text{-dimethoxy-4-} \]
\[ \text{amino-4'-nitrobiphenyl} \]
\[ \text{(Yellow-orange colour Chromogen)} \]
\[ \text{(Max. absorbance @ 370 nm)} \]

\[ 9.8\text{N} \cdot \text{HCl} \]
\[ \text{(Purple colour Chromogen)} \]
\[ \text{(Max. absorbance @ 540 nm)} \]

**Fig. 3-1:** Reaction sequence in DAO assay
3.2 MATERIALS

The modification of Gordon and Peters (273) of the procedure originally proposed by Aarsen and Kemp (272) was adopted with the additional modification of using aqueous 0.1M sodium phosphate buffer rather than glycerol-phosphate buffer.

All reagents were of analytical grade.

From crystalline histamine dihydrochloride (Fisher Sci., Toronto, Ontario), working solutions were prepared containing between 0.2 and 0.8 ммoles/ml distilled water. From salt-free (type II) horse-radish peroxidase powder (Sigma Chemicals, St. Louis, Mo., U.S.A.) a 0.04% solution (w/v) in 0.1M phosphate buffer pH 7.4 was prepared. 0-dianisidine (3,3'-dimethoxybenzidine dihydrochloride) (Eastman Kodak Co., Rochester, N.Y., U.S.A.) was dissolved in carbonyl-free ethanol (274) to a concentration of 0.50% (w/v).

As a preliminary to calibration, the molarity of a purchased stock solution of H₂O₂, 50% (Fisher Sci.), was determined by titration (275) with potassium permanganate (Fisher Sci.). This stock solution was then diluted serially with glass-distilled water to afford working solutions providing between 0.3 and 0.5 μMoles H₂O₂/ml. A constant volume (0.05 ml) of these solutions, containing between 15.6 and 250 nMoles H₂O₂, was part of the incubation mixture of the composition shown below which was used for calibration.
0.1M sodium phosphate buffer 0.80 ml
Histamine dihydrochloride (0.2 or 0.8M) 0.05 ml
0.04% horse-radish peroxidase (HRP) 0.05 ml
0.50% O-dianisidine 0.05 ml
\( \text{H}_2\text{O}_2, \text{ serially diluted/plasma} \) 0.05 ml

Total Volume: 1.00 ml

Phosphate buffer was prepared serially to obtain a pH dependence curve. Distilled water, 0.05 ml took the place of dilute \( \text{H}_2\text{O}_2 \) to afford a reagent blank. In the determination of DAO in plasma, sodium phosphate buffer substituted for the volume afforded by substrate to obtain a sample blank.

Mixtures were incubated at 37° C for 1 hour. The reaction was then stopped by chilling on an ice bath followed by the immediate addition of pre-chilled 9.8N HCl, 1.0 ml. After a minimum lapse of 5 minutes, absorbance (optical density; O.D.) reflecting oxidation of O-dianisidine by \( \text{H}_2\text{O}_2 \) generated in the oxidative deamination of histamine by DAO was read at 540 nm in a Gilford model 2400 spectrophotometer.
STATISTICAL METHODS

A programmable electronic calculator (Texas Instrument, Model SR-51-II) was used for computation of means and standard deviations, coefficients of variance and linear regression analyses.

Analysis of variance (ANOVA) with repeated measures between plasma DAO values determined in 3 defined groups of subjects were performed by computer in the Laboratory of the Statistical and Actuarial Sciences Department, University of Western Ontario, based upon an established program (303, 304).
VALIDATION OF METHOD

Fig. 3-2 reveals linearity between the concentrations of H₂O₂ in the standard incubation system and optical density.

Fig. 3-3 contrasting the rates of oxidation of O-dianisidine by H₂O₂ in the standard incubation system in the presence and absence of horse-radish peroxidase (HRP), illustrates that the catalytic effect of the latter subsidiary enzyme is immediate and dramatic.

In the determination of DAO activity in human plasma, duplicate incubation mixtures containing 100.9 nMoles H₂O₂ were routinely used as a standard.

Fig. 3-4 shows that the O.D. at 540 nm given by a standard mixture of the foregoing specification does not change with pH over the pH range encompassed, and that a pH optimum of 6.0 emerges for the determination of DAO in plasma. By contrast, the optimum pH is 7.6 for a commercial hog kidney preparation.

Fig. 3-5 shows that at both pH 6.0 and 7.4, curves of comparable shapes and hence, of comparable chromogenic characteristics were obtained over a wavelength range extending from 500 to 580 nm for an incubation mixture containing hydrogen peroxide as a standard and one of human-plasma to which a known amount of histamine had been added. As revealed later (cf. Fig. 3-10, page 105), these characteristics were shared by plasma incubation mixtures containing methacholine both in
Fig. 3-2: Relationship between known concentrations of $\text{H}_2\text{O}_2$ and optical density in standard incubation system for determination of DAO
Fig. 3-3: Rate of chromogen formation in standard incubation system during oxidation of O-dianisidine by $\text{H}_2\text{O}_2$ in the presence and absence of horse-radish peroxidase (HRP) (O-dianisidine, 0.25 mg/ml; $\text{H}_2\text{O}_2$, 100.9 nmoles/ml; HRP, 20 µg/ml).
Fig. 3-4: Changes in O.D. 540 nm with pH in the oxidation of 0-dianisidine by a H₂O₂ standard, human plasma DAO and hog kidney DAO.
the presence and absence of added histamine.

From Fig. 3-5, 540 and 535 nm emerge as optimum wavelengths for measurement of DAO activity in plasma at pH 6.0 and 7.4, respectively. For pH 6.0, this is in agreement with the data of Gordon and Peters (273). The working wavelength chosen by the writer for routine measurements was 540 nm whether incubation mixtures were buffered at pH 6.0 or 7.4.

Fig. 3-6 reveals sharply delineated optimum concentrations of substrate, histamine, in this assay system due to apparent inhibition by substrate at higher concentrations (276) as already reported by Gordon and Peters (273). These optimum concentrations are 0.2 and 0.8 μMoles of histamine/ml plasma at pH 6.0 and 7.4, respectively. For K_m determinations, cf. Fig. 3-9.

While sample blanks buffered at pH 6.0 gave readings warranting correction to obtain net activity, readings of sample blanks buffered at physiological pH were, as a rule, negligible.

Fig. 3-7 shows that at optimum concentrations of substrate and for a given plasma sample, there is linearity between plasma DAO activity and plasma volume.

Since as mentioned earlier, heparin may significantly increase DAO activity in vivo (173,174), the effect of heparin and other anticoagulants on this activity was studied in vitro. Fig. 3-8 shows
Fig. 3-5: Absorption spectra of chromogen formed in oxidation of 0-dianisidine

A: H₂O₂ standard containing 100.9 nmoles/ml
B: H₂O₂ generated in oxidative deamination of histamine by plasma DAO
Fig. 3-6: Effect of histamine substrate concentration on human plasma DAO activity
Fig. 3-7: Relationship between DAO activity and plasma volume/ml incubation mixture
that for a given incubation time, values for DAO in serum were of comparable orders of magnitude for serum proper and for serum to which heparin (25 units), citrate (1.5 mg) or oxalate (0.65 mg) had been added prior to incubation. Values were some 20% lower in the presence of EDTA (0.34 mg).

Fig. 3-9 shows that the known property of aminoguanidine of being a specific inhibitor of DAO of various species (277, 278, 264, 266) extends to human plasma DAO and is of the competitive type at pH 6.0 as well as at physiological pH. After correction for $H_2O_2$ generation at pH 6.0 in the absence of histamine, the apparent $K_m$ values for histamine were $0.18 \times 10^{-3}$M (pH 6.0) and $0.40 \times 10^{-3}$M (pH 7.4). Apparent $K_i$ values for aminoguanidine were $2.6 \times 10^{-6}$M and $2.5 \times 10^{-6}$M, respectively. In agreement with the scanty data afforded by Gordon and Peters (273), aminoguanidine at a $10^{-5}$ concentration per ml reaction mixture ($10^{-8}$ Moles/ml plasma) inhibited DAO by about 80% at pH 6.0. The inhibition at physiological pH (hitherto not reported) at that concentration is by about 90%. $H_2O_2$ generation occurring at pH 6.0 in plasma incubation mixtures devoid of added histamine would seem to reflect DAO activity, as judged by its suppression by aminoguanidine (Fig. 3-12).

Data already quoted to the effect that CMTD inhibits certain HNMT preparations (209), thus invalidating enzymic isotopic determination of histamine proper relying on such preparations (cf. page 50).
Fig. 3-8: Effect of anticoagulants on DAO activity on human serum
Fig. 3-9: Inhibition of plasma DAO by aminoguanidine
prompted a study of the effect, if any, of CMTD and DAO in vitro.

Table 3-1 shows that CMTD did not affect the optical density given at 540 nm by the incubation system containing H₂O₂ as a standard, and that CMTD added to the incubation system to yield concentrations ranging from 2.4 × 10⁻¹² to 2.4 × 10⁻⁶ Moles/ml of plasma had no effect on the amount of H₂O₂ generated in the oxidative deamination of added histamine by DAO.

Since the effect of DSCG on FEV₁, plasma histamine and DAO activity was to be ascertained during incremental bronchial inhalation challenge with methacholine (cf. Appendix), known amounts of DSCG were added to assay mixtures to judge its interference, if any, with the determination of plasma DAO activity in vitro. Table 3-2 shows that neither the commercial preparation of DSCG used for inhalation, nor its inhalation vehicle, lactose, affected the oxidation of O-dianisidine by a standard concentration of H₂O₂ or the oxidative deamination of added histamine by plasma DAO.
Table 3-1: Effect of CMTD on oxidation of O-dianisidine by H$_2$O$_2$ standard and on DAO Activity in Human Plasma

<table>
<thead>
<tr>
<th>CMTD ADDED (Moles)</th>
<th>ABSORBANCE @ 540 nm</th>
<th>pH 6.0</th>
<th>pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIL</td>
<td>0.886</td>
<td>0.839</td>
<td></td>
</tr>
<tr>
<td>2.4 x 10^{-6}</td>
<td>0.863</td>
<td>0.852</td>
<td></td>
</tr>
</tbody>
</table>

H$_2$O$_2$ STANDARD MIXTURE CONTAINING 1.0 x 10^{-6} Moles/ml

PLASMA INCUBATION MIXTURE

<table>
<thead>
<tr>
<th>CMTD ADDED (Moles)</th>
<th>DAO ACTIVITY (µMoles H$_2$O$_2$/ml plasma/hr @ 37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6.0</td>
</tr>
<tr>
<td>NIL</td>
<td>2.701</td>
</tr>
<tr>
<td>2.4 x 10^{-12}</td>
<td>2.742</td>
</tr>
<tr>
<td>2.4 x 10^{-11}</td>
<td>2.708</td>
</tr>
<tr>
<td>2.4 x 10^{-10}</td>
<td>2.763</td>
</tr>
<tr>
<td>2.4 x 10^{-9}</td>
<td>2.758</td>
</tr>
<tr>
<td>2.4 x 10^{-8}</td>
<td>2.797</td>
</tr>
<tr>
<td>2.4 x 10^{-7}</td>
<td>2.758</td>
</tr>
<tr>
<td>2.4 x 10^{-6}</td>
<td>2.811</td>
</tr>
</tbody>
</table>

Substrate (histamine) was present in optimum concentrations for pH, i.e. 0.2 µMoles/ml plasma at pH 6.0; 0.8 µMoles/ml plasma at pH 7.4.
Table 3-2: Effect of DSCG and Lactose on oxidation of O-dianisidine by H₂O₂ standard and on DAO activity in human plasma

<table>
<thead>
<tr>
<th>H₂O₂ STANDARD MIXTURE CONTAINING 1.0 x 10⁻³ Moles/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ADDITIONS</strong> (Moles)</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>NIL</td>
</tr>
<tr>
<td>DSCG*</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>LACTOSE*</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PLASMA INCUBATION MIXTURE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ADDITIONS</strong> (Moles)</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>NIL</td>
</tr>
<tr>
<td>DSCG*</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>LACTOSE*</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

* From 20 mg sodium cromoglycate cartridge containing lactose, supplied as "Intal" ® (Fisons Ltd., Don Mills, Ont.).
3.4 **IN VITRO EFFECTS OF METHACHOLINE ON PLASMA DAO ACTIVITY**

Since methacholine (MCH) was one of two agents to be inhaled by increments for bronchial challenge, its effect on human plasma DAO *in vitro* was studied separately.

Fig. 3-10 shows that addition of MCH to plasma incubation mixtures containing histamine in optimum substrate concentrations for pH yielded spectral absorption curves that were similar in shape but 26% (pH 6.0) and 15% (pH 7.4) lower in absorbancy than those obtained in the absence of this cholinergic agent (cf. Fig. 3-5). Fig. 3-10 also shows that when the same amount of MCH was incorporated in the absence of added histamine, spectral absorption curves were again similar in shape but absorbancy readings were still lower. Taken together, these findings suggested that MCH might be both an inhibitor and a weak substrate for DAO activity in plasma or may alternatively act as a negative and positive modifier of such activity, depending upon its concentration.

A substrate function of MCH was explored in three distinct plasma sources containing MCH in concentrations ranging from zero to 4.2 mMoles/ml plasma. Fig. 3-11 redemonstrates H$_2$O$_2$ generation at pH 6.0 in the absence of added substrate. In the case of plasma "H.L.H." and "P.T.", the rates of H$_2$O$_2$ generation increased as the concentration of added MCH was increased; in plasma "B.B.", 0.84 mMoles/ml plasma was an optimum concentration above which the rate of H$_2$O$_2$ generation declined.
Fig. 3-10: Absorption spectra of chromogen formed in oxidation of O-dianisidine by H$_2$O$_2$:
A: H$_2$O$_2$ generated in oxidative deamination of histamine added to plasma
B: H$_2$O$_2$ generated in oxidative deamination of histamine added to plasma in the presence of methacholine (4.2 mMoles/ml plasma)
C: H$_2$O$_2$ generated in plasma in the presence of added methacholine (4.2 mMoles/ml plasma)
Fig. 3-11: Top: Relationship between rate of \( \text{H}_2\text{O}_2 \) generation and methacholine (MCH) concentration at pH 6.0 and 7.4.

Bottom: Double-reciprocal plot of \( \text{H}_2\text{O}_2 \) generation (1/v) versus methacholine concentration (1/s) at pH 6.0 and 7.4.
At pH 7.4, H₂O₂ generation in the absence of MCH was negligible. Upon its addition in the amounts specified above, more H₂O₂ was generated in plasma "B.B." than in the case of "H.L.H." and "P.T.", with no evidence of an optimum concentration for this cholinergic agent in any of the three instances. After correction for velocities measured in the absence of added MCH, apparent K_m values determined by double reciprocal plot were 1.11 M and 1.14 M for pH 6.0 and 7.4, respectively.

Added evidence pointing to MCH as a substrate for human plasma DAO emerges from Fig. 3-12. At pH 6.0 and 7.4, H₂O₂ generation was suppressed by aminoguanidine, the specific DAO inhibitor, in incubation mixtures containing MCH in concentrations of up to 4.2 mMoles/ml plasma. A double reciprocal plot of data obtained at pH 6.0 after correction for blank activity measured in the absence of MCH (Fig. 3-12) afforded no possibility for further evaluation. A Dixon plot applying to the data obtained at that pH (Fig. 3-12) reveals the apparent inhibition exerted by aminoguanidine to be competitive at MCH concentrations ranging from zero to about 0.84 mMoles/ml plasma, with an apparent K_i of 0.83 x 10⁻⁵ M. At higher MCH concentrations, inhibition by aminoguanidine was non-competitive with distinct apparent K_i values of 1.17 x 10⁻⁵ M and 0.84 x 10⁻⁵ M for MCH concentrations of 2.1 and 4.2 mMoles/ml plasma, respectively.

A double reciprocal plot of data obtained at pH 7.4 (Fig. 3-12) did not permit computation of an inhibitor constant for the slight inhibition exerted by aminoguanidine in low concentrations. The
Fig. 3-12: Inhibition of $H_2O_2$ generation by Aminoguanidine with Methacholine (MCH) as substrate at pH 6.0 and 7.4;
(●) no inhibitor, (○) $10^{-10}$ Moles Aminoguanidine/ml of plasma, (○) $10^{-9}$ Moles Aminoguanidine/ml plasma, (▲) $10^{-8}$ Moles Aminoguanidine/ml plasma

Top: Reaction rate
Middle: Double-reciprocal plot ($1/v$ versus $1/s$)
Bottom: Dixon plot ($1/v$ versus $1/I$); (●) no MCH, (○) 0.42 mMoles MCH/ml plasma, (▲) 0.84 mMoles MCH/ml plasma, (○) 2.10 mMoles MCH/ml plasma, (▲) 4.20 mMoles MCH/ml plasma
inhibition exerted by the highest concentration of aminoguanidine (3.7 x 10^{-5}M) was non-competitive with an apparent K_i of 5.21 x 10^{-5}M. A Dixon plot of data obtained at pH 7.4 suggests competitive inhibition by aminoguanidine at concentrations of MCH ranging from zero to 0.42 mMoles/ml plasma with an apparent K_i of 7.87 x 10^{-5}M. Parallel lines emerging for the three higher MCH concentrations yielded apparent K_i values for aminoguanidine that were distinct for each MCH concentration encompassed, viz., 5.09 x 10^{-5}M for 0.84 mMoles MCH/ml plasma; 2.72 x 10^{-5}M for 2.10 mMoles MCH/ml plasma; 1.46 x 10^{-5}M for 4.20 mMoles/ml plasma.

Fig. 3-12 again illustrates the extent of H_2O_2 generation in the absence of any substrate in plasma buffered at pH 6.0 and shows that this blank activity is itself subject to inhibition by aminoguanidine, suggesting catabolism of endogenous substrate(s) by DAO at the optimum pH. Large variations in blank activities in a given subject emerge when the data obtained on "H.L.H." (Fig. 3-11, blank activity = 1.20 U) are compared to those shown in Fig. 3-12 (blank activity = 2.97 U). Presumably this difference accounts for the observation that in plasma "H.L.H." inhibition of H_2O_2 generation exerted by MCH in concentrations above 0.42 mMoles/ml plasma occurred only at the higher of the two levels of blank activity (Fig. 3-12). Similarly, inhibition of H_2O_2 generation at the higher MCH concentrations may have been confined to plasma "B.B." (Fig. 3-11) since its blank activity was much higher than that noted for plasma "H.L.H." and "P.T.". Finally, considerable differences
in blank activities in a given subject would seem to extend to pH 7.4.
as judged by a comparison of data obtained on plasma "H.L.H." shown
in Fig. 3-11 with those of Fig. 3-12.

Prompted by the suggestion emerging from Fig. 3-10 to the effect
that in the presence of both histamine and MCH, less $H_2O_2$ might be
generated than in the presence of histamine alone, the effect of
varying the concentration of added MCH on DAO activity in plasma
samples obtained from 23 adult volunteers was studied at optimum con-
centrations of histamine added at pH 6.0 and 7.4, respectively. Medical
histories of the volunteers permitted a subdivision into 3 groups;
asthmatic adults (n=7); allergic adults not suffering from asthma (N=10);
normal adults (N=6). Data were also obtained on plasma of a psychotic
boy (age 7) with multiple allergies.

