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THE INFLUENCE OF DRUGS OF ABUSE ON RAT BRAIN HISTAMINE: STUDIES WITH ETHANOL

A Thesis
Presented to the
School of Graduate Studies
University of Ottawa

Submitted by

George D. Prell

In partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Department of Pharmacology
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Ottawa, Ontario
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TABLE OF CONTENTS

TABLE OF CONTENTS

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et pour Denis

Table of Figures

Table of Tables

ABBREVIATIONS

Ald. Dehydro.	aldehyde dehydrogenase
BSA	bovine serum albumin
BST	bed nucleus of the stria terminalis
CNS	central nervous system
CX	cortex
cyclic AMP	cyclic 3',5'-adenosine monophosphate
cyclic GMP	cyclic guanosine 3',5'-monophosphate
DAO	diamine oxidase
DOPA	dihydroxyphenylalanine
DOPAC	dihydroxyphenylacetic acid
F_I	fluorescence intensity
FL	fluorescamine
α-FMH	alpha-fluoromethylhistidine
GC	gas chromatography
HA	histamine
HCl	hydrochloric acid
HD	histidine decarboxylase
His	histidine
HMT	histamine N-methyltransferase
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
HYPO	hypothalamus
LiCl	lithium chloride
LSD	lysergic acid diethylamide
MAO-B	monoamine oxidase (type B)
MB	midbrain
MeHA	methylhistamine

MeHis	methylhistidine
MFB	medial forebrain bundle
Mo	morphine
MS	mass spectroscopy
NEN	New England Nuclear
OPT	o-phthaldehyde
P_i	inorganic phosphate
QNB	quinuclidinyl benzilate
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
TEA	2-thiazolylethylamine
TLC	thin layer chromatography
TIQs	tetrahydroisoquinolines
Δ^9-THC	Δ^9-tetrahydrocannabinol
TMA	4-thiazolylmethoxyamine
t-MeHA	N-tele-methylhistamine

INTRODUCTION

I. HISTORICAL BACKGROUND

Histamine (HA) was first synthesized about 75 years ago as a chemical curiosity; it continues to hold the fascination of scientists attempting to discern its biochemical and physiological function in normal and pathophysiological states. At the time of its synthesis (Windaus and Vogt, 1907), histamine was termed imidazolylathylamin (in English, imidazolylethylamine). It was isolated from extracts of ergot or upon putrefaction of pure histidine (Barger and Dale, 1910). The ergot extract, the product of putrefaction of histidine and the synthesized compound were all biologically active since they stimulate the cat's non-pregnant uterus and reduce systemic arterial pressure in minute doses. While initially studied as a potent pharmacological agent with a profound spectrum of actions (Dale and Laidlaw, 1910; 1911), histamine acquired greater physiological significance following its isolation from the intestinal mucosa (Barger and Dale, 1911). However, the endogenous presence of this amine was thought to be merely the recovery product of histidine catabolism by bacterial enzymes. The latter had been shown to be a rich source of histamine (Ackermann, 1910).

Evidence for the endogenous presence of histamine was advanced by Abel and Kubota (1919) who found it localized in several mammalian tissues including the hypophysis cerebri. Yet convincing evidence was not forthcoming until investigators in Dale's laboratory succeeded in isolating histamine as its picrate salt from fresh ox lung and liver extracts by procedures which precluded histamine formation from bacterial sources (Best et al., 1927). In addition, the amine picrate was recovered in ethanol extracts of these tissues in sufficient quantities to account for the hypotensive effects produced by these tissue extracts. Thereafter, histamine's presence in mammalian tissues was established, although its precise function was uncertain.

The next several years witnessed the discovery of most of the major pharmacological activities of histamine. In the guinea pig bronchiolar constriction

and uterine, ileum and spleen muscle contraction were the principle responses while striatal muscles were largely unaffected. Histamine could be shown to slightly constrict cardiac and pulmonary arteries, while at the same time increasing both heart rate and contractility. Yet the overwhelming response was a generalized capillary dilation resulting in peripheral capillary pooling and as a consequence, systemic hypotension. There was also a substantial loss of plasma through the capillary epithelium. In retrospect, it appears that the main interest in this amine centered on histamine-induced shock-like symptoms. Based on almost a decade of research in several laboratories, Dale and Laidlaw (1919) advanced two general propositions concerning the effects of histamine. First, that local application of histamine causes redness, swelling and edema similar to that observed due to mild inflammation. The widening of the capillaries with subsequent plasma pooling and the exudation of this plasma through distended capillary walls could mimic wheal formation. Second, large doses of histamine injected into the circulation caused hemoconcentration due to plasma loss, profound fall in the arterial pressure and hypothermia. These effects were nearly identical to the clinical symptoms of shock produced by trauma, anaphylaxis, Witte's peptone and by several organ extracts. These highly suggestive concepts were furthered by Lewis (1927) who proposed that a substance similar to histamine, which he termed "H-substance," was released from cells in response to injurious stimuli or antigen-antibody interaction. Compelling evidence which joined these related concepts into one coherent theory came from studies of anaphylactic shock resulting from exposure of sensitized tissue to a specific antigen. Under these conditions, histamine was shown to be released into perfusates from which it was isolated and quantified in amounts sufficient to explain most of the observed effects (Dragstedt and Gebauer-Fuelnegg, 1932). Thereafter, histamine was indeed identified as Lewis' "H-substance." Since then, histamine has been acknowledged as one of the key agents in anaphylaxis, allergy, hypersensitivity

reactions and inflammation, although it is by no means the only endogenous substance involved. It is now established that histamine has effects on and may play a role in smooth muscle contraction, gastric secretion, tissue growth, cardiac rate and blood pressure (Best and McHenry, 1931; Dale, 1950).

It is important to acknowledge the early studies which led to the concept of histamine H_1 and later, the H_2 receptors and the agonists and antagonists which act upon them. Many of the earlier conclusions concerning HA's actions were either reinforced or discarded in light of more extensive experimentation utilizing these more recent available agents. Since HA had a large spectrum of activity, much of it detrimental to human health (e.g. anaphylaxis, hypersensitivity reactions, shock), it became clear that development of drugs that antagonize histamine actions would be of great benefit as both therapeutic and investigational agents. Fourneau and Bovet (1933) synthesized 2-isopropyl-5-phenoxyethyldiethylamine and later showed that it could antagonize histamine's actions in the guinea pig (Bovet and Staub, 1937). Symptoms of anaphylactic shock were reduced even after a dose of histamine equivalent to several lethal doses. In vitro antagonism of histamine-induced smooth muscle contraction could also be demonstrated. Unfortunately, toxicity limited the use of this agent and of diethylaminoethyl-N-ethylanaline, the second compound of this new class. However, a derivative prepared by Mosnier and subsequently studied by Halpern (1942) was an effective antagonist with acceptable toxicity and marketed as Antergan®. Neo-antergan®, a derivative of this drug (Bovet et al., 1944) was found to be a highly selective and effective histamine antagonist. Today, it is still used clinically and extensively utilized in research where it is known by its official name, pyrilamine maleate. Several derivatives of the Fourneau compounds were prepared in the United States which ultimately led to the development of diphenhydramine and tripelennamine. The history of these discoveries and discussions of the chemical synthesis of these histamine antagonists have been reviewed (Bovet, 1950).

Following world war two several more histamine antagonists were developed. Although an increasing number of these agents were made available, the concomitant research indicated three major limitations in the use of these "antihistamines". First, effects which were earlier ascribed to histamine blockade were sometimes found to be better explained by anticholinergic, local anesthetic or central nervous system depressant actions. Second, with more experience, it became evident that anaphylaxis and allergy were not exclusively histamine mediated events. Lastly, several effects which were well established to be elicited by histamine could not be attenuated by any of the available histamine antagonists. The suspicion arose that histamine may act through more than one receptor. For example, Folkow et al. (1948) observed, in the anesthetized cat, that there was a limit to the antagonism of histamine-induced vasodilation which could be produced by Benadryl® (diphenhydramine). They suggested that quantities of histamine which exceeded this limit may combine with a "receptive substance" different from that for which histamine and Benadryl in physiological doses were competing. They hypothesized that histamine may cause vasodilation by combining with two receptors, one of which is not antagonized by even lethal doses of histamine-blocking drugs. Furthermore, histamine-induced positive chronotropic effects on isolated atria and gastric acid secretion and its inhibitory action on the contracted rat uterus could not be specifically antagonized by the then available antihistaminic drugs.

Ash and Schild postulated that there were two receptors mediating histamine effects (1966). They clearly demonstrated that the histamine or histamine analogue-induced stimulation of isolated guinea pig ileum, when antagonized by mepyramine, exhibited the same pA_2 values. The pA_2 value (numerically equal to the pK_B, the negative logarithm of the antagonist dissociation constant) is the negative logarithm of the molar concentration of antagonist that reduces the effect of a given agonist concentration to half of that producing this

effect (Schild, 1947; 1949). The relative potencies of histamine analogues, common pA_2 values and the distribution of antagonist sensitive tissues suggested that a single population of "receptive substance" was antagonized. Therefore, mepyramine was probably acting on a homogeneous receptor population. They suggested the symbol "H_1" for the type of receptor which was specifically antagonized by low concentrations of histamine antagonists. A correlation was shown to exist ($r=0.95$) between the activity ratios of several ß-substituted ethylamines on the rat uterus and the rat stomach. Yet, no specific antagonists could be found to inhibit histamine action on the rat stomach or uterus and relative activities of several histamine analogues supported the contention that the HA receptors of the uterus and stomach were of a different variety and antihistamine insensitive (labeled non-H_1). They both differed from receptors of the guinea pig ileum, termed H_1.

In 1970, a breakthrough occurred in the United Kingdom at Smith Kline and French laboratories with the synthesis of burimamide, a competitive antagonist of the actions of histamine at non-H_1 receptors. This discovery also gave greater support to the existence of non-H_1 receptors, renamed H_2 receptors (Black et al., 1972). Due to the low potency of burimamide, an H_2 antagonist metiamide, with oral activity and ten times burimamide's potency, was developed (see Duncan and Parsons, 1980). The finding that both drugs produced agranulocytosis in humans stimulated further research and development which resulted in the manufacture of the now famous H_2 blocker, cimetidine (Brimblecombe et al., 1978), which is extensively prescribed around the world.

Since these rapid discoveries at Smith Kline and French, several other H_2 antagonists have been developed. During this period, another discovery particularly useful to research investigators was the synthesis of more compounds with greater selective agonist activities at H_1 and H_2 receptors, e.g. 2-methylhistamine and 4-methylhistamine, respectively (Black et al., 1972). More recent

information on H_1 and H_2 receptors is included in section XI of the literature background.

In summary, histamine is now established to be a ubiquitous amine; present in all mammals examined and widely distributed throughout their bodies. The discovery of H_1 and H_2 receptors along with chemical agents that selectively stimulate or antagonize their actions has provided valuable tools for more thorough studies of the possible roles of histamine in both health and disease. Unfortunately, we seem to have progressed little in precisely answering the questions first raised by the early investigators of this substance concerning its physiological role. Yet, investigators of histamine can credit themselves with having taken giant steps forward in attempting to answer this challenge initiated almost 75 years ago.

II. NOMENCLATURE: HISTORY AND PRACTICE

Confusion has occasionally arisen with respect to the identification of the specific atoms of the histamine structure (Fig. 1). Nomenclature ambiguities concerning the term histamine and the chemical terminology for its 5 membered ring structure have also surfaced. The "parent" ring was designated glyoxaline by Heinrich Debus (1858), who synthesized it in 1858. Owing to a controversy over various terminologies for nitrogen containing heterocyclic rings, Hantzsch (1888) suggested the name imidazole for 1,3-diazoles. Unfortunately, both terms were widely used, some of which carried over into histamine terminology. Imidazole was officially recognized in 1940 (Fox, 1943). Identification of the imidazole atoms begins with the imino nitrogen atom (bearing the hydrogen atom), as indicated in Figure 2. For imidazoles monosubstituted on either the 4- or the 5-carbon atom the term 4 (or 5) is used, an unresolved situation which itself has generated confusion in the literature.

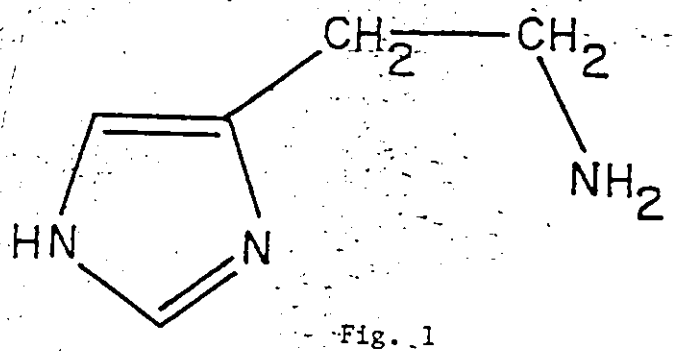


Fig. 1

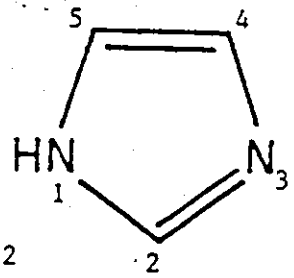
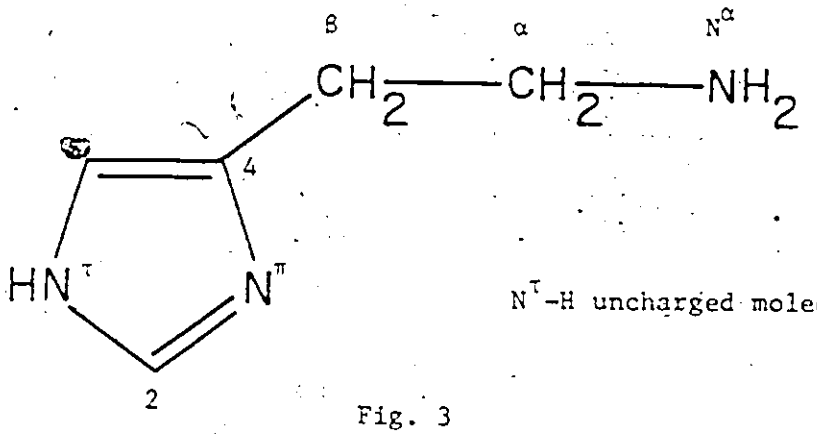


Fig. 2

As mentioned earlier, the original name for what we now call histamine was imidazolylethylamine, introduced by Windaus and Vogt in 1907 at the time of its synthesis. Dale and Laidlaw called it ß-iminazolylethylamine although others termed it ß-imidazolylethylamine or referred to it in empirical terms such as ergamine, histidine-base or vasodilation. The trivial term "histamine" was derived from the Greek word "histos" meaning tissue; thus histamine was tissue amine. It initially appeared in the publication of Frohlich and Pick (1912), although Dale claimed to have introduced the term but was requested not to use it so as to avoid confusion with a similarly named proprietary preparation (Dale, 1953). Several trivial names also arose from the fact that this amine was initially found in nature as a contaminant of ergot although other terms are of less certain origin, e.g. eramin, theramine, ergamine, ergotidine and lertigon (Marler, 1967; Merck Index, 1968). The chemical name for this small molecule became confused not only because the question concerning monosubstitution at C_4 or C_5 went unanswered, but also because it was not clear if the ethylamine was a substitution on the ring or if the ring was a substitution on the ß carbon of the alkylamine. Thus, the following chemical designations were all more or less legitimately employed (Fox 1943; Marler, 1967; Merck Index, 1968; Douglas, 1980; Black and Ganellin, 1974):

β-iminazolylethylamine
4-imidazoleethylamine
5-imidazoleethylamine
β-aminoethylglyoxaline
β-aminoethylimidazole
2-(4-imidazolyl)ethylamine
4-(2-aminoethyl)imidazole
5-(2-aminoethyl)imidazole

With bioscientists' general acceptance of the term histamine, the matter appeared resolved until substitutions on the imidazole moiety resurrected these unresolved questions. N-methyl substitutions on the protonated nitrogen (used as the reference atom) caused great debate and the tautomeric imidazole structures (see section III) served to promote this controversy. It was not officially addressed until the IUPAC-IUB Commission on Biochemical Nomenclature (1972) resolved this point for N-substitution of histidine and by inference, histamine (Fig. 3).



N^{τ}-H uncharged molecule shown

Fig. 3

The nitrogen atom nearest the side-chain residue was designated pros (symbolized by π) and the farther nitrogen as tele (τ). The ring carbon between the two nitrogens is position 2 whereas the remaining free position is carbon-4. A method for clearly identifying the side-chain ethylamine has been proposed by Black and Ganellin (1974) and appears to have been accepted. The carbon atom adjacent to the ring is termed the β carbon, that nearest the amino nitrogen is

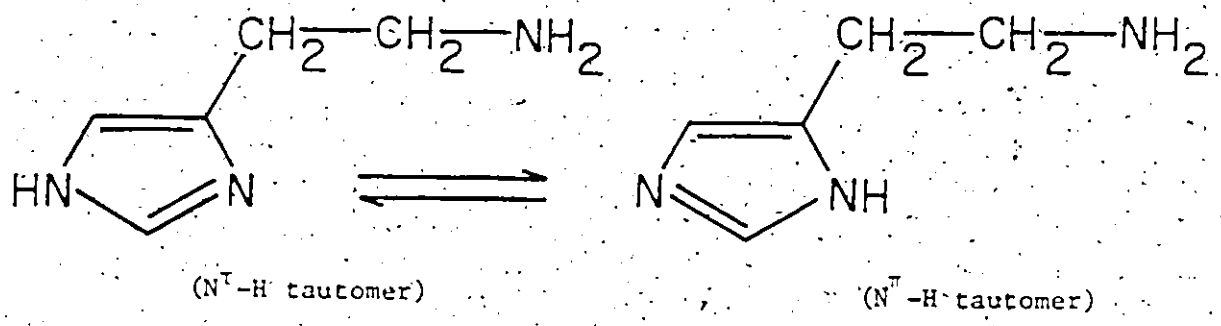
being called N^{α} such that the unambiguous atomic designation for histamine is then established.

III. CHEMICAL BACKGROUND

With the nomenclature for histamine (HA) having been established, some of its chemical properties can now be discussed. The empirical formula for histamine is $C_5H_9N_3$. It has a gram-molecular weight of 111.15. There is no evidence to suggest that it is other than a monomer. It exhibits aromatic heterocyclic character (Hill and Branch, 1940; Sundberg and Martin, 1974; Katritzky and Lagowski, 1968) since the imidazole moiety possesses $(4n+2)\Pi$ electrons in a planar array of atoms. HA does not have an asymmetric center, therefore the question of pharmacological activity versus optical activity is irrelevant. Commercial sources generally supply histamine as the dihydrochloride or diphosphate salts; free base preparations are also available. Synthesis is now generally by bacterial decarboxylation of histidine (Jones, 1966). The resulting HA is derivatized with 3,4-dichlorobenzenesulfonic acid which precipitates HA as the insoluble salt, histamine bis-3,4-dichlorobenzenesulfonic acid which precipitates HA as the insoluble salt, histamine bis-3,4-dichlorobenzene sulfonate. Histamine dihydrochloride, HA $(HCl)_2$, is recovered in about 90% yield (melting point 240-242^{0}C) after adding the insoluble salt to boiling n-butanol saturated with hydrogen chloride. The diphosphate salt is synthesized by first adding the bis-3,4-dichlorobenzenesulfonate plus one equivalent of barium hydroxide to hot water. After cooling, the precipitate of barium 3,4-dichlorobenzenesulfonate is removed by filtration and the filtrate (containing histamine) is treated with phosphoric acid and later precipitated by slow addition

of methanol. The histamine diphosphate is also recovered at about 90% yield with a melting point of 132-133°C. Both salts are highly soluble in water, alcohol and chloroform; they are stable in acid but unstable in alkaline media (Jones, 1966). Histamine is poorly soluble in ether.

Histamine, although generally represented as in Fig. 1, exists as an equilibrium mixture of two tautomeric forms (Hill and Branch, 1940; Sundberg and Martin, 1974; Katritzky and Lagowski, 1968; Jones, 1966) because the imidazole ring possesses two nitrogen sites capable of proton dissociation at physiological pH (Fig. 4).



(N^{τ}-H tautomer) (N^{π}-H tautomer)

Fig. 4

The relative population of tautomers has been estimated from pK_a data comparing the corresponding N-methyl or N-benzyl monocation histamine derivatives (Ganellin, 1973a). The ratio of the N^{τ}-H tautomer concentration to that of the N^{π}-H form is approximately 4.2, thus about 81% of the histamine monocation is in the N^{τ}-H form (Ganellin, 1973a) in aqueous solution at 7.4 pH. The ring nitrogens and the side-chain NH_3^+ group constitute the loci for the three stoichiometric pK_a values obtained when histamine is titrated in aqueous solution (Levy, 1935; Paiva et al., 1970; Ganellin et al., 1973). The overall titration scheme is depicted in Fig. 5. At low pH histamine is present as a dication.

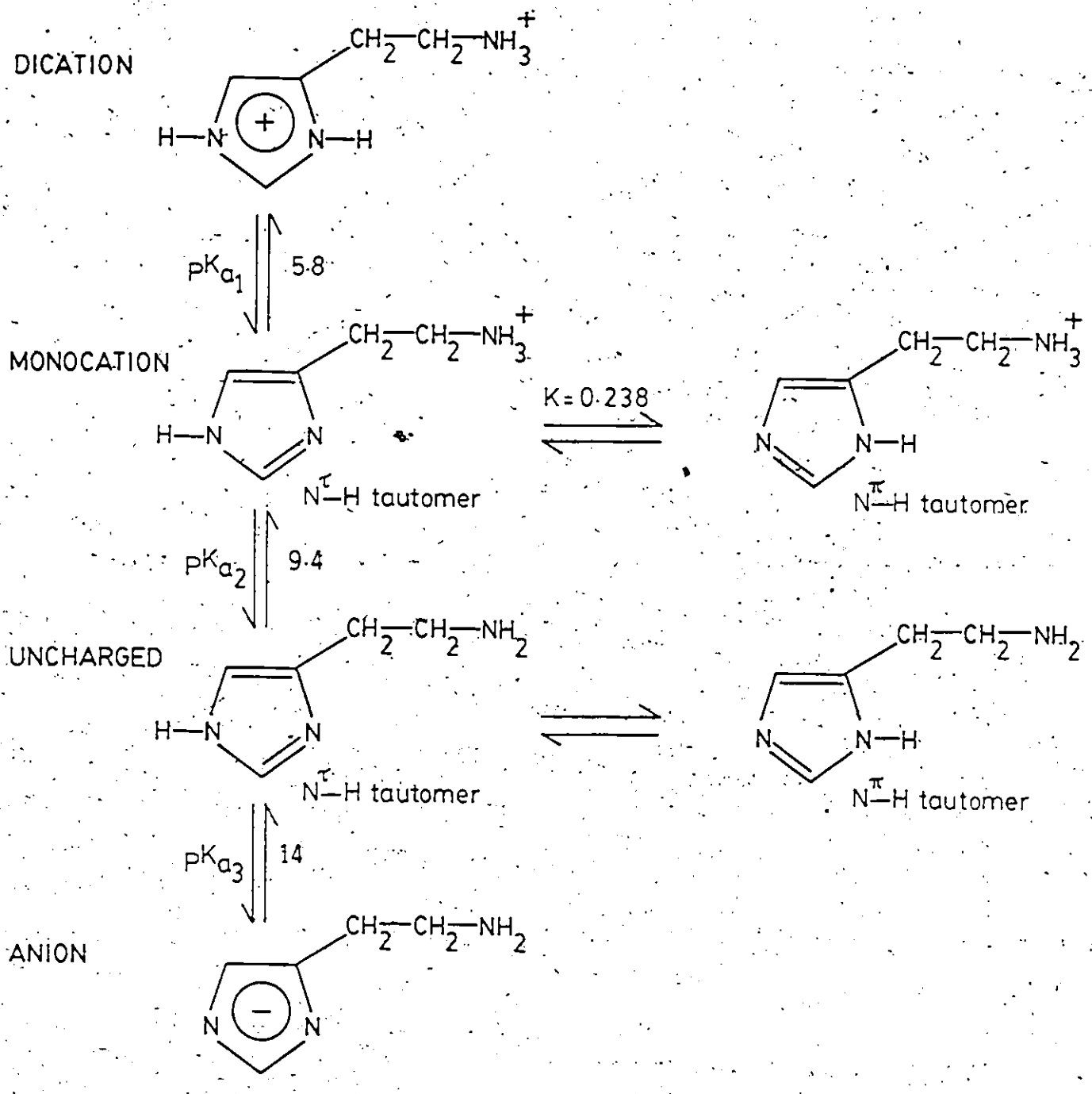


Fig. 5

Structures of various ionic species and tautomeric forms of histamine.

The first stoichiometric ionization constant (pK_{a1}) corresponds to the ring proton dissociation with a value of 5.8 whereafter higher pH values increase the monocation population according to the Henderson-Hasselbach relationship. The second ionization constant corresponds to deprotonation of the side-chain primary amine. Thus, with pH values above the pK_{a2} of 9.4, there is an increasing proportion of uncharged histamine molecules. This dichotomy of charged or uncharged histamine at low or high pH values, respectively, is the basis for some of the polar versus non-polar extraction methods for histamine determination (See Methods). Extreme pH values deprotonize the remaining H on the ring nitrogen exhibiting a pK_{a3} near 14. Increasing pH values above this only serves to increase the proportion of anionic species. However, due to the instability of histamine in strongly alkaline media (Jones, 1966) and the probability of side-chain amino-N^{π} interactions, histamine studies in this pH range are seldom carried out and of dubious analytical utility. The relative population of the major histamine ionic species as a function of increasing pH is graphically reproduced from Ganellin (1979) in Figure 6.

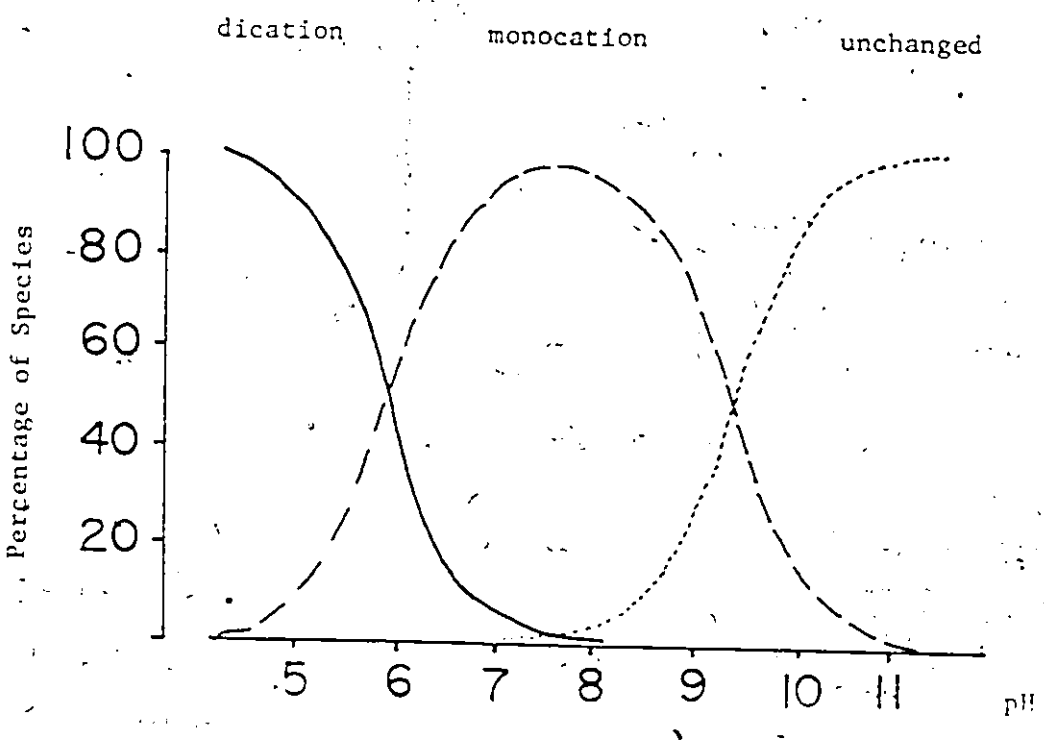
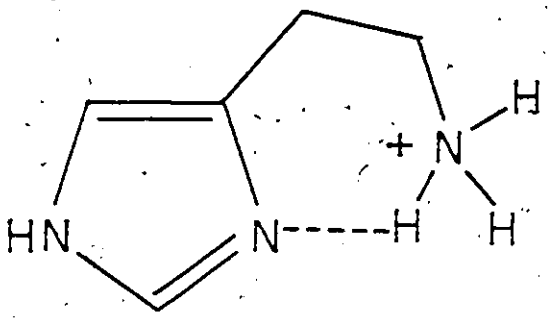


Fig. 6

From a medicinal chemistry standpoint, HA is a 1,2-disubstituted ethane derivative and is therefore a conformationally flexible molecule. Rotation occurs about the single C-C bond with the rotation of the C-NH_3 group of less importance because the protonated amino group is symmetrical. The imidazole ring is planar and relatively rigid. Extensive investigations using heterocyclic analogues of histamine have been carried out and a comparison of their various actions on histamine receptors has been determined. Thus, one can study their common structural features in order to deduce the conformational structure histamine must have in order to interact with H_1 or H_2 receptors (Ganellin et al., 1973 ,Ganellin, 1979; Durant et al., 1975). From such structure-activity relationships, it can be concluded that histamine acts as a monocation at both receptors with the presence of a proton on the N^{α}-ammonium group being obligatory. Tautomerism is probably not essential for H_1 receptor binding as 2-pyridylethylamine and 2-thiazolylethylamine which cannot tautomerize have H_1 agonistic activity. The N^{τ}-H tautomer is presently thought to be the active species. These observations and extensive molecular orbital calculations suggest that an "H_1-essential conformation" is a N^{τ}-H monocation in which the carbon and nitrogen atoms are coplanar with the imidazole ring (Fig. 7). Maximal activity occurs with a maximal separation of 5.1Å interatomic distance between the N^{π}-nitrogen atom and the ammonium group.



H_1-essential conformation

Fig. 7

Non-tautomeric heterocyclic ethylamines active at H_1 receptors only weakly stimulate H_2 receptors whereas compounds capable of 1,3-prototropic tautomerism are strong agonists of H_2 receptors. Further, electron withdrawing groups at position 4 alter the population of N^{τ}-H available such that reduced N^{τ}-H presence diminishes H_2 activity. Thus, H_2 stimulation requires tautomerism and suggests that a proton transfer reaction step occurs in the process of histamine-receptor interaction (Ganellin, 1979; Durant et al., 1975; Weinstein et al., 1976). The H_2 agonist dimaprit, an isothiourea resembling in part the imidazole ring system, probably undergoes charge transfer due to its 1,3-prototropic tautomerism. Pictorially, it may be represented as in Figure 8 where a proton is transfered from A to B.

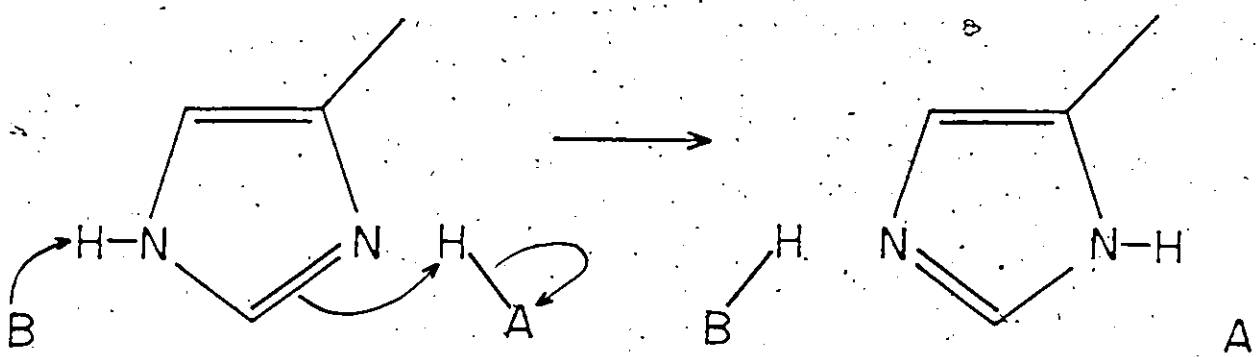


Fig. 8

Therefore, analysis of the probable molecular interactions between histamine and its receptors can lead to the synthesis of more agonists and antagonists of even greater selectivity. This careful analysis led directly to the synthesis of cimetidine, dimaprit and impromidine (Ganellin et al., 1973; Durant et al., 1977; 1978).