Tables 3-3 and 3-4 show that at both pH 6.0 and 7.4, plasma DAO
activity decreased each time the concentration of added MCH was increased.

Table 3-5 re-expresses the decrease in activity as percent changes
relative to the control values obtained in the absence of MCH. The
apparent mean inhibition at the highest MCH increment (4.2 mMoles/
ml plasma) was 34 (pH 6.0) and 17% (pH 7.4). Analysis of variance
(ANOVA) with repeated measures (303, 304) shows that for a given MCH
increment, there was no significant difference in relative values for
plasma DAO between any two of the three subject groups. The inhibition
achieved with each MCH increment was significant ($P < 0.001$). There
was no significant interaction between concentrations of MCH and subjects.
Table 3-3: Effect of Methacholine on plasma DAO activity at optimum* substrate (Histamine) concentration at pH 6.0

<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>AGE</th>
<th>SEX</th>
<th>PLASMA DAO ACTIVITY (U) AT pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>TW</td>
<td>42</td>
<td>M</td>
<td>1.994 1.958 1.924 1.637 1.328</td>
</tr>
<tr>
<td>PT</td>
<td>27</td>
<td>M</td>
<td>2.656 2.568 2.494 2.226 1.833</td>
</tr>
<tr>
<td>EMa</td>
<td>23</td>
<td>M</td>
<td>3.413 3.304 3.102 2.795 2.341</td>
</tr>
<tr>
<td>ADULTS</td>
<td>(N=7)</td>
<td></td>
<td>4.010 3.400 3.348 3.184 2.528</td>
</tr>
<tr>
<td>HLH</td>
<td>57</td>
<td>M</td>
<td>4.076 3.941 3.868 3.558 3.032</td>
</tr>
<tr>
<td>WJ</td>
<td>41</td>
<td>M</td>
<td>7.541 7.526 7.232 6.402 5.399</td>
</tr>
<tr>
<td>LG</td>
<td>25</td>
<td>M</td>
<td>3.544 3.353 3.147 2.722 2.084</td>
</tr>
<tr>
<td>PM</td>
<td>22</td>
<td>M</td>
<td>6.257 5.544 5.156 4.599 3.429</td>
</tr>
<tr>
<td>PK</td>
<td>44</td>
<td>F</td>
<td>3.537 3.335 3.272 2.830 2.331</td>
</tr>
<tr>
<td>GF</td>
<td>41</td>
<td>M</td>
<td>4.066 3.920 3.769 3.306 2.757</td>
</tr>
<tr>
<td>RS</td>
<td>35</td>
<td>M</td>
<td>3.320 3.313 3.227 3.164 2.603</td>
</tr>
<tr>
<td>GJ</td>
<td>30</td>
<td>F</td>
<td>5.214 4.969 4.682 4.129 3.556</td>
</tr>
<tr>
<td>SC</td>
<td>27</td>
<td>F</td>
<td>6.210 5.893 5.664 5.148 3.925</td>
</tr>
<tr>
<td>RB</td>
<td>33</td>
<td>M</td>
<td>3.899 3.727 3.536 3.076 2.546</td>
</tr>
<tr>
<td>AM</td>
<td>65</td>
<td>F</td>
<td>3.680 3.540 3.551 3.051 2.521</td>
</tr>
<tr>
<td>HP</td>
<td>28</td>
<td>M</td>
<td>2.930 2.779 2.647 2.289 1.977</td>
</tr>
<tr>
<td>DMc</td>
<td>38</td>
<td>M</td>
<td>2.967 2.887 2.572 2.421 1.920</td>
</tr>
<tr>
<td>IH</td>
<td>27</td>
<td>F</td>
<td>3.845 3.764 3.591 3.161 2.568</td>
</tr>
<tr>
<td>RS</td>
<td>23</td>
<td>M</td>
<td>3.001 2.875 2.695 2.338 1.753</td>
</tr>
<tr>
<td>BC</td>
<td>32</td>
<td>F</td>
<td>6.001 5.737 5.563 4.987 4.243</td>
</tr>
<tr>
<td>AMa</td>
<td>25</td>
<td>M</td>
<td>2.930 2.701 2.640 2.209 1.849</td>
</tr>
<tr>
<td>SB</td>
<td>20</td>
<td>M</td>
<td>3.350 3.209 3.079 2.687 2.231</td>
</tr>
<tr>
<td>BB</td>
<td>7</td>
<td>M</td>
<td>5.200 3.864 3.021 1.888 1.206</td>
</tr>
</tbody>
</table>

* 0.2 μMoles histamine/ml plasma
Table 3-4: Effect of Methacholine on plasma DAO activity at optimum* substrate (Histamine) concentration at pH 7.4

<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>AGE</th>
<th>SEX</th>
<th>METHACHOLINE ADDED (mMoles/ml plasma)</th>
<th>PLASMA DAO ACTIVITY (U) AT pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>TW</td>
<td>42</td>
<td>M</td>
<td>(NIL)</td>
<td>1.295 1.224 1.167 1.108 1.031</td>
</tr>
<tr>
<td>PT</td>
<td>27</td>
<td>M</td>
<td>(0.42)</td>
<td>1.381 1.355 1.341 1.264 1.149</td>
</tr>
<tr>
<td>EMa</td>
<td>23</td>
<td>M</td>
<td>(0.84)</td>
<td>2.241 2.214 2.062 1.947 1.782</td>
</tr>
<tr>
<td>PS</td>
<td>34</td>
<td>F</td>
<td>(2.10)</td>
<td>2.500 2.403 2.248 2.205 1.953</td>
</tr>
<tr>
<td>HLH</td>
<td>57</td>
<td>N</td>
<td>(4.20)</td>
<td>2.801 2.742 2.675 2.557 2.361</td>
</tr>
<tr>
<td>NJ</td>
<td>41</td>
<td>M</td>
<td></td>
<td>5.246 5.099 5.099 4.800 4.496</td>
</tr>
<tr>
<td>LG</td>
<td>25</td>
<td>M</td>
<td></td>
<td>2.351 2.262 2.229 2.121 1.942</td>
</tr>
<tr>
<td>PM</td>
<td>22</td>
<td>M</td>
<td></td>
<td>3.939 3.758 3.687 3.541 3.246</td>
</tr>
<tr>
<td>PK</td>
<td>44</td>
<td>F</td>
<td></td>
<td>2.512 2.341 2.266 2.180 1.939</td>
</tr>
<tr>
<td>GF</td>
<td>41</td>
<td>M</td>
<td></td>
<td>2.697 2.732 2.659 2.549 2.268</td>
</tr>
<tr>
<td>RS</td>
<td>35</td>
<td>M</td>
<td></td>
<td>2.082 2.082 2.030 1.963 1.763</td>
</tr>
<tr>
<td>SJ</td>
<td>30</td>
<td>F</td>
<td></td>
<td>3.390 3.285 3.207 3.054 2.787</td>
</tr>
<tr>
<td>SC</td>
<td>27</td>
<td>F</td>
<td></td>
<td>4.294 4.290 4.143 3.938 3.611</td>
</tr>
<tr>
<td>RB</td>
<td>33</td>
<td>M</td>
<td></td>
<td>2.156 2.057 2.024 1.893 1.604</td>
</tr>
<tr>
<td>DR</td>
<td>25</td>
<td>M</td>
<td></td>
<td>2.481 2.429 2.399 2.263 2.047</td>
</tr>
<tr>
<td>AM</td>
<td>65</td>
<td>F</td>
<td></td>
<td>2.440 2.411 2.330 2.359 2.196</td>
</tr>
<tr>
<td>HP</td>
<td>28</td>
<td>M</td>
<td></td>
<td>1.785 1.735 1.685 1.576 1.498</td>
</tr>
<tr>
<td>DMc</td>
<td>38</td>
<td>M</td>
<td></td>
<td>1.830 1.770 1.667 1.632 1.545</td>
</tr>
<tr>
<td>IH</td>
<td>27</td>
<td>F</td>
<td></td>
<td>2.384 2.351 2.348 2.177 2.007</td>
</tr>
<tr>
<td>RS</td>
<td>23</td>
<td>M</td>
<td></td>
<td>2.013 1.949 1.890 1.765 1.602</td>
</tr>
<tr>
<td>AMa</td>
<td>25</td>
<td>M</td>
<td></td>
<td>2.050 2.058 2.009 1.890 1.970</td>
</tr>
<tr>
<td>SB</td>
<td>20</td>
<td>M</td>
<td></td>
<td>1.950 1.870 1.829 1.720 1.570</td>
</tr>
</tbody>
</table>

* 0.8 mMoles histamine/ml plasma
Table 3-5: Effect of Methacholine on Relative Plasma DAO Activity at optimum substrate (histamine) concentration at pH 6.0 and 7.4.

<table>
<thead>
<tr>
<th>SUBJECT GROUP</th>
<th>METHACHOLINE ADDED (μMoles/ml plasma)</th>
<th>RELATIVE DAO ACTIVITY (%) AT pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(NIL)</td>
<td>(0.42)</td>
</tr>
<tr>
<td>ASTHMATIC</td>
<td>100.00</td>
<td>95.37±4.93</td>
</tr>
<tr>
<td>ALLERGIC</td>
<td>100.00</td>
<td>95.10±2.76</td>
</tr>
<tr>
<td>NORMAL</td>
<td>100.00</td>
<td>95.77±1.98</td>
</tr>
<tr>
<td>MARGINAL</td>
<td>100.00</td>
<td>95.36</td>
</tr>
</tbody>
</table>

ANOVA with repeated measures: P
Between Groups: 0.82
Between MCH Increments: < 0.001

* 0.2 μMoles histamine/ml plasma

---

<table>
<thead>
<tr>
<th>SUBJECT GROUP</th>
<th>METHACHOLINE ADDED (μMoles/ml plasma)</th>
<th>RELATIVE DAO ACTIVITY (%) AT pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(NIL)</td>
<td>(0.42)</td>
</tr>
<tr>
<td>ASTHMATIC</td>
<td>100.00</td>
<td>96.98±1.46</td>
</tr>
<tr>
<td>ALLERGIC</td>
<td>100.00</td>
<td>97.60±2.50</td>
</tr>
<tr>
<td>NORMAL</td>
<td>100.00</td>
<td>97.89±1.69</td>
</tr>
<tr>
<td>MARGINAL</td>
<td>100.00</td>
<td>97.49</td>
</tr>
</tbody>
</table>

ANOVA with repeated measures: P
Between Groups: 0.53
Between MCH Increments: < 0.001

* 0.8 μMoles histamine/ml plasma
For a given MCH increment, there was no significant difference in mean plasma DAO between male and female volunteers.

Fig. 3-13 shows that relative to the mean values for the 3 groups combined (N=24), inhibition by a given increment of added MCH was more dramatic in the case of "B.B.", the 7 year old psychotic boy with multiple allergies, such that the highest MCH increment depressed $H_2O_2$ by 75 and 50% at pH 6.0 and 7.4, respectively. Taken together, the data shown in Fig. 3-13 therefore suggest that there may be subject variation as to the extent of inhibition exerted by MCH on plasma DAO activity in vitro.
SUBSTRATE (HISTAMINE), 0.2 μM at pH 6.0
0.8 μM at pH 7.4

Fig. 3-13: Inhibition exerted by Methacholine on DAO activity in
24 distinct human plasma samples at optimum concentrations
of Histamine for pH
In the three plasma samples specified earlier (cf. Fig. 3-11), the effect of MCH on DAO activity at histamine concentrations below and above those optimum for pH was studied. As before, those of MCH ranged from zero to 4.2 mMoles/ml plasma. The left half of Fig. 3-14 redemonstrates the apparent substrate function of MCH in the absence of any added histamine at pH 6.0. However, for any given histamine concentration, increasingly less H₂O₂ was generated as the concentration of MCH was increased such that values eventually fell below those given by plasma blanks. At pH 7.4, MCH enhanced and inhibited H₂O₂ generation at the lower and higher concentrations of added histamine, respectively. In the case of plasma "Pşt." and "H.L.H.", the transition from an enhancing to an inhibitory effect of MCH suggests an isosbestic point at a concentration of histamine of about 0.50 µMoles histamine/ml plasma that was shared by the amount of H₂O₂ generated in the absence of any MCH. In the case of "B.B.", this transition occurred at a much lower concentration of added histamine although a sharply delineated isosbestic point was not ascertainable. At the optimum (0.8 µMoles/ml plasma) and the next higher concentration of histamine, activity decreased throughout as a function of the amount of MCH added. This effect of MCH was more pronounced in the case of plasma "B.B." such that at the highest MCH increment (4.2 mMoles/ml plasma), activity was depressed by 50%.

Since plasma DAO activity was to be measured serially during incremental MCH inhalation, the effect of its addition 47 minutes after
Fig. 3-14: Effect of varying concentrations of both Methacholine and Histamine on plasma DAO activity at pH 6.0 and 7.4 in three distinct subjects.
initiation of incubation was ascertained in a plasma incubation mixture containing histamine in optimum concentrations for pH. Parallel runs permitted comparisons of changes in $H_2O_2$ generation with time in the absence of MCH and in its presence prior to incubation. Simultaneous serial determinations of DAO activity were rendered possible by preparation of stock incubation mixtures comprised of plasma and reagents in 20 times the volume specified on page 88. The amounts of histamine and MCH added were increased commensurately to afford concentrations of 0.2 μMoles (pH 6.0) and 0.8 μMoles (pH 7.4) (optima for histamine) and 4.2 mMoles of MCH per ml of plasma, respectively.

Fig. 3-15 shows that at both pH 6.0 and 7.4, MCH addition at 47 minutes depressed $H_2O_2$ generation, the slopes of the respective curves being similar to the slopes applying to the inclusion of MCH in the plasma incubation system at zero time.
Fig. 3-15: Effect of Methacholine (MCh) on plasma DAO activity when added 47 minutes after initiation of prolonged incubation at pH 6.0 and 7.4.
3.5 SUMMARY & CONCLUSIONS

A modification of the method of Gordon and Peters (273) for determination of DAO activity was scrutinized for its validity when applied to human plasma. This method measures $\text{H}_2\text{O}_2$ generated in the oxidative deamination of added histamine based upon the colour produced by oxidation of 0-dianisidine. DAO was measured at the optimum pH of 6.0 as well as at physiological pH.

Optimum concentrations of added histamine, viz., 0.2 and 0.8 μMoles/ml plasma at pH 6.0 and 7.4, respectively, were sharply delineated due to marked inhibition of $\text{H}_2\text{O}_2$ generation at higher histamine concentrations. At the latter pH, the relationship between the concentration of histamine up to its optimum and the rate of its oxidation by DAO was sigmoidal. Apparent $K_m$ for histamine was $0.18 \times 10^{-3} \text{M}$ and $0.40 \times 10^{-3} \text{M}$ at pH 6.0 and 7.4, respectively.

At both optimum and physiological pH, $\text{H}_2\text{O}_2$ generation was suppressed by aminoguanidine. The inhibition was competitive with apparent $K_i$ values for aminoguanidine of $2.6 \times 10^{-6} \text{M}$ and $2.5 \times 10^{-6} \text{M}$ at pH 6.0 and 7.4, respectively. This specific inhibitor of DAO activity also suppressed $\text{H}_2\text{O}_2$ generation measured in the absence of added substrate.

$\text{H}_2\text{O}_2$ generation was unaffected by widely varying concentrations of DSCG, Lactose and CMTD.
MCH had distinct effects on $H_2O_2$ generation depending upon the following factors.

When MCH was added in the absence of histamine, $H_2O_2$ was generated as a function of the MCH concentration with an apparent $K_m$ of 1.11 and 1.14M at pH 6.0 and 7.4, respectively, suggesting this cholinergic agent to be a weak substrate for DAO. This conclusion received support from suppression of $H_2O_2$ generation by aminoguanidine. At pH 6.0, apparent $K_i$ for aminoguanidine in the presence of MCH ranged between $0.83 \times 10^{-5}M$ and $1.17 \times 10^{-5}M$ with a change from competitive to non-competitive inhibition when the MCH concentration was increased from 0.84 to 4.20 mMoles/ml plasma. The type of inhibition exerted by aminoguanidine at pH 7.4 remained ill defined by double-reciprocal plot. By Dixon plot, competitive inhibition was suggested at low concentration of MCH, changing to uncompetitive inhibition at the higher MCH concentration with apparent $K_i$ values ranging between 1.46 and $7.87 \times 10^{-5}M$.

By comparison to values given by histamine alone, addition of MCH enhanced $H_2O_2$ generation at the lower concentrations of added histamine; the degree of MCH-induced enhancement diminished with increasing concentrations of added histamine; this culminated in inhibition of $H_2O_2$ generation beginning at concentrations of histamine still below those optimum for pH. The degree of inhibition was a function of the amount of MCH added, values at pH 6.0 eventually falling below the blank level of $H_2O_2$ generation measured in the absence of both histamine and MCH.
Inhibition of plasma DAO exerted by MCH at concentrations of histamine optimum for pH was verified in plasma samples obtained from twenty-four subjects, with a suggestion of subject variation as to the extent of inhibition. With histamine present in concentrations optimum for pH, plasma DAO was also inhibited by MCH when added 47 minutes after initiation of incubation.

The well-known inhibition of DAO by aminoguanidine with histamine as a substrate is competitive (ref. 168, 273; cf. Fig. 3-9). The present data show that when MCH is present as a substrate in low concentrations, the inhibition of plasma DAO by aminoguanidine is likewise competitive. Taken together, these data suggest that histamine and MCH may share a common binding site on the enzyme, histamine being the better substrate due to its much lower $K_m$ value.

The sigmoidal curve applying to the relationship between concentrations of histamine as the only substrate and its rate of oxidation by human plasma DAO at pH 7.4 suggests this relationship to be more complex than that expressed by the Michaelis-Menten relationship and its modifications. There being no precedent to the determination of this relationship at physiological pH, this conclusion is in line with data by Fouts et al. (279) regarding partially purified hog kidney DAO; for most substrates of this enzyme, $K_m$ remained ill defined.

Present work implicating MCH as a substrate for human plasma DAO
may be linked to data by the same group of workers showing that monoamines may be attacked, although much less readily than diamines, by mammalian DAO (279). Apparently a second amino group of a potential substrate of DAO is not essential for formation of an enzyme-substrate complex although the affinity between the two moieties is greatly enhanced.

The fact that in the presence of histamine, MCH enhanced and inhibited plasma DAO activity at low and high concentrations of histamine, respectively, confirms and extends observations by Fouts et al. (279) to the effect that simultaneous oxidation of monoamines and diamines by DAO never leads to additive oxidation rates.

In vivo implications of the in vitro data obtained for plasma DAO at physiological pH may be as follows. If inhibition of plasma DAO exerted by histamine (substrate) proper in concentrations above a critical level extends to lung, then ongoing local histamine release may yield concentrations of this amine eventually inhibitory to its oxidation by DAO. This thesis might extend to exogenous histamine, especially when it is inhaled by increments for bronchial provocation. Similar implications may apply to the observation that MCH stimulated and inhibited DAO activity in vitro at low and high concentrations of histamine, respectively, and that the degree of inhibition was a function of the MCH concentration. Thus, inhalation of this cholinergic agent
by increments may eventually afford local concentrations inhibitory to the oxidation of released histamine by DAO. Singly or in combination, these mechanisms might contribute to bronchoconstriction via local histamine accumulation.

To test the validity of the foregoing considerations, serial determinations of plasma DAO during the bronchial provocations with histamine and MCH to be described were routinely obtained at physiological pH. The coefficient of variance between 296 duplicate assays was $1.41 \pm 1.52$ (S.D.) percent, thus permitting the encompassing of small changes. One unit (U) of DAO activity is defined as 1 µMole H$_2$O$_2$/ml plasma/hr at 37°C.
CHAPTER 4: BRONCHIAL INHALATION CHALLENGE

4.1. INTRODUCTION

Volunteer Subjects

Eighteen volunteers who enrolled for bronchial inhalation challenge with methacholine (MCH) by increments included fifteen allergic and two non-allergic individuals with definite histories of asthma and one normal subject.

In keeping with criteria approved by the Committee on Human Experimentation of the University of Western Ontario, only non-pregnant adult subjects free from disorders militating against serial blood withdrawals for determination of plasma histamine (HM) and diamine oxidase (DAO) activity were selected based upon medical history, physical examination, haemoglobin and haematocrit values and the outcome of any other clinically indicated laboratory test. An informed consent was obtained from each volunteer. All withheld medication and food overnight, as recommended by the Asthma and Allergic Disease Centres (AADC) (257).

Fifteen of the eighteen participants volunteered for only one bronchial provocation with MCH. Three male physicians (two asthmatics; one normal) were readily available for serial bronchial provocations with MCH before and after medication with Disodium Cromoglycate (DSCG) as well as Cimetidine. For control studies, these three physicians and one normal female volunteer underwent separate inhalations of saline
(diluent vehicle for inhalation of aerosol of MCH) under the controlled conditions underlying inhalations of the provocative agent proper. The latter four subjects underwent additional tests aimed at encompassing changes in Forced Expiratory Volume in the first second (FEV₁) and parameters of HM metabolism, if any, (a) during periods not involving aerosol inhalations; and (b) during periods of rest.

All tests began at 0900 hrs at the Clinical Investigation Unit at St. Joseph's Hospital under the clinical supervision of Dr. D.R.M. McCourtie or a staff physician.
BRONCHIAL INHALATION CHALLENGES WITH METHACHOLINE (MCH)

PROTOCOL: The standardized procedure for inhalation of MCH advocated by the AADC in conjunction with the National Institute of Allergic and Infectious Diseases (NIAID), National Institute of Health (NIH), Bethesda, Maryland, as published by Chai et al. (257) was extended to accommodate serial blood withdrawals for plasma HM and DAO activity. These and other agencies consider the FEV₁ as an adequate criterion for measurement of pulmonary function and accept a sustained minimum FEV₁ fall by 20% as evidence of its curtailment (250, 257, 280).

INSTRUMENTATION: All bronchial inhalation challenges were carried out using a "Nebulization Dosimeter" of advanced design developed by Dr. R.R. Rosenthal's Research Group, Johns Hopkins School of Medicine and Good Samaritan Hospital, Baltimore, Md. 21212. This dosimeter delivers consistent and reproducible amounts of aerosolized materials from a standard nebulizer (DeVilbiss #42). Its delivery system is adjustable by virtue of an electronic variable time circuit which opens a solenoid valve, triggered by the patient's own inhalation via a thermister. The valve allows air to flow at 20 psi from a compressed air tank to the nebulizer during a pre-set time interval of 8 seconds. All subjects inhaled from functional residual capacity to total lung capacity.

FEV₁ was determined using a Single Breath Wedge Spirometer (Vitalograph).
PROCEDURE FOR MCH INHALATION: Bronchial inhalation challenge was preceded by securing an average FEV₁ based upon two determinations obtained 1½ minutes apart. A blood withdrawal was performed 2 minutes after the second FEV₁ determination.

Five breaths of saline taken within half a minute, were followed by two FEV₁ determinations to obtain a post-saline FEV₁ average. Another blood withdrawal was performed 5 minutes after the last of the 5 breaths.

MCH inhalation was then initiated beginning with 5 breaths of the lowest concentration. FEV₁ was determined 1½ and 3 minutes after the last breath followed by a blood withdrawal at 5 minutes. This sequence was repeated with increasing concentrations of MCH until a sustained fall in FEV₁ of at least 20% occurred or the maximum permissible inhalation of 224.15 cumulative MCH breath units was attained (257).

Whenever a positive response occurred, serial FEV₁ determinations and blood withdrawals were continued until the FEV₁ value approximated baseline. The derivation of cumulative breath units (cu) for MCH is shown in Table 4-1 based upon dilution increments of 0.15, 0.31, 0.61, 1.25, 2.50, 5.00, 10.00 and 25.00 mg/ml.
<table>
<thead>
<tr>
<th>Methacholine Concentration (mg/ml)</th>
<th>Cumulative Number of Breaths</th>
<th>Units/Breath</th>
<th>Units/5 Breaths</th>
<th>Cumulative Units/5 Breaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>5</td>
<td>0.15</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>0.31</td>
<td>10</td>
<td>0.31</td>
<td>1.55</td>
<td>2.30</td>
</tr>
<tr>
<td>0.62</td>
<td>15</td>
<td>0.62</td>
<td>3.10</td>
<td>5.40</td>
</tr>
<tr>
<td>1.25</td>
<td>20</td>
<td>1.25</td>
<td>6.25</td>
<td>11.65</td>
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<td>2.50</td>
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<td>12.50</td>
<td>24.15</td>
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<tr>
<td>25.00</td>
<td>40</td>
<td>25.00</td>
<td>125.00</td>
<td>224.15</td>
</tr>
</tbody>
</table>

* One breath unit = 1 inhalation of 1 mg/ml (methacholine/0.9% saline)
Saline Inhalations and Other Control Tests

Three male physicians (2 asthmatics; one normal) and one normal female subject volunteered for the following additional tests:

(A) Serial timed blood withdrawals in the absence of any inhalation or FEV₁ determination served to encompass changes in plasma HM and DAO activity while subjects were at rest.

(B) Serial inhalations of the diluent vehicle (saline) accompanied by serial measurements of all of FEV₁, plasma HM and DAO activity were performed exactly as described for inhalation of MCH (cf. page 129).

(C) Serial inhalations of the diluent vehicle were performed, accompanied solely by timed serial blood withdrawals for determination of plasma HM and DAO activity, i.e., omitting FEV₁ measurements.

(D) Serial FEV₁ determinations accompanied by timed blood withdrawals for levels of HM and DAO activity in plasma were carried out in the absence of any inhalation requirement.

On a given subject, the foregoing four tests were performed in a random sequence seven days apart (257, 280).
BLOOD WITHDRAWALS

An arm vein cannulated with an indwelling Intracath for implementation of a continuous microdrip of 0.9% NaCl solution which was kept running at a slow rate, thus ensuring patency of the catheter. Immediately prior to removal of a timed blood sample, the drip was disconnected. Although visible dilution of blood by saline was no longer apparent before the first milliliter had escaped, an additional milliliter of blood was discarded as a precaution against saline dilution or contamination.

Ten milliliters of blood were then withdrawn into a glass syringe containing heparin (10 USP units). The timed blood samples were immediately transferred to pre-chilled polypropylene tubes provided with polypropylene snap caps. Tubes were then spun for 30 minutes at 600 x g in a refrigerated centrifuge. In no instance did the time between the withdrawal of a blood sample and its centrifugation exceed 3 hours. No more than 50% of supernatant plasma was gently removed from each sample with a pasteur pipette and partitioned into polypropylene vials. Plasma so partitioned was kept frozen at -60°C until ready for analysis.

Centrifugation of blood at 600 x g for 30 minutes and aspiration of the upper 50% supernatant plasma represented compromises between dissimilar recommendations by several workers (205, 208, 209, 224). They were chosen to minimize rupture of cells or platelets and also minimize variable leakage of histamine and Diamine oxidase from cells into plasma and the aspiration of cellular elements from the buffy coat.
In a two-stage procedure, Horakova et al. (206) first spun blood at 150 x g for 10 minutes to obtain platelet-free plasma which was then centrifuged separately at 2,000 x g for another 10 minutes. Other workers bypassing a two-stage centrifugation step have spun blood at 900 x g (209), 1,000 x g (208), 1,500 x g (224) and 2,000 x g (205).

In the present work, the adequacy of both the centrifugation step and mode of removal of supernatant plasma free from intact cells and platelets was verified separately and repeatedly on heparinized blood from hospital patients and staff volunteers. Red cells, white cells and platelets in whole blood and in both upper and lower portions of supernatant plasma were counted and hematocrit measured in a Coulter Model ZF-5 cell counter. In all instances, zero values for each were obtained in both portions of supernatant plasma.

In no instance was there visible haemolysis before or after the centrifugation step. The question of the dilutional artifact and of white cell and platelet rupture and/or leakage of histamine and Diamine oxidase into plasma will be raised again in Chapter 5 (cf. page 211).
STATISTICAL METHODS

A. Statistical analyses of data obtained on individual subjects

A programmable electronic calculator (Texas Instrument, Model SR-51-II) was used for analyses of variance and linear regression analyses of the changes occurring in FEV₁, plasma histamine and plasma diamine oxidase in each individual subject.

B. Group statistics

Comparisons within and between groups by paired and unpaired Student's t-test, respectively, Analysis of variance (ANOVA) with repeated measures and correlations by linear regression analyses were performed by computer in the Laboratory of the Statistical and Acturial Sciences Department, University of Western Ontario, based upon an established program (303, 304).
4.2 RESULTS

Saline Inhalations and Other Control Tests: Changes in Plasma Diamine Oxidase (DAO) Activity and Histamine (HM).

Four test periods encompassing fluctuations in plasma DAO activity and HM were involved, as outlined in Section 4.1 (cf. page 131). In Fig. 4-1 to 4-4, conditions for each period are re-specified in abbreviated form and identified by letter for quick reference as follows:

"A" = Resting
"B" = Serial saline inhalations and FEV₁ determinations ("NaCl + FEV₁")
"C" = Serial inhalations of saline ("NaCl only")
"D" = Serial determinations of FEV₁ ("FEV₁ only")

It is reiterated that for a given subject, the sequence of these four test periods was selected at random.

In the case of the two periods involving serial inhalations of saline (periods "B" and "C"), the first such inhalation was treated as the "Control inhalation" in analogy to the protocol underlying bronchial challenge with MCH. This permitted identification of plasma DAO and HM levels obtained immediately before and after the first saline inhalation as "Pre- and Post-saline control values". In the case of the two periods involving serial FEV₁ determinations
(periods "B" and "D"), the first such determination will be similarly referred to as the "Control Value".
Fig. 4-1A: Saline Inhalations and Other Control Tests in "T.W."- Time course of changes in plasma DAO and HM
T.W., Male, 46 years old

History

A non-smoking chest physician with mild asthma negative to MCH (cf. Fig. 4-22).

Saline Inhalations and Other Control Tests

Absolute control values for FEV\textsubscript{1} determined serially during "B" and "D" were the same. Those for plasma DAO ranged from 1.46 ("A") to 1.96 U/ml ("B"). Means for plasma DAO were 1.58 ± 0.08 ("A"), 1.89 ± 0.12 ("B"), 1.88 ± 0.08 ("C") and 1.68 ± 0.06 U/ml ("D"). By t-test, means were significantly different for any two periods (P < 0.001 in all instances) except "B" versus "C".

The preponderance of plasma HM determinations during "A", "B" and "C" yielded zero. Increases up to 16 ng/ml occurred during "D" which also revealed the highest mean (5.37 ± 5.03 ng/ml). This mean was significantly different from that obtained for "A" (150 ± 2.72 ng/ml; P < 0.05), "B" (0.31 ± 1.15 ng/ml; P < 0.01) and "C" (0.66 ± 1.45 ng/ml; P < 0.05).
Fig. 4-1B: Saline Inhalation Test in "T.W."—

Time course of changes in plasma DAO and HM
As shown in Fig. 4-1B, a single control test involving serial plasma HM and DAO determinations during a period of serial inhalation of NaCl accompanied by FEV₁ determinations preceded the above test series by two years. Conditions were thus comparable to those for period "B" on Fig. 4-1A except that intervals between NaCl inhalations and FEV₁ determinations were not consistent and the total number of these serial requirements was less (10 versus 12). By comparison with period "B" on Fig. 4-1A, plasma DAO was remarkably stable and plasma HM levels (mean, 10.5 ± 2.38 ng/ml) were significantly higher (P <0.001).
Fig. 4-2: Saline Inhalations and Other Control Tests in "H.L.H." -
Time course of changes in plasma DAO and HM
Fig. 4-2

H.L.H., Male, 61 years old

History

A heavy smoker and mild asthmatic with skin tests positive to grass pollens and molds. For the outcome of MCH challenges performed before and after administration of DSCG and CMTD cf. Fig. AP-1 and AP-3 of Appendix.

Saline Inhalations and Other Control Tests

Regrettably, data under the condition of period "A" were not obtained. Absolute control values for FEV₁ for periods "B" and "D" were comparable.

Those for plasma DAO ranged between 1.62 ("D") and 1.89 U/ml ("B"). The means for plasma DAO for the three periods were 1.85 ± 0.12 ("B"), 1.68 ± 0.05 ("C") and 1.61 ± 0.03 U/ml ("D"). These means were significantly different from any two periods (P < 0.001 in all instances).

Plasma HM values ranged between zero and 20 ng/ml. The greatest fluctuations in plasma HM occurred during "B" with a mean of 7.68 ± 6.04 ng/ml. Mean plasma HM values for "C" and "D" were 3.08 ± 2.73 and 1.36 ± 1.72 ng/ml, respectively. Only the mean for "B" was significantly different from that of "C" (P < 0.01) and "D" (P < 0.001).
Fig. 4-3: Saline Inhalations and Other Control Tests in "D.Mc."

Time course of changes in plasma DAO and HM
Fig. 4-3:

D. Mc., Male, 42 years old

History

A non-smoking, non-asthmatic internist whose FEV₁ was unaffected by methacholine, whether inhaled before or after administration of DSCG or CMTD (cf. Appendix).

Saline Inhalation and Other Control Tests

Absolute FEV₁ control values for "B" and "D" were identical. Those for plasma DAO ranged from 1.57 ("D") to 1.69 U/ml ("A"). Mean values for this enzyme were 1.67 ± 0.06 ("A"), 1.54 ± 0.05 ("B"), 1.55 ± 0.08 ("C") and 1.66 ± 0.06 U/ml ("D"). Means for any two periods were significantly different (P < 0.001 in all instances) except for "A" versus "D" and "B" versus "C".

Plasma HM ranged from zero to 15 ng/ml. A perponderance of values obtained during "B" and "D" was zero. Mean values were 3.75 ± 414 ("A"), 2.46 ± 4.97 ("B"), 3.03 ± 3.51 ("C") and 1.32 ± 2.86 ng/ml ("D"). There was no significant difference between any two means.
Fig. 4-4: Saline Inhalations and Other Control Tests in "L.V.":-

Time course of changes in plasma DAO and HM
Fig. 4-4:
L.V., Female, 31 years old

History

This lady is an habitual smoker free from asthma and allergies.

Saline Inhalations and Other Control Tests

Absolute control values for FEV₁ for periods "B" and "D" were comparable. Those for plasma DAO ranged from 2.95 ("D") to 3.93 U/ml ("A"). Means were 3.94 ± 0.05 ("A"), 3.99 ± 0.10 ("B"), 3.68 ± 0.09 ("C") and 3.05 ± 0.07 U/ml ("D"). Means for any two periods were significantly different except for "A" versus "B".

Plasma HM was zero throughout "A" and "B" and also during "C" except for a short-lived elevation to 7.11 ng/ml at the end of that period. Three spike-like increases in plasma HM from zero levels during "D" did not exceed 10.46 ng/ml. By t-test, means for plasma HM between any two periods were not significantly different.
Since of the 4 volunteers, "H.L.H." did not partake in the conditions underlying control period "A", comparisons of changes between periods are confined to period "B", "C" and "D".

For each of these three periods, the fourteen values for plasma DAO obtained 7 minutes apart on the two normal and two asthmatic volunteers were re-expressed as a percentage of the level found for this activity in the first blood sample. In the case of plasma HM, a similar manipulation was not possible since a preponderance of values in each of the four subjects had yielded zero.

Analysis of variance (ANOVA) with repeated measures by computer (303, 364) was applied to determine, for each plasma DAO and HM, the level of significance of differences between any two of the three periods. This was performed for over-all differences as well as for differences applying to timed blood samples, and also permitted the encompassing of any significant interaction between the test periods and timed values for the two variables.

The foregoing statistical analyses were performed on the four subjects as a single group and as separate groups comprised of two normals and two asthmatics for determination of sub-group differences. Table 4-2 to 4-5 show that there were no significant differences in mean plasma DAO and HM values by any of the above criteria.

By the same criteria, there were no significant differences when period "A" was included after elimination of all data obtained on "H.L.H." during periods "B", "C" and "D" (Tables 4-6, 4-7).
Table 4-2: Analysis of variance (ANOVA) with repeated measures* on serial relative plasma DAO levels obtained in 4 subjects** during 3 defined control periods not involving inhalation of bronchoconstrictive agents.

<table>
<thead>
<tr>
<th>PERIOD#</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLOOD SAMPLE</td>
<td>RELATIVE PLASMA DAO ACTIVITY (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Running Number</td>
<td>Time Lapse (minute)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>97.1 ± 8.5</td>
<td>93.3 ± 2.1</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>96.6 ± 9.9</td>
<td>91.7 ± 1.2</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>95.1 ± 6.7</td>
<td>91.5 ± 1.9</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>93.9 ± 11.7</td>
<td>90.7 ± 3.0</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td>93.6 ± 9.3</td>
<td>90.9 ± 3.7</td>
</tr>
<tr>
<td>7</td>
<td>42</td>
<td>95.4 ± 8.8</td>
<td>89.8 ± 3.3</td>
</tr>
<tr>
<td>8</td>
<td>49</td>
<td>94.4 ± 7.4</td>
<td>90.7 ± 3.9</td>
</tr>
<tr>
<td>9</td>
<td>56</td>
<td>93.4 ± 11.6</td>
<td>88.8 ± 2.0</td>
</tr>
<tr>
<td>10</td>
<td>63</td>
<td>93.1 ± 7.9</td>
<td>91.5 ± 1.2</td>
</tr>
<tr>
<td>11</td>
<td>70</td>
<td>93.3 ± 8.1</td>
<td>88.4 ± 3.1</td>
</tr>
<tr>
<td>12</td>
<td>77</td>
<td>96.4 ± 9.3</td>
<td>88.1 ± 4.3</td>
</tr>
<tr>
<td>13</td>
<td>84</td>
<td>96.2 ± 8.3</td>
<td>92.7 ± 1.5</td>
</tr>
<tr>
<td>14</td>
<td>91</td>
<td>99.4 ± 13.5</td>
<td>90.3 ± 0.8</td>
</tr>
</tbody>
</table>

ANOVA with repeated measures P

- Between Periods 0.39
- Between Timed Samples 0.26
- Interaction between Periods & Timed Samples 0.65

* cf. Ref. 303, 304
** two normals and two asthmatics
# Period "B" = Serial saline inhalations and FEV₁ determinations
"C" = Serial inhalations of Saline only
"D" = Serial determinations of FEV₁ only
@ Mean ± S.D.
Table 4-6. Analysis of variance (ANOVA) with repeated measures on serial plasma histamine levels obtained in 4 subjects during 3 defined periods not involving inhalation of bronchoconstrictive agents.