IV. HISTAMINE IN MAMMALIAN BRAIN - AN INTRODUCTION

Even before much was known about histamine in the mammalian brain, it was the relationship between the disparate fields of peripheral histamine research and brain research that triggered the critical investigations which were to give birth to the discipline of modern psychopharmacology. Antihistamines of the phenothiazine group were discovered to produce a "euphoric quietude" (Kety, 1978). The keen observations that another group of tricyclic antihistamines had some "antipsychotic" effects and that a derivative of this class had the additional property of improving endogenous depression gave rise to the development of the antidepressant agents (Kuhn, 1970). The importance of the phenothiazine, chlorpromazine, and the tricyclic antidepressant, imipramine, in biological psychiatry has been established. However, central histamine was not thought to play a major role in the pharmacological or therapeutic actions of the neuroleptic or antidepressant drugs. Only more recently has it become clear that these and other psychoactive drugs affect brain histamine.

The discovery of histamine's presence in brain antedates that of most of the other established biogenic amine neurotransmitters (Abel and Kubota, 1919). Kwaitkowski (1943) using the atropinized guinea pig ileum as a bioassay technique, was able to show histamine's presence and to quantify it in the central and peripheral nervous systems of several species, including man. Early investigations showed that the hypothalamus contained considerably higher concentrations of histamine than any other region (Harris et al., 1952). The local synthesis of brain histamine following decarboxylation of its immediate precursor, histidine, could be shown in vitro and in vivo by the use of labelled histidine. In the brains of the species studied, the greatest histamine forming capacity was in the hypothalamus (White, 1959;1960). The distribution of histamine in the major regions of the dog brain was determined by bioassay (Adam, 1961) where its uneven distribution and conspicuous presence in the hypothalamus confirmed

earlier observations. These facts and the sedative side effects following antihistamine administration suggested to many a neurotransmitter role for this biogenic amine. In fact, at this early date, Erspamer (1961) was prompted to state "Histamine in the CNS has been ignored for a long time by several investigators as a second-class amine. But this amine, however annoying the fact may be, has the same citizenship rights in the CNS as catecholamines and 5-HT; whose function in the CNS is approximately as obscure as that of histamine." While this statement may have been debatable then, it soon became obsolete because rapid advances in central catecholamine and 5-hydroxytryptamine research soon left brain histamine in relative obscurity.

There have been several obstacles to the effective study of the functions of this biogenic amine and how it may be altered and/or regulated in psychiatric disturbances or following psychoactive drug administration. Until recently, there was a lack of suitable methodology to accurately measure brain histamine and study its synthesis, release and metabolism. There was also a lack of sensitive and selective techniques to clearly demonstrate central histaminergic neurons. The complexity of characterizing the receptors upon which centrally released histamine acts made analysis of functional roles difficult to interpret. Yet several advances have been made in overcoming these technical impediments. Enough data may now be available to ascribe a neurotransmitter role to at least a portion of the histamine in mammalian brain. However, its status as a possible central neurotransmitter has for a long while been a particular troublesome issue for this biogenic amine. Like the catecholamines, 5-hydroxytryptamine (serotonin) and γ-aminobutyric acid (GABA), histamine has not been shown to be released upon physiological stimulation at a particular synapse in the mammalian central nervous system. As in the case of these "established" transmitters, the "criterion of transmitter collectibility" has not been fulfilled. Neither has it been shown that application of these transmitters (in amounts equivalent

to that released) to single, identified post-synaptic membranes cause actions identical to those normally observed in the CNS. This is due to the problems of isolating single, identifiable synaptic units and submitting them to biochemical or physiological analysis. The impermeability of the blood-brain barrier to the use of several agents with which to study these synaptic mechanisms, once isolated, make rigorous evaluation almost impossible. Moreover, the anatomical and neurochemical complexity of multiple inputs converging onto a single neuron and the lack of well characterized neuronal responses following stimulation have created additional technical problems.

A stringent application of these criteria to the vast majority of central synapses might preclude the existence of any central neurotransmitter in the mammalian brain. To deal with this situation, several indirect indications were established which are more of a biochemical, morphological, pharmacological and physiological nature. The criteria for neurotransmitter identification in both the periphery and the CNS and discussions of the rigor of their application have been reviewed (Werman, 1966; Kopin, 1968; Phillis, 1970; Hebb, 1970; Reis, 1972; Gerschenfeld, 1973; McGeer et al., 1978; Barchas et al., 1978). Neuroscientists have not yet fixed one set of rules as a standard to which all potential neurotransmitter candidates must be compared. The general consensus is that the other "established" central transmitters, unlike histamine, have apparently been more successful in gaining acceptance "by common-sense analysis" (Cooper et al., 1978). It may become apparent in the discussions to follow that histamine meets almost all the general requirements for central neurotransmitter status. Perhaps the confirmation of this field's most recent findings and the wider dissemination of these observations will finally herald the ascendancy of histamine from a putative to an established central neurotransmitter role.

V. TECHNIQUES OF HISTAMINE MEASUREMENT

A. Bioassay

Histamine (HA) is an ideal candidate for analysis by bioassay since it possesses biological activity, especially the ability to contract smooth muscle (e.g. isolated strips of guinea pig ileum) and heat stability, which destroys many bioactive substances and denatures proteins. While nanogram amounts of HA can be determined (Vugman and Rocha e Silva, 1966), the sensitivity of this technique is limited by the sensitivity of the particular tissue used and the volume of the organ bath. However, the applicability of bioassays of HA content in brain tissue is very limited. First, many biologically active substances may cause ileum contraction and must therefore be removed prior to analysis. Without such a separation step, for example the use of ion exchange chromatography and evaluation of recovery, the brain HA content will be much higher than the values obtained by other methods (Carlini and Green, 1963). Specificity to HA should be shown by blocking its effects with H_1 antagonists (Adam and Hye, 1966; Green, 1970). Whole brain and gross regional HA content may be determined, but the tedium and lack of sensitivity and precision have made this method unattractive.

B. Fluorometry

Condensation of HA with o-phthaldialdehyde (OPT) generates a highly fluorescent product (Shore et al., 1959), although the exact chemical nature of the reaction and product are still unknown (Hakanson et al., 1972). However, serious reservations have arisen for the use of this method in brain tissue due to the concomitant presence of other substrates for this reaction which exhibit fluorescence characteristics similar to the HA derivative (Carlini and Green, 1963; Kremzner and Pfeiffer, 1966; Hakanson et al., 1972). The diamines, in particular spermidine, which in the brain is at least 500-fold more abundant than HA must be analytically removed (Green, 1970) by such techniques as ion exchange chromatography to obtain levels in the range of those reported using other methods

(Michaelson and Coffman, 1969; Anton and Sayre, 1969; Endo and Ogura, 1973). A recent modification is the use of a combined enzymatic-fluorometric method (Neugebauer and Lorenz, 1981) in which the fluorescence is ascribed to histamine only after conversion to N^{τ}-methylhistamine following incubation with histamine N-methyltransferase. The decrease in fluorescence is due to histamine's removal from the samples since N^{τ}-methylhistamine does not fluoresce with OPT. Both ion exchange and enzymatic metabolism steps confer specificity to this analysis, but again, these additional procedures make analysis more tedious. The fluorometric methods also lack the sensitivity required to measure HA in small brain regions.

C. Enzymatic-Isotopic Methods.

The process of HA methylation by histamine N-methyltransferase (HMT) (Brown et al., 1959) confers enzymatic specificity to the determination of histamine in the brain. The radioactive labelled methyl group from S-adenosyl-L-methionine (SAMe) is transferred to the N^{τ}-nitrogen of the histamine molecule producing labelled N^{τ}-methylhistamine (N^{τ}-MeHA) and S-adenosyl-L-homocysteine (Snyder et al., 1966a). Boiling destroys the endogenous SAMe and denatures and partially precipitates protein and simultaneously inactivates synthetic and catabolic enzymes. This reaction uses exogenous HMT from guinea pig brain (Brown et al., 1959) or rat kidney (Shaff and Beaven, 1979). A double labelled isotopic method had been developed which uses trace amounts of ^{3}H-histamine to correct for the efficiency of methylation with ^{14}C as the methyl label. A single labelled method utilizing ^{3}H of the methyl group for transfer to HA has also been developed (Taylor and Snyder, 1972a; Beaven et al., 1972; Kobayashi and Maudsley, 1972; Verburg et al., 1983). Several of these methods have been reviewed and compared (Beaven and Horakova, 1978). The great advantage of these enzymatic-isotopic methods is that brain HA can be evaluated in small tissue samples with a high degree of specificity and sensitivity without the use of columns or multiple extractions.

D. Other Techniques

Several qualitative and quantitative methods are available for measuring histamine which utilize more classical chemical techniques such as colorimetry, spectrophotometry, gravimetric analysis and paper chromatography (McIntire, 1966). However, their reduced sensitivity and specificity preclude their routine use in measuring brain histamine. An isotope dilution technique using p-(^{131}I) iodosulfonylchloride to form dipipsylhistamine and requiring recrystallization was developed (Schayer et al., 1955; Schayer, 1971). However, the complexity of the method was immense and its sensitivity questionable for brain tissue. The most recent approaches for the measurement of brain histamine have utilized gas chromatography (GC), gas chromatography-mass spectroscopy (GC-MS) and high pressure liquid chromatography (HPLC) methods. Earlier GC methods based on the formation of volatile HA derivatives with which to quantitatively determine HA or N^{τ}-MeHA have been reported but were not widely accepted due to difficulties in sensitivity, specificity or baseline consistency (Brooks and Horning, 1964; Tham, 1966). The aqueous determination of minute concentrations of HA following derivatization has been successful using GC (Doshi and Edwards, 1979) and GC-MS (Mahy et al., 1978), but have not yet been applied in biological samples. Another GC-MS method which has been used for plasma and urine samples (Mita et al., 1980) gave values in agreement with parallel determinations by the radioenzymatic method. However, its sensitivity for analysis of brain histamine may not be sufficient without modification of the current method. Whole mouse brain HA has been measured fluorometrically after ion exchange chromatography and dansylation with the use of HPLC for final separation (Yamatodani et al., 1977). HPLC methodology has also been used to evaluate regional brain HA levels after derivatization with OPT in mice (Yamatodani et al., 1982; Maeyama et al., 1983) and guinea pigs (Nakamura et al., 1979). While this technique shows promise in specifically measuring global and regional HA levels, its sensitivity continues to be exceeded by the radioenzymatic methods.

VI. REGIONAL AND SUBCELLULAR LOCALIZATION OF HISTAMINE

Histamine has been found in the brains of all mammalian species examined, e.g. rat (Taylor and Snyder, 1972a; Blanco et al., 1973) (See Table I), mouse (Taylor and Snyder, 1972b), cat (White, 1966), dog (Adam, 1961), rabbit (Abou et al., 1973; Blanco et al., 1973), goat (Tuomisto and Eriksson, 1982), monkey (Michaelson et al., 1968; Taylor et al., 1972) and man (Lipinski et al., 1973). Whole brain concentrations in mammals generally range between 10 and 75 ng of histamine per gram of wet tissue (Green, 1970; Taylor, 1975) while the content of lower vertebrates are often outside this range (Almeida and Beaven, 1981). In mammalian brains, the regional distribution follows a similar pattern. The hypothalamus has the highest regional content while the medulla and cerebellum generally have the lowest global concentrations. Histamine concentration differs greatly among the various nuclei in individual brain regions of the monkey (Taylor et al., 1972) and man (Lipinski et al., 1973). The heterogeneity of hypothalamic HA distribution was confirmed and shown to be different from that of norepinephrine, dopamine and 5-hydroxytryptamine and choline acetyltransferase activity (Brownstein et al., 1974b).

The localization of brain histamine in various subcellular fractions has also been achieved. In almost all of the investigations of subcellular brain histamine, the classical scheme of separating tissue homogenates prepared in isotonic sucrose into four major fractions was applied (Michaelson and Whittaker, 1962; Carlini and Green, 1963; Michaelson and Dowe, 1963; Kataoka and DeRobertis, 1967; Michaelson and Coffman, 1967; Kuhar et al., 1971b; Young et al., 1971; Snyder and Taylor, 1972; Snyder et al., 1974; Dismukes et al., 1974; Pollard et al., 1974). Briefly, the brain tissue is generally homogenized in 0.32 M sucrose then centrifuged at 10,000 g-min to form a pellet which is termed the nuclear fraction or the P_1 pellet. It contains nuclei, plasma membrane fragments and blood cells in addition to whole cells and larger cellular debris resulting

Table I

Regional Distribution of Histidine, Histamine and Tele-methylhistamine and of Histidine Decarboxylase and Histamine N-Methyltransferase Activities in the Rat Brain (Adapted from Taylor and Snyder, 1972a)

Brain Region	Histidine (µg/g tissue)	Histidine decarboxylase (nmol/hr/g protein)	Histamine (ng/g tissue)	Histamine N-methyltransferase (µmol/hr/g protein)	Tele-methyl-histamine (ng/g tissue)
Hypothalamus	18.0	20.8	188	0.71	179
Thalamus-midbrain	12.6	9.1	62	0.63	35[a]
Corpus striatum	15.8	7.1	46	0.79	36[b]
Hippocampus	18.2	5.0	38	0.53	24
Cerebral cortex	7.9	4.8	27	0.67	30
Medulla-pons	15.1	3.0	16	0.41	10
Cerebellum	16.1	2.0	14	0.22	11

[a]Tele-methylhistamine values reported by Hough and Domino, 1979.

[b]Arithmetic mean of mesencephalon (23 ng/g) and thalamic (47 ng/g) values (Hough, personal communication).

[c]Composed of the globus pallidus and the anterior caudate nucleus (Hough, personal communication).

from incomplete homogenization. The supernatant is then transferred and centrifuged at 2-10 x 10^5 g-min to form the second pellet (P_2) termed the mitochondrial fraction and composed of mitochondria, lysosomes and synaptosomes. The synaptosomes, which may themselves contain mitochondria, are the membrane-bound sacs containing synaptic vesicles and cytoplasm from nerve terminals (Whittaker et al., 1964; Whittaker, 1965; 1969). Synaptosomes may in turn be subfractionated into their various subcomponents of mitochondria, vesicles, soluble material, membranes and other constituents. Submitting the intermediate (S_2) supernatant to higher centrifugal forces (6 x 10^6 g-min or greater) forms a P_3 pellet composed of an operationally defined class of subcellular particles termed microsomes which are the subcellular particles that require high centrifugal forces for complete sedimentation. Vesicles formed from the endoplasmic reticulum, Golgi membranes, small mitochondria and small free synaptic vesicles are recovered in the P_3 pellet. The final supernatant, often termed "the high speed supernatant," the S_3 supernatant or simply the supernatant fraction, consists of cytoplasm from cells lysed during homogenization and any material resistant to high speed sedimentation.

During the fractionation process, absorption, adsorption and various forms of trapping may occur such that the fractions obtained are far from pure. Thus, this methodology is not a rigorous biophysical or biochemical technique; but nevertheless, it has provided an approximate quantitative insight into the various locations for brain histamine.

In almost every study the majority of brain HA was localized to the mitochondrial (P_2), microsomal (P_3) or high speed supernatant (S_3) fractions. Less HA has been consistently found in the nuclear (P_1) fraction where it has been observed to contain between 8 and 40% of the recoverable HA regardless of the method of analysis. The subcellular distribution of HA, unlike that of acetylcholine, noradrenaline and serotonin, is bimodal (Whittaker, 1965). This is

significant for two reasons. First, the P_1 fraction may contain histamine from mast cell or mast cell-like granules which are much denser and consequently sediment at lower centrifugal forces than synaptosomes. The addition of rat peritoneal mast cells to guinea pig brain homogenates was shown to alter the distribution of HA such that almost all of the additional HA was recovered in the P_1 fraction. Also, comparison of histamine's subcellular distribution in the dog hypophysis (mast cell rich tissue) and hypothalamus (relatively poor in mast cell number) showed that almost all of the recoverable hypophyseal HA was in the P_1 fraction while hypothalamic HA was largely in the P_2 fraction (Michaelson and Whittaker, 1962; 1963; Michaelson and Dowe, 1963; Martres et al., 1975). Second, the recoverable HA in the P_2 fraction when subfractionated is much more abundant in the synaptosomal subfraction (B) than the myelin/membrane (A) or mitochondrial (C) subfractions (Whittaker, 1965; Kataoka and DeRobertis, 1967; Kuhar et al., 1971b; Snyder et al., 1974). This suggests a vesicular localization for a large portion of brain histamine. Unfortunately, detailed investigations with electron microscopy incorporating histamine-sensitive histofluorescence have not been reported. Thus, even though peripheral mast cell HA sediments in the nuclear layer, we cannot conclude with certainty that all P_1 HA is due exclusively to CNS mast cell sources. However, procedures used to isolate partially purified synaptosomal preparations, whose integrity was verified by electron micrographs and neurochemical markers, have been found to sequester most of the P_2 compliment of HA (Kataoka and DeRobertis, 1967; Kuhar et al., 1971b; Baudry et al., 1973; Snyder et al., 1974). Neurochemical evidence of P_2 subfractionation of rat hypothalamic tissue using norepinephrine, γ-aminobutyric acid, potassium and lactic acid dehydrogenase activity as synaptosomal markers confirmed that substantial recoverable HA was in synaptosomes. The mitochondrial layer, verified by a high monoamine oxidase activity, contained far less HA (Snyder and Taylor, 1972). The reduction of P_2 histamine content following

experimental lesions has been used as evidence for histaminergic neuronal pathways in the mammalian CNS (Dismukes et al., 1974; Barbin et al., 1976). The various techniques of fraction preparation and HA determination found in the literature make precise and consistent estimates of fractional HA content difficult; yet, a qualitative appraisal of these quantitative evaluations leaves little doubt that a major portion of brain histamine resides in a neuronal compartment.

VII. BRAIN HISTAMINE SYNTHESIS

Histamine in the mammalian CNS is almost exclusively formed locally after decarboxylation of its immediate precursor, L-histidine, since it poorly penetrates the brain from plasma (Halpern et al., 1959; Snyder et al., 1964; Schayer and Reilly, 1970; Oldendorf, 1971; Bulfield and Kacser, 1975). However, a stereoselective active uptake mechanism for L-histidine has been demonstrated in rat brain slices (Neame, 1964) and synaptosomes (Chudomelka and Murrin, 1983). In fact, L-histidine availability may constitute the rate-limiting step in the formation of brain histamine. While the histidine concentration inside cells containing histidine decarboxylase (HD) is not known, we can conclude by indirect evidence that it is not at sufficient concentrations to saturate HD. L-Histidine loading elevates brain histamine concentration in vivo while the D-isomer has no effect (Taylor and Snyder, 1972b; Schwartz et al., 1972; Bulfield and Kacser, 1975; Mazurkiewicz-Kwilecki, 1983). In addition, the mean levels of histidine in plasma and brain are approximately 60 μM whereas the K_M of HD for histidine is about 300 μM (Schwartz et al., 1970; 1980). The characteristics of the histidine uptake system and its direct relationship to the regulation of brain histamine are currently poorly understood. It is known that histidine alone is not involved in this process. There are probably some common uptake processes shared by phenylalanine, tryptophan and histidine since high levels of the two

former amino acids inhibit accumulation of the latter in rat brain slices (Neame, 1964; Barbosa et al., 1971). L-Tryptophan in high concentrations has been shown to compete with ^{3}H-histidine uptake into rat hypothalamic slices with a concomitant reduction in ^{3}H-HA formation (Verdiere et al., 1975). In vivo endogenous HA levels were reduced in the brains of mice treated with L-tryptophan (Taylor and Snyder, 1972b). On the other hand, L-histidine has also been shown to alter L-DOPA transport into slices of rat cortex and striatum (Herreros et al., 1978). This may have direct consequences on evaluation of endogenous transmitter systems using histidine loading techniques. For example, histidine loading was shown to lower mouse brain serotonin levels (Taylor and Snyder, 1972b). Therefore, the uptake of histidine and the relative abundance of other amino acids may have a regulatory role not only in brain histamine synthesis, but also in the synthesis of other neurotransmitters.

Two enzymes are capable of forming histamine by the single step process of histidine decarboxylation, aromatic L-amino acid decarboxylase (E.C. 4.1.1.26) (i.e. DOPA decarboxylase) and "specific" L-histidine decarboxylase (E.C. 4.1.1.22) (HD). The specific HD is so named since DOPA (dihydroxyphenylalanine) and 5-hydroxytryptophan act as very poor substrates in vitro while L-histidine but not D-histidine is effectively decarboxylated (Hakanson and Owman, 1966; Aures et al., 1970; Schwartz et al., 1970). In rat hypothalamic homogenates, the pH optimum (6.5-7.0), K_M values for histidine (approximately 3-4 x 10^{-4} M) and effects of various inhibitors indicated that it is the specific L-histidine decarboxylase that is physiologically involved in brain HA synthesis (Schwartz et al., 1970). The aromatic L-amino acid decarboxylase has a pH maximum in the range of 8.0-9.5 at which molar histidine concentrations may be required for saturation. For the specific enzyme, the pH optimum is inversely related to the substrate concentration, i.e. at lower histidine concentrations the optimum pH approaches 7.0 while high levels reduce the optimum pH to as low as 5.5

(Hakanson, 1967; Aures et al., 1970). The parallel regional distribution of specific HD activity and histamine concentration (Table I) and an abundance of lesion data (see Section X) strongly suggest that HA synthesis does not occur in catecholaminergic or serotonergic neurons.

The distribution of specific and nonspecific HD in a few brain regions show some similarities with relatively high activities in the hypothalamus and low activities in the cerebellum. Yet in the striatum (which has only about a third of the HD activity seen in the hypothalamus), the non-specific enzyme has its highest activity, nearly double that of the hypothalamus (Schwartz et al., 1979a). Histamine biosynthesis is not affected by the aromatic L-decarboxylase inhibitor, α-methyl-DOPA (Taylor and Snyder, 1971; Schwartz et al., 1972). Conversely, NSD-1055 (4-bromo-3-hydroxybenzyloxyamine) and α-hydrazinohistidine which compete with the cofactor pyridoxal phosphate (Levine et al., 1965) and 4-thiazolyl-methoxyamine are more potent inhibitors of specific HD than the non-specific enzyme (Taylor and Snyder, 1971; 1972a). While these latter agents inhibit HD in brain homogenates, they vary in their ability to penetrate the brain (Dismukes and Snyder, 1974), thus limiting their usefulness in the evaluation of histaminergic processes. Since pyridoxal phosphate is the cofactor for histidine decarboxylase, inhibitors acting at the cofactor site will also inhibit this enzyme, but the poor specificity of such agents (e.g. semicarbazide) are of dubious analytical value (Maslinski, 1975). A highly specific HD inhibitor acting by this mechanism has not been reported. A recent advance in this area was the development of the highly selective 'suicide' HD inhibitor, α-fluoromethylhistidine (Kollonitsch et al., 1978). This agent was shown to be a potent irreversible HD inhibitor both in rat brain homogenates and in the brains of mice following i.p. injection while histamine N-methyltransferase, DOPA decarboxylase and glutamate decarboxylase activities were unaffected (Garbarg et al., 1980b). Such treatment reduced endogenous brain histamine levels and required

up to 4 days to restore enzyme activity to control values. The high selectivity of this agent and its ability to pass the blood brain barrier make it a potentially powerful tool for the study of brain histamine synthesis.

The regulation of brain histidine decarboxylase activity under physiological conditions is poorly understood. Dialysis of homogenates was reported to reduce HD activity which was restored upon addition of exogenous pyridoxal phosphate. However, addition of exogenous cofactor to freshly prepared brain homogenates failed to alter enzyme activity (Palacios et al., 1976). While this suggests that the availability of pyridoxal 5'-phosphate may regulate HD activity, it argues against the presence of this cofactor as a rate-limiting process under normal conditions. End product inhibition of HA synthesis is also an unlikely mechanism since HA concentrations up to 10 mM have failed to affect HD activity in hypothalamic homogenates or rat brain slices and did not alter ^{3}H-HA formation from ^{3}H-histidine (Verdiere et al., 1975). Substrate availability may more effectively regulate HA formation since histidine probably does not saturate HD under normal conditions. While loading with L-histidine (500 mg/kg i.p.) raises endogenous HA levels (Schwartz et al., 1972; 1979a), N^{τ}-methylhistidine treated mice have been reported to undergo endogenous HA reduction due, at least in part, to competition for both L-histidine uptake and decarboxylation sites (Schwartz et al., 1972). N^{τ}-methylhistidine acts as substrate for brain HD, although its K_M far exceeds that of L-histidine by about thirty times (Schwartz et al., 1973).

The subcellular localization of HD activity has been studied in rat cortex (Baudry et al., 1973) and rat hypothalamus (Snyder et al., 1974). In both studies, over 80% of the recoverable enzyme activity was in the P_2 and supernatant (i.e. soluble) fractions. In the cortex, subfractionation of the P_2 fraction showed that 70% of the enzyme activity was in the synaptosomal subfraction. Osmotic shock released almost all the enzyme in soluble form suggesting that the enzyme was in the soluble cytoplasm of nerve terminals prior to homogenization

(Baudry et al., 1973). The finding that over 40% and over 60% of the activity was in the supernatant fractions of the cortex and hypothalamus, respectively is consistent with the suggestion that much of the HD recovered is in the cytoplasm of neurons and/or other cells of the brain. In both studies, no more than 15% of the total HD activity was recovered in the P_1 and P_3 fractions combined. From these observations, we can infer that a major portion of HD is localized in the neuronal cytoplasm and that at least a portion of this cytoplasic compartment is in synaptosomes.

The developmental pattern of HD activity in the brains of rat neonates is consonant with a neuronal presence which increases in parallel with age-related neuronal development. During the developmental process, the major portion of activity shifts from the supernatant fraction (70% of the recoverable HD at birth) to the P_2 fraction (46% in the adult), most of which is synaptosomal (Martres et al., 1975; Baudry and Martres, 1975). More importantly, the increase in total brain HD activity occurs almost exclusively in the P_2 fraction, a pattern similar to catecholamine neuronal development (Coyle and Axelrod, 1972). If the soluble HD represents a component which is largely derived from neuronal cell bodies, then the increase with age of the enzyme in the P_2 fraction may reflect its proliferation with that of neuronal fibers and terminals (Schwartz et al., 1979a). This is an attractive hypothesis which is consistent with the available data, but presently remains largely unproven.

VIII. FATE OF HISTAMINE IN THE MAMMALIAN CENTRAL NERVOUS SYSTEM

A. Release

An important function expected of a neurotransmitter is its release from presynaptic neuronal stores in response to depolarization of the axon terminal membrane. This rather obvious criterion for transmitter status has been more difficult to achieve for the central histaminergic stores for numerous reasons.

Almost certainly, much of the brain HA is non-neuronal as subcellular fractionation studies (section VI), a brain mast cell pool (section IX) and lesion studies (section X) indicate. A high density population of HA cell bodies with dense circuits projecting to well-defined brain regions which easily lends itself to experimental manipulation may not exist for this amine. Measurement of small amounts of HA released in vivo has also posed technical difficulties in evaluating (a) if HA is released and, (b) the quantity of HA released. Furthermore, in monoaminergic systems with established re-uptake mechanisms, incubation with radiolabelled transmitters has greatly facilitated studies of their release and metabolism. However, the lack of a high capacity system which can concentrate substantial amounts of exogenous HA against a concentration gradient in tissue stores has obviated such approaches for HA release analysis. Therefore, almost all investigations concerning HA release have been carried out in vitro using brain tissue slices.

Potassium-ion depolarization of mouse cerebral-hemisphere slices was reported to release endogenous HA (Atack and Carlsson, 1972); a stepwise elevation of K^+ concentration led to a stepwise enhancement of HA release as well as that of noradrenaline and serotonin. Measurement of HA using Dowex 50 W columns obtained by fluorimetric analysis compared favorably with those of others (Anton and Sayre, 1969; Green, 1970; Taylor and Snyder, 1972b). Taylor and Snyder (1972d) demonstrated HA release from rat hypothalamic slices depolarized by K^+ which was Ca^{++} and temperature dependent, augmented by higher K^+ levels and also released by reserpine. Quantitative recovery of HA under these conditions indicated that the sum of the medium HA content plus that remaining in tissue was unaltered. Histidine decarboxylase inhibition with NSD-1055 failed to affect K^+-evoked HA efflux. Similar results were observed in slices of rat hypothalamus pre-incubated with ^{3}H-histidine (Verdiere et al., 1975). The K^+-induced depolarization caused ^{3}H-HA to be released by a Ca^{++}-dependent mechanism(s) and

stimulated further synthesis of tissue ^{3}H-HA. Much of the radioactivity in the medium could be attributed to ^{3}H-methylhistamine which was further increased following isocarboxazid-induced monoamine oxidase inhibition.

Tritiated histamine release from tissue slices of several rat brain regions previously incubated with ^{3}H-HA indicated that K^{+} depolarization caused a rapid, Ca^{++}-dependent release of tritium (Subramanian and Mulder, 1976) although little net radioactivity could be sequestered in the tissues. Moreover, K^{+}- and compound 48/80-induced ^{3}H-HA efflux from hypothalamic slices were suggested to be two independent mechanisms. In addition, these authors stated that "part of, if not all, of the potassium-induced release of accumulated exogenous HA may be from glial cells." However, this statement was based exclusively on literature citations of GABA uptake by glial cells and subsequent Ca^{++}-dependent release. Hence, this speculation was not supported experimentally and the rationale for proposing that possibly all of the accumulated ^{3}H-HA came from glia remains obscure. Superfusion experiments using guinea pig hypothalamic slices previously incubated with ^{3}H-HA demonstrated ^{3}H-HA release by electrical field stimulation which was frequency- and Ca^{++}-dependent (Biggs and Johnson, 1980).

More recently, Mulder et al. (1983) reported that cortical, hippocampal and hypothalamic tissue slices, incubated with ^{3}H-HA, released tritium after depolarization induced by either high potassium ion concentration, electrical field stimulation or veratrine. The K^{+}-induced release increased with increasing concentrations of K^{+} and was Ca^{++}-dependent as was the release evoked by electrical stimulation and veratrine; the latter two were blocked by tetrodotoxin. Veratrine-induced tritium efflux was concentration-dependent and electrically-induced release was frequency-dependent. Investigators from this laboratory revised their previous view on glial uptake and release of ^{3}H-HA concluding from the above results that the profile of K^{+}, electrical stimulation and veratrine depolarization caused ^{3}H-HA to be released from neuronal sources

exclusively. However, the authors pointed out that they did not verify what fraction of the tritium could be attributed to HA. This may be important in light of previous studies in which at least 10% of the tissue tritium was ^{3}H-methylhistamine and no more than 75% due to ^{3}H-HA (Subramanian and Mulder, 1976). It also remains to be established if the cells involved, quite possibly neuronal, are histaminergic.

Much of the ambiguity concerning ^{3}H-HA uptake into histaminergic neurons was obviated by a study which demonstrated K^{+}- and veratridine-induced ^{3}H-HA release from rat cortical slices previously incubated with the precursor, ^{3}H-His (Arrang et al., 1983). This ^{3}H-HA release was dependent on both K^{+} and veratridine concentrations and the presence of extracellular Ca^{++}. In addition, ^{3}H-HA release was also tetrodotoxin sensitive (see section XI.I).

The release of endogenous HA was studied by superfusion of the posterior hypothalamus with push-pull cannulae and subsequent determination of the rate of HA released in the superfusate (Philippu et al., 1982). Endogenous HA was shown to be enhanced by electrical stimulation of the superfused area and hypothalamic superfusion with high K^{+} concentrations in artificial cerebrospinal fluid also increased endogenous HA release which was determined by a radioenzymatic method. The "resting" HA release rate in the anesthetized cat posterior hypothalamus and the rabbit anterior and posterior hypothalamus was approximately 50 picograms/minute. This release rate was not only physiological, but also sensitive to physiological changes in the body (see below); a response consistent with (and expected of) a neurotransmitter substance. In addition, the resting release rate oscillated rhythmically at approximately 70 min cycles, a pattern which argues against both anesthesia mediated release and a non-neuronal origin for the endogenous HA. The efflux rate was shown to be elevated, in some cases more than +100%, under conditions of increased or decreased arterial blood pressure. This suggested to the authors that HA release plays a role as an

immediate physiological signal of changes in peripheral blood pressure (Philippu et al., 1983). Whether this is part of a trigger for signalling that a change has occurred and/or a portion of the early responses remains to be established.

B. Histamine Inactivation: Uptake and Metabolism

As there are two enzymes capable of synthesizing histamine (see section VII), there are likewise two enzymes which metabolize it (Fig. 9), i.e. diamine oxidase (E.C. 1.4.3.6.) and histamine N-methyltransferase (HMT) (E.C. 2.1.1.8.) (Schayer, 1959). The action of the former enzyme produces imidazoleacetaldehyde and following further oxidation by aldehyde dehydrogenase, imidazoleacetic acid. HMT generates N-tele-methylhistamine (t-MeHA) which is ultimately oxidized by monoamine oxidase to N-tele-methylimidazole acetic acid. HA is not a substrate for the non-specific N-methyltransferase (Fong and Huang, 1983). Similar to the HA synthesizing enzymes, only one, the histamine N-methyltransferase, metabolizes HA in the mammalian CNS. The mammalian brain lacks the capacity to significantly oxidize histamine (Burkard et al., 1963; Schayer and Reilly, 1970; 1973; Schwartz et al., 1971). Although ^{3}H-HA injection into the rat lateral ventricle resulted in labelled imidazoleacetic acid formation has been reported (Snyder et al., 1966b), the large amounts of ^{3}H-HA used in this study and the lack of evidence that this substrate mixed with endogenous HA stores cast doubts on the significance of these findings (Taylor, 1975). Alternatively, it was suggested that HA is nearly quantitatively methylated (Schwartz et al., 1971). Intracisternal administration of moderate doses of ^{3}H-HA to rats showed that N-tele-methylation is the major, possibly the only, route of HA metabolism. It was observed that labelled t-MeHA content rose rapidly and pretreatment with the monoamine oxidase inhibitor, tranylcypromine, reduced the methylimidazole-acetic acid levels but increased ^{3}H-t-M-HA content still further, which accounted for more than 70% of the recovered radioactivity (Schwartz et al., 1971).

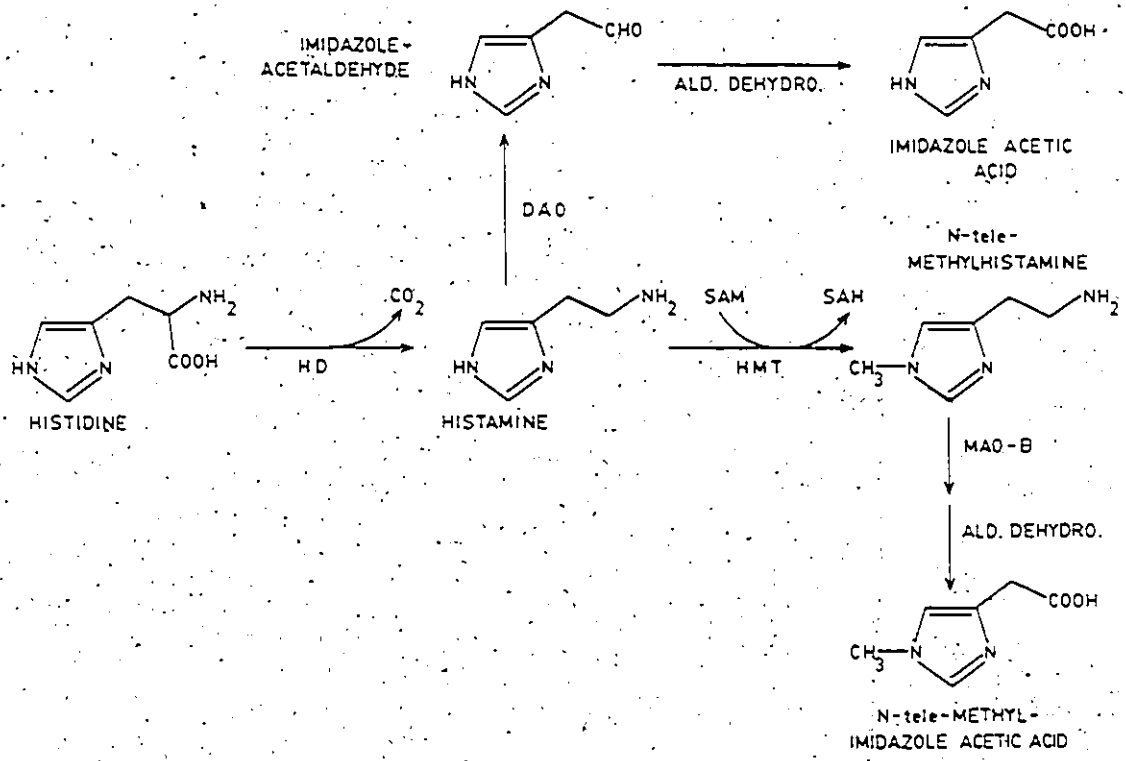


Fig. 9

Pathways of histamine synthesis and metabolism in the mammalian central nervous system.

Abbreviations used are as follows: HD, histidine decarboxylase
HMT, histamine N-methyltransferase
DAO, diamine oxidase
MAO-B, monoamine oxidase (type B)
Ald. Dehydro., aldehyde dehydrogenase
SAM, S-adenosyl-L-methionine
SAH, S-adenosyl-L-homocysteine

Isotope dilution analysis demonstrated that ^{3}H-imidazoleacetic acid, if formed, could not account for more than 2% of the recovered tritium. Also, the diamine oxidase inhibitor, amino-guanidine, given intracisternally failed to alter the levels of ^{3}H-deaminated metabolites. These findings were confirmed in rat brain when the metabolism of endogenously synthesized ^{3}H-HA from injected ^{3}H-His was studied. After pargyline-induced inhibition of monoamine oxidase, almost all of the radioactivity could be recovered as ^{3}H-t-MeHA (Pollard et al., 1974). Similar studies using both ^{3}H and ^{14}C labelled L-histidine have yielded consonant results (Reilly and Schayer, 1970; Dismukes and Snyder, 1974).

Gamma-glutamylhistamine formation from histamine has also been reported in both rat brain in vitro and in vivo studies (Konishi and Kakimoto, 1976). However, the small amounts formed and its short half-life following i.c.v. injection suggest that it is a quantitatively insignificant metabolic route in mammalian brain, although it has been shown to be the major route in the CNS of some mollusks (Weinreich, 1979; Stein and Weinreich, 1983).

While high-affinity, energy dependent re-uptake processes for several monoamine transmitters have been established, there is little firm evidence demonstrating that this occurs for histamine in the mammalian CNS. Early attempts to indicate the accumulation of labelled HA against large concentration gradients were unsuccessful (Neame, 1964; Snyder and Taylor, 1972; Honegger et al., 1974; Matthaei and Philippu, 1974; Schwartz et al., 1979a). More recent reinvestigations suggest that an uptake process may exist but none have yielded unequivocal conclusions. Accumulation of ^{14}C-HA has been shown in rabbit hypothalamic slices which could be inhibited by 5-hydroxytryptamine and desipramine (Tuomisto et al., 1975). However, this process required several hours and the highest concentration gradient attained was only 1.5. Bovine striatal synaptic vesicles were shown to accumulate ^{14}C-HA by a process that was independent of ATP and Mg^{++} concentrations (Philippu and Matthai, 1975). Accumulation of ^{3}H-HA in

slices from several regions of rat brain which was releasable upon K^+ depolarization was reported (Subramanian and Mulder , 1976). However, the maximum tissue-to-medium histamine ratio of approximately 1.5 and the non-parallel distribution of the regional tissue uptake with HD activity, the putative histaminergic marker, are inconsistent with a straightforward neuronal HA re-uptake scheme. Although a tissue-to-medium HA ratio of about 3 has been attained following a 90 minute incubation of ^{3}H-HA with guinea pig hypothalamic slices (Biggs and Johnson, 1980), the uptake characteristics, kinetics and energy requirements were not evaluated. Thus, the prevailing evidence indicates that the major process of histamine inactivation in the mammalian CNS is methylation by histamine N-methyltransferase.

The greater activity and relative stability of HMT compared to that of HD has made the study of the former much easier and more amenable to analysis of its mechanisms of enzymatic action. Kinetic evaluation of partially purified guinea pig whole-brain HMT activity suggested that HA was methylated by S-adenosyl-L-methionine through two half-reactions in a ping-pong mechanism (Thithapandha and Cohn, 1978). A more recent method of purification of guinea pig HMT to near homogeneity was reported but its enzymatic mechanisms were not kinetically evaluated (Matuszewska and Borchardt, 1983). Evaluation of HMT from human skin suggested an ordered sequential Bi-Bi mechanism (Francis et al., 1980). This is not surprising as the presence of HMT isozymes has been demonstrated in different tissues from the same species (Axelrod and Vesell, 1970; Suriyachan and Thithapandha, 1972). Unlike HD enzyme activity, histamine N-methylhistamine and S-adenosyl-homocysteine, the immediate products of histamine methylation are potent inhibitors of HMT activity (Brown et al., 1959; Baudry et al., 1973). Histamine itself exerts potent substrate inhibition on HMT activity. Various K_M values for histamine in the range of 8-43 μM have been reported (Brown et al., 1959; Zappia et al., 1969; Taylor and Snyder, 1972c; Thithapandha and

Cohn, 1978; Matuszewska and Borchardt, 1983). However, at concentrations near 100 μM or above, the HMT activity is reduced to about half of that obtained at 10 μM histamine concentrations (Taylor and Snyder, 1972c; Prell, unpublished).

While HMT appears to be ubiquitous throughout the mammalian central nervous system (Brown et al., 1959; Schwartz et al., 1973b; Saavedra et al., 1976), the distribution of N^{τ}-methylhistamine (t-MeHA) the inactive histamine metabolite, correlates well with that of histamine (Table 1, Hough and Domino, 1979). However, HA methylation is not found to occur at the same subcellular site(s) where HD and HA (neuronal or non-neuronal) are found since HMT activity and t-MeHA, unlike HA and HD, were both accumulated to a large extent in the supernatant (S_3) fraction (Snyder et al., 1974; Hough et al., 1982a). In addition, osmotic lysis of hypothalamic particulate fractions released HMT but not HA (Snyder et al., 1974). Indeed, there is evidence from lesion experiments that HMT may be localized to post-synaptic sites (Garbarg et al., 1973; Bischoff and Korf, 1978). Since its abundance in synaptosomal fractions suggests that it may reside in close juxtaposition to axonal endings, i.e. near the synaptic cleft (Snyder et al., 1974), HMT may have a function in metabolizing locally released HA. The demonstration that more than 50% of the enzyme activity is contained in neuronal elements of the striatum, although not necessarily exclusively in histamine-containing neurons (Sperk et al., 1981a) is consistent with this concept. However; the evidence from the cell culture studies suggests that some HMT may also reside in glial cells (Garbarg et al., 1975).

Drug-induced inhibition of HMT activity by metoprine and related diaminopyrimidines has been shown to increase in vivo HA levels in both whole brain (Duch et al., 1978) and in several regions of the rat brain, especially in the hypothalamus, thalamus and cortex (Duch et al., 1979). However, large decrements in HMT activity may be necessary before significant elevation of HA levels can be observed (Prell and Mazurkiewicz-Kwilecki, 1981). Consistent with such a

hypothesis, at equimolar concentrations, in in vitro studies, chlorpromazine and quinacrine inhibit HMT (Thithapandha and Cohn, 1978) to a lesser extent than metoprine (Duch et al., 1978) (-82%, -79% versus -93%, respectively). Yet, the two former drugs fail to alter brain HA levels in vivo (Taylor and Snyder, 1972b) whereas the latter more than doubles it (Duch et al., 1978; 1979).

Brain t-MeHA levels may be indicative of the locally released HA and as such, be used as an index of HA release, just as homovanillic acid and dihydroxyphenylacetic acid (DOPAC) concentrations reflect brain dopamine release (Cooper et al., 1978). However, HA release may not emanate from neuronal stores exclusively. In addition, t-MeHA may also be formed from decarboxylation of N^{τ}-methylhistidine (Schwartz et al., 1973a) although this may be a minor contribution to the total t-MeHA content since very little of the former may be present in mammalian brain (see appendix 3).

Endogenous N-tele-methylimidazoleacetic acid has recently been detected and measured in rat brain and human cerebrospinal fluid using gas chromatography/mass spectroscopy (GC/MS) (Khandelwal et al., 1982a; 1982b). Its measurement may prove useful in evaluating HA release and metabolism, but like t-MeHA, the exact source must first be ascertained. Using GC/MS, no imidazoleacetic acid was found in the brains of rats (Khandelwal et al., 1982a).

C. Turnover

Histamine turnover studies have been hampered by several factors which necessitated numerous qualifications and assumptions and made unequivocal estimates impossible. The existence of at least two major HA storage compartments, possibly equal in size, is a complicating issue which has been partially overcome by assuming that the HD activity/HA content ratio is much larger in the neuronal compartment than in the non-neuronal depot(s) (see sections VI and VII). In addition, while the measurement of brain HA is possible but technically difficult, techniques for the accurate quantification of brain methylhistamine

and other HA metabolites were not available until recently. Therefore, HA determination following histidine loading or HD inhibition with agents of dubious specificity were initially the only approaches available. Taylor and Snyder (1971) evaluated the decline in brain HA content following HD inhibition with NSD-1055 or α-hydrazinohistidine treatment in mice. However, only about half of the brain HA could be depleted while the remainder was insensitive to even higher doses. Although the half-life of brain HA turnover was determined to be 10 min, such drawbacks as lack of HA steady state, drug effects on other pyridoxal-5'-phosphate dependent enzymes and the induction of physiological effects such as hypothermia cannot be ignored.

Administration of ^{3}H-HA (Schwartz et al., 1971) or ^{14}C-His (Schayer and Reilly, 1973) intracisternally overcame some of these problems. While the latter group determined a half-life of 30 min to 3 hours in various brain regions, correction for ongoing synthesis during the catabolic process was not made. The former group observed that ^{3}H-HA may not be taken up by a neuronal pool and mix with endogenous stores, and therefore injected rats with ^{3}H-His intraventricularly. The expected precursor-product relationship was observed and ^{3}H-HA formation increased as ^{3}H-His levels declined such that a "conversion index" was derived, i.e. the ^{3}H-HA amount/^{3}H-His specific activity ratio (Pollard et al., 1974). Assuming a one compartment open model and a steady state HA concentration, whole rat brain turnover was estimated to be about 46 min (34 ng/g tissue/hr). There was an apparent correlation between the conversion index and brain HD activity; however, the non-physiological method of ^{3}H-His administration and the variable cerebral penetration to various regions were obvious limitations. Therefore, the more physiological approaches which used ^{3}H-His pulse plasma infusion or constant rate infusion were attempted. When injected into mice, the half-life of HA turnover in several brain regions varied between 30-70 min; the hypothalamus having the fastest turnover rate (Schwartz et al., 1979a).

Dismukes and Snyder (1974) reported that hypothalamic HA half-life was about 30 seconds in rats. This was based on the slope of brain HA content-depletion curves following i.v. injection of NSD-1055 and α-hydrazinohistidine. In a parallel study, the appearance rate of ^{3}H-HA formed from intraventricularly injected ^{3}H-His was determined in the hypothalamus and a value of about 60 seconds was obtained. However, several aspects of these studies merit further review. Even lethal doses of NSD-1055 and α-hydrazinohistidine depleted less than a third of the basal HA concentration in each region investigated. It was also assumed that HD inhibition did not alter the turnover rate, a fact which is still unproven. In addition, the selectivity of these agents is highly questionable.

Recent studies obtained, which used different approaches, values close to those determined by Pollard et al. (1974) and Schwartz et al. (1979a). Peripheral injection (i.p.) of the highly selective HD inhibitor, α-fluoromethylhistidine, into W/W^{V} mice (devoid of brain mast cells - see section IX.B), nearly totally depleted brain HA concentration. A half-life of 48-103 min was determined in the forebrain and midbrain regions, respectively, eliminating the limitations of the mast cell HA pool and the question of HD inhibition selectivity (Maeyama et al., 1983). Unfortunately, the tissue regions evaluated in this study (forebrain, midbrain and hindbrain) are not readily comparable to those used in other investigations nor can a rank comparison with regional HD activity be made. Another approach was to evaluate the accumulation of the primary HA metabolite, tele-methyl-histamine (t-MeHA), in whole rat brain after inhibition of monoamine oxidase with pargyline HCl (Hough et al., 1982b). Methylhistidine increased linearly for the first 4 hours and remained at about 3 times control levels for more than 12 hours. The slope of the linear increase (33 ng/g tissue/hr) approximated the rate of t-MeHA formation and reflects the HA turnover rate which was estimated to be about 50 minutes. Even using this

approach, several assumptions must apply. Brain HA and t-MeHA are assumed to be in a single compartment, that all HA is methylated and then oxidized and that t-MeHA is formed only from HA. In addition, pargyline is assumed to selectively inhibit monoamine oxidase nearly completely but not alter HA levels. Whole brain HA is probably not in a single compartment. However, if the rapidly turning over HA is in a single compartment, then this assumption may still have some validity. It is noteworthy that monoamine oxidase activity, assessed with phenylethylamine as a substrate by a dose of pargyline identical to that in the above mentioned studies, was more than 99% inhibited; although HA levels were little changed.

Until such time when the assumptions of the various kinetic models are clarified and validated, accurate estimates of brain HA turnover will be unattainable. However, the better designed studies tend to suggest a total and regional brain HA half-life in the general range of 45-90 minutes.

IX. POSSIBILITIES FOR MAST CELL HISTAMINE STORAGE

A. Mast Cells and Mammalian Brain

Paul Ehrlich discovered mast cells a little over a hundred years ago as a subgroup of "plasma cells" which appeared red or violet when exposed to a blue basic aniline dye. This "metachromasia" continues to be the characteristic feature for distinguishing and defining these large cells whose cytoplasm contains large metachromatic granules. This granular presence was originally attributed to over-feeding which was the basis for the term "Mastzellen" from the German root "Mastung" meaning masticate or chew. Even from their early discovery, mast cells were found by Ehrlich to be concentrated around blood vessels, nerves and glandular ducts as well as near neoplastic or inflammatory areas (Selye, 1965). In addition, present day technology indicated the presence of mast cells in the thymus, gut mucosa, peritoneal cavity and subcutaneous lymph nodes among other tissues (Dexter et al., 1981; Metcalfe et al., 1981). The mast cell basophil granules are at least partially composed of protein, heparin, histamine, 5-hydroxy-tryptamine, dopamine phospholipids, hyaluronic acid, chondroitin sulfate and other physiologically active substances in various proportions depending on the tissue and species investigated (Selye, 1965; Metcalfe et al., 1981; Uvnas, 1978). Since the original definition was essentially an operational one based on the original finding, ambiguities have developed as research on these and related cells has progressed. Therefore, the presence of basophilic granules or many of their constituents in a cell does not necessarily define a mast cell (Kiernan, 1976). The reference "metachromasia" used for evaluation can be altered by sulfation, oxidation and pH changes leading to artifactual observations (Selye, 1965). Likewise, the variation in responses to drugs, degranulating agents and other basic secretagogues and surface active agents make an unequivocal definition for a mast cell difficult (Pearce, 1983; Shanahan et al., 1983). To counter this situation, Selye suggested that the original definition be

utilized, i.e. "A mast cell is a connective-tissue element which possesses cytoplasmic granules that stain metachromatically under ordinary conditions."

Among all the substances present in mast cells, it is the presence and actions of histamine which most interests histamine investigators. Histamine release by injections of peptone or other chemical liberators (MacIntosh and Paton, 1949) or after induction of anaphylactic shock (Rocha e Silva, 1952) was long established, the latter has been accompanied by heparin release (Rocha e Silva, 1952). It was shown that the metachromatic positive material of mast cell granules are the sulfomucopolysaccharides, most notably heparin (see Metcalfe et al., 1981). Histamine releasing agents were shown to cause mast cell degranulation (Riley, 1953) and that a strong correlation existed between HA levels and mast cell content of several tissues (Riley and West, 1953). It was further indicated that following injection of HA-liberators, the recognizable mast cell population decreased as well as the tissue extractable HA content. Lastly, induction of carcinogenesis increased the HA levels and the number of mast cells. Tumors of mast cells were observed to contain exceptionally high concentrations of histamine (Riley and West, 1953). The presence of HA in mast cells and its release during the process of mast cell degranulation is now accepted and the subject of several reviews (Riley and West, 1966; Metcalfe et al., 1981; Pearce, 1982; Lagunoff et al., 1983).

Conversely, the presence of mast cells in nerve tissue, their type, abundance, regional distribution, susceptibility to degranulation and contribution to the measurable histamine pool are controversial issues. In many species, mast cells are abundant near peripheral motor nerves where there is a close correlation between histamine and dopamine content and mast cell distribution (Olsson, 1968). In addition, histofluorescence studies have confirmed dopamine's and 5-hydroxytryptamine's presence in nerve mast cell populations (Olsson, 1968). Lesions of a peripheral nerve cause a concomitant increase in mast cell number

and histamine concentration (Olsson, 1968; MacDonald et al., 1981). Mast cells appear in regions normally void of mast cells following disease or surgical interruption. Their presence in human brain on the periphery of infarcts, plaques in multiple sclerosis and syphilitic lesions has been observed (Olsson, 1968). However, a large population was not evident in human brains post-stroke or lobotomy (Dropp, 1979). In adult monkeys undergoing experimentally-induced cerebral infarction, the observed elevation of histamine content (100% over the contralateral side) of the basal ganglia was attributed to mast cell proliferation (Subramanian et al., 1981).

In the normal mammalian brain, mast cells were considered absent except for regions surrounded by connective tissue, e.g. choroid plexus, area postrema, pineal body and pituitary gland (Olsson, 1968). The meninges are especially rich in mast cells, particularly around blood vessels (Edvinsson et al., 1976). Yet more detailed studies have demonstrated mast cell presence in the central nervous system of several species, including rat (Dropp, 1972; Kruger, 1974; Ibrahim, 1974a; Edvinsson et al., 1977). However, studies designed to detect mast cells in dog, monkey (Mulatta), human, rabbit and guinea pig brains have been negative (Ibrahim, 1974a), although claims for the absence in the latter two species have been challenged (Edvinsson et al., 1977). These apparent inconsistencies have yet to be clarified. In species where brain mast cells are known to exist, a great interspecies and intraspecies variation in mast cell number exists, even among littermates. For example, in one study, the albino rat total brain mast cell compliment was reported to range between 632 and 1,205 and between 333 and 4,358 for the hooded rat (Dropp, 1976). Wistar rats showed total mast cell numbers ranging from 5,230 to over ten thousand within the diencephalon alone (Persinger, 1977). Several factors seem to alter mast cell number including age, handling and treatment prior to weaning, particularly in the diencephalon although other regions such as cortex were

reported to remain unchanged (Persinger, 1979; 1980). This suggests a further complication as mast cells are not homogenously distributed throughout the adult brain. Several reports indicated the scarcity of mast cells within major tracts, cerebral cortex, hypothalamus or basal ganglia of rat brains (Dropp, 1972; Ronnberg et al., 1973; Dropp 1976; Goldschmidt et al., 1983). These studies attributed up to 98% of the brain mast cell population to the diencephalon, especially the thalamus. The thalamic nuclei showed an uneven distribution (Dropp, 1976) with 6 of the 32 identified nuclei reported to contain about 75% of the thalamic mast cell compliment (Goldschmidt et al., 1983). The latter authors suggested that although the population size is highly variable, the cells have a precise proportional distribution among the various areas of the thalamus.

The confusion over mast cell variation in species, number and regional distribution may be dwarfed by the potential heterogeneity in mast cell type. Mast cells display a heterogeneity in size, shape, morphology, staining characteristics, location, differential responses to drugs etc. which suggests the existence of multiple types (Pearce, 1983; Shanahan et al., 1983). At least two types of mast cells have been postulated to co-exist in mammalian brain, termed type I and type II cells (Ibrahim, 1974a). Type I cells are the ones reported by most investigators. They are spherical with round nuclei and uniform, metachromatic granules which are concentrated around blood vessels, in the meninges and most abundant in the thalamus. Type II cells are ovoid or spindle-shaped, containing heterogeneous sized cytoplasmic granules which are lipid-rich. Since they do not consistently react metachromaticly and are therefore nearly devoid of heparin, they have been rejected as mast cells (Kiernan, 1976). Using the criterion of positive metachromasia, Kiernan states that "true mast cells" (Ibrahim's type I) occur only in the parenchyma of hedgehog, tree-shrew and slow loris brains. However, Ibrahim observed that although they

have been previously termed adventitial cells, pericytes or perivascular cells, they have most of the chemical constituents of type I cells and similarly undergo degranulation following compound 48/80, hydrocortisone or ionizing radiation treatment (Ibrahim, 1974a; 1974b). In a more detailed light and electron microscopic study of this variant cell group, it was suggested that the type II cells be re-designated neurolipomastocytes or neurolipomastocytoid (NLM) cells because of their high lipid content (Ibrahim et al., 1979). They are metachromatic (purple) to normochromic (blue) which appear to have gray-blue granules probably due to the greater lipid content. The heparin-stain interaction which generates a strong metachromasia that characteristically defines mast cells may be altered in NLM cells due to lack of sufficient heparin or different intracellular binding properties. The similarity of the mast cell and NLM cell colors plus the variations of color and intensity with interlaboratory methodology combine to make an objective analysis difficult.

The functional role of brain mast cells and/or NLM cells is currently unknown. The concomitant increase in mast cell number and histamine content following pathological or experimental lesions suggests a role in conditions of neuronal injury e.g. tissue repair, inflammation or demyelinization (Olsson, 1968; 1974; MacDonald et al., 1981). Since they contain vasoactive substances and are in close proximity to blood vessels, this suggests that they may assist in the regulation of the microvasculature. Yet there is little firm evidence to confirm such a suggestion. Application of compound 48/80 has been shown to cause local pial vasoconstriction (Rosenblum, 1973). Mast cell presence in human meninges and relaxation of human pial artery, probably by H_2 receptor activation has been demonstrated. Mast cells contain H_2 receptors (Lichtenstein and Gillespie, 1973). Intraarterial injection of HA has been shown to increase the permeability of labelled sucrose in brain (Gross et al., 1981). Since metiamide but not mepyramine blocked this HA response, H_2 receptors may enhance blood-brain barrier permeability. H_1 and H_2 receptor distribution in the cerebral circulation and their possible

roles have been reviewed (Edvinsson and MacKenzie, 1977; Gross, 1981; 1982).

B. Brain Mast Cells and Brain Histamine

Pertinent to brain histamine analysis is its presence in brain mast cells and the presumed contribution of mast cell HA to the measurable brain HA pool. The levels of HA in peripheral mast cells and the estimates of mast cell numbers in brain suggest that significant quantities of brain HA could be derived from this source. Purified rat peritoneal mast cells have been reported to contain up to 20 pg of HA per cell (Humphrey et al., 1963), while another group reported up to 40 pg per cell in a similar preparation (Archer, 1958). Human basophils (Sampson and Archer, 1967) and partially purified mast cells from human lungs (Patterson et al., 1976) were reported to contain HA levels of about 1 up to 6 pg per cell, respectively. Multiplying these values by the reported brain mast cell number of several laboratories yields a large range of HA values, some exceeding the total brain histamine content. The variability in brain mast cell number and the difficulty of counting mast cells and measuring HA in the same sample have thwarted efforts to correlate HA content with mast cell number. Nevertheless, histochemical analysis has shown the presence of HA in brain mast cells (Edvinsson et al., 1977).

While firm data is lacking, several speculations can be advanced concerning brain mast cell histamine content. If rat brain mast cells' HA content is approximately that of the peritoneal mast cells, then the great variability in brain mast cell number must be artifactual as rat brain histamine levels are normally fairly constant. Alternatively, the brain mast cells may not contain amounts of HA equivalent to peripheral mast cells. Clearly, more research in this area is needed.

Several recent reports tend to suggest a significant mast cell histamine component in mammalian brain. Studies with genetically anemic mice of the W/W^V genotype were found to be deficient in mast cells, i.e. about 1% of the normal number were present in skin while none were found in the brain (Kitamura et al.,

1978). Whole body histamine levels were only about 17% that of control wild type (+/+) mice (Watanabe et al., 1980). Whole brain histamine content of the W/W^V genotype was about half that of congenic (+/+) mice, but still higher than any other organ or tissue examined (Yamatodani et al., 1982). This suggests a large non-mast cell compartment of brain-HA and a quantitatively equivalent compartment for mast cell HA. Inherent in this inference is the assumption that the pools of mast cell and non-mast cell histamine are the same in mutant and wild type mice, which has not yet been shown. Also, mast cell deficiency is not the only variable. Since this mutant is pleiotropic, other tissue anomalies exist which lead to anemia, sterility and coat pigment alterations (Russell, 1979). Grzanna and Schultz (1982) found male and female W/W^V mice devoid of brain mast cells; however, HA levels were only moderately lower (i.e. about 75 to 80% the HA content of +/+ mice) in the hypothalamus, thalamus, midbrain and cortex. The other brain regions had nearly the same HA levels. This would imply that in some brain regions, mast cells may contain up to a quarter of the measurable brain histamine. One major drawback to this report is that the control brain HA concentrations appear abnormally high and show little regional distribution, at variance with other reports in which several other methods to determine mouse brain histamine were used (Taylor and Snyder, 1972b; Lee and Fennessy, 1976; Schatz et al.,1978). In a recent report it was shown that brain histidine decarboxylase activity of normal (+/+) and mutant (W/W^V) mice were nearly the same (Maeyama et al., 1983) which is consistent with reports claiming that histidine decarboxylase activity in mast cells is low (Baudry et al., 1973; Martres et al., 1975). Injection of α-fluoromethylhistidine (α-FMH), a selective inhibitor of histidine decarboxylase activity (Kollonitsch et al., 1978), decreased HD activity in both strains to 10% that of untreated mice. Yet, while brain HA levels in (+/+) mice decreased to about half that of untreated, the measurable HA concentration in mutant mouse brains decreased by 50% in 30 minutes

and almost disappeared by 6 hours. This report is supported by that of Garbarg et al. (1980b) who reported brain HA content to be reduced by half following α-FMH in normal mice. Tissues where mast cell HA content is proportionately greater than in the "rapid-turnover HA pool," e.g. the skin, evidenced no significant changes in histamine levels after α-FMH. This is consistent with the idea that HD activity is an indicator of a pool of HA that is rapidly turning over, possibly neuronal, and that mast cells have low HD activity with a slow turnover of histamine. Brocresine and α-hydrazinohistidine, both HD inhibitors, have also decreased rat brain HA concentration to about one half of control levels (Taylor and Snyder, 1971). This is at variance with lesion data which indicated that unilateral medial forebrain bundle interruption caused rat hippocampal HA levels to decrease about 70% below that of contralateral levels (Garbarg et al., 1974).