<table>
<thead>
<tr>
<th>PERIOD #</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>mean</td>
<td>mean</td>
</tr>
<tr>
<td>BLOOD SAMPLE</td>
<td>runnig</td>
<td>time lapse</td>
<td>number</td>
</tr>
<tr>
<td>1</td>
<td>1.57 ± 3.14</td>
<td>5.29 ± 6.21</td>
<td>2.37 ± 2.60</td>
</tr>
<tr>
<td>2</td>
<td>3.51 ± 6.62</td>
<td>3.55 ± 3.01</td>
<td>3.73 ± 4.29</td>
</tr>
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<td>3</td>
<td>3.58 ± 4.32</td>
<td>2.78 ± 2.40</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.91 ± 6.20</td>
<td>0.86 ± 4.00</td>
<td>3.57 ± 3.01</td>
</tr>
<tr>
<td>5</td>
<td>5.06 ± 7.11</td>
<td>1.47 ± 7.70</td>
<td>2.94 ± 3.59</td>
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<tr>
<td>6</td>
<td>5.46 ± 6.85</td>
<td>1.54 ± 7.92</td>
<td>2.04 ± 3.01</td>
</tr>
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<td>0.76 ± 0.53</td>
<td>0.85 ± 2.70</td>
<td>0.79 ± 0.79</td>
</tr>
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<td>8</td>
<td>0.79 ± 1.12</td>
<td>2.26 ± 5.11</td>
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</tr>
<tr>
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<td>4.83 ± 9.65</td>
<td>4.98 ± 1.20</td>
<td>3.71 ± 4.69</td>
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<tr>
<td>10</td>
<td>2.71 ± 5.42</td>
<td>1.34 ± 2.30</td>
<td>1.78 ± 2.70</td>
</tr>
<tr>
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<td>0.70 ± 0.14</td>
<td>1.10 ± 2.27</td>
<td>5.37 ± 6.49</td>
</tr>
<tr>
<td>12</td>
<td>4.29 ± 2.38</td>
<td>0.58 ± 1.76</td>
<td>1.79 ± 1.96</td>
</tr>
<tr>
<td>13</td>
<td>2.58 ± 4.77</td>
<td>3.38 ± 3.17</td>
<td>2.57 ± 5.83</td>
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<tr>
<td>14</td>
<td>4.00 ± 5.11</td>
<td>0.42 ± 0.83</td>
<td>0.80 ± 1.62</td>
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ANOVA with repeated measures

<table>
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<tr>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>0.791</td>
</tr>
</tbody>
</table>

Between Periods: 0.791
Between Times: 0.28
Interaction between Periods & Times: 0.65

* cf. Ref. 303, 304
** two normals and two asthmatics
# Period "B" = Serial saline inhalations and FEV1 determinations
"C" = Serial inhalations of saline only
"D" = Serial determinations of FEV1 only
θ mean ± S.D.
Table 4-4: Analysis of variance (ANOVA) with repeated measures* on serial relative plasma DAO levels obtained separately in two sub-groups (2 normals; 2 asthmatics) during 3 defined 'control periods not involving inhalation of bronchoconstrictive agents.

<table>
<thead>
<tr>
<th>SUBJECT GROUP</th>
<th>BLOOD SAMPLE</th>
<th>RELATIVE PLASMA DAO- ACTIVITY (%)</th>
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<td>PERIOD #</td>
<td>Running Number</td>
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<td>Normals (N=2)</td>
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<tr>
<td></td>
<td>2</td>
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<tr>
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<tr>
<td></td>
<td>14</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>2</td>
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<tr>
<td></td>
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<td></td>
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ANOVA with repeated measures, \( P \)

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<td>Interaction between Periods &amp; Time Samples</td>
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</tr>
</tbody>
</table>

* cf. Ref. 303, 304

# Period "B" = Serial Saline inhalations and FEV1 determinations
"C" = Serial inhalations of Saline only
"D" = Serial determinations of FEV1 only

@ Mean ± S.D.
Table 4-5: Analysis of variance (ANOVA) with repeated measures* on serial plasma histamine levels obtained separately in two sub-groups (2 normals; 2 asthmatics) during 3 defined periods not involving inhalation of bronchoconstrictive agents

<table>
<thead>
<tr>
<th>SUBJECT GROUP</th>
<th>BLOOD SAMPLE Running Number</th>
<th>Time Lapse (minute)</th>
<th>PLASMA HISTAMINE (ng/ml)</th>
</tr>
</thead>
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<tr>
<td>Normals (N=2)</td>
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<td></td>
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<tr>
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<td>0</td>
<td>5.96 ± 8.43</td>
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<tr>
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<td>2.02 ± 2.86</td>
</tr>
<tr>
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<td>14</td>
<td>0</td>
<td>4.44 ± 6.27</td>
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<tr>
<td>4</td>
<td>21</td>
<td>6.46 ± 9.12</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>3.74 ± 5.29</td>
<td>1.56 ± 2.20</td>
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<tr>
<td>6</td>
<td>35</td>
<td>6.76 ± 3.55</td>
<td>1.10 ± 1.55</td>
</tr>
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</tr>
<tr>
<td>8</td>
<td>49</td>
<td>0</td>
<td>1.19 ± 1.68</td>
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<td>56</td>
<td>0</td>
<td>1.23 ± 1.73</td>
</tr>
<tr>
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<td>63</td>
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<td>0</td>
<td>2.05 ± 2.90</td>
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<td>13</td>
<td>84</td>
<td>0.30 ± 0.42</td>
<td>4.19 ± 4.14</td>
</tr>
<tr>
<td>14</td>
<td>91</td>
<td>0</td>
<td>0.85 ± 1.17</td>
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<td>Asthmatics (N=2)</td>
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<td></td>
</tr>
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<td>3.14 ± 4.44</td>
<td>4.63 ± 6.55</td>
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<tr>
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<td>6.62 ± 9.36</td>
<td>5.08 ± 3.11</td>
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<td>3.37 ± 4.76</td>
<td>1.72 ± 0.50</td>
</tr>
<tr>
<td>5</td>
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<td>7.43 ± 10.50</td>
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<td>4.17 ± 5.90</td>
<td>1.98 ± 2.80</td>
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<td>7</td>
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<td>56</td>
<td>9.65 ± 13.65</td>
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<td>0.14 ± 0.20</td>
<td>0.03 ± 0.04</td>
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<td>2.08 ± 2.93</td>
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<tr>
<td>14</td>
<td>91</td>
<td>8.00 ± 5.20</td>
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ANOVA with repeated measures

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Between Groups</td>
<td>0.62</td>
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<tr>
<td>Between Periods</td>
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<tr>
<td>Between Timed Samples</td>
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<tr>
<td>Interaction between Periods &amp; Timed Samples</td>
<td>0.52</td>
</tr>
</tbody>
</table>

* cf. Ref. 303, 304
# Period "B" = Serial Saline inhalations and FEV1 determinations
"C" = Serial inhalations of Saline only
"D" = Serial determinations of FEV1 only
@ Mean ± S.D.
Table 4-6: Analysis of variance (ANOVA) with repeated measures* on serial relative plasma DAO levels obtained in 3 subjects** during 4 defined control periods not involving inhalation of bronchoconstrictive agents

<table>
<thead>
<tr>
<th>PERIOD #</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RELATIVE PLASMA DAO ACTIVITY (% )</td>
<td>RELATIVE PLASMA DAO ACTIVITY (% )</td>
<td>RELATIVE PLASMA DAO ACTIVITY (% )</td>
<td>RELATIVE PLASMA DAO ACTIVITY (% )</td>
</tr>
<tr>
<td>BLOOD SAMPLE</td>
<td>Running Number</td>
<td>Time Lapse (minute)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>100.0 ± 0.0@</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>93.0 ± 3.3</td>
<td>96.5 ± 10.3</td>
<td>93.8 ± 2.4</td>
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<tr>
<td>3</td>
<td>14</td>
<td>93.5 ± 3.5</td>
<td>95.9 ± 12.1</td>
<td>92.0 ± 1.3</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>90.7 ± 6.0</td>
<td>94.7 ± 8.1</td>
<td>91.7 ± 2.2</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>92.0 ± 3.6</td>
<td>93.3 ± 14.3</td>
<td>91.4 ± 3.2</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td>88.5 ± 7.0</td>
<td>92.4 ± 11.0</td>
<td>90.8 ± 4.6</td>
</tr>
<tr>
<td>7</td>
<td>42</td>
<td>90.5 ± 6.3</td>
<td>94.5 ± 10.6</td>
<td>89.1 ± 3.6</td>
</tr>
<tr>
<td>8</td>
<td>49</td>
<td>89.8 ± 6.3</td>
<td>95.4 ± 8.8</td>
<td>90.3 ± 4.7</td>
</tr>
<tr>
<td>9</td>
<td>56</td>
<td>90.8 ± 4.9</td>
<td>93.1 ± 14.2</td>
<td>88.3 ± 2.2</td>
</tr>
<tr>
<td>10</td>
<td>63</td>
<td>89.4 ± 6.6</td>
<td>94.8 ± 8.9</td>
<td>91.5 ± 1.4</td>
</tr>
<tr>
<td>11</td>
<td>70</td>
<td>90.3 ± 7.5</td>
<td>95.2 ± 8.8</td>
<td>87.7 ± 3.3</td>
</tr>
<tr>
<td>12</td>
<td>77</td>
<td>89.2 ± 7.8</td>
<td>97.4 ± 11.1</td>
<td>87.3 ± 5.0</td>
</tr>
<tr>
<td>13</td>
<td>84</td>
<td>90.0 ± 6.2</td>
<td>94.5 ± 9.3</td>
<td>93.3 ± 1.2</td>
</tr>
<tr>
<td>14</td>
<td>91</td>
<td>89.5 ± 5.7</td>
<td>94.7 ± 11.9</td>
<td>90.2 ± 0.9</td>
</tr>
</tbody>
</table>

ANOVA with repeated measures

- Between Periods: 0.64
- Between Timed Samples: 0.33
- Interaction between Periods & Timed Samples: 0.40

* cf. Ref. 303, 304
** two normals and one asthmatic
# Periods "A" = Resting
    "B" = Serial Saline inhalations and FEV₁ determinations
    "C" = Serial inhalations of Saline only
    "D" = Serial determinations of FEV₁ only
@ Mean ± S.D.
Table 4-7: Analysis of variance (ANOVA) with repeated measures* on serial plasma Histamine levels obtained in 3 subjects** during 4 defined control periods not involving inhalation of bronchoconstrictive agents

<table>
<thead>
<tr>
<th>PERIODS#</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLOOD SAMPLE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Running Number</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time Lapse (minute)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLASMA HISTAMINE (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>3.05 ± 5.29†</td>
<td>0</td>
<td>3.97 ± 6.88</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>2.72 ± 2.45</td>
<td>0</td>
<td>2.50 ± 2.08</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>1.67 ± 2.90</td>
<td>0</td>
<td>2.95 ± 5.12</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>0.95 ± 1.64</td>
<td>0</td>
<td>0.50 ± 0.87</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>1.10 ± 1.91</td>
<td>4.30 ± 7.45</td>
<td>1.03 ± 1.79</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td>1.16 ± 1.30</td>
<td>2.49 ± 4.31</td>
<td>0.73 ± 1.26</td>
</tr>
<tr>
<td>7</td>
<td>42</td>
<td>0.79 ± 1.37</td>
<td>4.50 ± 7.80</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>49</td>
<td>3.40 ± 5.88</td>
<td>0</td>
<td>0.79 ± 1.37</td>
</tr>
<tr>
<td>9</td>
<td>56</td>
<td>1.45 ± 2.52</td>
<td>0</td>
<td>0.81 ± 1.41</td>
</tr>
<tr>
<td>10</td>
<td>63</td>
<td>0.42 ± 0.72</td>
<td>0</td>
<td>1.59 ± 2.76</td>
</tr>
<tr>
<td>11</td>
<td>70</td>
<td>0</td>
<td>0</td>
<td>1.52 ± 2.63</td>
</tr>
<tr>
<td>12</td>
<td>77</td>
<td>5.68 ± 8.05</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>84</td>
<td>2.06 ± 1.97</td>
<td>0</td>
<td>2.79 ± 3.79</td>
</tr>
<tr>
<td>14</td>
<td>91</td>
<td>0</td>
<td>1.44 ± 0.35</td>
<td>0.55 ± 0.96</td>
</tr>
</tbody>
</table>

ANOVA with repeated measures

Between Periods 0.60
Between Timed Samples 0.64
Interaction between Periods & Timed Samples 0.86

* cf. Ref. 303, 304
** two normals and one asthmatic
# Period "A" = Resting
  "B" = Serial Saline inhalations and FEV₁ determinations
  "C" = Serial inhalations of Saline only
  "D" = Serial determinations of FEV₁ only
@ Mean ± S.D.
Finally, the highest value for plasma HM obtained in each subject during the test periods was singled out for ANOVA between subjects (N=3 with elimination of HLH for periods B, C and D) and conditions (N=3 with elimination of period A of subject LV, DMC and TW) separately. No significant differences were revealed (Table 4-8).
Table 4-8: Analysis of variance (ANOVA) on highest Histamine values obtained in 4 subjects during defined control periods not involving inhalation of bronchoconstrictive agents

<table>
<thead>
<tr>
<th>PERIOD</th>
<th>SUBJECT</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L.V.</td>
<td>0</td>
<td>0</td>
<td>7.11</td>
<td>10.46</td>
</tr>
<tr>
<td></td>
<td>T.W.</td>
<td>9.17</td>
<td>4.32</td>
<td>4.78</td>
<td>14.08</td>
</tr>
<tr>
<td></td>
<td>H.L.H.</td>
<td>-</td>
<td>19.30</td>
<td>9.26</td>
<td>4.48</td>
</tr>
</tbody>
</table>

ANOVA

<table>
<thead>
<tr>
<th>Between Periods*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.81</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Between Subjects**</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.93</td>
<td></td>
</tr>
</tbody>
</table>

# Period "A" = Resting
"B" = Serial Saline inhalations and FEV1 determinations
"C" = Serial inhalations of Saline only
"D" = Serial determinations of FEV1 only
*N=3 with elimination of period A of subjects LV, DMc and TW
**N=3 with elimination of HLH for periods B, C and D
Effects of Methacholine (MCH) Inhalation on Forced Expiratory Volume in the First Second (FEV₁) and Plasma Diamine Oxidase (DAO) Activity and Histamine (HM)

Histories of individual subjects will precede brief descriptions of the results obtained. The following guidelines apply to the graphic presentation of data (Fig. 4-5 to 4-23):

Beginning at zero time, inhalation of MCH by increments is depicted in a step ladder fashion. The cumulative units (cu) of MCH administered are ascertainable from the logarithmic scale on the left. FEV₁ changes are represented as open circles. The average of the two control values for FEV₁ (dots within open circles) obtained immediately after inhalation of the diluent control (NaCl; arrow) is set at 100% on the right-hand scale. The 100% mark of this scale is in line with the left logarithmic mark of 225 cu, the upper permissible limit for MCH inhalation. The right-hand scale also serves to ascertain the relative change in plasma DAO (solid squares). Absolute control (100%) values for FEV₁ and plasma DAO are listed in litres (L) and enzyme units (U), respectively. Values for plasma HM (solid triangles) are plotted as ng/ml (left lower scale) and as percent change (right lower scale).

Fig. 4-5 to 4-23 afforded the basis for computation of PD₂₀-FEV₁, defined as the provocative dose of MCH causing a fall in FEV₁ by 20%. This dose is derived by "bracketing" as follows:
From the point of intersection of a horizontal corresponding to a 20% FEV₁ fall (= 80% on the right-hand scale) with the line joining measured FEV₁ values falling above and below that mark, a vertical drawn downward will intersect a line joining two increments of MCH (step ladder). A horizontal drawn from the point of the latter intersection permits to ascertain PD₂₀-FEV₁ for MCH from the logarithmic scale on the left.

Data will be presented in decreasing order of sensitivity of individual subjects to MCH, based upon "PD₂₀-FEV₁". Results on subjects inhaling MCH before and after administration of DSCG and CMTD are revealed in an appendix to this thesis (cf. page 235).
L.H. 44 yrs
Nov 21, 1975

\[ \Delta \text{DAO}, \% \]
(right scale)
(100\% = 1.37 U)

\[ \Delta \text{FEV}_1, \% \]
(right scale)
(100\% = 2.25 L)

MCH, cu
(log scale)

Plasma HISTAMINE

nG/ml
(left scale)

\[ \Delta \% \]
(right scale)

NaCl
Ventolin

Fig. 4-5: Methacholine Inhalation Challenge in "L.H."
Time course of changes in FEV\(_1\) and plasma DAO and HM
Fig. 4-5:

L.H., Male, 44 years old

History

This subject with Dermatographism and a negative RAST test suffers from asthmatic attacks of largely undefined etiology which have been treated with DSCG and bronchodilators. During his current admission for further investigation, he was positive to house-dust inhalation challenge.

MCH Challenge

The post-saline control value for HM was much lower than its presaline counterpart. A maximum fall in FEV1 (38%) occurred at 20 minutes with only 5.4 cu of MCH, accompanied by increases in plasma DAO and HM. When FEV1 regained baseline aided by Ventolin given to alleviate wheezing, a decline in both plasma DAO and HM was followed by their rebound. This patient's ongoing apprehension forfeited further blood withdrawals.

PD20-FEV1 for MCH = 1.3 cu
Fig. 4-5: Methacholine Inhalation Challenge in "W.J." :–

Time course of changes in FEV₁ and plasma DAO and HM
Fig. 4-6:

W. J., Male, 41 years old

History

This subject is an asthmatic with evidence of concomitant chronic obstructive pulmonary disease who stopped smoking in October, 1976. Skin tests to common inhalant allergens were negative. Medications included bronchodilators, Prednisone and inhaled Beclomethasone dipropionate.

MCH Challenge

With a total of 5.4 cu of MCH, FEV₁ fell by 40%. Its subsequent sluggish recovery was markedly improved by Ventolin.

At the end of the experiment, plasma DAO had declined by 10%. Plasma HM, which had decreased with the second MCH increment, rose steeply during the phase of sluggish FEV₁ recovery. After Ventolin, it decreased to 50% its post-saline level.