These studies suggest at least two compartments of brain histamine, a mast cell-like compartment and another that is rapidly turning over, possibly neuronal. Even so, while these findings are attractive for a neuronal presence and seem to confirm a mast cell presence, further qualification is warranted. The mast cell deficient mice have other obvious anomalies, the biochemical and neurochemical natures of which are still unknown. In addition, not all investigators agree. For example, Hough et al. (1983) also found W/W^{V} mouse brains devoid of mast cells, but found no difference in HA levels of any brain regions investigated compared to +/+ mice.

Lastly, the fact that α-FMH depletes a HA pool that is rapidly turning over does not prove that the remainder resides exclusively within the mast cell population. While the issue of HA presence has focused on mast cell and neuronal locations in the CNS, the possible existence of other HA storage sites has been little explored. A HA compliment in NLM cells (or type II mast cells) cannot be ignored (Ibrahim, 1974a). Non-mast cell HA was reported in vascular walls (El-Ackad and Brody, 1975) and in the capillary-rich fractions of the brain (Karnushina et al., 1980). The presence of HA in astrocytoma cells has received almost no attention.

X. PUTATIVE HISTAMINERGIC PATHWAYS IN THE MAMMALIAN BRAIN

A. Lesion Studies: Biochemical and Neurochemical Evaluation

The most abundant evidence for possible histaminergic pathways has been of a neurochemical nature. Ascending and descending histaminergic pathways have been localized in mammalian brain through the neurochemical evaluation of lesion-induced degeneration of neural tissue. Histochemical and immunofluorescence methods were previously unsuccessful due to an insufficient sensitivity to detect neuronal stores of histamine and the concomitant existence of non-neuronal histamine stores in vascular walls and mast cells (Baudry and Martres, 1975; Martres et al., 1975; El-Ackad and Brody, 1975; Edvinsson et al., 1977). Several successful approaches have utilized histidine decarboxylase (HD) and histamine (HA) as neurochemical markers of histaminergic neurons following mechanical (Garbarg et al., 1974; Dismukes et al., 1974) electrocoagulation (Garbarg et al., 1976) or kainic acid lesions (Garbarg et al., 1978). Histamine N-methyltransferase activity was initially evaluated as a possible neurochemical marker (Garbarg et al., 1973); however, it was found to be unsuitable since it was lesion insensitive. These experiments and an abundance of other data led to the conclusion that HMT is probably located post-synaptically (Garbarg et al., 1973; Bischoff and Korf, 1978) or non-neuronally (Garbarg et al., 1978). In contrast, histidine decarboxylase serves as a specific marker since it is the specific decarboxylase responsible for HA synthesis, roughly parallels the brain distribution of HA and has a sub-cellular distribution consistent with its presence in the cytoplasm of cortical nerve endings (Baudry and Martres, 1975; Baudry et al., 1973) and synaptic vesicles (Kataoka and DeRobertis, 1967). It was shown that while cortical norepinephrine and serotonin levels have decreased and DOPA decarboxylase and 5-hydroxytryptophan decarboxylase activities have been significantly reduced (both >45%) after intraventricular and intracisternal administration of 6-hydroxydopamine (250 µg), cortical histidine decarboxylase remained unaltered (Garbarg et al., 1976).

Reduced concentrations and enzyme activity of the former were also noted after 40 µg of 5,6-dihydroxytryptamine administration while again HD activity remained constant, demonstrating an anatomical independence from these monoaminergic systems. Histamine levels can also be monitored in various regions after deafferentation. Although the reductions of HD activity and HA levels parallel one another, they do not fall by similar magnitudes of percent because of a near constant amount of non-neuronal HA present in such pools as mast cells (Garbarg et al., 1976). The evaluation of HA content in P_2 and supernatant fractions can be used as a sensitive marker of non-mast cell and presumably neuronal HA (Dismukes et al., 1974); whereas that of the P_1 (crude nuclear) fraction, comprising mainly mast cell or mast cell-like HA (Baudry and Martres, 1975) remains essentially constant after lesion experiments. The decreases of HA concentration and HD activity have been correlated with decreased HA formation based on studies of the formation of ^{3}H-HA from ^{3}H-His (Pollard et al., 1974; Garbarg et al., 1976). This served as an index of the rate of HA synthesis and was another approach used to determine HA afferent fiber localization.

Lesions have been made unilaterally in the lateral hypothalamic area where the medial forebrain bundle (MFB) presents itself as a compact, uncrossed bundle in the rat (Guillery, 1957) and human (Carpenter, 1978). They resulted in the lowering of rat ipsilateral cortical HD activity throughout diffuse regions of the telencephalon as early as 2 days post-lesion; maximal reduction occurred by day 8 exhibiting a half-life of 2 days (Garbarg et al., 1974; Schwartz et al., 1975). This value was consistant with Wallerian degeneration and almost identical to that observed in the forebrain for tryptophan hydroxylase after lesioning of the midbrain raphe (Kuhar et al., 1971a). HD activity was not significantly altered caudal to the lesions. Reduced HD activity was most pronounced in the anterior hypothalamus, the pyriform, cingulate, frontal, parietal, temporal and occipital cortices, olfactory bulb, striatum and the

hippocampus (Schwartz et al., 1976). The latter region has been closely studied.

Selective lesions at dorsal and ventral sites demonstrated that HA synthesis in the hippocampus probably occurs in nerve terminals (HD activity being altered mainly in P_2 and soluble fractions) of axons of extrinsic origin (Barbin et al., 1976). The dorsal pathway to the hippocampus, comprising the fimbria, fornix superior and the cingulum, was believed to contain approximately 60% of the histaminergic input since HD activity decreased 62% after dorsal transection whereas the ventral system eminating from the amygdaloid area (by similar reasoning) holds about 35% of this input (Barbin et al., 1976). This is similar to the serotonergic and cholinergic input (Storm-Mathisen and Guldberg, 1974; Lewis et al., 1967) to the hippocampus whose afferent pathways are thought to be predominantly dorsal. This HA synthesizing capacity as reflected in HD activity showed a variation in the afferent density with the subiculum receiving the greatest input and the area dentata the least (Barbin et al., 1976). The time course for the reduction of enzyme activity of both DOPA decarboxylase and histidine decarboxylase after hippocampal deafferentiation is grossly similar to that observed in the cortex after interruption of the MFB, a process causing afferent degeneration. In contrast to HD and DOPA decarboxylase activities, it has been found that the hippocampal levels of HA first increased 2-3 days after unilateral MFB lesions (+43%), then fell below contralateral HA levels (-70%) by day 6 (Barbin et al., 1976). This was attributed to a diminished release of the amine after fiber interruption prior to degeneration, a profile similarly observed in the forebrain (Garbarg et al., 1976). Again, the regions caudal to the lesion sites did not show altered HD activity.

Kainic acid has also been useful in lesion studies because it is effective in selectively destroying neuronal cell bodies (Coyle, 1983). Injection of kainic acid (1.5 µg) into the hippocampus resulted in a decrease in the activity of glutamic acid decarboxylase, a selective marker of GABAergic neurons,

confirming the neurotoxicity of kainic acid towards the GABAergic neurons intrinsic to the hippocampus. However, HD activity was not significantly decreased (Garbarg et al., 1978b). The fibers containing HD activity are therefore most likely to be extrinsic to the hippocampus, suggesting an afferent neuronal localization for this enzyme (Garbarg et al., 1978a; 1978b).

Histamine afferents have also been found to innervate the amygdaloid complex and the bed nucleus of the atria terminalis (BST) (Ben-Ari et al., 1977). The higher HD activity in the various nuclei of these structures suggests a high degree of HA fiber input into the medial, central and basomedial nuclei of the amygdaloid complex and ventral segment of the BST below the anterior commissure. HD activity showed no alteration following stria terminalis lesions whereas combined stria terminalis and ventral pathway lesions decreased HD activity about 60% in all nuclei of the amygdala but not that in the BST. However, MFB lesions decreased HD activity in both the amygdala and BST. On the basis of these observations, two HA pathway divisions arising from the MFB have been suggested. One system projects to the BST of which the ventral input seems to be quantitatively the more important while the other course penetrates the amygdala ventromedially along the ansa peduncularis and preferentially innervates the more medially located nuclei. It is noteworthy that the ventral regions of the BST are particularly rich in norepinephrine (Brownstein et al., 1974a) and profusely innervated by fibers containing dopamine β-hydroxylase (Swanson and Hartmen, 1975). The central and basolateral nuclei of the amygdala have relatively high concentrations of dopamine and norepinephrine (Brownstein et al., 1974a) as well as a high tyrosine hydroxylase activity (Ben-Ari et al., 1975). This would be consonant with the idea of histaminergic fibers being one part of a major circuit of monoaminergic fibers.

A descending histaminergic pathway has also been postulated. Although the brainstem of the rat is a region of low global histamine concentration and

reduced HD activity, several individual nuclei have been found to have high HA levels and HD activity. HD activity and HA content appear to be highest in the substantia grisea centralis, nucleus interpenduncularis, area tegmentalis ventralis and substantia nigra suggesting HA fiber input and as such were monitored following lesion experiments (Pollard et al., 1977). Total mechanical sections made just rostral to the hypothalamus failed to modify hypothalamic or brainstem HD activity. However, when lesions were performed at either the posterior regions of the hypothalamus or in the rostral mesencephalon, HD activity sharply decreased in the substantia grisea centralis and in the surrounding ventral brain stem (Pollard et al., 1978). The time course for this decrease in activity was consistent with a neuro-degenerative process. On the other hand, when electrolytic lesions were made in the four discrete nuclei mentioned above, the telencephalic HD activity was not altered (Barbin et al., 1977) suggesting that these regions do not contain the cell bodies for a system ascending through the MFB. It therefore follows that the cell bodies of this descending pathway(s) (and those for an ascending system(s) based on the above data) may lie in the diencephalon. Indeed, 10 days after electrolytic lesions centered in the mammillary region, HD activity decreased (-15% to -35%) in all telencephalic areas investigated.

It was apparent that lesions made rostral to the hypothalamus had little effect on caudal HA content or HD activity, whereas lesions made caudal to the anterior midbrain had failed to alter hypothalamic or cortical histaminergic markers. This fact and the heterogeneity of hypothalamic and brainstem HA levels and HD activity made it likely that the cell bodies of these putative fibers lie in this vicinity. To test this hypothesis (Schwartz et al., 1979a), small selective lesions were made in restricted sections of the hypothalamus and upper midbrain of 80 rats. The neurochemical changes occurring in the cortex and striatum were then studied up to two weeks post-lesion. The exact location

and volume of each lesion were determined upon histological analysis and were correlated with the reduction of HD activity by computer analysis. By determining the minimal lesioned area responsible for the maximal depletion of HD activity in the cortex and striatum, the "posterior plane" and "efficient lesion areas" were elucidated. The posterior plane, a theoretical plane which defined the most anterior brainstem section failing to affect telencephalic HD activity, was caudal to the reticular formation near the level of the ventral tegmentum (Schwartz et al., 1979b). The efficient lesion areas were defined as ablated regions responsible for reduction of HD activity exceeding 30% with at least 75% efficiency. This was presumed to comprise the neuronal cell bodies of the ascending histaminergic pathway (Garbarg et al., 1980a). With this analysis, the projections to the cortex were postulated to eminate from the mesencephalic reticular formation. The striatal fibers came from a different region with their origin localized rostrally in the posterior mammillary bodies near where the cortical bound fibers are thought to join the MFB (Schwartz et al., 1978; 1979b; Gargarg et al., 1980a). These cell bodies and putative pathways are depicted from Schwartz et al. (1979b) in Figure 10.

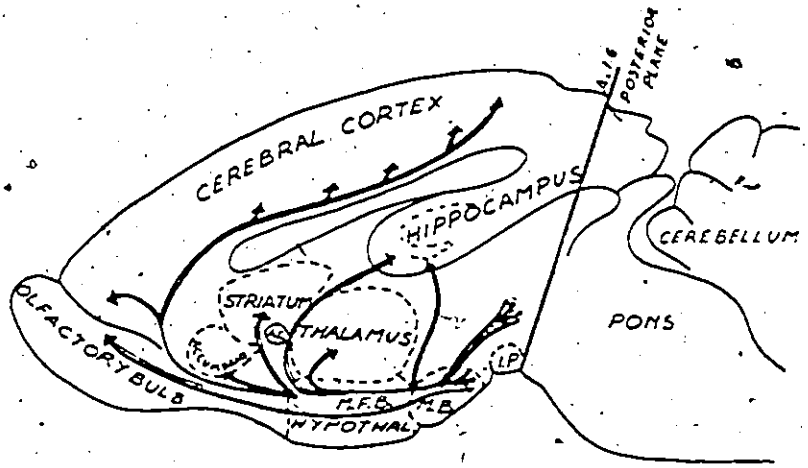


Fig. 10

To confirm these alleged localizations, four separate approaches were examined: (1) A hemisection performed just caudally to these putative cell bodies at A1.6 (Konig and Klippel atlas, 1970) did not affect cortical nor striatal HD activities (Garbarg et al., 1980a); (2) 48 hours after injection of 20 µg colchicine (i.c.v.) at a dose reported to inhibit axonal transport (Rose and Sinha, 1976), cortical and striatal HD activity were significantly decreased 25% and 42%, respectively. A concomitant increase in reticular enzyme activity (26%) was also observed; (3) Midbrain reticular infusion of 1 µg of kainic acid significantly lowered both cortical (18%) and reticular (49%) HD activity, yet striatal activity remained unaffected; (4) The ratio of soluble HD activity (presumed to be present largely in cell bodies) to "particulate" enzyme activity (enzyme occluded in nerve endings) was significantly higher in the mammillary bodies and reticular formation than in the cortex (Schwartz et al., 1979b; Garbarg et al., 1980a).

While the above appears to establish the localization of histaminergic cell bodies, it should be noted that critical evaluation of the above is difficult since a detailed description of the methodology and results is currently unavailable. A journal reference which cites experiments confirming cell body existence and localization, states that it was submitted for publication, yet it has not appeared. Writing to the first author went unanswered. While the data is highly provocative, some qualifications may be required. Colchicine, which is known to impair axonal transport, was not directly shown to do so in these experiments. In addition, kainic acid infusion into the mammillary bodies also reduced cortical HD activity. From these data, we can speculate that (a) the putative cell bodies for the cortex may also eminate from the mammillary bodies; (b) that at the dose of kainic acid used a significant portion of fibers traversing this cell group were also severely affected, or (c) that the hypothetical localization of at least some of these cell bodies is in error. Lastly, the soluble versus particulate HD activity is a function of the method of fraction preparation which was not mentioned. As these observations have appeared in review

articles only (Schwartz et al., 1979b; Garbarg et al., 1980a; Schwartz et al., 1981a) the description of the methodology used to obtain these results is of course limited. However, the data tend to confirm the concept of histaminergic pathways eminating from these regions. These conclusions have received some support from the studies of Chronister et al. (1982) which showed that nuclei lateral to the mammillary nuclei project to the nucleus accumbens in the rabbit. Serial sectioning following either iontophoretic or controlled pressure injection of horseradish peroxidase (HRP) into the nucleus accumbens revealed HRP positive neurons. The rank order of positive neurons were: nuclei gemini > supramammillary complex > lateral mammillary region > neurons scattered between the mammillary bodies and the ventral tegmental area. While this demonstrated that connections exist between the rostral mesencephalon/caudal diencephalon and the nucleus accumbens, they were not verified to be histaminergic. Nevertheless, they are reasonable candidates for HA cell bodies. In addition, Segal and Landis (1974) showed in the rat that areas adjacent to the mammillary nuclei project to the nucleus accumbens and other regions postulated to receive HA fiber input, e.g. hippocampus (Barbin et al., 1976).

Additional lesion data from other laboratories have supported the existence of histaminergic neurons. Sperk et al. (1981b) infused kainic acid (1 µg) into the left caudate of the rat and observed reduced striatal choline acetyltransferase and glutamate decarboxylase activities to 18 and 20% of controls, respectively. Striatal histamine levels more than doubled within 2 days but persisted up to 10 weeks post-lesion. The former observation is highly consistent with previous reports. However, the persistence of the HA elevation is not. A similar pattern of prolonged elevation has also been observed for striatal dopamine and serotonin (Andersson et al., 1980). These histaminergic changes were suggested to be in the neuronal compartment since MFB lesions abolished this effect and the synaptosomal HA levels rose while that of the P_1 fraction

was unaffected. Histidine decarboxylase activity was found to decrease. The disappearance rate of HA following administration of α-fluoromethylhistidine, a highly selective HD inhibitor (Kollonitsch et al., 1978) to lesioned rats was also reduced. Specific binding of ^{3}H-histamine in the striatum of the lesioned side decrease 40%, presumably due to cell body damage. Also, no accumulation of mast cells in the damaged area was observed using light microscopy (Sperk et al., 1981b). The authors speculated that there might be a decrease in HA release, presumably from afferent neurons, and a subsequent reduction in the rate of its synthesis leading to decreased rates of turnover. While the determination of HA levels per se in kainic acid treated tissue was a novel approach, the observed reduction in HD activity is at variance with similar lesions of the guinea pig hippocampus where no change in enzyme activity occurred (Garbarg et al., 1978a). Perhaps the elevation of HA content and the reduction of HD activity in kainic acid lesioned tissue represent, at least in the striatum, more complicated processes than are currently thought to occur. Further clarification of this issue is clearly warranted.

Sensitivity of the cyclic AMP generating system to histamine was shown to be altered in rat cortical slices for up to 3 weeks following interruption of the medial forebrain bundle (Dismukes et al., 1975). Formation of cyclic AMP in response to 100 μM histamine was significantly greater (43%) in anterior tip forebrain slices from the ipsilateral hemisphere, a response similar to that seen with norepinephrine (+39%) and isoproterenol (+42%). The response could be partially blocked by d-bromopheniramine, an H_1 antagonist, but was nearly abolished with metiamide, an H_2 antagonist, each at 10 μM concentration. The enhancement of this cyclic AMP accumulation was attributed to denervation hypersensitivity due to interruption of putative fibers in the MFB. Using a similar approach, but with guinea pig hippocampal and cortical slices (which show a greater cyclic AMP response to HA than rat brain), Garbarg et al. (1978b) were

unable to observe denervation hypersensitivity following MFB lesions.

B. Electrophysiological Studies

The biochemical and neurochemical studies are supported by electrophysiological evidence for histaminergic neuronal pathways. The cerebral cortical neuron discharge rate is known to diminish following iontophoretic application of histamine (Phillis et al., 1968). This reduction occurred if 4-methylhistamine, an H_2 receptor agonist, was applied but was unaffected upon application of the H_2 agonists, 2-pyridylethylamine or 2-methylhistamine. The failure of mepyramine to selectively block these depressant actions and the effective antagonism of histamine-induced depression by metiamide infer that these actions may be mediated by the H_2 receptor (Sastry and Phillis, 1976a). This observation was used as the basis for a general test for a possible physiological response to neuronally released histamine. The ipsilateral cortical neuronal firing rate of anesthetized rats was evaluated following MFB stimulation (Sastry and Phillis, 1976b). MFB stimulation with 4 (0.1 ml, 200 Hz) pulses inhibited spontaneous firing for a duration of 179 ms. Metiamide (30-50 nA) reduced the duration of inhibition by 35% on 21 neurons. Exogenous HA (40-60 nA) was evaluated on 14 of these neurons and found to depress the firing which was reversibly antagonized by metiamide. The results are clearly consistent with the hypothesis that HA is released from stimulated neuronal fibers which are part of the medial forebrain bundle. However, this data could also be consistent with the notion that another transmitter fiber system stimulates cortical histamine release locally from mast cells, interneurons or other possible histamine stores. The failure to use other H_2 agonists and antagonists and the inability to precisely determine the concentrations of histamine and metiamide applied do not negate the possibility of artifactual results.

Studies of Haas and Wolf (1977) confirmed the antagonistic effects of metiamide on cortical inhibition resulting from MFB stimulation which presumably

led to local HA release. Direct cortical inhibition was not strongly influenced by metiamide. Exogenous HA at the currents applied also showed little effect. This suggested that it was the intimate contact of locally released HA with its receptor which was antagonized by metiamide. Stimulation of the fornix inhibited hippocampal neurons and microelectrophoretically applied metiamide reversibly reduced this effect. In a combined biochemical and electrophysiological study (Haas et al., 1978), electrolytic lesions of guinea pig medial forebrain bundle reduced DOPA decarboxylase and HD activities and histamine content in the cortex. However, after 8-20 days post-lesion, the depression of cortical neurons by histamine and norepinephrine showed a clear hypersensitivity. Lower HA ejection currents were required to reach threshold (-78%) and a greater neuronal depression occurred (-47%) on the side ipsilateral to the lesion compared to the intact side. Norepinephrine had similar effects (-72% and -54%, respectively) while responses to GABA were less marked. These hypersensitivity effects of HA were more likely due to post-synaptic changes since, unlike noradrenaline, there is no presynaptic rapid HA uptake system which is impaired following lesions nor was HMT activity affected (Haas et al., 1978). Conversely, a hypersensitivity to HA-stimulated cyclic AMP formation in the cortex or hippocampus was not observed as the maximal response and EC_{50} of HA were the same on ipsilateral and contralateral sides. These findings are in agreement with those reported by others (Garbarg et al., 1978b; Dismukes et al., 1976) using guinea pig brain slices. As both responses were due to H_2 receptor activation, it was suggested that the receptors mediating these actions are distinct. The presence of non-neuronal cyclic AMP synthesizing systems which are unaffected by lesions may be another possibility (Clark and Perkins, 1971; Karnushina et al., 1980).

C. Histofluorescence Studies

Fluorescence techniques have recently added more credibility to the neurochemical data derived mostly from lesion experiments. Quinacrine HCl

binding to mouse brain tissue (in vitro) was suggested to be a potential indicator of histamine receptors and/or histaminergic neurons (Orr and Wordinger, 1980). However, a strict neuronal interaction is in doubt since the quinacrine-derived fluorescence did not correlate with regions known to contain substantial HA levels nor HD activity. Its lack of binding in the presence of mepyramine but not cimetidine (each 100 μM) suggest it may be binding to an H_1 receptor. Quinacrine is also known to have a high affinity for HMT (Thithapandha and Cohn, 1978), which most likely has a post-synaptic localization (Bishoff and Korf, 1978).

The recent development of a fluorescent antibody to histidine decarboxylase from rabbit plasma following its near total purification has great potential for the examination of the distribution of the histaminergic system in the mammalian CNS. Using this probe, HD was reported to be localized in the hypothalamus, mammillary body, amygdala and hippocampus dentate gyrus and in fibers of the bed nucleus of the stria terminalis in rat brain (Tran and Snyder, 1981). A more extensive evaluation has yet to be achieved. Another preparation may be possible using whole fetal rat body HD which, when injected into rabbits, elicits antibodies that have been reported to cross-react immunologically with the adult rat brain enzyme (Fukui et al., 1980). However, rat fetus and rat brain HD may in fact be isozymes as the latter investigators showed that enzymes from these sources had different isoelectric points, although similar K_m values for histidine and similar molecular sizes.

Recently, histamine-like immunoreactivity was localized in rat brain sections fixed in formaldehyde using a histamine-methylated bovine serum albumin (BSA) complex generated antisera from guinea pigs (Wilcox and Seybold, 1982). Specificity for histamine was deduced since preincubation of the primary antiserum with this new antigen, HA-methylated BSA, eliminated staining but antiserum incubation with methylated BSA alone did not. Thus, the positive immuno-

staining due to antibodies recognizing the endogenous histamine/protein complex was the sole criterion for histamine specificity. Loading rats with histidine (1 g/kg i.p.) 30 minutes prior to sacrifice enhanced the immunoreactivity. To evaluate the existence of putative histaminergic neurons and cell bodies, several approaches were attempted. Rats treated with colchicine (50 µg, i.c.v.) showed immunoreactive cell bodies localized in the lateral hypothalamus at the level of the median eminence. These cell bodies were arranged in an arched configuration extending to the optic tracts from the dorsal tip of the third ventricle. Positive staining for fibers was observed in the hypothalamus, cortex, amygdala and hippocampus. The highest density of fibers was in the hypothalamus, particularly in the median eminence and areas lateral to it. A very dense network of beaded-like fibers was observed in the mammillary nuclei. Pretreating the rats with compound 48/80 (administered i.p. in increasing doses up to 2.5 mg/kg over 3 days) did not alter the staining characteristics suggesting that mast cells or other non-neuronal HA stores were not interfering with neuronal immunostaining. Treating the rats with colchicine, compound 48/80 and histidine had the effect of enhancing cell body immunoreactivity only (Wilcox and Seybold, 1982). Reasons why neuronal but not non-neuronal HA stores are sensitive to this technique were not stated by the authors nor was there an in depth analysis clearly indicating that this method is highly specific for HA only. Furthermore, it was not ascertained if the 48/80 dosing schedule effectively degranulated brain mast cell histamine reserves. Nevertheless, these observations are supported by the abundance of lesion data presented earlier and are consistent with the known electrophysiological evidence. The general pathways although not elaborated upon by the authors, were asserted to have a distribution different from that of the catecholamines (Falck et al., 1962) and serotonin (Steinbush, 1981). The rather diffuse pattern of putative cell bodies and the fibers which emanate from them may possibly account for some of the difficulties

previously encountered by investigators searching for a densely compacted bundle of histaminergic neurons. While such observations are a necessary first step in firmly localizing these putative pathways, this technique needs confirmation. Data will then be required to prove that the HA and presumably HD contained in these fibers are not merely part of another fiber system, but subserve a unique neuronal presence and possible function of their own.

XI. CENTRAL HISTAMINE RECEPTORS

A. Overview of Histamine Receptor Agonists and Antagonists

There are several points which must be borne in mind when discussing the pharmacological evaluation of central histamine receptors. It is important not to overestimate the selectivity of various agents acting as agonists or antagonists at histamine receptors. For example, dimaprit (Durant et al., 1977; Parsons et al., 1977) and impromidine (Durant et al., 1978) are highly selective H_2 receptor agonists with negligible H_1 stimulatory activity (Table 2). The relative activities of dimaprit and impromidine for stimulating the guinea pig atrium is 10^6 times greater than its stimulation of guinea pig ileum contraction, an H_1 mediated event (Parsons et al., 1977; Durant et al., 1978). However, the remaining agonists (which are often used to define H_1 or H_2 receptor actions) are full agonists on both receptor populations. Therefore, at low concentrations they may appear to be somewhat selective but at high enough concentrations they stimulate both receptors. For example, at a concentration of 100 μM, 2-methylhistamine produces 97% of the maximal response at H_1 receptors. Yet at the same concentration this selective H_1 agonist stimulates the H_2 receptor to 80% of its maximal response. Under such circumstances attributing an observed response to specific H_1 receptor activation would be erroneous since at this concentration both receptors could be stimulated to a large extent (Green et al., 1978). Even greater ambiguity would result if H_1 and H_2 stimulation independently caused

Table 2

Relative Selectivity and Potency of Several Histamine Agonists[a]

Agonist	Selectivity	Relative Agonist Potency[b]	
		Guinea pig Ileum (H_1)	Guinea pig Right Atrium (H_2)
Histamine	H_1 and H_2	100	100
2-Methylhistamine	H_1	17	5
2-Thiazolylethylamine (TEA)	H_1	26	0.3
4-Methylhistamine	H_2	0.2	43
Dimaprit	H_2	$<10^{-4}$	70
Impromidine	H_2	$<10^{-3}$	4800

a) Adapted from Schwartz (1979).

b) Relative agonist potency calculated from the equation

$$\frac{EC_{50}\ \text{Histamine}}{EC_{50}\ (\text{agonists})} \times 100$$

actions which were physiologically antagonistic. Even when cognizant of such factors the identification of receptors solely by selective agonist stimulation is limited by many events, often unidentified, which take part in eliciting an eventual effect. Such unidentified events could be one agonist having tissue uptake properties which are different from another or an unequal rate of metabolism. In addition, endogenous histamine, acetylcholine or catecholamine release may account for apparent histamine agonist-like activity.

Competitive drug antagonism of established agonist effects leads to less ambiguity in identification and classification of receptors if such studies are approached judiciously. Parallel dextral displacement of the agonist dose-response curve by a potential antagonist demonstrates that both agents are acting on the same receptor. By Schild analysis, one can estimate the apparent dissociation constant of the antagonist, K_B. This is the concentration of antagonist needed to give a dose ratio of 2, i.e. the concentration of an antagonist that requires twice the agonist concentration to obtain the identical response as obtained in the antagonist's absence. Antagonism is assessed by determining the dose-ratios of agonist required to produce equal responses in the absence and presence of various concentrations of a particular blocker. The K_B can be obtained graphically on a Schild plot whereby the logarithm of the dose-ratio minus 1 is plotted on the ordinate against the logarithm of the antagonist concentration. A slope of 1 indicates competitive antagonism and the intercept of the abscissa yields the pA_2 value which is numerically equal to the negative logarithm of the antagonist dissociation constant, K_B. Greater confidence in evaluating competitive antagonism and identifying receptors is obtained by determining the K_B values using different agonists since the dextral shift of dose-response curves is a function of antagonist affinity for the receptor and is independent of the agonist affinity (Black et al., 1982). For example, mepyramine was shown to have approximately the same pA_2 values on the guinea

pig ileum H_1 receptors when stimulated by six different histamine analogues having agonist activities (Ash and Schild, 1966). Burimamide was confirmed as an H_2 competitive antagonist partially because its pA_2 values were nearly indistinguishable regardless if the agonists histamine, 4-methylhistamine or 2-methylhistamine were used to stimulate guinea pig atria (Black et al., 1972). In a situation analogous to receptor stimulation, exaggeration of antagonist selectivity can also lead to difficulties. This has sometimes been the case when the various H_1 and H_2 antagonists have been used at concentrations high enough to generate doubts about their selective action (Green et al., 1978). The H_1 antagonists generally have dissociation constants in the 0.1 to 10 nanomolar range. See Table 3. On the other hand, the H_2 blockers generally have less affinity for the H_2 sites than the H_1 blockers have for the H_1 receptors. K_B values of H_2 antagonists for their receptors are almost always higher, sometimes in the micromolar range, i.e. a hundred to a thousand times greater than the K_B values of H_1 antagonists (Table 3). Higher concentrations of H_2 antagonists are therefore required to block H_2 receptors than are concentrations of H_1 antagonists required to block H_1 receptors. It is apparent that an examination of H_1 or H_2 receptor involvement by comparing H_1 and H_2 antagonists both at the same single concentration could yield misleading results. At micromolar antagonist concentrations H_1 receptor blockade is far more complete than H_2 blockade. Investigations utilizing an antagonist at concentrations of more than a hundred times its dissociation constant profoundly increase the possibility of blocking other receptors or affecting other processes.

The histamine blockers are also known to influence systems far removed from the histamine receptor, a property for which the H_1 antihistamines are already well known (Douglas, 1980). Elevated concentrations of H_1 antagonists have anticholinergic and local anesthetic properties. Concentrations greater than 10^{-6} M also block H_2 receptors of the guinea pig atria and H_2 activated

Table 3

Relative Selectivity and Affinity of Several Histamine Antagonists[a]

Antagonist	Selectivity	Antagonist Affinity (K_B, pA_2)	
		Guinea Pig Ileum (H_1)	Guinea Pig Right Atrium (H_2)
Mepyramine (Pyrilamine)	H_1	0.5×10^{-9} (9.3)	-
Burimamide	H_2	3×10^{-3}	7.8×10^{-6} (5.1)
Metiamide	H_2	10^{-3}	0.9×10^{-6} (6.0)
Cimetidine	H_2	45×10^{-3}	0.8×10^{-6} (6.1)
Ranitidine[b]	H_2	10^{-4}	1.5×10^{-8} (7.8)
Tiotidine[c]	H_2	10^{-3}	3.7×10^{-8} (9.4)
Doxepin[d]	H_1 and H_2	60×10^{-12} (10.2)	1.7×10^{-8} (7.8)

a) Adapted from Schwartz (1979)

b) Yellin et al. (1979)

c) Gajtkowski et al. (1983)

d) Kanof and Greengard (1978)

adenylate cyclase in brain homogenates (Green et al., 1977). H_2 antagonists also demonstrate "extra-histamine" effects. Burimamide blocks α-adrenergic receptors and releases catecholamines (Brimblecombe et al., 1976). Cimetidine is a non-competitive blocker of β-adrenergic receptors in the uterus and can block muscarinic and H_1 receptors in the ileum (Brimblecombe et al., 1975). Thus, a background knowledge of these factors which limit a careful analysis of histamine receptors must be applied to surveys of the literature and future research in this area. Lastly, failure to use several antagonists to study a receptor mediated event leaves an important question unanswered, i.e. is a change due to receptor blockade or an effect mediated by the antagonist at non-receptor sites?