PD₂₀-FEV₁ for MCH = 3.0 cu
Fig. 4-7: Methacholine Inhalation Challenge in "J.C."

Time course of changes in FEV₁ and plasma DAO and HM
Fig. 4-7:

J.C., Female, 32 years old

History

This hairdresser associated breathing difficulties with the use of hair sprays.

MCH Challenge

With four increments affording 11 cu, FEV₁ fell by some 35%. The subject's marked apprehension due to chest tightness was further aggravated by difficulties in blood sampling for which reason the test was discontinued prematurely.

With the first MCH increment, plasma DAO rose some 20% above its control level. At 1.03 U, this level was much lower than that noted in any other subject. With the two subsequent MCH increments, activity fell to an even lower value. Its subsequent rise once more exceeded the control level.

During the entire period of study, there was a virtually continuous increase in plasma HM from 9 to 22 ng/ml.

PD₂₀-FEV₁ for MCH = 6.5 cu
Fig. 4-8: Methacholine Inhalation Challenge in "E. Mac."

Time course of changes in FEV₁ and plasma DAO and HM
Fig. 4-8:

E. Mac. Male, 23 years old

History

This medical student with allergic bronchial asthma and perennial allergic rhinitis has positive skin tests to several allergens including pollens, house-dust and molds. He is a non-smoker. Treatment at the time of study was confined to antihistaminics and was withheld.

MCH Challenge

Inhalation of MCH to 11 cu evoked a fall in FEV₁ by nearly 35%, followed by its spontaneous return towards baseline.

An erratic decline in plasma HM to trace levels was accompanied by a decline in plasma DAO.

PD₂₀-FEV₁ for MCH = 7.2 cu.
A.M. σ, 25 ys  
Oct 23, 1975

\[ \Delta \text{DAO, \%} \]  
(right scale)  
(100\%=1.73 U)

\[ \Delta \text{FEV}_1, \% \]  
(right scale)  
(100\%=4.55 L)

\[ \text{MCH, cu} \]  
(log scale)

\[ \text{Plasma HISTAMINE} \]  
(nG/ml)  
(left scale)  
\[ \Delta \% \]  
(right scale)

Fig. 4-9: Methacholine Inhalation Challenge in "A.M.":-  
Time course of changes in FEV$_1$ and plasma DAO and HMM
Fig. 4-9:

A.M., Male, 25 years old

History

This non-smoking resident in Internal Medicine has mild allergic and infectious asthma. During previous inhalation challenges, he was positive to house-dust and MCH.

MCH Challenge

With a total of four MCH increments affording 11 cu, FEV₁ decreased by nearly 40%, followed by its spontaneous recovery.

During the MCH inhalation phase, a swift increase in plasma HM coincided with a modest decline in plasma DAO.

During FEV₁ recovery, a short-lived fall in plasma HM and discernible rise in DAO was followed by a HM spike, accompanied by a renewed fall in plasma DAO.

A negative correlation between plasma HM and DAO \( r = -0.52; N = 11 \) was not significant \( (P < 0.1) \).

\( \text{PD}_{20}-\text{FEV}_1 \) for MCH = 9 cu.
Fig. 4-10: Methacholine Inhalation Challenge in "R.L."

Time course of changes in FEV₁ and plasma DAO and HM
Fig. 4-10:

R.L., Female, 48 years old

History

This subject has asthma and hypertension. By skin tests, she was positive to dog danders, dusts and molds. The etiology for asthma is not clear.

MCH Challenge

The patient's low FEV₁ prior to testing (1.63 ± 0.15 L) necessitated administration of Choledyl, 200 mg and Prednisone, 5 mg following which the post-saline FEV₁ control average rose by some 60% to 2.60 L. This gain was virtually lost when MCH was inhaled to 11.65 cu, associated with wheezing. Ventolin was given as a precaution, entailing a rise in FEV₁ above the post-saline control average.

The post-saline control value for plasma HM was appreciably higher than its pre-saline counterpart. During MCH inhalation, plasma DAO rose while plasma HM fell steeply from a value more than double its post-saline control level. After MCH inhalation, a short-lived fall in plasma DAO was associated with a new and erratic rise in plasma HM to values above control levels.

\[ \text{PD}_{20}-\text{FEV}_1 \text{ for MCH = 9.0 cu.} \]
G.R. 6, 29 ys
Aug 5, 1976

Fig. 4-11: Methacholine Inhalation Challenge in "G.R."
Time course of changes in FEV₁ and plasma DAO and HM
Fig. 4-11:

G.R., Male, 29 year old

History

This non-smoking subject has allergic and infectious asthma with skin tests positive to animal danders, house-dusts and pollens. Treatment included bronchodilators plus removal of animals from his environment.

MCH Challenge

FEV₁ fell by a maximum of 42% in response to a total of five MCH increments affording 24 cu. Although its subsequent recovery appeared to be swift, Ventolin was administered to alleviate chest tightness and wheezing. There was no major change in plasma DAO.

Changes in plasma HM occurred within a low range of values. With the first three MCH increments affording 5.4 cu, it dropped virtually to zero. The 4th (penultimate) increment entailed a HM spike. During the recovery phase, plasma HM dropped to levels below the control values.

PD₂₀-FEV₁ for MCH = 24 cu.
Fig. 4-12: Methacholine Inhalation Challenge in "J.H.";
Time course of changes in FEV₁ and
plasma DAO and HM
Fig. 4-12:

J.H., Male, 54 years old

History

This chicken and cattle farmer who until December 1976 smoked 6-10 cigarettes per day, has allergic asthma. Skin tests to common allergens were negative. Asthmatic attacks are precipitated by undefined allergens in his chicken barn. He has been treated with DSCG, bronchodilators and inhaled Beclomethasone dipropionate.

MCH Challenge

With six MCH increments affording 49 cu, FEV₁ eventually dropped by some sustained 21%. Chest tightness, coughing and laboured breathing cleared with administration of Ventolin following which FEV₁ rose above control levels.

Oscillations in plasma DAO did not exceed the two control values at any stage. There was a biphasic rise in plasma HM from zero nanogram (pre-saline control), yielding the highest value ten minutes after the last MCH increment. Plasma HM then fell to the post-saline control level.

PD₂₀-FEV₁ for MCH = 49 cu.
Fig. 4-13: Methacholine Inhalation Challenge in "K.B.":

Time course of changes in FEV₁ and plasma DAO and HM
Fig. 4-13:

K.B.*, Female, 29 years old

**History**

This non-smoking nurse has allergic and infectious asthma with emotional factors. By skin test, she was positive to animal danders, house-dust, ragweed and molds. Treatment included bronchodilators and DSCG.

**MCH Challenge**

Sudden chest tightness was already felt immediately prior to inhalation of the diluent control when plasma HM spiked dramatically from a trace to a control value of 78.5 ng/ml.

During MCH inhalation to 100 cu, a significant and sustained FEV₁ fall occurred only with the last two increments and was associated with chest tightness, wheezing and coughing.

During the initial stages of MCH inhalation, plasma DAO briefly rose from a control value much higher than that of any other subject. This was associated with a precipitous drop in plasma HM to sustained low values.

Coincident with the eventual FEV₁ fall, there was a fall in plasma DAO together with another dramatic spike in plasma HM to 95 ng/ml.

When Alupent was administered to alleviate symptoms, DAO rose again to plateau at the control level; although FEV₁ likewise rose,
the control level was not regained and there was another sudden and prominent rise in plasma HM.

Changes in FEV₁ and plasma DAO yielded a significant correlation \((r = 0.70, N = 12, P < 0.01)\).

An inverse relationship between plasma DAO and HM was not significant \((r = -0.40, N = 12, P < 0.2)\).

\(PD_{20} - FEV₁\) for MCH = 78 cu.
Fig. 4-14: Methacholine Inhalation Challenge in "G.K."

Time course of changes in FEV₁ and plasma DAO and HM
Fig. 4-14:

G.K., Male, 28 years old

History

This patient is a physician allergic to furs. He has exercise-induced asthma and took DSCG for prolonged periods of time.

MCH Challenge

A progressive fall in FEV$_1$ exceeded the 20% mark only with the 7th (last) MCH increment affording 100 cu. Wheezing beginning after the penultimate MCH increment together with an apparent delay in the return of FEV$_1$ to baseline prompted administration of Ventolin.

With the initial MCH increment, plasma DAO first rose, then fell some 10% below the post-saline control level. A renewed rise above that level yielded the highest value immediately after the last MCH increment. Plasma DAO then returned to baseline, followed by another rise. The post-saline control value for plasma HM (58.49 ng/ml) was exceedingly higher than its pre-saline counterpart.

Plasma HM fell with the 1st MCH increment, rose as high as 136 ng/ml with the second, then dropped steeply to values below 10 ng/ml. There was no correlation between plasma HM and DAO.

PD$_{20}$-FEV$_1$ for MCH = 100 cu.
Fig. 4-15: Methacholine Inhalation Challenge in "D.G." -
Time course of changes in $\text{FEV}_1$ and
plasma DAO and HM.
Fig. 4-15:

D.G., Male, 28 years old

History

This patient, a physician, is allergic to ragweed and experienced attacks of wheezing during mountain climbing.

MCH Challenge:

A fall in FEV₁ by some 20% occurring with the penultimate increment, was barely sustained with the highest increment affording 174 cu.

A phase of throat irritation, chest tightness and coughing which began after the 5th increment and reached its climax after the last, was associated with a rise in plasma HM. The subsequent erratic return of this amine to control levels was accompanied by a discernible fall in plasma DAO.

This volunteer refused any medication for alleviation of his symptoms.

PD₂₀-FEV₁ for MCH = 165 cu.
D.M. 24 yrs
Dec 15, 1977

\[ \Delta \text{DAO,}\% \]
\[ \text{(right scale)} \]
\[ (100\% = 1.72\text{U}) \]

\[ \Delta \text{FEV}_1,\% \]
\[ \text{(right scale)} \]
\[ (100\% = 4.82\text{L}) \]

\[ \text{MCH, cu} \]
\[ \text{(log scale)} \]

\[ \text{Plasma HISTAMINE} \]
\[ \Delta \text{nG/ml} \]
\[ \text{(left scale)} \]
\[ \Delta \% \]
\[ \text{(right scale)} \]

---

**Fig. 4-16:** Methacholine Inhalation Challenge in "D.M."

Time course of changes in FEV\(_1\) and plasma DAO and HM
Fig. 4-16:

D.M., Male, 24 years old

History

This subject has allergic rhinitis and allergic asthma with positive skin tests to 3 pollen groups and house-dust. He is a non-smoker. Treatment has been confined to bronchodilators.

MCH Challenge

A gradual decline in FEV₁ during MCH inhalation culminated in a short-lived 25% drop only with the highest permissible MCH increment. Wheezing at that stage prompted Ventolin administration.

There were no major changes in plasma DAO. Those in plasma HM occurred within an exceedingly low range such that it spiked from zero to 3 ng/ml five minutes after inhalation of the penultimate MCH increment.

PD₂₀-FEV₁ for MCH = 220 cu.
H.L. H. 58 yrs.
Jan 17, 1978

Fig. 4-17: Methacholine Inhalation Challenge in "H.L. H."
Time course of changes in FEV₁ and plasma DAO and HM
H.L.H\textsubscript{1}, Male, 58 years old

**History**

This laboratory physician is a heavy smoker and has mild asthma. He has positive skin tests to grass pollens and molds. He has been on no therapy for asthma.

**MCH Challenge**

The permissible limit of MCH inhalation to 224.15 cu was required to elicit a significant fall in FEV\textsubscript{1} by 21%. The subsequent FEV\textsubscript{1} return towards baseline was sluggish.

With no major changes in plasma DAO, plasma HM was 8 ng/ml or less until 15 minutes after inhalation of the highest MCH increment when it dramatically spiked to 56.2 ng/ml.

PD\textsubscript{20} - FEV\textsubscript{1} for MCH = 220 cu
Fig. 4-18: Methacholine Inhalation Challenge in "H.L.H."

Time course of changes in FEV₁ and plasma DAO and HM

* repeat Methacholine Inhalation Challenge
Fig. 4-18:

H.L.H₂, Male, 58 years old

History:

This heavy smoker and mild asthmatic with skin tests positive to
grass pollens and molds and somewhat lessened sensitivity to MCH after
taking DSQ, as described in Appendix (cf. page 235, Fig. AP-1), was
re-challenged with MCH some 7 months later.

MCH Challenge

On this repeat occasion, he complained of malaise, with a respira-
tory infection and pain in his throat. The post-saline control average
for FEV₁ (3.20L) was comparable to values obtained during his earlier
MCH challenge. Inhalation of MCH proceeding again to the upper per-
missible limit, this time did not affect a significant FEV₁ fall. As
before, there were no major changes in plasma DAO.

Changes in plasma HM extended over 3 logarithmic cycles. The
post-saline control level already exceeded 200 ng/ml and was very
much higher than its pre-saline counterpart. Plasma HM dropped pre-
cipitously with the 1st MCH increment and spiked drastically to
479 ng/ml when 11.65 cu of MCH were attained; coincident with a spo-
tonaneous swift return of FEV₁ to its own control level, plasma HM then
increased to even higher levels (521.6 and 658.3 ng/ml, respectively).

These confirmed changes in plasma HM are unique in that there
would seem to be no precedent to such drastic elevations.

PD₂₀-FEV₁ for MCH = not definable.
Fig. 4-19: Methacholine Inhalation Challenge in "D.Mc.":
Time course of changes in FEV₁ and plasma DAO and HM
Fig. 4-19:

D.Mc., Male, 38 years old

History

This non-smoking internist and specialist in allergy is a normal subject free from asthma.

MCH Challenge

While FEV\textsubscript{I} was not significantly affected by MCH inhaled to the permissible maximum, there was a short-lived rise in plasma DAO with the attainment of 11.65 cu. followed by one in plasma HM, when the penultimate MCH increment was inhaled.

PD\textsubscript{20}-FEV\textsubscript{I} for MCH = not definable.
M.M. ♂, 20 ys
Mar 31, 1976

Fig: 4-20: Methacholine Inhalation Challenge in "M.M.":
Time course of changes in FEV₁ and plasma DAO and HM
Fig. 4-20:

M.M., Male, 20 years old

History

This science student has exercise-induced asthma and possible allergic rhinitis related to house-dust. Skin tests were negative to diverse allergens. Treatment included desensitization attempts and administration of bronchodilators prior to exercise.

MCH Challenge

MCH inhaled to the highest permissible increment did not effect a significant FEV$_1$ fall. Plasma DAO did not exceed its post-saline control level at any stage.

Oscillations in plasma HM afforded values some 40% above and below control levels.

PD$_{20}$-FEV$_1$ for MCH = not definable
B.P., 23 yrs
Dec 18, 1975

\[ \Delta \text{DAO} \%
\]
(right scale)
(100\% = 1.34 U)

\[ \Delta \text{FEV}_1 \%
\]
(right scale)
(100\% = 5.43 L)

\[ \text{MCH, cu}
\]
(log scale)

\[ \text{Plasma HISTAMINE}
\]
(nG/ml)
(left scale)
\[ \Delta \%
\]
(right scale)

Fig. 4-21: Methacholine Inhalation Challenge in "B.P.":
Time course of changes in FEV\(_1\) and plasma DAO and HM.
Fig. 4-21:

B.P., Male, 23 years old

History

This patient has had asthma and perennial rhinitis since he was 14 years old, with skin-tests positive to cat fur, house-dust and ragweed. Asthma has become much less of a problem since he quit smoking.

MCH Challenge

FEV$_1$ was not affected until MCH was inhaled to 99.15 cu. Its subsequent maximum drop, occurring with the permissible maximum of 224.15 cu, was short-lived and not significant (-18.5%).

With the 1st MCH increment, plasma DAO rose some 15% above control. A prominent fall to 70% the control level was followed by a steep rise, occurring with the 6th MCH increment. The highest value measured during the recovery phase exceeded the control level by some 20%.

The post-saline value for HM was much lower than its pre-saline counterpart. With the lower MCH increments, plasma HM dropped to yield zero ng/ml when 24.15 cu of MCH were inhaled. This drop coincided with the major fall in plasma DAO. Plasma HM then rose although the highest level (pre-saline control value) was not regained.

There was no significant correlation between changes in plasma HM and DAO.

PD$_{20}$-FEV$_1$ for MCH = not definable.
Fig. 4-22: Methacholine Inhalation Challenge in "T.W."—
Time course of changes in FEV₁ and plasma DAO and HM
Fig. 4-22:

T.W., Male, 42 years old

History

This non-smoking chest physician is a mild asthmatic with skin tests positive to diverse inhaled allergens. Asthmatic attacks have not recurred for many years.

MCH Challenge

Inhalation of MCH to the upper permissible limit did not affect FEV\textsubscript{1} significantly.

After a minor fall in plasma DAO with the earlier MCH increments, this activity regained the control level at the stage of 24.15 cu of MCH. It then fell again.

A negative correlation between changes in plasma DAO and those in plasma HM was not significant ($r = -0.52$, $N = 10$, $P < 0.1$).

$PD_{20} - FEV_{1}$ for MCH = not definable.
Fig. 4-23: Methacholine Inhalation Challenge in "E.McL."

Time course of changes in FEV₁ and plasma DAO and HM
Fig. 4-23:

E. McL., Male, 23 years old

History

This medical student with exercise-induced asthma and skin tests positive to house-dust and *D. farinae* was on no specific therapy.

**MCH Challenge**

FEV₁ dropped by some sustained 10% with MCH inhalation to a total of 99.15 cu. His emergency call for duty forfeited completion of his challenge to a desirable endpoint. With the 2nd MCH increment, plasma DAO increased some 10% above the control level, then declined erratically, the value pertaining to the last blood withdrawal being some 7% below that level.

Plasma HM changes yielded values 40% above and below its post-saline control level.

PDₓ₀-FEV₁ for MCH = not definable.
Fig. 4-24: Comparison of all Methacholine Challenges
Fig. 4-24 compiles results previously shown as time courses of changes in FEV₁ and plasma DAO and HM for the total 19 instances of MCH inhalation by increments. Results are given in decreasing order of the subject's sensitivity to this cholinergic agent, as judged by PD₂₀-FEV₁.

For each subject, absolute values for FEV₁ and plasma DAO and HM prior to inhalation of the diluent (saline) control are plotted as open circles together with the control values obtained after such inhalation (solid dots). The range within which changes occurred during the subsequent inhalation of MCH and ensuing recovery from this agent is given by the hatched area. For FEV₁, the distance between a solid dot and the lower limit of the hatched area then reflects the maximum FEV₁ fall (arrow) achieved with MCH. The FEV₁ percent fall in each instance is listed numerically irrespective of whether or not such fall was significant. To permit appreciation of the unusually large spread in plasma HM values obtained for "K.B.", "G.K." and especially "H.L.H." relative to that observed in other subjects, data for plasma HM are plotted on a logarithmic scale.

By this sequential arrangement, Fig. 4-24 then comprises two groups of subjects, viz., those responding to MCH inhalation with a significant
FEV<sub>1</sub> fall ("Responders"; N=13) and those not revealing such a fall ("Non-Responders"; N=6). By this criterion, "H.L.H." having inhaled MCH twice, emerges as both a "Responder" and a "Non-Responder".