While the preceeding discussion draws mainly from the evaluation of peripheral histamine receptors due to their relative ease of study, the same principles apply to the CNS where quantitative, well characterized responses are seldom observed and difficult to assess. The precise local drug concentrations at receptor sites in vivo are generally not known, enhancing our need for critical evaluation of receptor data.

B. Limitations of Binding Studies

While abundant data is being promulgated on the scientific community concerning receptor binding in mammalian brain, one must remember that a clear distinction exists between binding sites and receptors. Only after an exhaustive study which successfully links functional responses of receptors to carefully amassed binding data can we infer the former from the latter. In brain, the difficulties in demonstrating this linkage are hampered in many instances by failure to show a clear, quantifiable response to agonists and their blockade with antagonists. Technical difficulties of ligand-receptor non-selectivity and non-specific binding to membrane fragments, filters or glassware (which may appear at first to be specific) are some of the artifacts to be dealt with.

Furthermore, an unambiguous, kinetically distinguishable population of antagonist binding sites may be shown to exist. Yet such binding which is saturable, stereospecific and antagonist displaceable may not be receptor linked. They may instead bind to their own sites of which the receptor undergoing analysis may comprise but a small portion. Binding studies designed to detect histamine H_1 and H_2 receptors are a case in point. False inferences have been derived from such data as well as successful linkage of tissue binding characteristics to receptor-ligand interactions. Therefore, while an in-depth analysis would not be appropriate here, these issues have been dealt with in some detail concerning H_1 and H_2 binding studies.

C. H_1 Receptor Binding Studies

In the guinea pig, rat and rabbit, the tissue with the highest H_1 receptor density, determined by ^{3}H-mepyramine binding, appears to be the brain (Chang et al., 1979a; 1979b). Scatchard analysis of the mepyramine binding to whole guinea pig brain in the presence of triprolidine, another H_1 antagonist, demonstrated a homogeneous population of binding sites. A mepyramine dissociation constant (K_B) of 0.5 nM was consistent with H_1 receptor binding and correlated well with pharmacological effects in the guinea pig ileum, an H_1 mediated response (Tran et al., 1978; Hill et al., 1977; 1978). However, the K_B values for whole rat, rabbit, mouse brains and human frontal cortex differed, i.e. 4.0, 3.9, 3.0 and 1.0 nM, respectively. Binding to all the above tissues was saturable, homogeneous in nature and could be displaced by several H_1 antagonists of diverse structures. Furthermore, binding in these tissues could discriminate between d- and l-chlorpheniramine similarly to the pharmacological response of guinea pig ileum contraction examined in parallel studies (Tran et al., 1978). It appears that in the rat brain membrane preparation, affinities of H_1 antagonist for H_1 receptors are better correlated with K_B values of guinea pig ileal receptors than those of guinea pig brain membranes (Chang et al., 1979b;

Hill and Young, 1980). This may be related to the possible H_1-H_2 receptor interactions to be discussed below. Differential K_B values of mepyramine among species is not unique as other H_1-antagonists share this property (Chang et al., 1979b).

In whole brain membranes of rat, guinea pig, mouse, rabbit and membranes of human frontal cortex, the B_{max} values were similar (4-9 picomoles of ^{3}H-mepyramine bound/g of wet tissue) (Chang et al., 1979b). Yet, the regional brain distribution of these sites differs among species. Adult rats (Tran et al., 1980) showed the highest density in the cerebral cortex, the lowest in the cerebellum while in the guinea pig (Tran et al., 1978) the cerebellum was the highest and the corpus striatum the lowest. In calf brains (Tran et al., 1978), the parietal and occipital cortices had the highest density while the brain stem regions had the lowest. In the human brain (Chang et al., 1979b) (dissected and frozen 3-10 hours after death; age and sex unreported), the frontal, parietal and cingulate cortices had the highest mepyramine binding density, the brain stem regions the lowest. The brain distribution of ^{3}H-mepyramine B_{max} values following intravenous administration to mice was similar to its *in vitro* distribution. The potency of displacement of this ^{3}H-mepyramine binding by other H_1 antagonists in brain was similar to their affinities for the H_1 receptor (Quach et al., 1979; 1980a). In *in vitro* experiments, the subcellular distribution of ^{3}H-mepyramine binding in whole rat brain (Tran et al., 1980) was found to be localized predominantly to the synaptosomal fraction. This profile was similar to quinuclidinyl benzilate (QNB) binding to brain muscarinic receptors (Tran et al., 1980). In a related *in vivo* experiment, several H_1 antagonists displaced ^{3}H-mepyramine binding in particulate fractions with a displacement potency similar to these drugs' affinities for the H_1 receptor (Quach et al., 1980a). However, the correlation was not exact, possibly a reflection of other *in vivo* drug effects such as general drug distribution and blood-brain barrier penetration.

Autoradiography of guinea pig brain slices labeled with ^{3}H-mepyramine showed a high density in the cerebellar molecular layer (Palacios et al., 1979). In the hippocampus, the dentate gyrus was shown to be rich in ^{3}H-mepyramine density while the CA4 area had less and regions CA1-3 the least. Slices of guinea pig and rat brain yielded binding parameters similar to those of homogenates (Palacios et al., 1979; 1981). In the rat, the labelling of the cerebellar molecular layer was far less (Palacios et al., 1979), findings consonant with binding data from homogenates (Tran et al., 1980). In rat brain hypothalamic slices, the supraoptic, suprachiasmatic and ventromedial nuclei had the highest apparent density. Injections of kainic acid into the guinea pig cerebellum caused a decrease in ^{3}H-mepyramine labelling in the molecular layer (Palacios et al., 1981a; 1981b), a region of exceptionally high density binding (Palacios et al., 1979). Since kainic acid is rather selective for neuronal cell bodies (Coyle, 1983), this suggests that the ^{3}H-mepyramine was binding to neurons.

Thus, the evidence that ^{3}H-mepyramine is binding to H_1 receptors in slices and homogenates is rather strong. However, such an inference requires some qualification. The largest body of studies of possible H_1 receptor binding has used ^{3}H-mepyramine exclusively as the labelling ligand. This is almost certainly due to the lack of other suitable ligands from commercial sources, although doxepin and mianserin have been touted as relatively selective H_1 antagonists. A larger spectrum of other antagonists would have enhanced our confidence in selective H_1 receptor binding. Yet the numerous H_1 antagonists of diverse structures which displace ^{3}H-mepyramine *in vitro* and *in vivo* partially overcome this reservation. Failure of non-H_1 active agents to inhibit ^{3}H-mepyramine binding (Quach et al., 1979; 1980a) reinforces the association between mepyramine binding and H_1 occupation. The binding parameters (K_B and B_{max} values) and their correlation with peripheral H_1 receptor studies, uneven regional brain distribution and *in vivo* labelling of general regions and subcellular fractions

strongly argue for H_1 receptor labelling. Nevertheless, we lack firm proof that mepyramine is labelling H_1 receptors exclusively although the greatest portion is probably H_1 associated binding. ^{3}H-Mepyramine binding and/or H_1 receptors are probably not unique to neuronal membranes. This may explain why ^{3}H-mepyramine binding in rat brain has been found to sustain lesions induced by tissue transection, cerebral cortical ablation, 6-hydroxydopamine nigral and intrahippocampal kainate injections (Chang et al., 1980). The discrete H_1 localization argues against blood vessel H_1 receptor association as does mast cell involvement whose regional distribution is quite different (Edvinsson et al., 1976; 1977). H_1-receptor association with glial cells is a possibility consonant with this "lesion survival" (Chang et al., 1980). Also, in none of the species studied does mepyramine binding distribution correlate well with the distribution of endogenous HA nor HD activity. Yet, similar independence between regional endogenous transmitter distribution and receptor binding has been reported for GABA, serotonin and NE, both α and β receptors (see Chang et al., 1979b). The significance of these observations is not clear, yet shows histamine to be similar to established neuroregulators.

Hill et al. (1978) have partially confirmed some of the above observations. They bound ^{3}H-mepyramine with promethazine as the masking ligand to membranes sedimenting at 6,000 x g. Following incubation, they collected the ligand by centrifugation rather than by filtration. They showed ^{3}H-mepyramine density to be greatest in the cerebellum of guinea pig, the region of lowest density in rat brain (Hill and Young, 1980). A major difference was the finding of a second H_1 site with lower affinity for mepyramine when the concentration of this ligand was increased. The K_B of the high-affinity site in guinea pig membranes was almost equal to the mepyramine K_B values of guinea pig intestinal membranes (Hill and Young, 1980). But Hill et al. (1978) reported a B_{max}

substantially higher than Snyder's group in guinea pig preparation. While stereoselectivity was demonstrated, unity Hill coeficient were not. The use of a different type of preparation may account for some of these discrepant findings. A variation in binding and a failure to correlate binding with histidine decarboxylase activity or endogenous histamine levels was also found (Young, 1979).

H_1 receptors prepared by 50,000 x g centrifugation and evaluated after filtration (Chang et al., 1979a; 1979b) are sensitive to proteolytic enzymes and phospholipase A and C. In vitro binding of H_1 agonists to this receptor appears to be regulated by guanine nucleotides and cations, properties encountered with other receptors (Chang and Snyder, 1980). Sodium decreased affinity for histamine and 2-thiazolylethylamine, an H_1 agonist, by over 90 per cent. GTP, its analog, GMP-PNP and GDP decreased H_1 affinity by a half; the GTP and Na^+ effects were additive. In contrast, enhanced affinity by the receptor for the agonists occurred in the presence of Mn^{++} or Mg^{++}. Ca^{++} was essentially without effect (Chang and Snyder, 1980). In contrast, the affinity for H_1 antagonists shows negligible alteration when incubated with these nucleotides or cations. Much of this regulatory behavior was evident in soluble histamine H_1 receptors prepared from guinea pig brain using digitonin as a detergent (Gavish et al., 1979). The potencies of agents competing for ^{3}H-mepyramine binding were reported to be similar to membrane bound receptors suggesting that recognition sites undergoing the solubilization process remain largely intact. However, GTP related regulation was lost (Toll et al., 1980). The isolated H_1 receptor as such awaits purification and reconstitution in addition to confirmation of these general observations.

D. H_1 Receptors Linked to Adenylate Cyclase in Brain Slices

Strong evidence for histamine receptors in mammalian brain came in 1968 with the breakthrough discovery that histamine stimulated cyclic AMP formation

in brain slices (Kakiuchi and Rall, 1968). Histamine receptors were not investigated per se as the clear demonstration of H_2 receptors had not yet been reported. Therefore, the early literature on HA interactions with adenylate cyclase assumes this interaction as if it occurred through a homogeneous receptor population. It is now understood that this is not the case. Also, the inappropriate use of HA antagonists at concentrations several orders above their known K_B values generated added confusion. The H_1 and H_2 receptor involvement in adenylate cyclase activation of brain slices is not evident in homogenates where only the H_2 receptor has been unambiguously shown to be coupled to this enzyme (see section XI.H). The fact that H_1 coupling to the cyclase in slices cannot be shown in homogenates of the same brain regions suggests that conditions for eliciting this effect are inappropriate or that the homogenizing process itself may somehow impair the receptor-enzyme interaction. The exact cause for this has not been ascertained.

The presence of H_1 receptors coupled to brain adenylate cyclase is somewhat tenuous. Enzyme activity could be elicited in guinea pig cerebellar cortical and hippocampal slices, but not in homogenates containing Tris or tris buffer with Mg^{++} and Ca^{++}. Only cell-free preparations in Krebs-Ringer under the mildest of conditions retained histamine-sensitive adenylate cyclase activity (Chasin et al., 1974). Since homogenization abolished this activity and H_2-linked cyclases survive this preparation, the adenylate cyclase active in slices may have been H_1 linked.

Antagonism of histamine-sensitive adenylate cyclase in slices and homogenates of rabbit cerebral cortices was compared after addition of various neuroleptic agents (Spiker et al., 1976). Several neuroleptics and promethazine, which were powerful inhibitors of cyclic AMP stimulation by HA in slices, were very weak antagonists in homogenates. Conversely, thioridazine was a strong antagonist in homogenates but weak in slice preparations (Spiker et al., 1976). This too suggests that the HA-stimulated cyclase in slices differs from that

in homogenates. In special cell-free preparations of guinea pig whole brain, cerebral cortex and hippocampus both H_1 and H_2 receptor linkage to adenylate cyclase was reported (Psychoyos, 1978). However, the tissue preparation was designed to disrupt the tissue mass rather than to form an homogeneous as possible mixture of disrupted tissue constituents. Under such mild conditions, this cell-free mixture may more nearly approximate a mince or a slice than a "standard" homogenate. Unfortunately, the concentrations of 2-methylhistamine and 4-methylhistamine used to stimulate cyclic AMP formation exceeded 500 μM - almost certainly causing non-specific receptor activation. Tripelennamine and metiamide, H_1 and H_2 antagonists, respectively, were both used in concentrations of 0.3 μM to 10 μM (Psychoyos, 1978), again at concentrations casting doubts on selective antagonism.

Antagonism of HA-stimulated cyclic AMP elevation by H_1 blockers could be shown in slices of guinea pig cerebral cortex and hippocampus (Chasin et al., 1973). These elevations could be completely blocked by the H_1 blockers, pyrilamine, chlorpheniramine and SQ 10648, but at concentrations of 1-100 μM. These antagonists were not as effective in the hippocampus where they inhibited about 50% of the cyclic AMP elevation in the same concentration range (Chasin et al., 1973). In experiments more mindful of antagonist concentrations, HA-induced accumulation of cyclic AMP in slices of guinea pig cerebral cortex required both H_1 and H_2 receptor occlusion for maximal inhibition (Baudry et al., 1975). Mepyramine and metiamide were both able to block the effect of HA in a concentration-dependent manner; yet only about 50% in each case. 4-Methylhistamine was able to elicit a response that could be abolished by metiamide, but not by mepyramine. This suggested that both H_1 and H_2 receptors mediate the HA response in slices from guinea pig brain (Baudry et al., 1975). A similar conclusion was reached simultaneously in studies using several antagonists to inhibit HA-elicited accumulation of cyclic AMP in slices of guinea pig cortex and hippocampus

(Rogers et al., 1975). In this study, about 80% of the HA-response was attributed to H_1 receptor-linked mechanisms. This may be due to the excessive concentrations of H_1 antagonists used, e.g. d-brompheniramine at 100 μM, which probably also occluded a significant portion of H_2 receptors. Metiamide (10 μM) inhibited about 35% of the cortical and 55% of the hippocampal histamine-stimulated response (Rogers et al., 1975).

Dismukes et al. (1976) also suggested that two populations of HA receptors mediate the HA-cyclic AMP response based on differential responses to H_1 and H_2 agonists and adenosine in guinea pig cortical and hippocampal slices. The H_1 agonist, 2-thiazolylethylamine (TEA), stimulated cyclic AMP formation in the absence and presence of adenosine (Huang et al., 1971). Addition of the latter increased TEA's maximal response and decreased the EC_{50} by about 90%. Without adenosine, TEA was antagonized by both H_1 and H_2 antagonists while only H_1 antagonism was evident in adenosine's presence. The H_2 agonist, 4-methylhistamine, stimulated a smaller accumulation of cyclic AMP than TEA in both tissues. Addition of adenosine caused less synergism. Without adenosine, 4-methylhistamine stimulation was blocked by metiamide, but the presence of adenosine necessitated both H_1 and H_2 antagonism for maximal inhibition. These studies confirmed previous reports of enhanced cyclic AMP elevation by HA in the presence of adenosine. It also suggested that adenosine may have a direct effect on the H_1 and H_2 receptors mediating this effect. Dismukes et al. (1976) concluded that adenosine increased the affinity of the H_1 receptor for both H_1 and H_2 agonists. However, in none of the above studies which suggested a dual receptor involvement were dissociation constants or relative agonist potencies examined. In addition, several reports (Psychoyos, 1978; Chasin et al., 1973; Rogers et al., 1975; Dismukes et al., 1976) cited the use of enormous antagonist concentrations which vitriated specific receptor effects.

Investigetors in Schwartz's laboratory proposed that both H_2 receptors and those similar or identical to H_1 receptors are involved in the histamine response and moreover, may be sequentially activated (Palacios et al., 1978a). The alteration of HA stimulation of cyclic AMP in guinea pig hippocampal slices was investigated through evaluation of antagonist affinities (pA_2 values), Schild analysis and relative agonist potencies. Furthermore, the concentrations of the agents used were not excessive and therefore more selective in their actions. Metiamide (1-30 μM) competitively antagonized stimulation elicited by histamine or TEA, an H_1 agonist. The parallel dextral shift without modification of the maximal response and a Schild slope indistinguishable from unity confirmed this competitive inhibition. The dissociation constant of 6.04 indicated metiamide's interaction with a homogeneous population of H_2 receptors (Palacios et al., 1978a). H_2 receptor participation was also demonstrated by the relative potencies of the H_2 agonists, 4-methylhistamine and dimaprit, compared to the effects on peripheral tissues. Yet two factors suggested possible H_1 involvement as well. First, the relative potencies of the H_1 agonists, TEA and 2-methylhistamine, were not those observed for homogeneous H_1 receptors but instead were intermediate between those of H_1 and H_2 receptors. Second, in the presence of moderate concentrations of mepyramine (5-100 μM), the effect on HA stimulation was more complex. At low concentrations (<10 μM) of HA, mepyramine was inactive while at higher HA concentrations, it induced a competitive antagonism. Schild analysis revealed two separate components of this complex response. The first one, insensitive to mepyramine, had an EC_{50} of 6 μM while the second one, antagonized by mepyramine (pA_2 = 8.2) had an EC_{50} of 14 μM for histamine. Each component contributed about 50% to the maximal response (Palacios et al., 1978a). Lastly, the maximal response seen with dimaprit alone was progressively elevated still further by TEA in a concentration-dependent manner. This additional elevation was sensitive to 100 nM mepyramine while the dimaprit response was unaltered. The total inhibition of

the HA response due to metiamide and its partial antagonism by mepyramine suggests that the accumulation of cyclic AMP following H_1 activation may be an indirect response. The ability of HA to generate cyclic AMP through H_1 receptors would then depend on the previous or simultaneous stimulation of H_2 receptors (Palacios et al., 1978). This indirect H_1 effect may be somehow associated with Ca^{++} translocation since H_1 activation of cyclic AMP formation is dependent on external Ca^{++} concentrations (Schwartz et al., 1980). Only the additional TEA-induced cyclic AMP accumulation in the presence of dimaprit-induced maximal response is largely prevented in a calcium-free medium, consonant with such a hypothesis. The exact role of Ca^{++} in this mechanism(s) remains to be explored.

The cells bearing histamine receptors which are linked to adenylate cyclase in slices of guinea pig hippocampal and cortical tissue are probably post-synaptic neurons since the response was nearly totally abolished following intrahippocampal kainic acid injection (Barbin et al., 1976; Garbarg et al., 1978a). However, after interruption of putative histaminergic fibers to the hippocampus, histamine-induced cyclic AMP accumulation was not modified, suggesting an absence of denervation hypersensitivity to this response (Garbarg et al., 1978b). A similar sequential or facilatory role was proposed for H_1 receptors linked to adenylate cyclase in Krebs-Ringer homogenates of guinea pig cerebral cortex preparations. The particulate fractions containing "sac-like entities" which retained much of their membranal adenylate cyclase were studied for H_1 and H_2 effects on cyclic AMP generation (Daly et al., 1980). Blockade of both adenosine and H_2 receptors abolished histamine (H_1 and H_2) responses. However, in the absence of adenosine blockade, HA effects were more effectively antagonized by H_1 than H_2 antagonists. This may be because d-brompheniramine and metiamide were both used at 100 μM concentrations. Nevertheless, the data suggested that in the absence of functional H_2 receptors, H_1 receptors have no effect on cyclic AMP generating systems in Krebs-Ringer homogenates. It was proposed that H_1 receptors are

primarily involved with potentiation of adenosine responses rather than direct potentiation of H_2 responses (Daly et al., 1980). Unfortunately, in the absence of a more precise receptor evaluation, these conclusions may be rather speculative. A possible H_1 association with Ca^{++} was not investigated.

Earlier investigations which used tissue slices and homogenates employed inappropriate agonist and antagonist concentrations to infer H_1 coupling to adenylate cyclase. Studies mindful of the relative selectivity of such agents failed to observe a direct or major role for the H_1 receptor in this system. This leaves the hypothesis of direct H_1 receptor activation of adenylate cyclase in the mammalian CNS very much in doubt.

E. H_1 Receptor Linked to Guanylate Cyclase

H_1 receptor activation stimulates cyclic GMP accumulation in cultured mouse neuroblastoma (clone N1E-115) cells (Taylor and Richelson, 1979; Richelson, 1979a,b). This response is similar to that of muscarinic receptor activation in the same preparation which was used as a model for analysis of guanylate cyclase linked to receptors (Richelson, 1979b). Cyclic GMP content increases up to 50-fold in response to 100 μM histamine. Mepyramine, at doses less than 10 nM, dextrally shift the dose-response curve without altering the slope of maximal response. The K_B values of H_1 antagonists acting both on the histamine receptors of the mouse neuroblastoma and guinea pig ileum are highly correlated (Figge et al., 1979; Richelson, 1980). 2-Methylhistamine, an H_1 agonist, stimulated cyclic GMP formation whereas 4-methylhistamine, an H_2 agonist, at concentrations up to 1 mM was ineffective (Richelson, 1978). Excessive H_1 agonist or prolonged agonist exposure (up to 30 minutes) reversibly desensitizes the cyclic GMP response by a mechanism that likely uncouples guanylate cyclase from the receptor (Richelson, 1981). Mepyramine (50 nM) blocked histamine (100 μM)-induced desensitization, yet 15 μM metiamide could not block this effect (Taylor and Richelson, 1979). In addition, 4-methylhistamine could not cause desensitization. Both histaminic

and muscarinic stimulation of cyclic GMP elevation are dependent on extracellular calcium concentration and do not occur in broken cell preparations. In the presence of external Ca^{++}, verapamil, a calcium channel blocker, inhibits cyclic GMP formation, suggestive that Ca^{++} channels are involved in the coupling of the receptor to guanylate cyclase (Richelson, 1981).

Histamine enhances cyclic GMP formation in the mammalian central nervous system of several species. Unfortunately, the receptor(s) mediating this response has not been evaluated. Histamine elevates cyclic GMP levels in cortical slices of guinea pig (Schwabe et al., 1978) and rabbit (Kuo et al., 1972), although guinea pig, mouse and rabbit cerebellar slices fail to respond (Ohga and Daly, 1977; Ferrendelli et al., 1975; Kuo et al., 1972). Rat pineal and bovine superior ganglion cyclic GMP content also rise in response to histamine (O'Dea and Zatz, 1976; Study and Greengard, 1978). In these preparations, the HA sensitive guanylate cyclase appears to require Ca^{++} (Schwabe et al., 1978).

F. Other H_1 Mediated Cellular Events

1. H_1 Receptor activation of glycogenolysis

A method for quantifying glycogen hydrolysis was developed as an experimental model for the evaluation of H_1 receptors in mammalian brain (Quach et al., 1978). In this model, mouse cortex slices are incubated with ^{3}H-glucose and the ^{3}H-glycogen formed in tissues is determined by computing the ethanol-insoluble radioactivity (due to unhydrolyzed glycogen) on filter paper. Following attainment of steady state ^{3}H-glycogen levels (about 30 min), addition of histamine profoundly depletes the ^{3}H-glycogen store (Quach et al., 1978). In a few minutes, as much as 80% of the polymer is hydrolyzed. This response to HA is similar to the glycolytic action of noradrenaline acting on β-receptors (Quach et al., 1978). The HA response was shown to be concentration-dependent (Quach et al., 1978) with an EC_{50} of 3 μM (Quach et al., 1980b) close to the value for H_1 mediation of cyclic AMP formation in guinea pig brain. 2-Thiazolylethylamine stimulated

hydrolysis but 200 μM dimaprit, an H_2 agonist, did not. That H_1 receptors are selectively involved in this effect was demonstrated by the relative agonist potencies and apparent K_B values of the H_1 antagonists, all less than 6 nM (Quach et al., 1980b). Schild analysis for mepyramine inhibition yielded a line with unity slope and a pA_2 value of 8.0; clearly, antagonism of a competitive nature. Adrenergic, serotonergic and H_2 receptor antagonists were unable to inhibit the HA-stimulated hydrolysis. Yet, at concentrations ranging from 3-300 nM (+)chlorpheniramine although not its (-)isomer antagonized this response. The process of HA-induced glycogenolysis was also sensitive to extracellular calcium ion concentration (Quach et al., 1980b).

The dependency of Ca^{++} ion availability for H_1-induced cyclic GMP accumulation in neuroblastoma, brain slices and superior cervical ganglia and for mouse cortex glycogenolysis suggests that H_1 receptors may interact with Ca^{++} channels (Schwartz, 1979). The absence of a major change in glycogen hydrolysis in the presence of a phosphodiesterase inhibitor, isobutylmethylxanthine, argues against a direct cyclic AMP involvement in this process (Quach et al., 1980b). The finding that the Ca^{++} channel blocker, verapamil, inhibits H_1 stimulation of cyclic GMP formation in the presence of abundant extracellular Ca^{++} (Richelson, 1981) is consonant with this view. Also in line with this hypothesis is the failure to produce glycogenolysis or elevate cyclic GMP levels in cell-free preparations. Under such conditions receptors, channels and other spatially oriented systems necessary for these responses could be separated. To date, this remains only an attractive prospect to explain much of the data; however, a more comprehensive explanation awaits.

2. H_1 stimulation of P_i incorporation into phospholipids

Histamine has been found to stimulate incorporation of inorganic phosphate (P_i) into phospholipids of whole rat brain in a dose-related manner (Friedel and Schanberg, 1975). Histamine (50 μg) stimulated incorporation of $^{33}P_i$ into phosphatidic acid five minutes after intracisternal injection. After 30 minutes,

$^{33}P_i$ was found in phosphatidic acid, phosphatidyl inositol and phosphatidyl choline when extracted in neutral organic solvent (Friedel and Schanberg, 1975). Since tripelennamine HCl (100 µg) had no effect alone, but inhibited HA stimulation by 85%, the authors attributed this response to histamine stimulation of H_1 receptors. These findings are in line with another in vivo study which demonstrated HA-induced $^{33}P_i$ incorporation into phospholipids of whole rat brain in a dose-dependent manner (Subramanian et al., 1980b). Prior reserpine treatment was without effect, obviating possible HA mediated catecholamine release. H_1 activation with 2(2-pyridyl)ethylamine stimulated incorporation which was refractory to cimetidine, but antagonized by pyrilamine, all given at 100 nmoles/g brain. H_2 stimulation with 4-methylhistamine had no effect (Subramanian et al., 1980b). The effect could not be mimicked by 48/80 (intracisternally) at a dose of 50 µg/g brain, injected 5 minutes prior to $^{33}P_i$ and agonist administration. The authors suggested that these HA actions were neuronally mediated because this mast cell degranulator did not elicit any effect. From these findings, two hypotheses may be advanced. First, HA may not act directly to cause P_i incorporation since 48/80-induced HA released from mast cells or similar structures should have equivalent effects as exogenous HA at equal concentrations. Second, that at the site of 48/80 injection and regions where it rapidly diffuses, mast cells are not present or in such small numbers as to have negligible effect. This is supported by the fact that dopamine, which also stimulates P_i incorporation into phospholipids in vivo (Friedel et al., 1974) is known to be present in mast cells. Unfortunately, the site of injection was not reported.

The nature of these studies, the inability to determine local drug concentrations and, in one instance, use of H_1 and H_2 agonists and antagonists all at the same dose (Subramanian et al., 1980b)make a critical evaluation of these results difficult. It may be noteworthy that these observations

are at variance with an in vitro investigation of $^{33}P_i$ incorporation into phospholipids of the pineal body in which HA (100 μM) was without effect (Muraki, 1972). However, norepinephrine and acetylcholine at the same concentration caused significant incorporation.

G. H_2 Receptor Binding Studies

Binding studies utilizing selective H_2 antagonists have demonstrated saturable binding sites in mammalian brain preparations. However, clear identification of these sites as H_2 receptors is probably still lacking. Specific binding of tritiated amitriptyline, a potent inhibitor of H_2 stimulated adenylate cyclase (Green and Maayani, 1977), in mouse brain (P_2 pellets) could be inhibited by 100 μM metiamide (Rehavi and Sokolovsky, 1978), an H_2 antagonist. Specific, saturable binding of ^{3}H-cimetidine in guinea pig brains that were homogenized, centrifuged (1.8×10^5 g-min) and resuspended was reported to have a K_D of 42 nM (Burkard, 1978). Kendall et al. (1980) described a ^{3}H-cimetidine binding site in crude synaptic membrane preparations derived from P_2 pellets from rat brain. Kandel et al. (1980) showed saturable ^{3}H-cimetidine binding in P_2 pellets from pooled rat cerebral cortices; however, a paradoxical separation and mutual independence of H_2 agonist and H_2 antagonist sites was observed. Using resuspended pellets from whole rat brains, Smith et al. (1980) reported ^{3}H-cimetidine binding which was saturable, specific (inhibited by 500 μM metiamide) comprised of a single population of sites as determined by Scatchard analysis and had a dissociation constant of 0.2 μM. In addition, a correlation could be shown between IC_{50} values (concentration of a substance which inhibits 50% of the specific binding of another substance) of imidazole H_2 antagonists and the K_B values of these antagonists on peripheral H_2 receptors. However, potent non-imidazole H_2 antagonists were inactive (IC_{50} values exceeding 1 mM) in displacing specific ^{3}H-cimetidine binding. Several antidepressant drugs were also without inhibitory effect, although they have been shown to be potent

inhibitors of H_2 stimulated (dimaprit) adenylate cyclase (Green and Maayani, 1977; Kanof and Greengard, 1978). Dimaprit, a highly selective and potent H_2 agonist, was also inactive. They concluded that the ^{3}H-cimetidine recognition site was in fact an imidazole binding site. Indeed, Kendall et al. (1980) showed in their preparation that imidazole itself had nearly the IC_{50} value of histamine and about three times the IC_{50} value of 4-methylhistamine for displacing ^{3}H-cimetidine. ^{3}H-Cimetidine binding of guinea pig cerebral cortex and gastric mucosal membrane preparations were found to be time dependent, saturable and composed of a single population of binding sites yet exhibited different K_D values (Rising et al., 1980). These K_D values differed from the cimetidine K_D values obtained from the atrium and gastric mucosa and here too dimaprit failed to displace this radioligand binding at concentrations exceeding 1 mM. Further, Warrander et al. (1983) have questioned the need for copper ions in ^{3}H-cimetidine binding studies who reported that Cu^{++} significantly increased total and specific binding. Moreover, neither ranitidine, tiotidine nor dimaprit displaced ^{3}H-cimetidine from guinea pig cortical preparations in the presence or absence of Cu^{++}. Therefore, while binding studies in brain tissue reveal a possible imidazole binding site, they have not clearly characterized central H_2 receptors. Specific ^{3}H-cimetidine binding has also been reported in the rat anterior pituitary (Devoto et al., 1980). However, confirmation of H_2 binding which they suggested was only due to both cimetidine and metiamide binding. This leaves doubts that these too may be non-H_2 receptor binding sites. The suggestion that ^{3}H-cimetidine may be binding to high affinity sites unrelated to the H_2 receptor was bolstered by reports describing similar binding anomalies in guinea pig gastric mucosa (Norris et al., 1980) and heart membrane preparations (Bristow et al., 1980). The latter study also showed ^{3}H-ranitidine to be unsuitable for labelling H_2 recognition sites since it could not be displaced by histamine itself, cimetidine, tiotidine nor dimaprit.

Most recently, ^{3}H-tiotidine (a non-imidazole H_2 antagonist) has been claimed to specifically label H_2 sites in homogenates of guinea pig cerebral cortex (Gajtkowski et al., 1983). This claim was based on the observations that i) specific ^{3}H-tiotidine binding was inhibited by several known H_2 agonists and antagonists of diverse structure; ii) these agents failed to displace non-specific binding; iii) K_B data from the cortex agreed with that determined in guinea pig gastric mucosa (adenylate cyclase activity) and guinea pig atrium (chronotropic activity); and iv) non-H_2 agents did not significantly alter ^{3}H-tiotidine binding even at concentrations exceeding by a thousand-fold tiotidine's K_B (30 nM). In addition, binding was shown to be saturable, heat labile and resistant to the filter adhesion reported by others (Maayani et al., 1982). These findings appear more credible as this group has questioned earlier claims of specific H_2 receptor labelling. However, even with this discovery, these authors were unable to demonstrate specific ^{3}H-tiotidine binding to established pharmacological H_2 receptors in the guinea pig's gastric mucosa and atrium or the rat's uterus or cerebral cortex (Gajtkowski et al., 1983). The reasons for this drawback remain unknown and this general finding awaits confirmation.

H. H_2 Receptors Linked to Adenylate Cyclase in Brain Homogenates

Adenylate cyclase stimulation due to H_1 receptor activation, which can be elicited in brain slices (Schwartz et al., 1980) is not observed in brain homogenates (Green et al., 1977; Hegstrand et al., 1976). There is however, an abundance of evidence that histamine-stimulated adenylate cyclase in brain homogenates is associated with the H_2 receptor. Computer models evaluating possible H_1 and H_2 receptor involvement in homogenate cyclase preparations have suggested that an H_1 linked system cannot contribute more than 15% to the total enzyme activity (Hough et al., 1980). The evidence for H_2 receptor participation is based on both agonist and antagonist H_2 effects compared to their peripheral actions. The failure of H_1 antagonists to competitively inhibit homogenate adenylate cyclase also supports such a hypothesis.

Homogenates from guinea pig brain have been the model system most utilized since greater adenylate cyclase enzyme activation occurs with this preparation than those from the rat. The relative agonist activities (as evaluated by their relative EC_{50} values) for cyclase stimulation coincide with their activities on peripheral H_2 receptors (Green et al., 1977; Hegstrand et al., 1976). Several antagonists, among them metiamide, cimetidine and tiotidine, had similar affinities for the histamine linked cyclase and the guinea pig atrium, an H_2 mediated system (Green et al., 1977; 1979; Maayani et al., 1982). These antagonists were shown to be competitive since they provoked a parallel dextral displacement of the dose-response curve and exhibited slopes of approximately 1.0 upon Schild analysis. Their dissociation constants were independent of the agonist used to stimulate the enzyme as activation by dimaprit (a highly selective H_2 agonist possessing negligible H_1 activity) or histamine yielded nearly the same K_B values. Also, cimetidine antagonism of adenylate cyclase activity in hippocampal or cortical homogenates yielded the same Schild values inferring that these sites are homogeneous and H_2 receptor linked (Green et al., 1977) or could not be distinguished from the H_2 receptor. In guinea pig cortical homogenates, pyrilamine, an H_1 antagonist, also shifted to the right the dose response curve of dimaprit stimulated adenylate cyclase at a concentration of 10 μM. However, when this enzyme in both cortical and hippocampal preparations was stimulated by histamine as well as dimaprit and then subjected to Schild analysis, it was found that pyrilamine's antagonism was non-competitive (Maayani et al., 1982). The slope of the Schild curve was significantly less than 1.0 and the pA_2 value of 5.5 was far different from the usual value of 9.3 observed in the periphery (Ash and Schild, 1966). This example underscores again the importance of using antagonist concentrations closer to established K_B values and the false inferences that can be derived in the absence of furtber receptor analysis, in this case, multiple agonists and Schild evaluation.

Guinea pig hippocampal homogenates were used to evaluate some of the various regulatory mechanisms which may interact with histamine H_2-stimulated adenylate cyclase (Kanof et al., 1977). This enzyme may require guanosine triphosphate (GTP) or a structural analogue since incubation with GTP increases the basal activity and the V_{max} for histamine. It is of interest that GTP has been reported to alter the affinity of adenylate cyclase for its activating ligand (Rodbell et al., 1974; Birnbaumer et al., 1974), although this view is not universally held (Hanoune et al., 1975). While such a hypothesis may explain this effect of GTP, the low enzyme activity in the absence of GTP precludes accurate analysis for measuring half-maximal histamine stimulation (Kanof et al., 1977). The possibility that histamine may be augmenting GTP-enzyme interactions is also tenable but has not been examined. Magnesium ions and adenosine triphosphate (ATP) also likely regulate the H_2 associated adenylate cyclase. Mg^{++} enhance histamine-stimulated cyclase activity and kinetic analysis suggests that this enhancement may be accomplished by free Mg^{++} binding to an allosteric site. Histamine (100 µM) was shown to increase the affinity of free Mg^{++} for this site by reducing its K_M for Mg^{++} by two-thirds (Kanof et al., 1977). ATP exhibits a biphasic action on HA-stimulated adenylate cyclase as lower concentrations (<2 mM) augment and higher concentrations (>4 mM) attenuate enzyme activity. Mg^{++} may elevate activity also be chelating free ATP under conditions when ATP is at higher levels. Histamine was shown to increase the enzyme's V_{max} without altering its apparent affinity (K_M) for its substrate, Mg-ATP (Kanof et al., 1977).

Results from <u>in vitro</u> and <u>in vivo</u> experiments using neonate chick brains support central HA-stimulated adenylate cyclase activity linkage to H_2 receptors. The 2-3 day old chick is a useful model since it lacks a blood-brain barrier to peripherally administered HA (Edwards et al., 1974). Injections of HA (10 mg/kg, i.v.) increased the cyclic AMP content of cerebral hemispheres 3-4 fold. Pretreatment with mepyramine (20 mg/kg, s.c.) 30 minutes before failed

to antagonize this histamine-stimulated effect, while burimamide (10 and 20 mg/kg s.c.) and metiamide (2 and 5 mg/kg, s.c.) both antagonized this increase in a dose related manner (Nahorski et al., 1974). In parallel in vitro experiments, slices of neonate chick cerebral hemispheres were shown to produce a 25-30 fold increase in cyclic AMP formation after exposure to 100 μM histamine. This response is greater than in any species studied at any age. Mepyramine (50 μM) was without effect while burimamide (10 and 50 μM) and metiamide (1 and 5 μM) both antagonized this HA-induced cyclic AMP production in a dose related manner (Nahorski et al., 1974). Mepyramine was shown to attenuate HA-stimulated cyclic AMP production at a concentration of 100 μM, yet further analysis demonstrated this antagonism to be insurmountable. Metiamide (10 μM) was shown to be competitively inhibiting this HA action. This confirmed the association of histamine stimulated adenylate cyclase in brain to central H_2 receptors in yet another species (chicken). It emphasized again the non-specificity of action when large concentrations of antagonists are used. Stimulation of cyclic AMP in chick neonate cortex slices by the H_2 agonist, 4-methylhistamine, showed an EC_{50} similar to histamine (4 μM), but also only about half the maximal histamine response at 100 μM concentration (Nahorski et al., 1977). 4-Methylhistamine was therefore only a partial agonist while 2-methylhistamine had no significant stimulant actions except at 100 μM. Mepyramine (10 μM) antagonized only 2-methylhistamine (100 μM)-induced cyclic AMP accumulation. However, metiamide (5 μM) blocked histamine (10 μM), 4-methylhistamine (10 μM) and 2-methylhistamine (100 μM) stimulated cyclic AMP formation (Nahorski et al., 1977). The distribution of this HA-activated cyclase was non-uniform in the chick neonate with a rank order of activity of the cortex>cerebellum>medulla and diencephalon> optic lobes (Nahorski and Smith, 1976).

Histamine-sensitive adenylate cyclase has been found in homogenates of other species as well. A significant increase following 100 μM histamine was

observed in the homogenates from rat cortex, but not from rat hippocampus, corpus striatum nor cerebellar tissues (Hegstrand et al., 1976). Rat and rhesus monkey hypothalamic homogenates were unresponsive to 100 μM histamine (Ahn and Makman, 1977). In a separate study (Green et al., 1977), rat hippocampal adenylate cyclase was responsive to 10 μM histamine stimulation, but was less sensitive than guinea pig hippocampal preparations (higher EC_{50} and reduced maximal responses). Homogenates from rabbit frontal cortex, anterior limbic cortex and hypothalamus were shown to be sensitive to histamine stimulation (Makman et al., 1980) although receptor identification was not investigated. HA produced concentration-dependent increases in adenylate cyclase activity from superior frontal cortex brain homogenates in monkey (Newton et al., 1982). The responses were mediated through H_2 receptors as verified by Schild analysis. The rank order of activation of tissue cyclase sensitive to HA was frontal cortex>hippocampus>putamen. However, the adenylate cyclases of homogenates from the monkey caudate nucleus, globus pallidus, amygdala and hypothalamus were unresponsive to HA (Newton et al., 1982). Adenylate cyclases from gerbil or hamster whole brain homogenates were not significantly stimulated by histamine (Hough and Green, 1981).

Histamine activation of H_2 receptor sensitive adenylate cyclase is not a unique phenomenon limited to neurons in the mammalian CNS. Glial cells (astrocytoma) maintained in culture C and some mast cells L have H_2 receptors which have been shown to be linked to adenylate cyclase. However, there is probably no connection between H_2 stimulated increases in cyclic AMP in mast cells and those actions observed previously in brain homogenates. In fact, there is an inverse correlation between the distributions of H_2 linked adenylate cyclase activity and mast cell population. Mast cells are most abundant in the hypothalamic and thalamic areas (Dropp, 1976; Goldschmidt et al., 1983), whereas H_2 stimulated cyclase in these regions is very meager (Makman et al., 1980; Green et al., 1978) or non-existent

(Ahn and Makman, 1977; Newton et al., 1982). Conversely, cyclase activation is most evident in the cortex and hippocampus (Hegstrand et al., 1976; Green et al., 1978b; Makman et al., 1980; Newton et al., 1982), regions where mast cells are extremely scarce (Dropp, 1972; 1976). Nevertheless, histamine (500 μM)-stimulated cyclic AMP formation has been demonstrated in cerebral mast cells, but only in the choroid plexus (Hammers et al., 1977). Kainic acid induced lesions (selective for neuronal cell bodies) (Coyle, 1983) of guinea pig hippocampal tissue almost destroys HA-stimulated cyclase activity (Garbarg et al., 1978). Subcellular fractionation studies indicated that the distribution of HA-sensitive adenylate cyclase activity prepared from guinea pig cortical homogenates was primarily in the mitochondrial subfraction (Kanof et al., 1977). This fraction accounted for over 70 per cent of the total recoverable activity. Further fractionation of this layer resulted in cyclase activity almost entirely (99%) in the M_1 mitochondrial subfraction containing mitochondria, membranes and nerve endings (synaptosomes). Sucrose density gradient centrifugation of the M_1 fraction caused this cyclase to sediment predominantly in the synaptic fragment and mitochondrial layers (Kanof et al., 1977). Therefore, the subcellular distribution of HA-sensitive cyclase paralleled the distribution of the synaptic membrane fragments. Mast cells, their granules and incompletely homogenized material generally sediment out with the nuclear (P_1) fraction. This accounted for no more than 10% of the initial homogenate enzyme activity. These findings support a neuronal locus for the H_2 receptor linkage to histamine-stimulated adenylate cyclase activity.

To date, the histamine sensitive cyclic AMP formation in homogenates of mammalian brains can be overwhelmingly attributed to H_2 receptor mediation. Yet failure of HA to stimulate adenylate cyclase activity should not infer an absence of H_2 receptors. The biochemical and biophysical interactions which link the H_2 receptor to the enzyme in some tissues may be labile or inhibited during the process of homogenization. Thus, the ability to observe HA stimulation

may partially be a function of the assay technique and therefore be somewhat artifactual. For example, this may explain the clear observation of a HA sensitive cyclase in homogenates of guinea pig hypothalamus in some laboratories (Ahn and Makman, 1977; Green et al., 1978b) while others fail to see an effect (Hegstrand et al., 1976). Likewise, the ability to stimulate H_1 and H_2 receptors and generate effects in tissue slices is often lost in the same brain regions after homogenization. More importantly, H_2 receptors may not always be associated with adenylate cyclase.

I. Histamine H_3 Receptors - The Proposed Autoreceptor

Investigators in Schwartz's laboratory have recently proposed an autoreceptor which, when stimulated by exogenous HA, can inhibit potassium- or veratridine-induced ^{3}H-HA release from rat cerebral cortical slices (Arrang et al., 1983). These slices were previously incubated with ^{3}H-histidine in order to endogenously form ^{3}H-histamine (before the excess substrate was washed away). The depolarization-induced ^{3}H-HA release increased 5-fold over basal amine release in Krebs-Ringer bicarbonate buffer, but was nearly abolished in Ca^{++}-free buffer or media containing 10 mM Mg^{++}. Potassium (30 mM)- or veratridine (10 μM)-induced ^{3}H-HA release was inhibited by about 50% in the presence of exogenous HA (1 μM). This inhibitory process which was saturable, reversible, competitively antagonized and highly selective pharmacologically (see below) suggested to the authors a receptor mediated response which they designated H_3. The HA inhibitory action was concentration dependent with an EC_{50} for HA of 41 nM when added before K^+ and about 125 nM when added with K^+, although under both conditions, maximal inhibition (61% reduction compared to control ^{3}H-HA release) was the same. Histamine was postulated to display a direct action since the HA-induced inhibition was not prevented by tetrodotoxin, could be elicited in cortical synaptosomes and was antagonized by stronger depolarization stimuli, e.g. higher levels of K^+ or veratridine.

Perhaps the strongest evidence for this proposed H_3 receptor was the difference in potency (compared to both H_1 and H_2 receptor mediated effects) of both agonists and antagonists and the demonstration of competitive antagonism of the HA-induced inhibition of ^{3}H-HA release. N^{α},N^{α}- and N^{α}-methylated histamine derivatives had 2- and 3-fold greater inhibitory potency than HA, respectively, although at both H_1 and H_2 receptors, they are slightly less potent than the parent amine. The H_1 agonists, 2-methylhistamine and 2-thiazolylethylamine, and the H_2 agonists, 4-methylhistamine and dimaprit, had no effect. However, impromidine, another H_2 agonist, reproducibly and in a concentration dependent manner enhanced K^+-stimulated ^{3}H-HA release by 20%. In addition, impromidine dextrally shifted in a parallel manner, the dose-response curve of HA-induced inhibition of ^{3}H-HA release without altering the maximal response. Schild plots were indistinguishable from unity with a pA_2 value of 7.5 whether exogenous HA concentration was 1 μM or 100 μM. Arrang et al. (1983) concluded that impromidine antagonized the histamine negative feedback system that was activated by the newly released ^{3}H-HA. While H_1 antagonists showed no effect, several H_2 antagonists inhibited the HA (1 μM)-induced reduction in a dose-dependent manner, shifting the response curve parallel to the right. Burimamide also competitively antagonized HA inhibitory effects as verified by Schild analysis (with unity slope) and was found to enhance ^{3}H-HA release, but only at a concentration of 5 μM. This too suggested an antagonism of HA-induced inhibition. The rank order of potencies of H_2 antagonists in eliciting the above antagonistic effects (as evaluated by K_B values) were radically different from those observed for H_2 receptors. For example, at H_2 receptor in the guinea pig atrium, the K_B ratio of burimamide (Black et al., 1972) to tiotidine (Yellin et al., 1979) is 520 whereas the same ratio at these putative H_3 receptors is less than 6×10^{-3}.

Arrang et al. (1983) noted that HA-induced inhibition of ^{3}H-HA release occurred at histamine concentrations which were far lower than those required to stimulate H_1 receptors which mediate glycogenolysis (Quach et al., 1980b)

or H_2 receptors eliciting cyclic AMP accumulation (Palacios et al., 1978a). However, they also observed that even the maximal inhibition attained reduced HA release not completely, but by approximately 60%. Since impromidine was proposed to block the HA-induced negative feedback which resulted in a 20% increase in HA release, the authors concluded that endogenously released HA may normally inhibit 20% of its own release. This is physiologically more relevant than the pharmacological concentrations of HA used in these studies. Thus, HA was postulated to exhibit an auto-inhibition similar to that postulated for other neurotransmitters (Langer, 1977; Starke, 1981).

The different agonist and antagonist potencies and the Schild evaluation which confirmed competitive antagonism argue for a receptor mediated mechanism subserving this apparent inhibition of HA release. However, several reservations to this recent and singular report should be kept in mind in light of data from previous reports, many from the authors' own laboratory which argued against presynaptic histamine receptors (see below). While there is an abundance of evidence to suggest the existence and function of autoreceptors, it is only a hypothesis at the present time that is by no means universally accepted. Experiments exploring the mechanisms involved and agonists and antagonists used to demonstrate this phenomenon have mounted strong challenges to this concept (Kalsner, 1982). While the concept suggesting H_3 receptor existence seems well supported, it could be strengthened further. Unfortunately, as this receptor is a novel proposition, it would be difficult to evaluate the reported antagonism by H_2 antagonists by using other established H_3 agonists which are not available. However, as the authors showed the N^{α}-methylated HA derivatives to be potent agonists, they could have been used for this purpose. One must also consider that we have no firm evidence that any neurons, in particular histamine neurons, are directly involved in this response. Such a demonstration is necessary since other brain cells, e.g. mast cells, are known to sequester and

release HA and synthesize ^{3}H-HA from ^{3}H-His (Bauza and Lagunoff, 1983). The uptake of ^{3}H-HA into brain slices and the demonstration of regional variation in their uptake has been previously reported; however, this uptake distribution did not parallel that of histidine decarboxylase activity, the putative HA neuronal marker (Subramanian and Mulder, 1976). Mast cells are known to bear H_2 receptors (Lichtenstein and Gillespie, 1973), and the alleged H_3 receptor is sensitive to H_2 antagonists. Incidentally, H_2 stimulation of mast cells inhibits histamine release. Prior use of degranulating agents would obviate some of this ambiguity should mast cells be abundant in the cortical slices (Joo et al., 1975; Karnushina et al., 1980). The capillary rich fraction of mammalian brain also contains H_2 receptors. Although the H_2 antagonists in the report of Arrang et al. (1983) exhibited actions distinct from classical H_2 receptor interactions and the rapid decarboxylation of ^{3}H-His to ^{3}H-HA argue against mast cell involvement, one cannot firmly conclude that exclusive neuronal action is involved in ^{3}H-HA release. Chemical depolarization by K^+ (30 mM) or veratridine (10 μM), an agent stimulating sodium channel permeability, is obviously not a physiological process; electrical stimulation may be more relevant for the study of HA release and possible autoinhibition. The 3-fold elevation of HA EC_{50} when given simultaneously with K^+ may indicate that this HA-induced inhibition during K^+ depolarization may not be as straightforward as suggested. The authors offerred no explanation to account for this elevation.

The stores of releasable ^{3}H-HA may or may not be neuronal and if neuronal, we have no evidence that they are identical to the releasable stores that may be normally active physiologically. The absence of any significant interference due to methylation by histamine N-methyltransferase bears further scrutiny. Arrang et al. (1983) assert that inhibition of ^{3}H-HA metabolism by exogenous HA would have resulted in an increased recovery of ^{3}H-HA in the medium. This is a valid assumption; however, at concentrations of 1 μM HA, which was used by

the authors, HMT activity which is highly substrate dependent is approaching its maximal activity (Taylor and Snyder, 1972b). Yet, the surmounting of H_2 antagonist inhibition by administration of HA at a concentration of 2 mM (not described in detail by the authors) certainly must have strongly inhibited HMT by the process of substrate inhibition which is evident above 50-100 µM levels (Taylor and Snyder, 1972b; Matuszewska and Borchardt, 1983; Prell, unpublished) (section VIII.B). Burimamide probably had no effect on HMT activity as it noncompetitively inhibits this enzyme in rat with a K_I of about 300 µM (Beaven and Shaff, 1979). The failure to detect changes under various conditions known both to strongly enhance and markedly inhibit HA methylation suggests that under these conditions, the proposed autoinhibition may act independently of processes of HA metabolism. This therefore casts some doubts upon the validity of this model as accurately representing the normal physiological mechanisms and synaptic processes thought to be involved in autoreceptor mechanisms.

One methodological limitation in this particular study is that the ^{3}H-dpm that were recovered were ascribed to ^{3}H-HA because methylation of the collected labelled material by (partially purified) histamine N-methyltransferase incubated in the presence of S-adenosyl-L-methionine yielded ^{3}H-N^τ-methylhistamine. However, we have no way of knowing how much of the ^{3}H-HA was methylated <u>in situ</u> before the exogenous methylation occurred. This distinction may be significant as ^{3}H-HA but not N^τ-methylhistamine is claimed by the authors to act on the putative H_3 receptors.

It should be emphasized that Arrang et al. (1983) postulated the existence of autoinhibitory HA receptors without attributing them to presynaptic sites, a localization this research group previously suggested not to exist for HA receptors. Using ^{3}H-HA as a label for high affinity HA binding sites in cerebral membranes, they concluded that the localization and developmental pattern of ^{3}H-HA binding in rat brain are consistent with the view that the sites are post-

synaptic receptors (Schwartz et al., 1980a). There was no strict correlation between ^{3}H-HA binding sites and presynaptic markers such as histidine decarboxylase activity (Palacios et al., 1978b). Furthermore, there is a marked loss of ^{3}H-HA binding sites in the striatum following local kainic acid injection suggestive that these sites are intrinsic to neuronal bodies rather than nerve terminals (Schwartz et al., 1980a). Lesions of the MFB which interrupts HA-synthesizing afferents did not reduce the density of the ^{3}H-HA high affinity sites (Schwartz et al., 1980a) suggestive that HA does not bind to axon terminals of putative histaminergic neurons (see section X). There was however, a significant increase in these binding sites 10-12 days post-lesion which was attributed to post-synaptic denervation hypersensitivity (Haas et al., 1978). This raises an interesting dilemma; if HA autoinhibition is not localized to pre-synaptic sites, then how is it manifested physiologically?

The hypothesis for an autoinhibitory mechanism mediated by H_3 receptors needs confirmation and explanation before it can be widely accepted. Its demonstration in the peripheral tissues using model systems which are better characterized than the brain might be helpful, although even in these tissues, exclusive neuronal participation would also be difficult to prove unequivocally. This hypothesis offers additional intriguing and more complex mechanisms for controlling brain histamine levels and turnover. But the fundamental questions of neuronal versus non-neuronal localization and conflicts with data obtained from lesion experiments must be addressed before we can more confidently consider autoinhibition a genuine locus of brain histamine regulation.

XII. EFFECTS OF SEVERAL PSYCHOACTIVE DRUGS ON THE CENTRAL HISTAMINERGIC SYSTEM

A. Antidepressants and the Central Histaminergic System

Antidepressant agents appear to influence the histaminergic system at two stages of its regulatory processes, i.e. the histaminergic catabolic system and

histamine receptors. Tricyclic antidepressants have been shown to exert a biphasic action on HMT activity, elevating it in small doses and causing inhibition at high doses (Taylor and Snyder, 1972c). Non-tricyclic or "atypical" antidepressants such as doxepin, mianserin, nomifensin and maprotiline also show this biphasic effect (Prell, unpublished). However, in vivo changes in HMT activity or HA concentration were not observed in the hypothalamus, midbrain or cortical regions of adult rats sacrificed 60 min after administration of doxepin (20 mg/kg, p.o.) (Mazurkiewicz-Kwilecki and Prell, unpublished). Acute administration of the tricyclic antidepressant, imipramine, to rats caused elevation of cortical and midbrain HA levels as well as increased HD activity although neither were altered in the hypothalamus (Mazurkiewicz-Kwilecki and Bielkiewicz, 1977). The effects of antidepressants on brain HD activity in vitro have not been reported.

Significant elevations of endogenous rat brain tele-methylhistamine (oxidized by monoamine oxidase B) levels have been reported following pargyline or deprenyl (Hough and Domino, 1979; Hough et al., 1982b). Brain histamine levels were slightly raised in the latter two studies and, following ipromiazid treatment, in cat hypothalamus (Adam and Hye, 1966). In clinical studies, increased urinary tele-methylhistamine levels were reported in patients receiving isocarboxazid, a monoamine oxidase inhibitor (Fram and Green, 1968). Yet, the increased levels in the latter study and another reporting decreased urinary tele-methylhistamine levels in clinically depressed non-medicated patients (Gagne et al., 1982), one cannot ascribe the observed changes exclusively to processes of the central nervous system. In the latter cases, the alteration of peripheral tele-methylhistamine metabolism may have been a larger contributor to these altered urinary levels.

In addition to their influence on HA catabolic processes, antidepressants also competitively antagonize H_1 and H_2 receptors (Green and Maayani, 1977;

Kanof and Greengard, 1978; Schwartz et al., 1981b; Richelson, 1979b; Maayani et al., 1982). Competitive antagonism of brain H_2 receptors, induced by antidepressants, has been shown to reduce HA-stimulated adenylate cyclase activity (Green and Maayani, 1977), H_2-linked stimulation of guinea pig hippocampal CA3 cell firing rate (Olianas et al., 1982) and H_2-linked depression of cortical neuron firing rate in rats (Haas, 1979). A reduction in dimaprit-induced hypothermia was reported following pretreatment with amitriptyline or imipramine (Nowak et al., 1979). However, the antidepressants also antagonize H_1 receptors. Competitive blockade of H_1-stimulated guinea pig ileum (Figge et al., 1979) and H_1-stimulated cyclic GMP formation in mouse neuroblastoma cells (Richelson, 1981) have been confirmed by Schild analysis (see section XI.E). Antidepressant blockade of *in vitro* mepyramine binding (presumably to H_1 receptors) in brain homogenates of rat (Taylor and Richelson, 1980) and guinea pig (Coupet and Szuchs-Myers, 1981) has been demonstrated. Labelled antidepressants also bind to H_1 sites in rat and guinea pig brain homogenates (Taylor and Richelson, 1982). Since amitriptyline and imipramine have been shown to block both H_1 and H_2 receptors *in vivo* (Haas, 1979; Taylor and Richelson, 1982), this blockade has been proposed as one of the mechanisms for some of the clinical effects associated with antidepressant therapy. However, to date, it is not the therapeutic effects but several of the side effects such as sedation, drowsiness, hypnosis and inability to concentrate which seem to be the best correlated with central histamine receptor blockade (Richelson, 1979b; Schwartz et al., 1981b).

B. Opiates and Brain Histamine

Morphine (Mo) has been found to be a potent liberator of histamine (HA) (Brashear et al., 1974). The well known hypotension following intravenous injection of Mo and codeine in cats is due at least in part to the peripheral release of HA (Nasmyth and Stewart, 1950). This depressor action was found to be resistant to atropine but was abolished by mepyramine, a H_1-receptor antagonist

(Feldberg and Paton, 1951). There is increasing evidence implicating HA in the central mechanisms of morphine tolerance and physical dependence. Narcosis induced by i.v. administration of morphine to dogs was reported to be blocked by the H_1-receptor antagonists, chlorcyclizine and diphenhydramine, indicating possible H_1-receptor involvement (Gershon and Shaw, 1958). Central administration of HA to dogs shortened the onset of morphine narcosis and analgesia, improved the level of consciousness and shortened the duration of narcosis (Feldberg and Paton, 1951). HA administered peripherally caused effects similar to those observed with morphine such as vomiting, defecation, loss of balance and occasionally a slight analgesia. Respiratory depression was always observed (Gershon and Shaw, 1958). These HA effects must be critically reviewed since little, if any, HA passes the blood-brain barrier (Snyder et al., 1964; Schayer and Reilly, 1970; 1974) (see Section VII). However, it is noteworthy that both HA and Mo have at least two common central sites of action, the choroid plexus and the area postrema (emetic center) Adam, 1961; Hug, 1971; Bhargava et al., 1976). Mo-induced release of brain histamine (0.18 µg/ml) could be detected in the efflux after perfusion of rabbit lateral ventricles with artificial cerebrospinal fluid containing 1 µM Mo (Tanaka and Lin, 1969). Both HA or Mo added to the perfused CSF produced respiratory depression followed by tachypnea and a sudden rise in blood pressure followed by a prolonged hypotension. In both cases, pretreatment with diphenhydramine (10 nM) blocked these effects. Similar effects were reported in mice after intracerebroventricular (i.c.v.) injection of compound 48/80 (Rocha e Silva, 1959), a highly potent mast cell HA liberator (Morrison et al., 1975; Johnson and Moran, 1969). These experimental results are thus attributable to either mast cell or neuronal HA release or to both.

In chronic studies from this laboratory, decreased HA levels were demonstrated in the hypothalamus of rats treated with increasing doses (30, 60, 90 mg/kg) of Mo for 9 days and in the midbrain and cortex following 21 days of treat-

ment (Mazurkiewicz-Kwilecki and Henwood, 1976). Acute treatments with Mo (30 mg/kg), methadone (5 mg/kg) or naloxone (0.4 mg/kg) failed to alter the HA content in the above regions of rats sacrificed one hour later. Induction of Mo withdrawal by either abstinence for 2 days in chronically treated rats or by naloxone resulted in a further depletion of hypothalamic HA concentration to levels significantly lower than those after chronic Mo alone. The time-course of HA depletion during withdrawal showed a maximal depletion at 66 hours and a return to control levels at about one week post-treatment (Henwood and Mazurkiewicz-Kwilecki, 1977; Henwood, 1977). Replacement of Mo by methadone (15 mg/kg) for 2 days following 21 days of Mo treatment resulted in essentially the same low hypothalamic HA levels as those observed in rats treated for 23 days with Mo alone. Histidine decarboxylase activity remained unchanged while HA levels were unaltered following acute Mo treatment but a sharp increase (+70%) in the HD activity was noted when HA was depleted after chronic opiate administration and during Mo withdrawal (Mazurkiewicz-Kwilecki and Bielkiewicz, 1978). This is consistent with a biochemical scheme in which brain HA synthesis is enhanced in response to a fall in HA levels (Schwartz et al., 1972; Verdiere et al., 1975).

L-Histidine (which is known to markedly increase brain HA levels (see section VII) administered concomitantly with morphine for 21 days was able to reverse the Mo-induced decline in hypothalamic HA to control levels (Henwood and Mazurkiewicz-Kwilecki, 1977). L-Histidine could also significantly prevent the HA depletion observed during Mo withdrawal although a complete return to control values was not seen. Hui and Roberts (1974) demonstrated that L-histidine and HA administered in the 'withdrawal' phase strongly inhibited the development of Mo dependence when naloxone induced jumping in dependent mice was used as a test; however, d-histidine had no effect (Wong and Roberts, 1977). Thus, not only do L-histidine and histamine influence Mo effects, but this influence is

stereospecific for the L-isomer; the precise molecular interactions of which remain to be elucidated.