Absolute pre- and post-saline control values for both FEV<sub>1</sub> and plasma DAO were not significantly different for Responders and Non-Responders separately as well as for the entire series.

In most instances, plasma levels of DAO in a given subject appeared to be stable characteristics. Changes observed during the MCH inhalation and subsequent recovery phase yielded fluctuations by 7.4 ± 6.7 above, and by 7.9 ± 5.9% (mean ± S.D.) below the post-saline control value. The highest increase above that value (21.3%) occurred in a Responder (JC). The greatest fall (-28.7%) occurred in a Non-Responder (BP).

Plasma DAO levels in one female Responder (KB) were much higher than those noted in any other volunteer. In this subject, changes in plasma DAO correlated directly and significantly with those in FEV<sub>1</sub> (P<0.01); an inverse correlation between plasma DAO and HM was not significant although the lowest value for plasma DAO coincided with a prominent plasma HM spike.

In 9 instances [7 Responders (WJ, AM, KB, GR, JH, DM, HLH); 3 Non-Responders (HM, EMCL, TW)] changes in plasma DAO correlated inversely with those in plasma HM; in no instance was this inverse relationship significant. The remaining 10 instances [6 Responders (RL, EMAC; JC,
LH, GK, DG); 3 Non-Responders (DMc, BP, HLH₂) revealed a direct relationship between plasma DAO and plasma HM. Only in one Responder (EMac) was this relationship significant.

In the case of the thirteen Responders, the mean of pre-saline control values for plasma HM (5.16 ± 5.10 ng/ml) was significantly lower (P<0.02) than that of the post-saline controls (14.74 ± 24.42 ng/ml). For the six Non-Responders, means of pre- and post-saline values for plasma HM (12.95 ± 8.89 and 44.98 ± 88.11 ng/ml, respectively) were not significantly different. There was no significant difference in means for pre- and post-saline HM levels when the two groups were combined (7.62 ± 7.29 versus 24.29 ± 52.56 ng/ml; N=38; P<0.2).

Absolute values for plasma HM extended over the widest range (<1.0 - 658.3 ng/ml) during repeat inhalation of MCH by the asthmatic who became a Non-Responder during an acute respiratory infection (HLH₂). In the remaining five Non-Responders (DMc, MM, BP, TW, EMcL), the range of changes extended from 2 - 60 ng/ml. In 2/13 Responders (KB, GK), one or more values exceeded 100 ng/ml. In 4/13 Responders (AM, GR, JH, DM), plasma HM at no stage exceeded 10 ng/ml. In 7/13 Responders (EMac, GR, JH, KB, GK, DM, HLH₁), plasma HM yielded one or more values below 1 ng/ml.

Changes in plasma HM concentrations during the MCH inhalation and subsequent recovery phase yielded discernible spikes in 12 Responders (LH, WJ, EMac, AM, RL, GR, JH, KB, GK, DG, DM, HLH₁) and 6 Non-Responders (HLH₂, DMc, MM, BP, TW, EMcL). In 3 Responders (WJ, DM, HLH₁) and 1 Non-Responder
(DMc), there was one single spike towards the end of the MCH inhalation phase. In 9 Responders (LH, EMac, AM, RL, GR, JH, KB, GK, DG) and 5 Non-Responders (HLH₂, MM, BP, TW, EMcL), there were additional spikes; in 4 Responders (AM, KB, GK, DG) and 1 Non-Responder (HLH₂), only one additional spike occurred during the FEV₁ recovery phase; in 2 Responders (EMac, GK) and 1 Non-Responder (BP), several spikes confined to the MCH inhalation phase yielded successively lower orders of magnitude for plasma HM.

In 10 Responders (LH, EMac, WJ, AM, GR, RL, JH, DG, DM, HLH₁) and 5 Non-Responders (DMc, MM, EMcL, TW, DMc), HM spikes during MCH inhalation exceeded the post-saline control by a minimum of 25 and a maximum of 2500%. In 1 Responder (KB) and 1 Non-Responder (BP) in whom HM had fallen during the earlier MCH increments, the subsequent spike yielded orders of magnitude comparable to the post-saline control. In the case of another Responder (GK) in whom plasma HM had similarly fallen after an original spike, the level of the post-saline control value was not regained by the subsequent spikes.

In one Responder hitherto unconsidered (JC), HM changes did not follow any of the above patterns in that there was a continuous HM rise throughout the MCH inhalation and subsequent spontaneous recovery phase.
Responders to Methacholine: Within group comparison of relative changes in plasma-DAO and HM by paired t-test

In order to eliminate variation between subjects in timed plasma DAO and HM values expressed in their absolute units of measurements, post-saline control levels were set at 100%.

Pre-saline levels and values found in blood removed immediately after inhalation of the pen-ultimate MCH increment (affording maximum cumulative MCH units smaller than PD_{20\text{-FEV}}{1}) were then re-expressed as a percentage of post-saline controls. Values obtained 3-5, 7-10 and 15-20 minutes following inhalation of the last (highest) MCH increment (which afforded the maximum cumulative MCH units greater than PD_{20\text{-FEV}}{1}) were similarly re-expressed. The choice of these time intervals permitted the resorting to, say, a 3-minute value in a subject in whom a 5-minute value was not obtained or vice versa.

The foregoing manipulations, yielding N=13 for each blood withdrawal as defined above, eliminated differences between subjects as to their sensitivity to MCH and were a preliminary to the performance of paired t-tests by computer (303).

Table 4-9 summarizing the changes in plasma DAO and plasma HM expressed as a percentage of post-saline control levels, reveals mean relative changes to be very small in the case of plasma DAO; those in plasma HM were much higher so as to indicate its increase with MCH inhalation; for both variables, p-values attached to each defined group of blood withdrawals show mean relative changes not to be significant.
<table>
<thead>
<tr>
<th>BLOOD WITHDRAWAL</th>
<th>N</th>
<th>PLASMA DAO</th>
<th>PLASMA HM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( % of Post-saline control)</td>
<td>( % of Post-saline control)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Mean Change</td>
</tr>
<tr>
<td>Before diluent</td>
<td>13</td>
<td>100.87</td>
<td>0.87</td>
</tr>
<tr>
<td>Saline control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After pen-ultimate MCH increment*</td>
<td>13</td>
<td>101.08</td>
<td>1.08</td>
</tr>
<tr>
<td>3-5 min after last MCH increment**</td>
<td>13</td>
<td>99.35</td>
<td>-0.65</td>
</tr>
<tr>
<td>7-10 min after last MCH increment</td>
<td>13</td>
<td>97.65</td>
<td>-2.35</td>
</tr>
<tr>
<td>15-20 min after last MCH increment</td>
<td>13</td>
<td>98.77</td>
<td>-1.23</td>
</tr>
</tbody>
</table>

* maximum cumulative MCH units smaller than PD_{20}-FEV_1

** maximum cumulative MCH units greater than PD_{20}-FEV_1
As far as other timed values for plasma DAO and HM are concerned, within group comparisons by paired t-test were not feasible since the number of timed blood withdrawals obtained from the subjects varying inversely with their bronchial sensitivity to MCH was not uniform.

Using independent measurements for plasma DAO and HM, there was no significant correlation between these two variables for any of the blood withdrawals defined in Table 4-9.

Finally, the highest values for plasma HM relative to the post-saline control level were grouped for Responders irrespective of after what stage of MCH inhalation up to and including the pen-ultimate increment they occurred. The highest values for this amine relative to the post-saline control level after inhalation of the last MCH increment by Responders were similarly grouped, affording N=13 in both instances.

Table 4-10 shows that by paired t-test (303, 304) both sets of maximum mean values were significantly higher than those of post-saline controls set at 100%; as indicated by foot-note (Table 4-10), there was no significant difference between these two HM maxima.

Responders versus Non-Responders to Methacholine: Between group comparisons of relative changes in plasma DAO and HM by unpaired t-tests

For Non-Responders, N was reduced to 5 with elimination of the MCH challenge obtained on "H.L.H." on Aug 3, 1978. This was necessary because
Table 4-10: Maximum plasma Histamine values relative to post-saline control levels found in MCH Responders—before and after inhalation of the last (highest) MCH increment

<table>
<thead>
<tr>
<th>Maximum plasma Histamine value found*</th>
<th>N</th>
<th>Mean</th>
<th>Mean Change</th>
<th>S.D.</th>
<th>S.E.M.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before highest MCH increment</td>
<td>13</td>
<td>234</td>
<td>134</td>
<td>124</td>
<td>34</td>
<td>0.002</td>
</tr>
<tr>
<td>After highest MCH increment</td>
<td>13</td>
<td>263</td>
<td>163</td>
<td>235</td>
<td>65</td>
<td>0.028</td>
</tr>
</tbody>
</table>

* expressed as % of post-saline control level

By paired t-test, maximum plasma HM values found Before and After the highest MCH increment were not significantly different (P=0.73)
on that date, he was suffering from an acute respiratory illness, and also because that test was a repeat of the MCH challenge of Jan 17, 1978 (when HLH was a Responder).

Responders shared with Non-Responders a common stage of MCH inhalation, namely, the attainment of maximum cumulative MCH units not yielding a drop in FEV₁ by 20%. In the case of the Responders, that stage was afforded with inhalation of the pen-ultimate MCH increment; in the case of Non-Responders, it was limited to the inhalation of the maximum permissible MCH increment ( = the attainment of 224.15 cu of MCH), thus forfeiting the computation of a value for PD₂₀-FEV₁.

Accordingly, respective values for plasma DAO and HM obtained in Responders and Non-Responders after these stages were amenable to comparison by unpaired t-test following their re-expression (and that of pre-saline values) as a percentage of post-saline control levels (303, 304).

Table 4-11 shows that there was no significant difference in these values between Responders and Non-Responders.
Table 4-11: Relative changes in plasma DAO and plasma HM in MCH Responders and Non-Responders

<table>
<thead>
<tr>
<th>BLOOD WITHDRAWAL GROUP</th>
<th>SUBJECT</th>
<th>PLASMA DAO (% of post-saline control level) Mean ± S.D.</th>
<th>P</th>
<th>PLASMA HM (% of post-saline control level) Mean ± S.D.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Responders (N=13)</td>
<td>100.87 ± 3.72</td>
<td>0.99</td>
<td>97.0 ± 79.5</td>
<td>0.54</td>
</tr>
<tr>
<td>Before diluent saline control</td>
<td>Non-Responders (N=5)</td>
<td>101.52 ± 3.65</td>
<td>124.8 ± 90.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;Responders (N=13)</td>
<td>After critical MCH increment*</td>
<td>101.08 ± 5.59</td>
<td>181.0 ± 162.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Responders (N=5)</td>
<td>99.12 ± 7.05</td>
<td>150.0 ± 63.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* pen-ultimate MCH increment for Responders; maximum permissible MCH increment for Non-Responders
4.3 SUMMARY

(A) Control Periods Not Involving Exposure to Methacholine. Plasma DAO and plasma HM were determined every 7 minutes for a total of 91 minutes in 2 asthmatic and 2 normal subjects during 4 conditions not involving MCH inhalation. Conditions involved resting; serial inhalations of 0.9% NaCl accompanied by FEV₁ determinations; serial inhalations of 0.9% NaCl only; serial determinations of FEV₁ only. On a relative basis, timed values for each plasma DAO and HM were not significantly different from the first measurement within the 4 subjects, between the 2 asthmatic and 2 normal volunteers, and between the 4 conditions. On an absolute basis, a preponderance of plasma HM values during each conditions yielded zero; other values were afforded by short-lived increases to maxima ranging between 4.3 and 19.3 ng/ml.

(B) Methacholine Inhalation Challenges. Of eighteen (17 asthmatic; 1 normal) subjects inhaling MCH by increments to maximum permissible limits of 224.15 cu, 13 asthmatics were bronchial Responders; 4 asthmatics and the normal volunteer were Non-Responders.

In the Responder group, PD₂₀-FEV₁ for MCH ranged from 1.3 to 220 cu. In the case of both plasma DAO and HM, neither mean pre-saline levels nor any timed mean value obtained during and after the MCH inhalation phase were significantly different from the post-saline control set at 100%. On a relative basis, maxima for plasma HM found at variable stages and grouped separately according to their occurrence before and after inhalation of the highest MCH increment were in each instance significantly
higher than the post-saline control. There was no significant difference between plasma HM maxima so defined.

Relative to the post-saline control for both plasma DAO and HM, mean pre-saline values of Non-Responders were not significantly different from those of Responders. Inhalation of the penultimate and maximum permissible MCH increment by Responders and Non-Responders, respectively, yielded a stage common to both groups characterized by the absence of a significant FEV\textsubscript{1} fall. Mean relative plasma HM increases by 81 (Responders) and 50% (Non-Responders) above the post-saline control found in blood removed immediately after that stage were not significantly different.
CHAPTER 5: GENERAL DISCUSSION

A wealth of in vitro information implicates HM released from lung as a primary chemical mediator of immediate hypersensitivity affecting bronchomotor tone. In vivo evidence of the release of this amine from lung in acute human asthma is conflicting.

Using the acute asthma attack induced by incremental inhalation of MCH as a human model, this thesis attempted to define this role by studying relationships between changes in FEV₁, plasma HM and plasma DAO. Changes in these variables were also determined during four self-contained periods not involving MCH inhalation. As a preliminary to a discussion of the results obtained, the adequacy of conditions for testing, serial blood sampling and determination of plasma HM and DAO will be reviewed.

Condition of Subjects. With 2 exceptions, conditions under which the volunteers presented for testing were comparable. All subjects were fasting; asthmatic subjects were asymptomatic; any medications were withheld over-night. Since all tests began at 09:00, conditions were also comparable with respect to the diurnal rhythm for plasma HM (yielding the highest values at 04:00) quoted earlier (140). The exceptions concern an acute respiratory illness suffered by one mild asthmatic (HH) during a second MCH challenge; Choledyl and Prednisone administration was necessary to achieve an acceptable baseline for FEV₁ as a prelude to a similar challenge in a severe asthmatic (RL).
Blood Sampling. Heparinized plasma rather than serum (308) was used for HM determinations to exclude possible interference by vasoactive amines released from platelets during the clotting process (311). The reported absence of HM proper from human platelets (310) has recently been challenged with the finding of some 20 nanograms of HM per $10^9$ platelets in normal volunteers (307).

Since serial timed blood withdrawals were part of the protocol underlying both the MCH challenge tests and self-contained control periods, it was ill-advised to obtain samples by repetitive venipuncture. The insertion of an indwelling intracath ensuring patency of veins by a saline drip raises the question of a minimal dilutional artifact when it was disconnected prior to each timed blood withdrawal. Added precautions were taken to obtain blood undiluted with 0.9% NaCl solution by permitting the escape of double the volume at which visible blood dilution was no longer ascertainable. Furthermore, centrifugation at 600 x g for 30 minutes provided more gentle conditions than those chosen by other workers to minimize cell rupture. This step and subsequent gentle removal of only the upper one-half of supernatant plasma were found to be adequate for exclusion of non-ruptured cells and platelets, as judged by zero counts in plasma obtained from heparinized blood centrifuged under identical conditions.

Determination of Plasma Histamine. As discussed below, a number of plasma-HM values found by the writer during MCH inhalation were much higher than those reported by other workers. While this difference in findings should further militate against the possibility of dilutional
artifacts, it may be argued that the higher values found by the writer may be due to differences in methodology arising from the inclusion of the plasma ultrafiltration step. With equal justification, it may be argued that with plasma ultrafiltration, a preponderance of plasma HM values found during the four control periods not involving MCH inhalation measured zero; it is highly unlikely that dilutional artifacts were confined to these tests.

The inclusion of the plasma ultrafiltration step would not seem to have a published precedent. As revealed earlier in this thesis, this step effectively retained substances, presumably immunoglobulins (208, 309), shown by the writer and others to interfere with the double isotopic enzymatic assay for HM. The equivalent step of prior plasma boiling (found to be impractical by the writer) was given only casual consideration by Beaven et al. (205). Higher values for plasma HM found by the writer with prior ultrafiltration would seem to stress the necessity of removing interfering substances routinely.

The assay as modified by the writer retained its validity in the presence of methacholine and Disodium cromoglycate. Cimetidine, reported by others to interfere with the assay by inhibiting the critical subsidiary enzyme HNMT, did not affect results when this enzyme was harvested from rat brains. Presumably HNMT from that source is devoid of Cimetidine binding sites. Finally, the HM assay as modified by the writer yielded acceptable results when applied to reconstituted lyophilisates of HM prepared by the Mayo Clinic. Since the writer's work reached completion, it has been reported that the sensitivity of this assay is
enhanced by isolation of the radioactive methylhistamine generated by HNMT by thin-layer chromatography (304, 306).

**Determination of Plasma Diamine oxidase.** Due to high blank activity at pH 6.0 (optimum), serial assays for DAO were performed at the optimum concentration of substrate (HM) on plasma samples buffered at physiological pH. Disodium cromoglycate and Cimetidin did not interfere when added in vitro. Known inhibition effected at optimum pH by substrate (HM) in concentrations beyond a critical level was shown to extend to physiological pH. Moreover, at both optimum and physiological pH, MCH was found to function as a weak substrate in the absence of added HM and to enhance and inhibit plasma DAO activity at low and high concentrations of HM, respectively.

MCH-induced inhibition of plasma DAO at optimum concentrations of added HM was separately verified in vitro in 3 groups of adult (7 asthmatic; 10 allergic without asthma; 6 normal) subjects and also in a psychotic boy with multiple allergies. Beginning with a critical concentration of added MCH, mean inhibition achieved by further increasing its concentration was significant for each MCH increment. Although there was no significant difference in relative mean values between any two of the above three groups, a suggestion of subject variation emerged from the more dramatic inhibition effected with each MCH increment in the psychotic boy with multiple allergies.
If the hitherto unsuspected concentration-dependent effect of MCH of inhibiting DAO; \textit{in vitro} extends to lung \textit{in vivo}, inhalation of this cholinergic by increments may eventually afford local concentrations inhibitory to the oxidative deamination of released HM. Such a mechanism might contribute to bronchoconstriction \textit{via} local HM accumulation. This might more readily occur in subjects whose DAO activity is most sensitive to the inhibitory effect of MCH. As discussed further below, data obtained on one of 13 Responders to MCH were suggestive of the operation of such a mechanism \textit{in vivo}.

Control Periods not Involving \textit{Exposure} to Methacholine. Marked discrepancies in values for HM concentrations arrived at by 22 laboratories participating in the large-scale quality control study conducted by Gleich and Hull (241) emerged as a major deterrent to the acceptance of HM values reported in the literature. This prompted the application of the improved assay for HM described in this thesis to serial measurements of plasma HM concentrations, complemented by those of plasma DAO, in normal and asthmatic subjects during four specified periods not involving inhalation of bronchoconstrictive agents.