Collier et al. (1972) suggested that neurotransmitters and their regulation may play different roles during the 'induction phase' of morphine tolerance than during the 'withdrawal phase' from morphine dependence. In mice implanted with morphine pellets for 24 hours, mepyramine, administered during the induction phase, had no effect on the development of tolerance to analgesia (measured by the tail-flick test (D'Amour and Smith, 1941). However, both mepyramine and L-histidine significantly affected development of physical dependence; 35 times and 50 times, respectively, more naloxone was required to elicit withdrawal as compared to mice given Mo alone. Although these results are difficult to interpret, the above data clearly indicate HA involvement in central morphine action. In these studies, brain Mo levels remained unaltered in all groups, but total brain HA levels were significantly increased in both tolerant (+37%) and non-tolerant (+25%) mice given mepyramine (Hui and Roberts, 1975). It is important to note that less than 5 mg of morphine was absorbed in the above studies. This confirms the observations of Lee and Fennessy (1976) who showed a rise in whole brain HA ($p < 0.005$) in mice injected with less than 6 mg/kg Mo, but a significant fall when given more than 7.5 mg/kg. It is of paramount importance to consider that at all acute doses of Mo, brain HA returned to control levels within 60 minutes of injection. Although a different species was studied in the above investigation, it partially supports previous observations from this laboratory in which no alterations in rat brain HA levels (Mazurkiewicz-Kwilecki and Henwood, 1976; Henwood and Mazurkiewicz-Kwilecki, 1975; 1977) nor HD activity (Mazurkiewicz-Kwilecki and Bielkiewicz, 1978) occurred one hour after acute Mo administration. Lee and Fennessy (1976) observed an inverse relationship between Mo-induced changes in brain HA and changes in locomotor activity. It may be relevant that an inverse relationship between hypothalamic HA and locomotor activity has been observed by Schwartz et al. (1976).

More recently, analgesia and catalepsy resembling acute opiate effects was reported following microinjection of HA into the dorsal raphe nucleus or the periaqueductal grey region of rats (Glick and Crane, 1978). Analgesia, catalepsy and hypothermia were observed after intraventricular HA injection while only hypothermia occurred with a rostral hypothalamic injection. Of greater interest was the profound withdrawal-like behavior following HA injection (6.25-25 µg) into the dorsal hippocampus such as teeth chattering, facial tremors, head shakes, 'wet dog shakes', writhing, salivation, yawning, chewing and grooming. Milder withdrawal-like behavior occurred after ventral hippocampal injections. Although one cannot verify the accuracy of the injection nor can diffusion be totally ruled out, it is noteworthy that Barbin et al. (1976) postulated that the dorsal histaminergic pathway was of quantitatively greater importance than ventral hippocampal input. If one can infer that a greater receptor population exists where there is an abundance of HA fiber input, the stimulation of more HA receptors at dorsal sites may account for these results. Another salient point is that injections near the hippocampus, i.e. the cortex, corpus callosum, lateral ventricle, lateral thalamus or superior colliculus failed to elicit any abstinence-like effect, thus making an argument for HA diffusion from dorsal to ventral hippocampal sites less tenable.

Further important support for the role of brain histamine in the central effects of Mo was obtained from other studies involving the use of H_1- and H_2-receptor agonists and antagonists. Glick and Crane (1978) could show that the same time was required to elicit abstinence-like effects after hippocampal injection of HA as after injection of dimaprit (0.3 µmol), a specific H_2-agonist (see section XI). This suggests that H_2-receptors play a role. Indeed, cimetitine (0.2 µmol), a specific H_2-antagonist, blocked the dorsal hippocampal withdrawal effects elicited even with large doses of HA (100 µg); however, the H_1-antagonist, mepyramine had no effect. In the dorsal raphe dimaprit could

elicit analgesia and catalepsy but not the hypothermia associated with acute opiate actions. Cimetidine could block the HA-induced alangesia but not the catalepsy while H_1 blockade with mepyramine inhibited catalepsy but had no effect on analgesia. Thus, in the dorsal raphe H_1-receptors may participate in analgesia whereas the catalepsy may be attributed to H_2-receptor stimulation. While these observations appear attractive, it should be noted that comparatively large doses of HA were injected directly into the brain tissues.

It is also of interest that reduction of histamine formation following 4-thiazolylmethoxyamine (TMA) administration, a highly selective histidine decarboxylase inhibitor (Menon et al., 1971) resulted in the inhibition of morphine tolerance development to analgesia (Hui, 1976). TMA also affected physical dependence development since more naloxone was required to produce withdrawal jumping in dependent mice. Wong and Roberts (1977) were also able to inhibit tolerance to Mo analgesia by the use of another specific HD inhibitor, 4-imidazolyl-3-amine-2-butanone, but unfortunately, as in the report of Hui (1976), brain HA levels were not reported. The above results thus support the concept that morphine tolerance and dependence both have underlying mechanisms which may in part depend on central histaminergic system.

C. Ethanol and Brain Histamine

a) HA-stimulated adenylate cyclase

Brain HA and ethanol interactions have to date been relatively unexplored. This may be partially attributed to the fact that ethanol tends to exert rather non-specific effects, and lacks a specific receptor and a pharmacological antagonist. Nevertheless, alterations of the central histaminergic system in response to ethanol have been reported. A significant increase in cortical HA-sensitive adenylate cyclase activity (+39%) was demonstrated in brain slices of mice maintained for two weeks on an ethanol containing diet. Mice receiving sucrose served as controls (Israel et al., 1972). However, norepinephrine in

brain slices was without effect. In other studies, the activity of gastric mucosal and hippocampal HA-stimulated adenylate cyclase was examined in vitro at ethanol concentrations of 1-5% (v/v) (Szucs and Karnuchina, 1981). Ethanol in increasing concentrations proportionately enhanced cyclic AMP formation in both tissues in a dose-dependent manner. Also, both basal and the HA-stimulated enzyme activities were increased; a 5% (v/v) concentration caused the HA (100 μM) stimulated cyclase to increase nearly four-fold. Neither cimetidine or mepyramine (each 100 μM) affected this ethanol-elicited elevation. However, since HA-stimulated adenylate cyclase in guinea pig brain homogenates is almost certainly H_2-mediated (section XI.H) and yet in this case, was insensitive to 100 μM cimetidine, these stimulatory effects may not have been HA receptor linked. Further, while a 5% (v/v) ethanol concentration was considered to be low by the authors, in pharmacological terms this would represent approximately 4 grams of ethanol per 100 ml or 4,000 mg%. Such concentration in vivo would be incompatible with mammalian systems.

An increase in HA (10 μM)-stimulated adenylate cyclase activity was also observed in cerebral cortical slices of rats withdrawn (72 hours) from ethanol (15 g/kg/day for 11 weeks) when compared to those withdrawn from (presumably equicaloric) dextrose treatment (French et al., 1975). This enhanced cyclic AMP formation was blocked by the H_1 antagonist, chlortripolon, as well as by phenoxybenzamine or dl-propranolol (each 100 μM). Unfortunately, H_1 receptor antagonism at this concentration could not be considered specific and may even have had effects unrelated to the HA receptor (see section XI.B). Further receptor characterization was not carried out. Although HA (10 μM) and norepinephrine (100 μM) each significantly enhanced adenylate cyclase in cortical slices from ethanol withdrawn rats, their combined stimulation failed to cause an additive response, suggesting that both HA and norepinephrine act at a common site or that the cyclase stimulation is already maximal at 100 μM

norepinephrine concentrations (French et al., 1975). Since this HA-induced effect was sensitive to phenoxybenzamine and dl-propranolol blockade, the authors concluded that a nonspecific supersensitivity may have occurred, possibly due to changes in membrane structure and/or physiology.

b) Brain HA levels

In a study of maternal alcoholism and its influence on brain HA (Rawat, 1980), pregnant rats were administered ethanol (6%, v/v in Sustac® liquid diet) beginning at conception. Maternal ethanol consumption resulted in a significant increase (+22%) in fetal whole brain HA concentration at 18 days of gestation. Brain HA levels of 10 day old neonate rats suckling on ethanol-fed mothers were also elevated (+36%) (Rawat, 1980). Withdrawal of ethanol for one week resulted in a return of brain HA to control levels. However, in both experiments, brain HD activity was not significantly affected. Acute ethanol (2 g/kg) or acetaldehyde (78 mg/kg) administered intragastrically elevated adult male rat brain HA content (+49% and +73%, respectively) by 60 min, although the levels returned to control (saline) values after 2 hours. In addition, elevated plasma HA levels were observed in fetuses and neonates of mothers chronically fed ethanol as well as in ethanol and acetaldehyde acutely treated adult rats. It was suggested (Rawat, 1980) that the elevated brain HA concentration was a direct consequence of the release of brain HA due to the presence of ethanol and its metabolic product, acetaldehyde. The lack of any significant change in HD activity through these experiments was asserted to support this hypothesis (Rawat, 1980).

Since other dynamic aspects of the histaminergic system were not studied such as HA turnover, HMT activity or HA metabolite concentrations, the conclusion that the ethanol- or acetaldehyde-induced elevation in brain HA was due to a general release of HA may be rather speculative. The elevation of plasma HA levels could have been a non-specific effect caused by gastric irritation following ethanol administration by intubation. Since the concentration of

ethanol administered was not stated, nor were these effects correlated with plasma ethanol or acetaldehyde levels, it is difficult to make further conclusions.

Dose-related elevations of hypothalamic (Subramanian et al., 1978), thalamic, cortical and striatal HA levels (Subramanian et al., 1980a) have been reported in adult male rats sacrificed one hour after administration of ethanol (80-160 mg/100 g While HMT activities remained unchanged in all four regions studied, hypothalamic HD activity increased (Subramanian et al., 1978). Striatal HD activity was unchanged. Plasma HA levels were reported to rise significantly (+61% and +92% at doses of 0.8 and 1.6 g ethanol/kg, respectively). In chronically treated rats (15%, v/v in drinking water for 4 weeks) HA levels were significantly elevated in the hypothalamus, thalamus and cortex while HD and HMT activities were reported to be unchanged (Subramanian et al., 1978; 1980a). The effect of various ethanol concentrations (20-200 mM) on the in vitro release of ^{3}H-HA from slices of these four regions of untreated rats was also studied. The brain slices were superfused with ^{3}H-HA as described earlier (Subramanian and Mulder, 1976; see section VIII.A) and the basal and potassium (40 mM)-stimulated ^{3}H-HA release determined. While ^{3}H-HA efflux at ethanol concentrations of 20 and 40 mM were not significantly changed, the basal hypothalamic ^{3}H-HA release significantly decreased (approx. -41%) at 80 and 160 mM ethanol. Potassium-stimulated ^{3}H-HA release was significantly lower (approx. -23%) in the presence of 100 mM ethanol (Subramanian et al., 1978). Depolarization-induced efflux of ^{3}H-HA from the thalamic and cortical slices was inhibited by 200 mM ethanol (Subramanian et al., 1980a).

This study of ethanol-induced inhibition of ^{3}H-HA release shares many of the drawbacks of the original ^{3}H-HA release experiments which were dependent on ^{3}H-HA tissue uptake (Subramanian and Mulder, 1976; Mulder et al., 1983; see section VIII.A). For example, the lack of specific ^{3}H-HA uptake mechanisms, the tissue accumulation of ^{3}H-methylhistamine and its possible release upon

K^+-induced depolarization and the inability to store ^{3}H-HA against a large concentration gradient are all distinct disadvantages. A further aspect of this study worth consideration is that the chronically administered ethanol was introduced in the drinking water as a 15% (v/v) solution. However, this method of ethanol administration, termed "forced" or "no choice" has been strongly challenged since it generally leads to variable day to day doses per rat and fails to achieve high blood ethanol concentrations (Cicero, 1978; Goldstein, 1983).

D. Effects of Other Psychoactive Drugs on Brain Histamine

Several other psychoactive agents were reported to alter endogenous HA levels, the activities of its synthesizing or metabolizing enzymes or to antagonize H_1 or H_2 receptors. Neuroleptics were found to affect brain histaminergic system. Acute haloperidol administration resulted in reduced rat hypothalamic and cerebral cortical HD activity without altering endogenous histamine concentration while chronic treatment (20 days) depleted hypothalamic and cortical HA levels (Mazurkiewicz-Kwilecki and Bielkiewicz, 1977). High doses of chlorpromazime were reported to elevate whole brain histamine content of the rat (Green and Ericksson, 1964). Although the mechanism for this elevation was not precisely determined, the finding that chlorpromazine (at higher doses) inhibits histamine-N-methyltransferase activity (Brown et al., 1959; Taylor and Snyder, 1972c; see section VIII.B) may possibly explain this increase in brain HA levels.

Both H_1 and H_2 receptors are blocked by several neuroleptics. H_1-receptor antagonism was demonstrated *in vitro* using H_1-dependent cyclic GMP formation in mouse neuroblastoma cells (Richelson, 1980; 1981) and by the use of labelled mepyramine in competitive binding studies in brain homogenates from rat, guinea pig and man (Tran et al., 1978; 1980). Neuroleptics (e.g. trifluoperazine, haloperidol, pimozide, chlorpromazine) have also been shown to displace ^{3}H-mepyramine binding in mouse brain *in vivo* (Quach et al., 1979; 1980a). Several

neuroleptics block HA-stimulated H_2 receptor-dependent adenylate cyclase activity of guinea pig and rabbit brain slices and homogenates (Spiker et al., 1976; Palmer et al., 1977; Palmer, 1981; see section XI.H) and homogenates of guinea pig hippocampus (Kanof and Greengard, 1978; Coupe and Szuchs-Myers, 1981; Maayani et al., 1982). However, Schild analysis of data indicated that of all neuroleptics investigated, only chlorpromazine exhibited competitive antagonism (Maayani et al., 1982). Nevertheless, even though neuroleptics caused non-competitive antagonism, the potencies of these agents (evaluated by IC_{50} data) for inhibition of H_2 receptor-dependent cyclic AMP formation in homogenates appears to be nearly the same. Surprisingly, among the neuroleptics, the most potent H_2 receptor blockers are the least potent H_1 receptor antagonists (Maayani et al., 1982). However, a positive correlation for antidepressant drug potencies on H_1 (Figg et al., 1979; Richelson, 1980) and H_2 (Green and Maayani, 1977; Kanof and Greengard, 1978) receptors was observed. The affinity of neuroleptic agents for histamine receptors (H_1 and H_2) generally appears to be less than their affinity for α-adrenergic, dopaminergic (receptor subgroup not clarified), cholinergic (muscarinic) or serotonergic receptors (Richelson, 1980). However, at clinically used doses, all of these receptors would be, at least partially, occluded by most neuroleptic agents (Green, 1983).

The action of hallucinogenic drugs on the brain histaminergic system has also been studied. Both H_1 and H_2 receptors are competitively antagonized in vitro by D-lysergic acid diethylamide (D-LSD) (Green et al., 1977). The pharmacologically inactive stereoisomer, L-LSD exhibits little in vitro antagonism (K_B value of 25 μM) of H_1-stimulated cyclic GMP formation in mouse neuroblastoma cells (Frederickson and Richelson, 1979) and no antagonism of H_2-stimulated adenylate cyclase activity in guinea pig hippocampal homogenates (Green et al., 1977). The derivative, 2-bromo-LSD has about ten times greater affinity for the H_2 receptor than its parent compound, D-LSD, although both are nearly equipotent at the H_1 receptor.

Other hallucinogens such as mescaline and psilocin also antagonize the H_1 receptor. However, the latter two failed to antagonize H_2 receptor-stimulated adenylate cyclase activity in guinea pig hippocampal homogenates (Green et al., 1977). Also, other groups of psychoactive drugs (i.e. opiates, anticonvulsants, anxiolytics, Δ^9-THC and barbiturates) at concentrations up to 100 μM failed to cause H_2 receptor antagonism (Maayani et al., 1982; *see below).

Acute administration of diazepam (2.5-10 mg/kg p.o.) failed to alter endogenous hypothalamic, midbrain or cortical HA concentration; however, it did attenuate a stress-induced elevation of hypothalamic HA content (Mazurkiewicz-Kwilecki, 1980). Thiopental (75 mg/kg i.p.) did not affect endogenous levels of both histidine and histamine in the hypothalamic and cortical brain regions (Pollard et al., 1974). However, a reduction in histamine turnover, evidenced by decreased HA synthesis and reduced ^{3}H-HA metabolism (following ^{3}H-HA administration, i.c.v.) was observed.

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*Psilocin, bufotenine, mescaline, barbital, phenobarbital, carbamazepine, phenytoin, valproate, ethosuximide, paramethadione, Δ^9-THC, chlordiazepoxide, morphine, naloxone, phencyclidine, picrotoxin, procaine, LiCl, pilocarpine, and phentolamine.

XIII. BASIS FOR THE THESIS RESEARCH

In general, the overall objectives were to determine if ethanol, administered in acute or chronic schedules, would alter rat brain histamine levels. If this occurred then a more detailed study of the processes responsible for regulation of histamine content (see Fig. 9) would be undertaken to determine those which are altered by ethanol. Such a study of the mechanisms responsible for histamine synthesis and catabolism would have two immediate beneficial effects. First, it would necessitate that this investigator develop, adopt or modify methods with which to investigate these mechanisms; methods not presently available in this laboratory. Second, a detailed investigation of the influence of ethanol on the brain histamine system could potentially yield significant findings for other investigators studying the ethanol, drug abuse or brain histamine fields.

Since histamine N-methyltransferase (HMT) is the major, possibly only, route of histamine inactivation in brain (see VIII. B), the effects of ethanol on *in vitro* HMT activity would be useful. Such data could indicate if ethanol or its primary metabolite, acetaldehyde, directly alter this metabolic step. In *in vivo* studies acute and chronic ethanol administration schedules would be carried out. In acute studies doses up to and including those which produce plasma ethanol levels associated with human intoxication would be used. Histamine levels would be determined as well as the activities of its synthesizing and metabolizing enzymes. Since brain histidine availability may also influence brain histamine content, a method for its determination would have to be developed for subsequent evaluation during ethanol studies.

In chronic studies ethanol would be administered on a daily basis in gradually increasing doses for three to four weeks. The effect of ethanol withdrawal on the histaminergic system would also be investigated. Histidine decarboxylase and histamine N-methyltransferase activities and brain histidine

content would also be studied. As histamine is known to be bimodally distributed in mammalian brain (see VI and IX) subcellular fractionation studies following ethanol administration could be useful. The results obtained might suggest if a neuronal or "neuronal-like" histamine pool is preferentially affected by ethanol or if the "mast cell-like" pool is more sensitive to ethanol-induced changes. Unfortunately, one component of brain histamine regulation, the process of histamine release, cannot be evaluated at the present time. However, a thorough evaluation of the above processes might lead to inferences concerning the possibility of brain histamine release due to ethanol.

METHODS

XIV. GENERAL METHODOLOGY

A. Biochemical and Neurochemical Methods

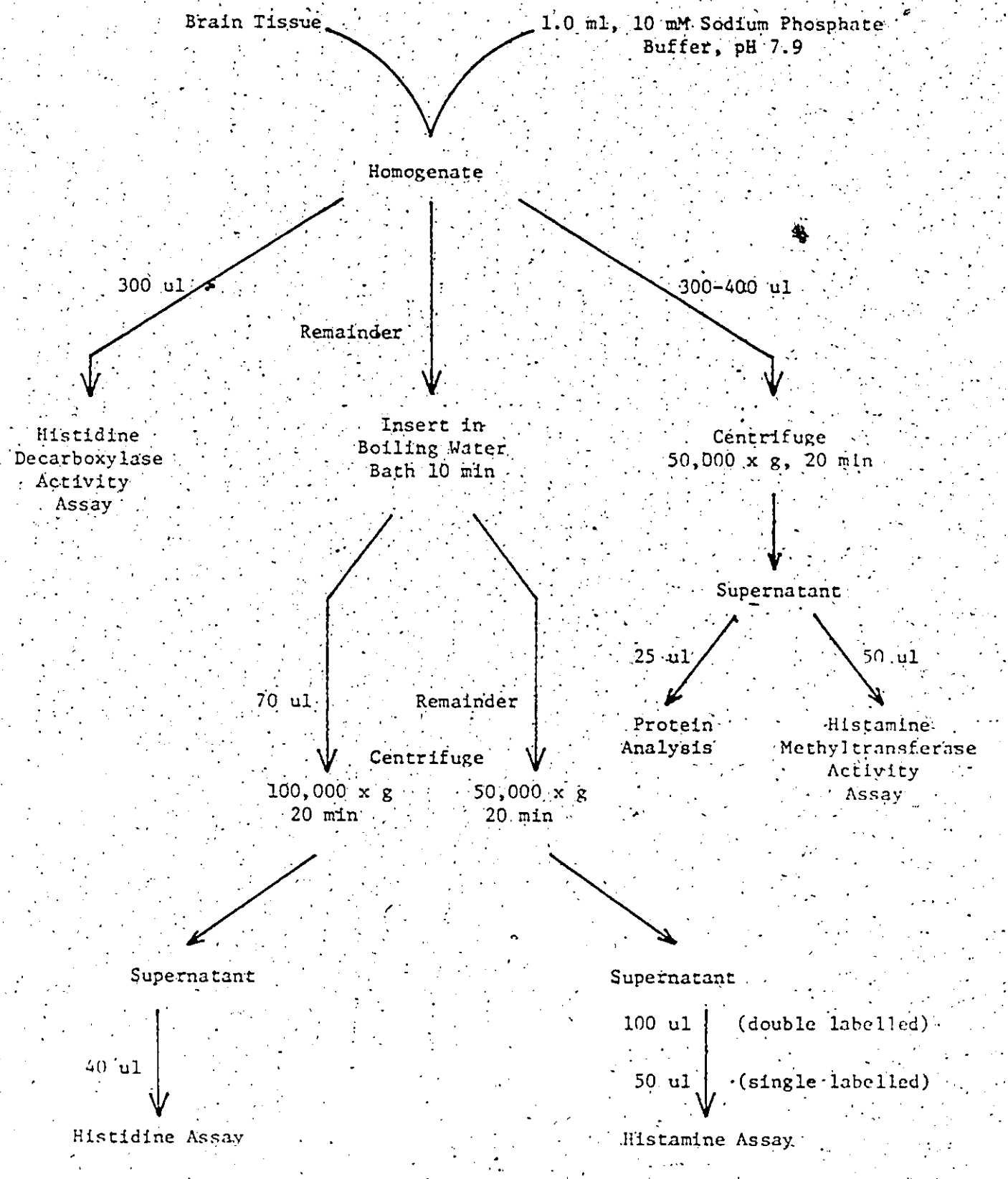
1. Determination of brain histamine concentration: double labelled method

Male Sprague-Dawley rats were sacrificed by decapitation and their brains were rapidly removed and washed with ice cold saline. The hypothalamus, midbrain and right cerebral cortex regions were dissected on an ice-cooled plate according to the method of Glowinski and Iversen (1966). The tissues were weighed, added to 1.0 ml of 10 mM sodium phosphate buffer (pH 7.9) and homogenized for 20 sec with a Brinkmann Polytron Homogenizer (type PT10, set at 7). The homogenates were heated in boiling water (see V. C) for 10 min, cooled and transferred to polycarbonate centrifuge tubes maintained in an ice bath. The boiled homogenates were centrifuged at 50,000 x g for 10 min at 4°C in a Beckman L5-50 preparative ultracentrifuge. Triplicate supernatant aliquots of 100 µl each were transferred to polypropylene culture tubes and frozen at -80°C until the time of analysis (see Fig. 11).

Histamine content was assayed by a modification of the Taylor and Snyder (1972a) double isotopic microassay technique (see V. C). This method depends on the methylation of endogenous HA by exogenous histamine N-methyltransferase (HMT) using labelled S-adenosyl-L-methionine. After thawing the frozen supernatants (stored in the culture tubes and prepared as described above) 100 µl of 50 mM sodium phosphate (pH 7.9) buffer were added followed by a 25 µl solution of ^{3}H-histamine (10.5 Ci/mmole) from New England Nuclear (NEN). To this was added 50 µl of a guinea pig brain HMT preparation, previously isolated and partially purified through the ammonium sulfate step of the method of Brown et al. (1959). After vortexing, 25 µl of an adenosyl-L-methionine, S(methyl-^{14}C) (^{14}C-SAM) (approx. 55 mCi/mmole, NEN) solution was introduced. The mixture was vortexed again then incubated for 60 min at 37°C. The final exogenous ^{3}H-HA and ^{14}C-SAM concentrations and their specific activities per sample were 0.82 nM (2.6 nCi) and 3.0 µM (49.7 nCi), respectively. The reaction was terminated with the addition of 1 N NaOH saturated with NaCl. The newly formed single and double labelled N^{τ}-methylhistamine was extracted into 3.0 ml of chloroform and vigorously vortexed. After 2 minutes of centrifugation at 1,000 x g, the aqueous layer was removed

Figure 11

General Scheme of Brain Tissue and Sample Preparation



by aspiration and the remaining organic phase was washed with 1 ml of the NaOH/NaCl solution, vortexed and re-centrifuged. After removal of the aqueous layer, the washed organic layer was transferred to polyethylene liquid scintillation counting vials and evaporated under a stream of nitrogen gas. The residue was dissolved in absolute ethanol then 10 ml of Econofluor (NEN) were added. Both ^{14}C and ^{3}H were simultaneously counted in a Beckman LS 8100 liquid scintillation spectrometer. Sterile polypropylene culture tubes, polypropylene pipette tips and polycarbonate centrifuge tubes were used so as to minimize histamine or methylhistamine adsorption onto glass.

Histamine standard curves were obtained by plotting histamine (Baker) base (ng) versus the ratio of $^{14}C/H^{3}$ disintegrations per minute. This ratio was used to determine the ng of histamine in the 100 µl supernatants from the boiled homogenates. Tissue concentrations were expressed as the ng of histamine per gram of wet tissue weight.

2. Determination of brain histamine N-methyltransferase activity: basal activity and its alteration due to drugs in vitro.

Aliquots from homogenates previously prepared for histamine determinations were transferred to polycarbonate centrifuge tubes kept in an ice bath prior to being centrifuged at 50,000 x g for 10 minutes in a Beckman L5-50 preparative ultracentrifuge. Histamine N-methyltransferase (E.C. 2.1.1.8) (HMT) activity in 50 µl supernatant volumes buffered with 50 µl of 50 mM sodium phosphate (pH 7.4) was determined by a modification of the isotopic microassay method of Taylor and Snyder (1972a) as described earlier (Prell and Mazurkiewicz-Kwilecki, 1981). The supernatants at all states of analysis were maintained in an ice bath and centrifugation procedures were at 4°C. Exogenous histamine was methylated by supernatant HMT in a total incubation volume of 250 µl using 9 nCi of adenosyl-L-methionine, S-(methyl-^{14}C) (NEN) as the methyl donor at final concentrations of 10 µM and 80 µM, respectively. Following 120 min of incubation at 37°C,

200 µl of ice cold sodium borate buffer (500 mM, pH 10.5) were added and the newly synthesized ^{14}C-methylhistamine was extracted into 2.5 ml of (1:1) toluene-isoamyl alcohol solution, vortexed vigorously, and centrifuged at 15,000 x g for 20 min in a Sorvall RC2-B refrigerated centrifuge. For analysis, 2 ml of the organic layer were added to polyethylene scintillation counting vials containing 2 ml of ethanol followed by 8.5 ml Econofluor (NEN) then counted for 15 min in a Beckman LS 8100 liquid scintillation spectrometer. Protein estimation of 50 µl supernatant samples was determined by a modification of the method of Lowry et al. (1951). HMT activity is expressed as µmoles of methylhistamine formed per gram of supernatant protein per hour of incubation.

Twelve male Sprague-Dawley rats (250-350 g) were sacrificed by decapitation between 10:00-13:00. Brains were rapidly removed and the hypothalamus, mid-brain and cerebral cortical regions were dissected and homogenized as described earlier. After homogenization, all homogenates of the same brain region were pooled, vortexed and centrifuged for 10 min at 50,000 x g in a Beckman L5-50 preparative ultracentrifuge. The supernatants were pooled, vortexed and frozen at -80°C in small volumes until the time of analysis.

For analysis of drug effects, 50 µl samples of the thawed supernatants containing endogenous brain HMT were used. To each 50 µl of a solution containing either ethanol, acetaldehyde, morphine sulfate or naloxone HCl prepared in 10 mM sodium phosphate buffer (pH 7.9) was added. This mixture (100 µl) was pre-incubated for 10 min at 37°C then the 150 µl mixture of exogenous histamine and adenosyl-L-methionine, S-(methyl-^{14}C) (prepared as described above) was added and the entire mixture (250 µl) was incubated for 120 min at 37°C. The incubation concentrations of exogenous histamine and S-adenosyl-L-methionine were kept constant at 10 µM and 80 µM, respectively. The final incubation concentrations of the various agents ranged between 0.1 µM and 0.5 mM except that of ethanol which ranged from 0.1 µM up to 10 mM. The effects of 1 µM morphine sulfate were

studied in the presence of variable ethanol concentrations while the effects of 1 μM naloxone were examined with various morphine concentrations. Controls contained drug free 10 mM sodium phosphate buffer (pH 7.9) and were analyzed simultaneously. Each determination of HMT activity was carried out in triplicate and replicated in 2 to 4 separate experiments with different enzyme preparations.

RATIONALE OF THE PROCEDURE FOR DETERMINATION OF HISTAMINE N-METHYLTRANSFERASE ACTIVITY

General Aspects of the Methodology for Determination of HMT Activity and Improvements Introduced

a) Histamine concentration

Histamine exerts a potent substrate inhibition upon its own methylation. Concentrations of 10 µM were used routinely whereas those near 30 µM were not reliable, producing inconsistent HMT activity values. Concentrations of 100 µM were clearly inhibitory (less than half the activity than at 10 µM concentrations) and values determined at 1,000 µM concentrations were indistinguishable from blanks. The K_M value for S-adenosyl-L-methionine (SAM) was empirically determined to be about 13-18 µM under the assay conditions used. Since 50 µM and 100 µM SAM concentrations yielded nearly the same activity, 75-80 µM was routinely used in the assay.

b) Blanks

Several potential blanks were investigated for their suitability. Boiled homogenates were slightly but consistently higher than blanks using homogenizing sodium phosphate buffer. The non-incubated tissue blank (maintained in ice water) yielded cpm values several-fold higher than buffer blanks and the values increased with time. Another possibility was the use of S-adenosyl-L-homocysteine, a potent HMT inhibitor (Baudry et al., 1973b), even at concentrations which saturated the phosphate buffer, inhibited HMT activity no more than 70%. Aside from these limitations, these modified tissue blanks would require larger portions of the homogenates, nearly doubling the number of samples for analysis. Therefore, the relatively constant sodium phosphate buffer (10 mM, pH 7.9) was routinely used as blanks.

Duration of Incubation at 37°C; Its Effect on HMT Activity

The incubation time varied between 15 and 180 minutes; triplicate supernatant samples from the hypothalamus, midbrain and cortex were used. The HMT activity within each region was approximately the same over the course of the

experiment. The formation of ^{14}C-tele-methylhistamine was directly related to the duration of incubation ($r > 0.99$ in all three regions) (Fig. 12). The hypothalamic and hippocampal HMT activity was linear for up to 4 hours (not shown). Blank values also increased with increasing incubation times, from about 25 cpm at 15 min up to 60 cpm at 3 hours, but not in parallel with tissue generated cpm. Therefore, in order to balance the need for high enough number of dpm against reasonable conditions for routine enzymatic analysis, a two hour incubation period was chosen. This is longer than that suggested by Taylor and Snyder (1972a).