Requirements during the four test periods each of 91 minutes duration, are respecified as involving resting ("A"); serial inhalation of saline accompanied by FEV\textsubscript{1} determinations ("B"); serial inhalation of saline ("C") and serial determination of FEV\textsubscript{1} ("D"). Two normal\textsuperscript{*} and two asthmatic\textsuperscript{**} subjects volunteered for 14 blood withdrawals, obtained

\textsuperscript{*} "D.Mc." and "L.V." (LV did not undergo any bronchial challenge)
\textsuperscript{**} "T.W." and "H.L.H." (HLH did not undergo period A)
7 minutes apart, during each of these requirements.

Although in each subject there were spike-like increases in plasma HM to values as high as 19.3 ng/ml (cf. Table 4-8), a preponderance of values obtained during the 4 periods yielded zero. By analysis of variance with repeated measures, mean values for any of the 4 periods and for any two timed samples were not significantly different, and there was no significant difference between mean values of the 2 normal and 2 asthmatic subjects. This indicated that plasma HM was unaffected by any of the conditions differentiating the 4 periods.

The occasional occurrence of plasma HM spikes during conditions not involving the inhalation of a bronchoconstrictive agent is intriguing. While in "T.W.", "L.V." and "D.Mc.", a preponderance of values for plasma HM yielded zero, spike-like changes occurred in each of the four volunteers during at least one of the periods specified. By comparison of data between subjects, spikes were not confined to a given period. In one subject (TW) undergoing an additional test period under conditions nearly identical to those underlying period "B", the mean value for plasma HM was significantly higher (10.52 ± 2.38 ng/ml) than that for the latter period (0.31 ± 1.15 ng/ml; P < 0.001). In each of the four subjects, there were significant differences between mean plasma HM values for at least two of the four test periods. Even in "L.V.*", a non-asthmatic female in whom plasma DAO remained

* Due to her reluctance to undergo further tests, comparisons of these changes with those occurring during inhalation of bronchoconstrictive agents are not feasible.
remarkably constant and as many as 52 of the total 56 HM values measured serially during the four test periods yield zero, there was a terminal spike to 7.1 ng/ml during period "C"; three distinct spikes occurred during period "D", the highest value affording 10.5 ng/ml.

If the sampling artifact is excluded by precautions already discussed, these data indicate that in the absence of any exposure to MCH, the plasma HM level is not necessarily a stable characteristic.

If non-exposure to these agents is taken as a common denominator of the four test periods and the total number of plasma HM determinations obtained is pooled separately for normal and asthmatic subjects, then by paired t-test, the mean for the two normal volunteers, 1.56 ± 3.26 ng/ml; N=112, is significantly lower (P < 0.02) than that for the two asthmatics (2.83 ± 4.22 ng/ml; N=97). Inclusion of results additionally obtained on "T.W." under conditions nearly identical to those for period "B" raises the mean for plasma HM for the two asthmatics to 3.80 ± 4.77 ng/ml (N=111; P < 0.01). The large standard deviation attached to each mean should caution against the acceptance of 1 ng/ml as the upper normal HM concentration in plasma, as suggested by some workers (133, 234, 302).

Eiser et al. (299) quoted indirect evidence to the effect that asthmatics may differ from normal subjects by exhibiting continuous HM release in the resting state, as suggested by differences in basal bronchomotor tone. Of the four test periods, only "A" truly reflected that state. The occurrence during that period of three successively greater plasma HM spikes from zero values to a maximum of 14.9 ng/ml
in "D.Mc.", a normal subject with no demonstrable bronchial sensitivity to MCH, may indicate that normal subjects are not exempt from continuous HM release while resting.

If the resting state is less rigidly defined, then the data obtained on "H.L.H." during period "B" may support the hypothesis to the effect that continuous HM release in asthmatics may account for an increase in basal bronchomotor tone (299): Three plasma HM spikes to successively higher orders of magnitude suggested such release and the mean plasma HM value was significantly higher than that for periods "C" and "D". FEV$_1$ at the same time was some 20% lower than any baseline value obtained before bronchial challenge with MCH (cf. Figs. 4-2 and 4-17).

Of the four periods, "B" by virtue of the requirement for serial inhalation of NaCl accompanied by FEV$_1$ determinations closely mimicked the protocol underlying the bronchial inhalation challenge with MCH as described in this thesis. Accordingly, this period should serve, theoretically at least, as an added and self-contained control period for evaluation of results obtained during these challenges. However, as illustrated in "D.Mc." and "H.L.H.", this period may not necessarily afford the lowest value for plasma HM; as shown in "T.W.", the mean and range of these values may differ significantly even in a given subject undergoing the condition underlying period "B" twice.

It follows that if the possibility of the occurrence of plasma HM spikes during period "B" (or any other of the four test periods) were ignored, similar spikes observed during bronchial inhalation challenge with MCH, to be discussed subsequently, might be spuriously considered to be an effect of the provocative agent.
Methacholine Inhalation Challenges. In the present work, 13 of 18 volunteers inhaling MCH by increments to defined limits were responders to this cholinergic agent, as judged by a fall in FEV₁ by at least 20%. By unpaired t-test, the pre-saline plasma HM mean of the 13 Responders was not significantly different from that of the 5 Non-Responders when expressed as a percentage of the post-saline control (cf. Table 4-12). This suggests that, in both groups, HM levels remained unaffected by inhalation of the diluent control. This is in line with data already discussed, viz., no significant changes in plasma HM occurred in 2 asthmatic and 2 normal subjects during defined periods not involving MCH exposure (cf. Table 4-5).

Inhalation of the penultimate and maximum permissible MCH increment by Responders and Non-Responders, respectively, yielded a stage common to both groups characterized by the absence of a significant FEV₁ fall. By unpaired t-test, mean plasma HM increases by 81% (Responders) and 50% (Non-Responders) above post-saline controls immediately after that stage were not significantly different.

Comparisons of relative plasma HM values of Responders by paired t-test showed that neither the pre-saline level nor any timed value obtained during and after the MCH inhalation phase was significantly different from the post-saline control.

Ignoring the time factor, only maximum HM levels found in each Responder both before and after inhalation of the highest MCH increment were singled out and grouped separately for comparison by paired
t-test. In both instances, mean maximum values were significantly higher than the post-saline control (P=0.002 and 0.028, respectively); there was no significant difference between relative maximum HM values obtained before and after inhalation of the highest MCH increment.

Using a constant-breath technique, Atkins et al. (135) ascertained plasma HM changes only after the highest increment had been given or a sustained decrease in FEV₁ by at least 20% had taken place. Under these circumstances, increases in plasma HM sustained for 60 minutes were observed in both atopic and non-atopic subjects and occurred only in association with bronchospasm; individual maximum values did not exceed 35 ng/ml (135). Statistical analysis of data was not attempted due, presumably, to the small (N=6) number of MCH Responders. In 6 MCH Responders, Lee et al. (305) likewise omitting the sampling of blood during the phase of the patients' exposure to MCH by an unspecified protocol, found plasma HM levels (measured for one hour after challenge) not to be significantly different from pre-challenge controls; maximum values did not exceed 0.25 ng/ml. Bhat et al. (134) administering single pre-determined threshold doses of MCH for bronchospasm failed to demonstrate any changes in plasma HM; maximum values reached 2.8 ng/ml. An additional report published in abstract form (281) related to MCH challenges of asthmatic subjects with "low" concentrations and of normal subjects with both "low" and "high" concentrations of this cholinergic agent. Plasma HM in systemic arterial and mixed venous blood was elevated in 6 of 7 asthmatics, peak levels ranging from 1.5 to 8.0 ng/ml. To produce comparable elevations in normal subjects apparently required "ten to one hundred times more MCH" (sic).
Valid comparisons of the present data with those just quoted are hampered by differences in protocol for MCH inhalation and timing for serial blood withdrawals, by lack of essential detail (281) and by recognized methodological discrepancies in values for plasma HM (241).

To the writer's knowledge, there is no published precedent describing changes in plasma HM occurring during the phase of incremental MCH inhalation, i.e., before inhalation of the highest increment. The significant mean increase by 134% above the post-saline control revealed for maximum values measured in Responders during that phase was based upon individual maxima ranging between 2.6 and 136 ng/ml. The similarly significant mean relative increase by 163% in maximum values after inhalation of the highest MCH increment, based upon individual levels ranging between 0.1 and 94.9 ng/ml, may be in line with the data reported by Arkins et al. (135) excepting differences in protocol and methodology.

In the present work, unusually high plasma levels were confined to 3 MCH Responders with relatively high values for PD<sub>20</sub>-FEV<sub>1</sub>. It remains to be ascertained whether this association was by chance. In "K.B." (PD<sub>20</sub>-FEV<sub>1</sub> = 78 cu; cf. Fig. 4-13), the post-saline control amounted to 78.5 ng/ml, and the highest value found in this subject, 94.9 ng/ml, was measured after inhalation of the highest MCH increment. In "G.K." (PD<sub>20</sub>-FEV<sub>1</sub> = 100 cu; post-saline control for plasma HM, 58.5 ng/ml; cf. Fig. 4-14), the maximum rise to 136.0 ng/ml occurred during the phase of incremental MCH inhalation. In "H.L.H." (PD<sub>20</sub>-FEV<sub>1</sub> = 220 cu; cf. Fig. 4-17), plasma HM rose to 56.2 ng/ml after inhalation of the highest MCH increment, an increase above the post-saline control (2.15 ng/ml) by 2,514%. Presumably, such high order of magnitude for plasma HM were revealed by
performing HM assays on plasma ultrafiltrates. Still higher values, to be discussed separately, were found in "H.L2." (cf. Fig. 4-18).

By various criteria, the fraction of inhaled MCH undergoing absorption into the systemic circulation is considered to be slight (135, 282), and evidence suggests that cholinergic agents do not enhance HM release from sensitized human basophils (39). This strengthens the possibility that a major source of the unusually high HM levels found in the 3 Responders with a high bronchial threshold for MCH was HM released from lung where it may have afforded local concentrations capable of triggering bronchoconstriction at the higher MCH increments. Much lower plasma HM levels were found in 2 other Responders with a high bronchial threshold to MCH (DG & DM; PD_{20}-FEV_{1} = 165 & 220 cu, respectively) and in all of the 8 Responders in whom this threshold was low (PD_{20}-FEV_{1} < 50 cu). Therefore, the amounts of HM released from lung by these Responders would seem to have been small unless the bulk of HM so released was metabolized before reaching the venous sampling site and HM may not have been directly involved in the mediation of MCH-induced bronchoconstriction.

The suggestion emerging from the present data to the effect that plasma HM levels are little affected by MCH in subjects with a low bronchial threshold to this cholinergic agent whereas in subjects with a high threshold, they may rise to unusually high values, deserves further investigation. The upper permissible limit to the inhalation of MCH by increments precludes information as to whether cumulative doses of MCH in excess of this limit might elicit greater increases in plasma HM than those currently found.
McFadden et al. (141) have presented evidence suggesting that the plasma HM level may be a marker for bronchial inflammation. The latter possibility cannot be overlooked as a factor accounting for the unusually high orders of magnitude for plasma HM in "K.B.", "G.K." and "H.L.H.1".

It may be appropriate at this point to refer to the data obtained on "H.L.H.2" during his repeat MCH challenge when he was suffering from an acute respiratory illness and became a Non-Responder. Three dramatic plasma HM spikes of increasing orders of magnitude culminated in a maximum value of 658 ng/ml, and the post-saline control level for this amine prior to MCH inhalation already exceeded 200.0 ng/ml. These drastic elevations may have reflected the degree of airway inflammation although findings by Charles et al. (132) indicated that acute illness per se may be associated with high plasma HM concentrations whether or not the patient is an asthmatic.

The fact that "H.L.H." was a Responder to MCH during the earlier MCH test but was a Non-Responder upon its re-inhalation during an acute respiratory illness has only one published precedent (245). Several other reports point to increased sensitivity to MCH after certain viral infections or vaccines (284-287). Presumably, acute respiratory illness may affect bronchial permeability to MCH by either increasing or decreasing it. In the latter instance, significant amounts of MCH may not reach target sites, thus explaining the possibility of a negative bronchial response to its inhalation by an asthmatic. Assuming the slight fraction of inhaled MCH undergoing absorption into the systemic circulation to increase during acute respiratory illness, the unusually high plasma HM values noted in "H.L.H.2" may be explained via MCH-induced
HM release from sites other than lung although peripheral basophils are unlikely to be a possible source (93).

An increase in plasma HM from 9.0 ng/ml (post-saline control) to 26.1 ng/ml occurred before inhalation of the highest permissible MCH increment in "D.Nc.", the only normal Non-Responder. Not only did this increase represent the highest absolute value found at any stage of MCH inhalation in asthmatic Non-Responders (excepting HLH₂), but also did it exceed any value found in the 8 Responders with a low bronchial threshold to MCH (PD₂₀-FEV₁ = 50 cu or less).

Data reported by Atkins et al. (135) imply that no increase in venous plasma HM occurred in association with MCH inhalation by 5 non-atopic subjects not responding to this agent. From rises in arterial plasma HM with exercise in patients with exercise-induced asthma as well as in normal individuals, Hartley et al. (283) concluded that HM had no direct role in the mediation of exercise-induced bronchoconstriction.

In the present work, performance of paired t-tests on each set of timed DAO values obtained in Responders and Non-Responders to MCH revealed relative changes in this activity to be small and not significant (cf. Tables 4-9, 4-11). This suggests that unlike plasma HM, plasma DAO is a more stable characteristic.

On an individual basis, changes in plasma DAO activity occurring during MCH inhalation were more marked in 2 Responders (JC & KB) and also in 1 Non-Responder (BP). In the case of "J.C." and "B.P., no clear-
cut relationship between these changes and those occurring in plasma HM and FEV₁ was revealed. However, relationships between these variables in "K.B.", a MCH Responder already discussed for the finding of unusually high increases in plasma HM, deserve added comment:

At a PDₐ₀-FEV₁ of 78 cu for MCH, changes in FEV₁ (cumulating in a sustained 23% fall) uniquely and significantly correlated with those in plasma DAO; absolute levels of the latter activity were within a much higher range than that noted for any other subject, and the lowest DAO value coincided with one of three prominent plasma HM spikes. Unless these relationships occurred by chance, data support the thesis that the DAO-inhibiting effect of MCH demonstrated in vitro may extend to inhaled MCH, contributing to bronchoconstriction via delayed inactivation of HM released in lung in vivo. Implicit in this hypothesis is the assumption that the changes in plasma DAO reflected parallel changes of this activity in lung. Since in no other subject was there a significant negative correlation between plasma DAO and FEV₁, bronchospasm due to delayed HM inactivation may be rare, but may add to the heterogeneity of pathogenetic mechanisms inferred by Austen and Orange to cause acute human asthma (18). As indicated earlier, only in one of 24 subjects exhibiting concentration-dependent MCH-induced inhibition of plasma DAO in vitro was this inhibition marked. Presumably, it is such subjects in whom inhalation of this cholinergic might contribute to bronchoconstriction via delayed HM inactivation. The validity of this hypothesis is testable by inhalation of the specific DAO inhibitor, aminoguanidine. Precedents of its administration to human subjects in other situations (172,177) were revealed earlier. As already indicated, opposite changes in plasma DAO and total amines, pointing to a cause-and-effect relationship, have been demonstrated in patients given heparin during haemodialysis (181).
In the present work, relative maxima for plasma HM singled out for Responders and grouped separately according to their occurrence before and after inhalation of the highest MCH increment were in each instance significantly higher than the post-saline control; in absolute units of measurement (ng/ml), these maxima were small in all of the 8 Responders with a low bronchial threshold to MCH. Although plasma HM rose to unusually high values in 3 of 5 Responders in whom this threshold was high, work by others indicates that the plasma HM level may mark the degree of bronchial inflammation and may rise during acute illness other than asthma. The latter possibility seems to be dramatically illustrated by the data obtained on "H.L.H2."

Taken together, these data militate against an unique association of high plasma HM levels with bronchoconstriction. Furthermore, if the plasma HM level is taken as an index of HM released from lung, then no clear-cut evidence emerges from the present work suggesting that HM so released was directly involved in MCH-induced bronchoconstriction unless the subject's bronchial threshold to inhaled MCH varied directly with their bronchial sensitivity to endogenous HM. Pertinently, dramatic variations in the bronchial response to exogenous HM emerged from large-scale investigation by Specter and Farr (246). It may be reiterated that the upper permissible limit to the inhalation of MCH by increments precludes information as to whether cumulative doses of MCH in excess of this limit might elicit greater increases in endogenous HM in plasma than those currently found.

Observations suggesting that HM proper may trigger the release of other mediators, such as SRS-A (84), cannot be ignored to account for MCH-induced bronchospasm especially in the 8 Responders whose bronchial
threshold to MCH was low, but in whom changes in plasma HM were not marked. Kaliner and co-workers found cholinergic stimulation to enhance antigen-induced release of both HM and SRS-A from IgE sensitized human lung (84) and nasal polyps (82). Much less SRS-A relative to HM was released from polyps than from lung (84). It has been theorized that this difference may underlie both the beneficial effect of antihistamines in allergic rhinitis and their limited effectiveness in the alleviation of human bronchospasm (82).
CHAPTER 6: SUMMARY & CONCLUSIONS

Conflicting evidence as to the role of HM in the mediation of acute human asthma in vivo prompted an investigation of relationships between changes in FEV₁, plasma DAO and plasma HM in subjects during incremental inhalation of MCH. Control tests compared changes in these variables in non-medicated subjects under 4 defined conditions not involving inhalation of bronchoconstrictive agents.

HM was determined by enzymatic double isotopic derivative dilution analysis performed on plasma ultrafiltrates in order to exclude factors shown by the writer and others to interfere with this assay. Results were unaffected by MCH and DSGC. Preparation of the subsidiary enzyme, HNMT, from rat brains ensured non-interference by CMTD. The modified assay yielded acceptable results when applied to reconstituted lyophilates of HM prepared by the Mayo Clinic. Due, presumably, to the inclusion of the plasma ultrafiltration step, some HM levels measured with this assay in human plasma were of hitherto un-reported high orders of magnitude.

DAO was determined at optimum concentrations of substrate (HM) on plasma samples buffered at physiological pH due to high blank activity at pH 6.0 (optimum). The colour change of 0-dianisidine effected by H₂O₂ generated in the oxidative deamination of HM was used as a criterion for quantitation. DSGC and CMTD did not interfere when added in vitro.

MCH, however, was found to function as a weak substrate in the absence of added HM with an apparent Kₘ of about 1.11 M and 1.14 M at
pH 6.0 and 7.4, respectively. At low and high concentrations of HM, MCH enhanced and inhibited DAO activity, respectively.

Concentration-dependent MCH-induced inhibition of plasma DAO in vitro at optimum concentrations of added HM was separately verified in 24 subjects. Decreases in activity effected with each MCH increment were significant at final concentrations of MCH ranging from 0.42 to 4.2 mMoles/ml plasma. The apparent mean inhibition at the highest MCH increment was 34% (pH 6.0) and 17% (pH 7.4). Plasma sources included 7 asthmatic adults, 10 allergic adults not suffering from asthma, 6 normal adults and 1 psychotic boy with multiple allergies. While for a given MCH increment, there was no significant difference in relative DAO decreases between any two of the above three groups, inhibition was more pronounced in the psychotic boy with multiple allergies (75 and 50% at the highest MCH increment; pH 6.0 and 7.4 respectively), suggesting subject variation.

In order to assess the extent to which plasma HM and plasma DAO may vary under conditions not involving MCH inhalation, both were determined at intervals of 7 minutes in 4 subjects (2 asthmatic; 2 normal) during 4 distinct control periods each of 91 minutes duration. On a relative basis, timed values of either variable were not significantly different from the first measurement for the 4 subjects, the 2 subject groups and the 4 test conditions. On an absolute basis, a preponderance of plasma HM values yielded zero. Short-lived maximum increases to levels ranging between 4.3 and 19.3 ng/ml occurred during the four test conditions.
Eighteen subjects (17 asthmatic, 1 normal) volunteered for bronchial challenge with MCH inhaled incrementally; 13 asthmatics were Responders; 4 asthmatics and 1 normal subject were Non-Responders.

In the Responders, PD_{20} - FEV\textsubscript{1} for MCH ranged from 1.3 to 220 cu. In the case of both plasma DAO and HM, neither mean pre-saline levels nor any timed value obtained during and after the MCH inhalation phase were significantly different from the post-saline control set at 100%. Maximum plasma HM levels found at variable stages and grouped separately according to their occurrence before and after inhalation of the highest MCH increment, were in each instance significantly higher than the post-saline control. There was no significant difference between relative maximum plasma HM levels so defined.

Relative to the post-saline control for both plasma DAO and HM, mean pre-saline values for Non-Responders were not significantly different from those of Responders. Inhalation of the penultimate and maximum permissible MCH increment by Responders and Non-Responders, respectively, yielded a stage common to both groups characterized by the absence of a significant FEV\textsubscript{1} fall. By un-paired t-test, mean relative plasma HM increases by 81% (Responders) and 50% (Non-Responders) above the post-saline control found in blood removed immediately after that stage were not significantly different from the post-saline control.

On an absolute basis, peak values for plasma HM ranged from 3 to 658 ng/ml. The latter value was obtained when a previous asthmatic Responder was re-challenged with MCH during an acute respiratory illness and became a Non-Responder, his post-saline control value for plasma HM already exceeding 200 ng/ml. While these orders of magnitude are
without published precedent, they support the thesis that acute illness *per se* may be associated with abnormal elevations in plasma HM.

Maxima for plasma HM by far exceeding those reported by others were also found in 3 of 5 Responders with a high bronchial threshold for MCH (PD_{20}-FEV_{1} 78 to 220 cu). In the remaining 8 Responders in all of whom this threshold was much lower (PD_{20}-FEV_{1} < 50 cu), maxima for plasma HM did not exceed 21.6 ng/ml. The latter value was itself exceeded by an increase in plasma HM occurring with inhalation of the maximum permissible increment in the only normal Non-Responder.

One major objective of this thesis was to infer a role for endogenous HM as one of several mediators implicated in acute bronchospasm from relationships between plasma HM and FEV_{1} changes during inhalation of the bronchoconstrictive agent, MCH, by increments. This objective pre-supposed that the order of magnitude of the level of HM in plasma is an index of the magnitude of its release from lung. This hypothesis is itself contingent upon the assumption that inhaled MCH does not promote HM release from other sites.

Since plasma HM levels in all of the 8 Responders with a low bronchial threshold to MCH were much lower than those found in 3 of the remaining 5 Responders in whom this threshold was high, the subjects' bronchial sensitivity to inhaled MCH would not seem to have depended upon the magnitude of HM release from lung. Endogenous HM could then only be implicated as a major factor responsible for their bronchospasm.
if their bronchial sensitivity to inhaled MCH varied directly with the sensitivity to the histamine so released.

Since the subjects' bronchial threshold to endogenous HM is unknown and since plasma HM elevations in 2 of the 3 Responders with a high bronchial threshold to MCH were not marked, a major role for HM released from lung in the causation of acute bronchoconstriction would not seem to be convincingly demonstrated. This conclusion receives support from the present work and findings by others suggesting that the plasma HM level may rise during acute illness per se and may also mark the degree of bronchial inflammation, thus militating against a unique association of high plasma HM levels with acute bronchoconstriction.

In keeping with data emerging from this thesis to the effect that MCH inhibits plasma DAO in vitro, an added objective was to determine whether this inhibitory effect might extend to inhaled MCH so as to contribute to MCH-induced bronchospasm via delayed HM inactivation in vivo. In the 13 Responders and 5 Non-Responders, relative changes in plasma DAO grouped for each time interval were small and not significant, suggesting than unlike plasma HM, plasma DAO during incremental MCH inhalation is a more stable characteristic. Thus, assuming changes in lung DAO to have been similar to those in plasma, between group and within group analyses afforded no evidence suggestive of an inhibitory effect of inhaled MCH on plasma DAO in vivo.

On an individual basis, plasma DAO changes in 2 Responder and 1 Non-Responder were more marked. In one of the 2 Responders whose
$PD_{20} - FEV_1$ for MCH was 78 cu and whose plasma HM spiked to unusually high values, plasma DAO levels were much higher than those noted in any other subject and correlated directly and significantly with changes in \( FEV_1 \) \((P < 0.01)\); an inverse correlation between plasma DAO and HM was not significant although the lowest plasma DAO level coincided with one of three prominent plasma HM spikes. These relationships may be a case in point suggesting that the DAO-inhibiting effect of MCH revealed in this thesis \textit{in vitro} may extend to inhaled MCH so as to contribute to bronchospasm \textit{via} delayed HM inactivation \textit{in vivo}. However, this possibility is based upon data obtained in only one subject. Therefore, the general conclusion emerging from this thesis would seem to be unaffected, \textit{viz.}, changes in plasma DAO and plasma HM occurring during MCH inhalation by increments revealed no clear-cut evidence suggesting that endogenous HM is a major factor mediating MCH-induced bronchoconstriction.
APPENDIX I:

Bronchial Inhalation Challenges Preceded by Administration of Disodium Cromoglycate (DSCG) and Cimetidine

This section gives an account of the limited number of instances permitting a comparison of the changes in FEV₁ and plasma HM and DAO occurring during incremental MCH inhalation before and after administration of DSCG and CMTD.

Only two subjects were involved, viz., "H.L.H.", the mild asthmatic, and "D.Mc.", the normal volunteer.

Protocols for premedication with DSCG and CMTD were as follows:

DSCG: After completion of the first challenge with MCH, DSCG, 20 mg was inhaled as 'INTAL' (Fisons Canada Ltd., Don Mills, Ont.) four times a day to a total of 80 mg/day for a minimum of six days using a "Spinhaler" (supplied by the manufacturer). The last single 20 mg dose of this agent was inhaled on the day of the repeat challenge immediately after pre-saline values for FEV₁, plasma HM and DAO activity had been secured. During the ensuing 30 minutes, at least one other timed set of values for these variables was obtained prior to inhalation of the diluent control (saline), followed by the securing of post-saline values of the variables under scrutiny. The lowest concentration of MCH was then inhaled, as already described (cf. Chapter 4.1).

CMTD: Only one dose of this H₂ blocking agent, 600 mg, was taken orally as 'TAGAMET' (Smith Kline & French Canada Ltd., Mississauga, Ont.)

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together with a light breakfast on the day of the repeat challenge, i.e., immediately after pre-saline values for FEV₁, plasma HM and DAO activity had been secured. Within the ensuing 30 minutes, further timed values for these variables were obtained prior to inhalation of MCH.
Fig. AP-1: Methacholine Challenge in "H.L.H." before and after Disodium cromoglycate (DSCG):—
Time course of changes in FEV₁ and plasma DAO and HM
APPENDIX II:

Effect of Methacholine (MCH) Inhalation on Forced Expiratory Volume in the First second (FEV₁) and Plasma Diamine Oxidase (DAO) Activity and Histamine (HM) Before and After Administration of Disodium Cromoglycate (DSCG).

Figures AP-1 and AP-2 compare the effects of inhaled MCH on FEV₁ and plasma HM and DAO activity before and after DSCG in each of the two physicians involved. It is re-iterated that prior to the second MCH challenge, DSCG, 20 mg was inhaled four times a day to a total of 80 mg/day for a minimum of six days. Only the last of these doses is depicted by arrow.

Fig. AP-1:

H.L.H., Male, 58 years old

History

As mentioned earlier (cf. page 184), this laboratory physician is a heavy smoker and has mild asthma. He has positive skin tests to grass pollens and molds. He has been on no therapy for asthma.

MCH Challenges

A. Before DSCG (Fig. AP-1, left half; re-demonstration of Fig. 4-17)

As already described, the permissible limit of incremental MCH inhalation of 224.15 cumulative units (cu) was required
to elicit a significant fall in FEV₁ by 21%. The subsequent FEV₁ return towards baseline was sluggish.

With no major changes in plasma DAO, plasma HM was 8 ng/ml or less until 15 minutes after inhalation of the highest MCH increment when it dramatically spiked to 56.2 ng/ml.

$PD_{20}$-FEV₁ for MCH = 220 cu.

B. After DSCG (Fig. AP-1, right half)

After a lapse of only 7 days during which DSCG was taken, as described, MCH challenge was repeated preceded by a control average for FEV₁ of comparable order of magnitude. A continuous fall in FEV₁ reached a short-lived maximum (-21.3%) with the highest permissible MCH increment. As opposed to the data obtained before DSCG was taken, the return of FEV₁ to baseline was swift and complete.

With the earlier MCH increment, plasma DAO decreased, then increased to yield a final value above baseline.

Contrary to the data obtained before DSCG, there was no spike in plasma HM 15 minutes after inhalation of the highest MCH increment.

$PD_{20}$-FEV₁ for MCH = 224 cu.
Fig. AP-2: Methacholine Challenge in "D.Mc." before and after Disodium cromoglycate (DSCG):
Time course of changes in FEV₁ and plasma DAO and HM
Fig. AP-2:

D. M., Male, 38 years old

History

As mentioned earlier (cf. page 188), this non-smoking internist and specialist in allergy is a normal subject free from asthma.

MCH Challenges

A. Before DSCG (Fig. AP-2, left half; re-demonstration of Fig. 4-19)

FEV$_1$ was not affected by MCH inhaled to the permissible maximum. There was a short-lived rise in plasma DAO with the attainment of 11.65 cu, followed by one in plasma HM when the penultimate MCH increment was inhaled.

PD$_{20}$-FEV$_1$ for MCH = not definable

B. After DSCG (Fig. AP-2, right half)

Preceded by daily inhalation of DSCG to a total of 80 mg/day for 7 days, MCH inhalation was repeated some 2 years later when control data for FEV$_1$ were unchanged; those for plasma DAO were higher and those for plasma HM were much lower.

As before, FEV$_1$ was not affected when MCH was inhaled to the upper permissible limit.
Plasma DAO, however, fell with the first MCH increment. Plasma HM remained low throughout such that the previous increase occurring with the penultimate MCH increment was blunted.

$PD_{2h}/FEV_1$ for MCH = not definable.
Fig. AP\#3: Methacholine Challenge in "H.L.H." before and after Cimetidine (CMTD):
Time course of changes in FEV$_1$ and plasma DAO and H$	ext{M}$
APPENDIX III:

Effects of Methacholine (MCH) Inhalation on Forced Expiratory Volume in the First Second (FEV\textsubscript{1}) and Plasma Diamine Oxidase (DAO) Activity and Histamine (HM) Before and After Administration of Cimetidine (CMTD)

The protocol for this phase of investigations involved the oral administration of a single dose of CMTD (600 mg) on the day of the second challenge with MCH, as described on page 233.

Fig. AP-3:

H. L. H., Male, 58 years old

History

This heavy smoker and mild asthmatic with skin tests positive to grass pollens and molds and somewhat lessened sensitivity to MCH after taking DSCG, as just described (cf. page 236, Fig. AP-1), was challenged with MCH some 7 months later.

MCH Challenges

A. Before CMTD (Fig. AP-3, left half; re-demonstration of Fig. 4-19)

As already described, on this repeat occasion, he complained of malaise, with a respiratory infection and pain in his throat. The post-saline control average for FEV\textsubscript{1} (3.20 L) was comparable to values obtained during 2 earlier MCH challenges. Inhalation of MCH proceeding again to the upper permissible limit, this time did not affect a significant FEV\textsubscript{1} fall. As before, there were no major changes in plasma DAO.
Changes in plasma HM extended over 3 logarithmic cycles. The post-saline control level already exceeded 200 ng/ml. Plasma HM dropped precipitously with the 1st MCH increment and spiked drastically to 479 ng/ml when 11.65 cu of MCH were attained; coincident with a spontaneous swift return of FEV₁ to its own control level, plasma HM then increased to even higher levels (521.6 and 658.3 ng/ml, respectively).

As mentioned earlier, there would seem to be no precedent to such drastic elevations.

\[ \text{PD}_{20} - \text{FEV}_1 \text{ for MCH} = \text{not definable.} \]

B. After CMTD (Fig. AP-3, right half)

The post-saline control value for FEV₁ exceeded its pre-CMTD counterpart obtained 7 days earlier by only 3.0%. FEV₁ fell with MCH inhaled to the permissible maximum, but the 80% mark was not reached.

The post-saline control value for plasma DAO was unchanged from its pre-CMTD counterpart obtained 7 days earlier. With MCH inhalation, plasma DAO declined swiftly but regained its post-saline control level when FEV₁ had returned to baseline. Plasma DAO then declined again.

Plasma HM changes did not afford the drastic elevations observed 7 days earlier. There was a single spike to 56 ng/ml with the first MCH increment, all other values falling between 5 and 13 ng/ml.

\[ \text{PD}_{20} - \text{FEV}_1 \text{ for MCH} = \text{not definable.} \]
DISCUSSION

Methacholine inhalation before and after disodium cromoglycate.

During his first MCH inhalation challenge in the non-medicated state, "H.L.H." responded with a sustained FEV\(_1\) fall by more than 20%, associated with a prominent plasma HM spike occurring after inhalation of the highest permissible MCH increment. Upon re-inhaling MCH under comparable conditions subsequent to DSCG administration, he became a "Non-Responder" (by definition) and plasma HM failed to rise at any stage. Mean plasma HM levels during MCH inhalation before (7.58 ± 13.11 ng/ml) and after this medication (4.46 ± 1.42 ng/ml) were not significantly different, thus placing emphasis upon the abolishment of the terminal spike as the only apparent effect of DSCG. "In M.D. Mc. ", the normal Non-Responder, DSCG likewise may have abolished the terminal plasma HM spike occurring during the control MCH inhalation challenge test performed nearly two years earlier.

The data on "H.L.H." are in line with the known antiasthmatic properties of DSCG as well as with evidence showing that this agent may decrease bronchial sensitivity to inhaled MCH in children (289) although it failed to do so in adults (290, 291). The absence of a plasma HM spike during MCH inhalation after DSCG may be in keeping with the known \textit{in vitro} properties of DSCG of being an inhibitor of HM release (103) and stabilizer of mast cell membranes (110). The apparent effect of DSCG of attenuating MCH-induced HM release \textit{in vivo} may be confined to lung since Assem and Mongar (292) have shown that inhibition of HM release from human lung exerted by DSCG \textit{in vitro} does not extend to human leukocytes.
MCH inhalation before and after cimetidine. Data are confined to one asthmatic volunteer (HLH). As discussed earlier, the validity of the MCH challenge test scheduled to serve as a control for MCH re-inhalation after a single dose of CMTD (0.6 g) was forfeited by his acute respiratory illness. "H.L.H." however, had inhaled MCH in the non-medicated state some 7 months earlier when he was free from respiratory illness and when he was a Responder, a plasma HM spike to 56.2 ng/ml coinciding with the maximum FEV₁ fall (21%). Three groups of workers have afforded evidence to the effect that the bronchial threshold for a positive response to MCH does not change for prolonged periods of time (245,290,293). Therefore, it would seem to be justifiable to use the first MCH challenge test undergone by "H.L.H." in the non-medicated state for comparison. Baseline values for FEV₁ and plasma DAO were nearly identical. Those for plasma HM were below 10 ng/ml.

After administration of CMTD half an hour prior to inhalation of the diluent control, "H.L.H." inhaling MCH to the highest permissible increment became a Non-Responder. After CMTD administration, a short-lived spike-like increase in plasma HM from values below 10 to 56.2 ng/ml occurred with inhalation of the first MCH increment. Since all subsequent values were below 15 ng/ml, CMTD would seem to have shifted the plasma HM spike occurring with inhalation of the highest permissible MCH increment during the control test to this early stage of MCH re-inhalation.
In an attempt to account for this apparent shift, one is reminded of the negative feedback mechanism described by Lichtenstein and Gillespie (151) in which HM controls its own release via the H₂ receptor. Provided this mechanism operates in a non-immunological situation, it is possible that blockade of this receptor exerted by CMTD disrupted this mechanism such that HM release was, temporarily at least, promoted. The current suggestion to the effect that CMTD may increase the bronchial threshold for MCH is based upon data obtained on a single subject. The validity of this suggestion may be hampered by the long interval between the MCH challenge chosen as a control and its repeat after CMTD administration.

If alternately the scheduled control MCH inhalation challenge test complicated by acute respiratory illness and associated with unusually high plasma HM levels is used as a basis for comparison, it is apparent that the plasma HM levels obtained 7 days later during repeat inhalation of MCH after CMTD were significantly lower, and bronchial reactivity was unaltered. Although an effect of CMTD of attenuating HM release cannot be excluded, the markedly lower values for plasma HM after CMTD administration may then be a mere reflection of the subsidence of acute respiratory illness.
As indicated earlier, Shaff and Beaven (209) found the analytical principle for HM determinations chosen by the writer to be invalid in the presence of CMTD due to its interference with the subsidiary enzyme, HNMT, when prepared from conventional sources (guinea pig brain and rat kidney). Work reported in this thesis has shown that this analytical drawback can be overcome when HNMT is prepared from rat brain. Furthermore, plasma ultrafiltration emerged from this thesis as a separate critical preliminary to HM determinations in human plasma, and the assay as modified by the writer retained its validity in the presence of DSCG and MCH. Regrettably, no volunteers other than "H.I.H." and "D.Mc." were prepared to re-inhale MCH after premedication with DSCG or CMTD, thus precluding statistically valid information as to the effect of the latter two agents on the level of HM in plasma during incremental MCH challenge.
REFERENCES


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power of plasma in endometrial adenocarcinoma. Cancer, 15: 
271-275.

Diamine oxidase activity in plasma and urine in uremia. Nephron, 

oxidase (DAO) activity in human plasma in vitro by hydralazine. 

183. Sjaastad, O.V. (1967). Potentiation by aminoguanidine of the 
sensitivity of sheep to histamine given by mouth. Effect of 
aminoguanidine on the urinary excretion of endogenous histamine. 

The effect of histamine and progestational agents on plasma 


mechanism of anaphylaxis in the rat. J. Physiol., 159: p. 61 
(abstract).


303. BMDP Biomedical Computer Program (1974) Health Sciences Computing Facility, Department of Biomathematics, School of Medicine, University of California, Los Angeles, U.S.A.


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