Dependence of Supernatant Volume on HMT Activity Estimation

To evaluate ^{14}C-tele-methylhistamine formation, different volumes of the supernatant were tested; 10, 25, 50, 75 or 100 µl from the hypothalamus, thalamus-midbrain or cortex were used. Each sample was brought to 100 µl with addition of 10 mM sodium phosphate buffer (pH 7.9). In all regions, there was a volume dependent increase in the formation of tele-methylhistamine presumably due to an increase in the amount of enzyme present. However, the elevation was strictly linear only in the thalamus-midbrain (Fig. 13). In the hypothalamus and cortex the tele-methylhistamine increased with increasing supernatant volume. This may be attributed to a reduced buffering capacity at higher volumes although the absence of a similar effect on thalamus-midbrain HMT is an enigma. This non-linear profile for these two brain regions was reproducible, but was not markedly different from linearity; therefore, 50 µl supernatant aliquots were chosen for routine analysis. This obviated pipetting errors at low supernatant volumes and provided a volume for an additional buffer at a pH (of 7.4) more compatable with elevated HA levels (Snyder et al., 1966a).

Influence of Borate Buffer Volume and the Composition of Organic Solvent on HMT Activity

The volume of 500 mM sodium borate buffer (pH 10.5) used in HMT assays

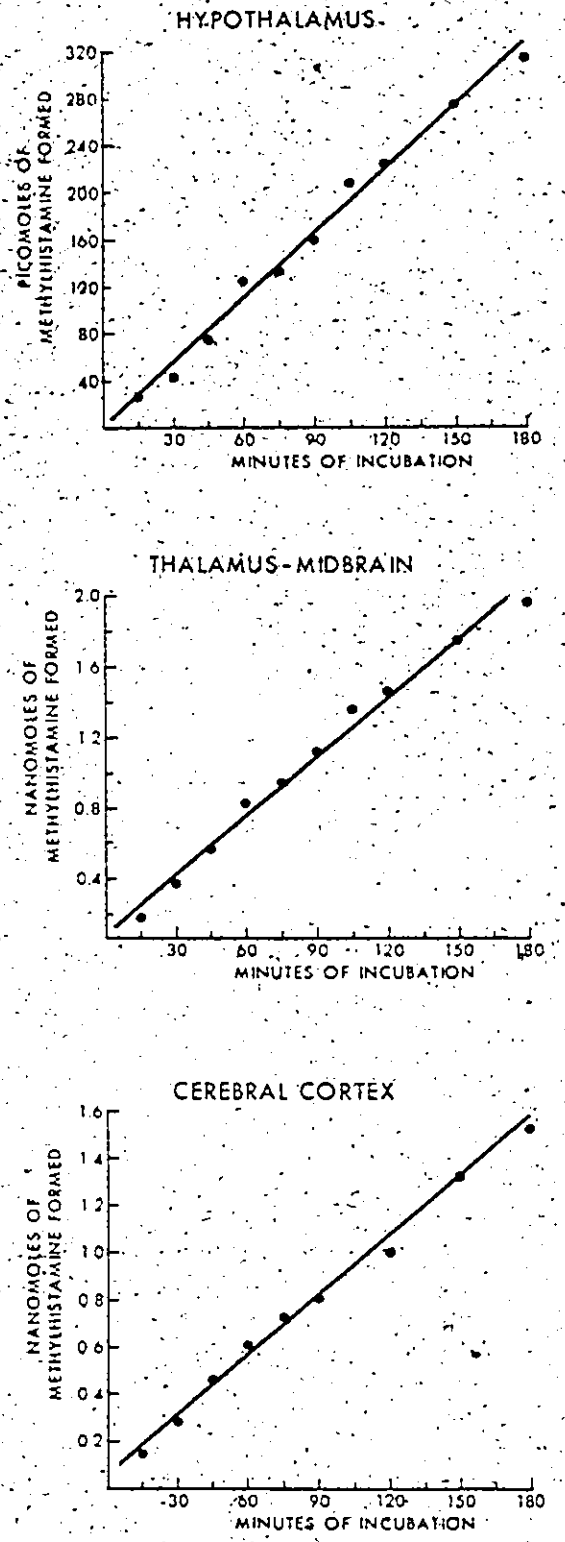


Fig.12

Dependence of incubation time on the formation of methylhistamine in samples prepared from the rat hypothalamus, midbrain and cortex.

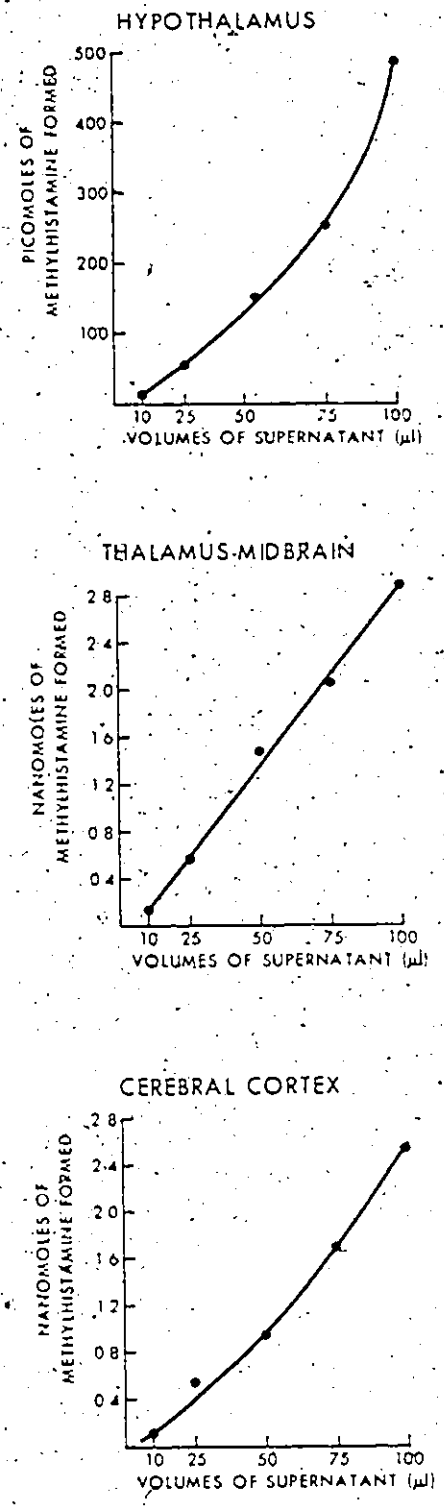


Fig. 13

Dependence of supernatant volume on the formation of methylhistamine in samples prepared from the hypothalamus, midbrain and cortex.

was initially 100 µl, which was added after the termination of incubation. This was varied to examine its effects on HMT activity and to see whether better assay conditions could be achieved. Volumes ranging between 50 µl and 1,000 µl were evaluated and found to alter the values for enzyme activity. However, further study revealed that while the cpm obtained with brain supernatants did not vary greatly, the blank values as much as quadrupled. The lowest blank value occurred with a borate buffer volume of 200 µl, which was subsequently used in the HMT determinations.

The toluene-isoamyl alcohol solution was used to extract methylhistamine since chloroform, used in the HA assay in order to selectively extract methylhistamine from histamine containing solutions (Taylor and Snyder, 1972a) is not necessary in this assay and difficult to transfer analytically. Adjusting the relative proportions of these organic constituents over a wide range generated an apparent change in the values for HMT activity. However, a volumetric ratio of 1 to 1, the proportion suggested by Taylor and Snyder (1972a) yielded the lowest blanks and highest net dpm for tissue samples.

Preparation, Isolation and Purification of ^{14}C-Tele-Methylhistamine

The extraction of ^{14}C-tele-methylhistamine into toluene-isoamyl alcohol solution during the HMT assay procedure was reported to be less than quantitative (Snyder and Axelrod, 1965). Since radiolabelled t-methylhistamine was not commercially available, it was synthesized in order to determine fractional recovery during the assay under present conditions.

Exogenous histamine (10 ng base) was methylated by exogenous guinea pig brain histamine N-methyltransferase and adenosyl-L-methionine, S(methyl-^{14}C) (^{14}C-SAM) (NEN) by a procedure analogous to the double labelled method of Taylor and Snyder (1972a). However, tritiated histamine was not introduced. This amount of HA was chosen since it was the upper limit of the histamine used in the double labelled standard curve; a procedure that was well established in our laboratory.

The newly generated ^{14}C-methylhistamine was extracted into chloroform, washed twice with 1 N NaOH saturated with NaCl. Following the aspiration steps, as described before, the chloroform was evaporated. The polypropylene culture tubes were capped and frozen at -80°C until the time of analysis.

At the time of assay, the samples were defrosted and for initial separation, 200 µl of absolute ethanol was added to several tubes to dissolve the ^{14}C-tele-methylhistamine residue. The ethanol was then evaporated to 10-30 µl under a N_2 gas stream. This volume was applied to 20x20 cm (250 µ)-prescored multi-channeled silica gel G thin layer chromatography (TLC) plates and eluted for about 60 min with a solvent system of chloroform-methanol-ammonia (12:7:1) (Aures et al., 1968). Authentic t-methylhistamine was run on parallel channels for comparison. 1 cm segments along the length of the plate were scrapped from several channels then placed into polypropylene LSC vials and 2 ml ethanol and 10 ml Econofluor (NEN) added. The ^{14}C-radioactivity in each tube was counted for 20 min. The radioactivity was plotted as a function of distance along the plate and compared to authentic tele-methylhistamine (Sigma Chemical Co.) developed with ninhydrin (0.2% in acetone) (Fig. 14a). Scrappings from the remaining channels near the area corresponding to the R_f for methylhistamine were placed in filter funnels, eluted with 6-10 ml of ethanol and evaporated to 10-30 µl volumes under N_2 gas. This volume was applied to TLC plates under identical conditions as above. Following elution and drying, 1 cm segments of the silica gel were scraped into LSC vials, eluted with ethanol and counted after addition of Econofluor as described above. The radioactivity of this purified ^{14}C-tele-methylhistamine was plotted as a function of the distance along the chromatogram (Fig. 14b).

The single peak of the rechromatographed material attests to the lack of contaminants of the newly formed ^{14}C-methylhistamine. The large unidentified and unexpected peak which was initially eluted from the solvent front (localized at 14-16 cm) (Fig. 14a). was a cause for concern for it seemingly raised doubts

THIN LAYER SEPARATION OF ^{14}C-METHYLHISTAMINE

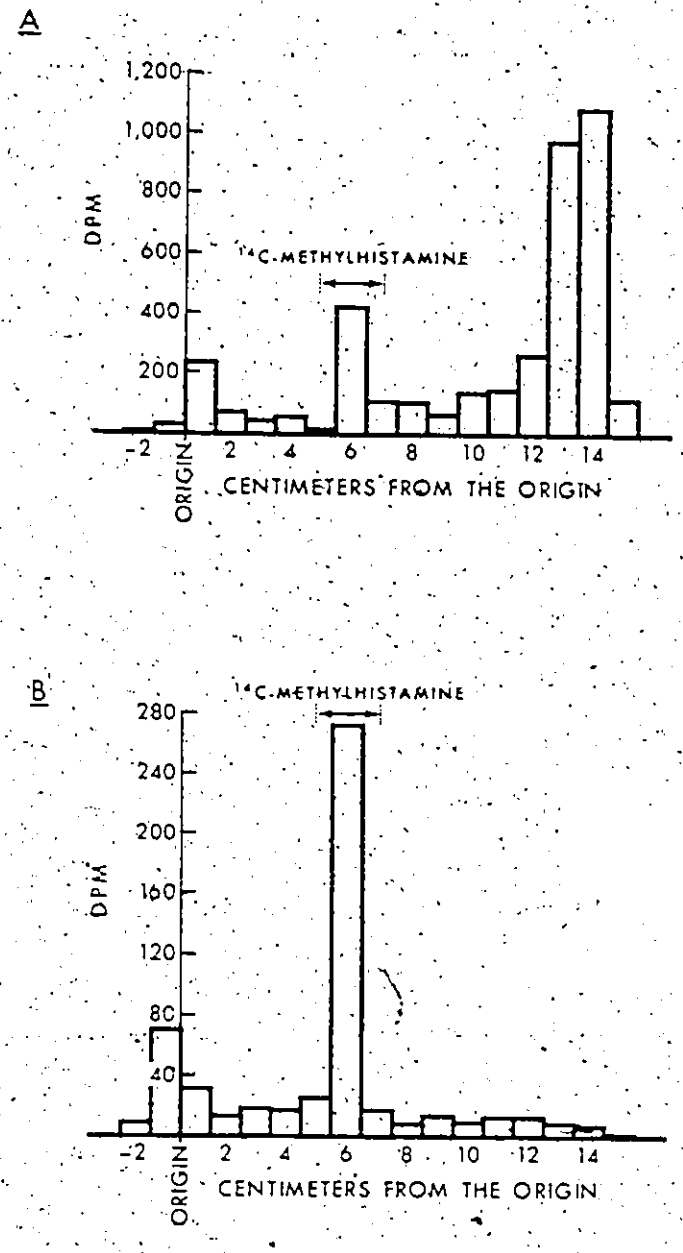


Fig. 14

Radiochromatographic separation of ^{14}C-methylhistamine on silica gel after multiple elutions with ethanol. A. Separation of ^{14}C-radioactivity generated in a modified Taylor and Snyder (1972a) procedure. B. Separation of the methylhistamine region of the primary chromatogram.

on the validity of the procedure for histamine determination. However, further scraping and elution of this zone of radioactivity subsequently revealed that no less than 90% of this ^{14}C-region was attributable to ^{14}C-tele-methylhistamine. Replicating this experiment led to similar results. While the co-migration of a substantial portion of the methylhistamine and the solvent front may question the suitability of this method, in retrospect the absence of unlabelled tele-methylhistamine to act as a carrier may explain much of this apparent inconsistency.

Chloroform, initially used to elute the ^{14}C-tele-methylhistamine from the silica gel and the sides of the collecting tubes was judged unsuitable since it was found to break down methylhistamine after several hours. The second chromatograph ultimately exhibited no less than 6 peaks using this solvent. n-Butyl alcohol has also been claimed to be an effective solvent by this method (Hough, personal communication).

Extraction of ^{14}C-Tele-Methylhistamine During the HMT Assay

In order to approximate the fraction of ^{14}C-tele-methylhistamine extracted and recovered into the toluene-isoamyl alcohol solution during the determination of HMT activity, the HMT assay was performed as described earlier but devoid of ^{14}C-SAM. Ten minutes before the end of the incubation, 10 µl aliquots of isolated and purified ^{14}C-tele-methylhistamine, previously dissolved in 50 mM sodium phosphate buffer (pH 7.4) and pooled, were added to 5 samples and 5 blanks. Thereafter, the procedure for determining HMT activity continued as described earlier. After accounting from the removal of 2.0 ml from the 2.5 ml organic layer, the fraction extracted averaged 80.7% (range 97.6-81.5). Replicating this procedure (n=4) led to similar findings (79.8%).

Since ^{14}C-tele-methylhistamine was added near the end of the incubation period, its non-specific or enzymatic metabolism would be unlikely. A 70% extraction of methylhistamine was reported by Snyder and Axelrod (1965), although their methodology in determining this value was not elaborated. It may be

important that their method was carried out using glass tubes, a surface upon which Beaven et al. (1972) suggested methylhistamine adheres. Perhaps this accounts for their reduced fractional extraction. In their micro-method, Taylor and Snyder (1972a) made no correction for the efficiency of methylhistamine extraction.

3. Determination of protein concentration in homogenates and supernatants from brain tissue

Protein content was determined by a modification of the colorimetric method of Lowry et al. (1951). Various homogenates or supernatant samples previously stored at -80°C were thawed and diluted with distilled water such that 5-200 µg of protein were present in a volume of 500 µl. Blanks were composed of distilled water. Five milliliters of a solution containing 80 mM copper sulfate and 213 mM sodium tartrate in 4 % sodium carbonate was added to the diluted samples at room temperature and vortexed immediately. The mixture was then incubated at 45°C after which 500 µl of a phenol (Folin-Ciocalteau) solution diluted to one-third with distilled water were added and the mixture cooled for 5 min in an ice water bath. The transmittance was determined at 640 mµ in a Bausch and Lomb Spectronic 20 colorimeter 30 min after the addition of phenol. A standard curve relating the per cent transmittance to known amounts of protein (Bovine Albumin, Fraction V; J. T. Baker Chemical Co.) ranging from 5-200 µg was used with each group of samples to determine protein concentration.

4. Determination of brain histidine decarboxylase activity

Histidine decarboxylase (E.C. 4.1.1.22) (HD) activity was determined in several brain regions by a modification of the method described earlier (Mazurkiewicz-Kwilecki and Bielkiewicz, 1978). Aliquots (300 µl) from homogenates prepared for the determination of histamine were transferred to polypropylene tubes and buffered with 50 µl of 60 mM potassium phosphate buffer (ph 7.0) which contained 2.6 nmoles of histamine in order to inhibit histamine

N-methyltransferase. Fifty microliters of freshly prepared pyridoxal 5'-phosphate were added and the mixture was pre-incubated for 10 min at 37°C; then, 10 µl of L-histidine, (^{14}C-uniformly labelled, 343 mCi/mmole) (NEN) were added followed by 50 µl of 1.5 mM aminoguanidine nitrate, a diamine oxidase inhibitor. This mixture was incubated in a nitrogen atmosphere for 90 min at 37°C. The final concentrations of exogenous histidine and pyridoxal 5'-phosphate in the 460 µl reaction volume were 6.3 µM and 8.8 µM, respectively. Blanks were composed of homogenizing buffer as a substitute for tissue homogenates.

The reaction was terminated by insertion into an ice bath and addition of 50 µl of an aqueous histidine/histamine solution (containing 10 mg of each salt in 5 ml) followed by 50 µl of 1 N perchloric acid. The mixture was vortexed and then centrifuged for 20 min at 10,000 x g. The supernatant was retained and spotted onto WHATMAN #3 chromatographic paper for electrophoretic separation in a formate-acetate (1:4 by vol.) buffer (pH 1.9) at 3,000 V on a LOCARTE flat plate high voltage electrophoretic apparatus. Histidine and histamine markers were run in parallel with the labelled supernatants and after drying, identified with 0.2% ninhydrin in acetone.

The region near the histamine positive spot was cut out and inserted into polyethylene counting vials. The newly formed ^{14}C-histamine was eluted with 1 ml of water to which 10 ml of Bray's solution (NEN) or Aquafluor (NEN) were added then counted in a Beckman LS 8100 liquid scintillation counter. The enzyme activity is expressed as the nanomoles of histamine formed per 90 min per g of wet tissue weight to compare with previous values (Mazurkiewicz-Kwilecki and Bielkiewicz, 1978).

5. Determination of plasma ethanol concentration

Plasma ethanol levels were determined by a modification of the ultraviolet enzymatic method for ethyl alcohol analysis (Sigma Tech. Bull. 331-UV). Plasma samples, previously frozen at -80°C, were thawed and re-centrifuged for 10 min at 2,000 x g. Since the plasma was generally not hemolyzed, 100 µl samples were

transferred to 12x75 mm polypropylene tubes and buffered with 400 µl of 75 mM tetrasodium pyrophosphate buffer (pH 9.2) which contained 30 µmoles of semicarbazide. After vortexing, 100 µl of this 5-fold diluted sample were transferred to test vials containing 3.0 ml of 1.8 µM nicotinamide adenine dinucleotide (NAD) and 150 units* of alcohol dehydrogenase buffered with 75 mM pyrophosphate. Blanks contained 100 µl of 10 mM sodium phosphate buffer (pH 7.9). The reaction mixture was shaken, capped and incubated for 20 min at 37°C. Upon cooling, the mixture was transferred to square cuvettes (1 cm light path) and the absorbance was determined at 340 nm in a Unicam SP 800B ultraviolet spectrophotometer.

In cases where the extent of hemolysis was questionable, the plasma samples (500 µl) were deproteinized with 2.0 ml of 0.4 M trichloroacetic acid and mixed by inversion. After centrifugation for 10 min at 2,000 x g, 100 µl supernatant aliquots were transferred to the test vials and the procedure continued as above. Under both conditions, samples run in parallel produced almost identical results. The values obtained were compared to a standard curve which related absorbance to known ethanol concentrations ranging from 20 to 160 mg ethanol per 100 ml solution. Under no circumstances did the ethanol concentrations determined by graphical analysis differ by more than 6% from those obtained by theoretical evaluation of absorbance measurements. The plasma ethanol concentrations are expressed as milligrams of ethanol per 100 ml of plasma, i.e. mg%.

6. Determination of plasma corticosterone concentration

Following decapitation, rat blood was collected from the severed cervical area into heparinized tubes and centrifuged at 2,000 x g for 20 min. Duplicate plasma samples (500 µl each) were retained. Corticosterone concentration was determined by a modification of the method of Givner and Rochefort (1965) which is based on the capability of corticosterone to fluoresce in sulfuric acid.

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*One unit of alcohol dehydrogenase enzyme converts 1.0 µmole of ethanol to its product, acetaldehyde, per minute at a pH of 8.8 at 25°C.

The samples and standards (40-400 nanograms of corticosterone) were transferred to 12 ml glass stoppered tubes to which 6 ml of methyl chloride (Fisher Scientific, certified A.C.S.) were added. After vortexing, 500 µl of a 0.1 N NaOH solution were added, vortexed again and centrifuged for 10 min at 1,000 x g. The aqueous layer was removed by aspiration and 4.0 ml of the remaining organic phase were transferred to another set of tubes to which 2.0 ml of a sulfuric acid solution (70% in ethanol by volume) were added. After vortexing and centrifugation at 1,000 x g for 5 min, the organic phase was removed and the acidic phase was read in an Aminco-Bowman (model J4-8962) spectrophotofluorometer (excitation and emission wavelengths of 470 nm and 530 nm, respectively). A standard curve which related known amounts of corticosterone (Sigma) to fluorescence intensity was used to determine the plasma corticosterone levels which are expressed as micrograms of corticosterone per 100 ml of plasma.

7. Determination of brain histidine concentrations: a method using fluorescamine

Brain tissue homogenates were prepared by the same procedure as for the double labelled histamine determination except that the initial centrifugation was at 100,000 x g for 20 min in a Beckman L5-50 preparative ultracentrifuge. Supernatants (40 µl) were transferred to Brinkmann 500 µl polypropylene micro tubes and frozen at -80°C until the time of analysis. The samples were thawed and the remaining proteins were precipitated by sequential addition of 60 mM $ZnSO_4$ and 0.12 N NaOH, each in a volume of 5 µl. The mixture was vortexed then centrifuged at 12,000 x g for 10 min in an Eppendorf model 5412 centrifuge. Blanks, composed of 40 µl of 10 mM sodium phosphate buffer (pH 7.9) were similarly filtered. Forty microliter aliquots of the filtrate were introduced into polypropylene culture tubes and buffered with 10 µl of 0.5 M sodium borate buffer (pH 10.5). Histidine hydrochloride monohydrate standards (1-500 µM) were prepared in sodium phosphate buffer and then buffered by the addition of borate.

After vortexing the mixture, 50 µl of fluorescamine (100 mg% in acetonitrile) were added, then vortexed again and allowed to stand 10 min after which 20 µl of 4 N HCl was added.

The entire mixture was heated for 45 min at 80°C. Following cooling and centifugation at 1,000 x g for 10 min, a 20 µl aliquot was removed and added to 1.2 ml of distilled water. After 30 min, the fluorescence intensity of the histidine-fluorescamine adduct was measured in an Aminco-Bowman spectrophotofluorometer (excitation and emission wavelengths of 369 and 465 nm, respectively).

DEVELOPMENT OF THE METHOD FOR DETERMINATION OF BRAIN HISTIDINE CONCENTRATION USING FLUORESCAMINE*

Rationale for the Development of this Method

The regulation of brain histamine levels is, in part, controlled by the histidine decarboxylation process. However, since the histidine decarboxylase enzyme is not saturated by endogenous histidine (His) in mammalian brain (see section VII) and because the uptake of histidine in the brain may be altered by drugs, the determination of brain histidine content appeared essential in elucidating the mechanisms of drug-induced changes in brain histamine levels. The established and most implemented methods for brain His measurement utilize amino acid analyzers or high pressure liquid chromatography. These methods have the marked advantage of simultaneously determining several amino acids which leads to important inferences concerning similar groups of amino acids or the amino acid population as a whole. A set-back is that prior derivatization is usually required and each sample run may take 1-4 hours before another sample can be evaluated. More importantly, the above mentioned equipment must be available. Since such equipment was not available during the course of this study, alternative methods were considered. Fluorescence techniques using o-phthalaldehyde (OPT) are available (e.g. Hakanson et al., 1974); however, this approach necessitates multiple and tedious extractions to remove potential contaminants. The radioenzymatic microassay of Taylor and Snyder (1972a) utilizes bacterial histidine decarboxylase (Clostridium perfrinigens, Sigma Chemical Co.) and conversion of HA is a measure of the histidine originally present in a sample. This method is reported to be extremely sensitive; however, the residual presence of drugs and/or metabolites in the tissues, especially following acute drug administration schedules, may

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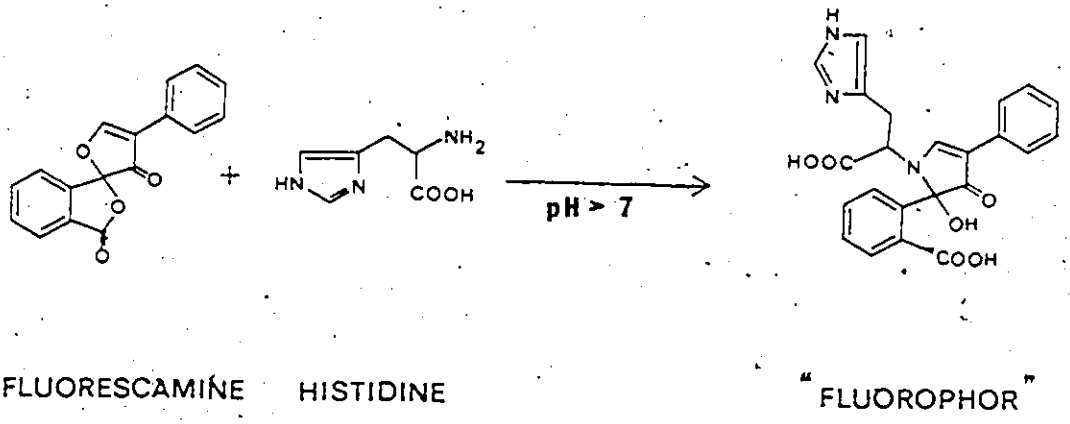
*Part of this investigation was presented at the Twenty-Second Annual Meeting of the Society of Toxicology, Las Vegas, Nevada (March 1983) and the Twenty-Sixth Annual Meeting of the Canadian Federation of Biological Societies, Ottawa, Ontario (June 1983).

influence this enzymatic-dependent process. Altering HD activity directly would lead to false inferences concerning the endogenous histidine levels. Therefore, a reliable chemical method that is sensitive, rapid and cheap was sought which would not confound the specificity of histidine analysis.

Experimental Conditions Which are Selective for Histidine

Since its development in 1972, fluorescamine (FL), 4-phenylspiro(furan-2(^{3}H), 1'-phthalan)-3,3'-dione, has been increasingly used as a reagent for the qualitative and quantitative fluorescence analysis of primary amines, amino acids, peptides and proteins at sub-nanomole levels (Weigele et al., 1972; Stein et al., 1973; Imai et al., 1974) (see Fig. 15 for an example). Several properties make



FLUORESCAMINE HISTIDINE "FLUOROPHOR"

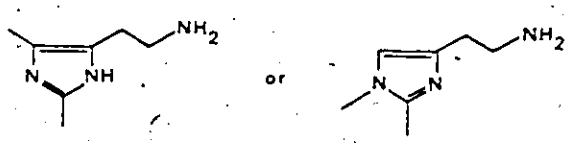


Fig. 15

General reaction for derivatization of fluorescamine with histidine in basic media and the structural requirements for fluorescamine-imidazolylethylamine derivative fluorescence.

fluorescamine a highly useful reagent. It is cheap and stable for prolonged periods (weeks) when stored below 0°C in water-miscible, nonhydroxylic solvents. (DeBernardo et al., 1974). Fluorescamine derivatizes with primary amines at room temperature in seconds while excess fluorescamine is simultaneously hydrolyzed (Udenfriend et al., 1972). It has a marked advantage over OPT in that neither fluorescamine nor its hydrolysis product(s) generate significant fluorescence, yet appropriate fluorescamine derivatives exhibit intense fluorescence intensity (Udenfriend et al., 1972). Although broad in its spectrum of application, the original methodology is far from selective for any particular group of primary amines or amino acids and therefore prior or subsequent separation is needed in order to quantify a single substance in biological systems (Taylor and Lieber, 1977). More recently, it was reported that heating fluorescamine-primary amine derivatives in hydrochloric acid selectively abolishes the fluorescence of those derivatives that do not possess the 2-(4-imidazolyl)-ethylamine moiety as their complete or amino-terminal structure (Nakamura and Pisano, 1976; Nakamura, 1977) (see structures in Fig. 15). Unfortunately, histidine is not the only imidazolylethylamine present in mammalian tissue nor in the brain in particular. Yet, it was also reported that the various fluorescamine-imidazolylethylamine derivatives had chemical properties sufficiently dissimilar to allow silica gel thin layer chromatographic separation of these adducts using several solvent systems (Nakamura, 1977). Therefore, the initial approach in finding conditions highly selective for His was to study the excitation/emission spectra of His and some related compounds, i.e. histamine (HA), tele-methylhistidine (MeHis) and tele-methylhistamine (MeHA).

Fluorescamine (FL) derivatives of these agents were prepared from 1 mM stock solutions in distilled water by the method outlined earlier for preparation of the histidine standard curve. The excitation (Fig. 16) and emission

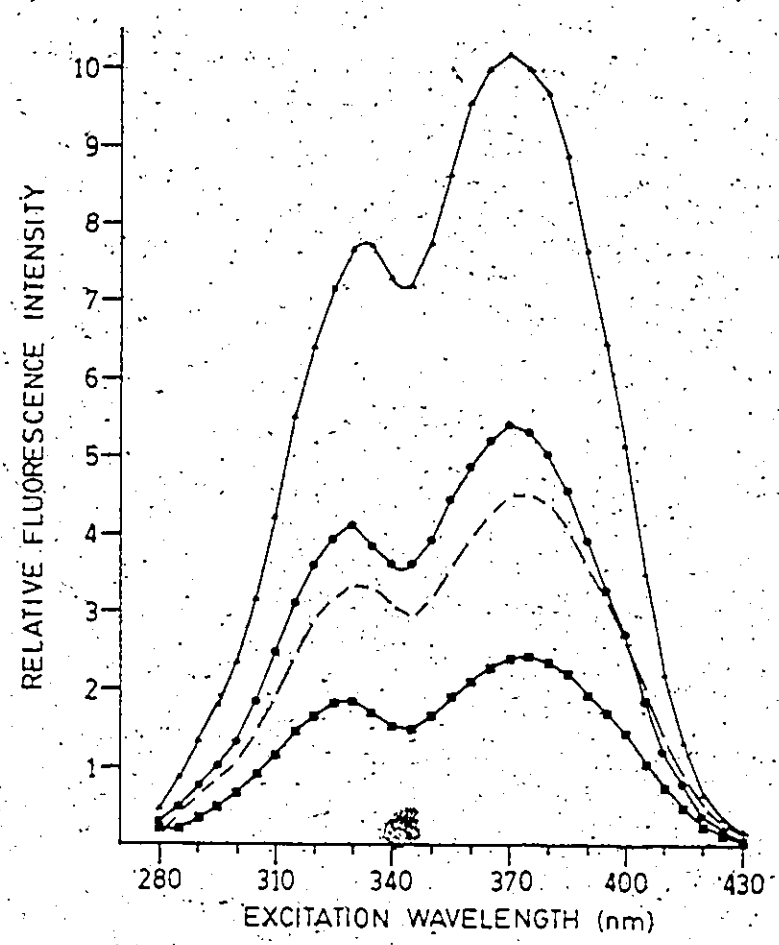


Fig. 16

Excitation spectra of fluorescamine-imidazolylethylamine derivatives measured at 465 nm. Relative fluorescence intensity (machine units) was determined every 5 nm for histidine (●), methylhistidine (▲), histamine (■) and methylhistamine (□).

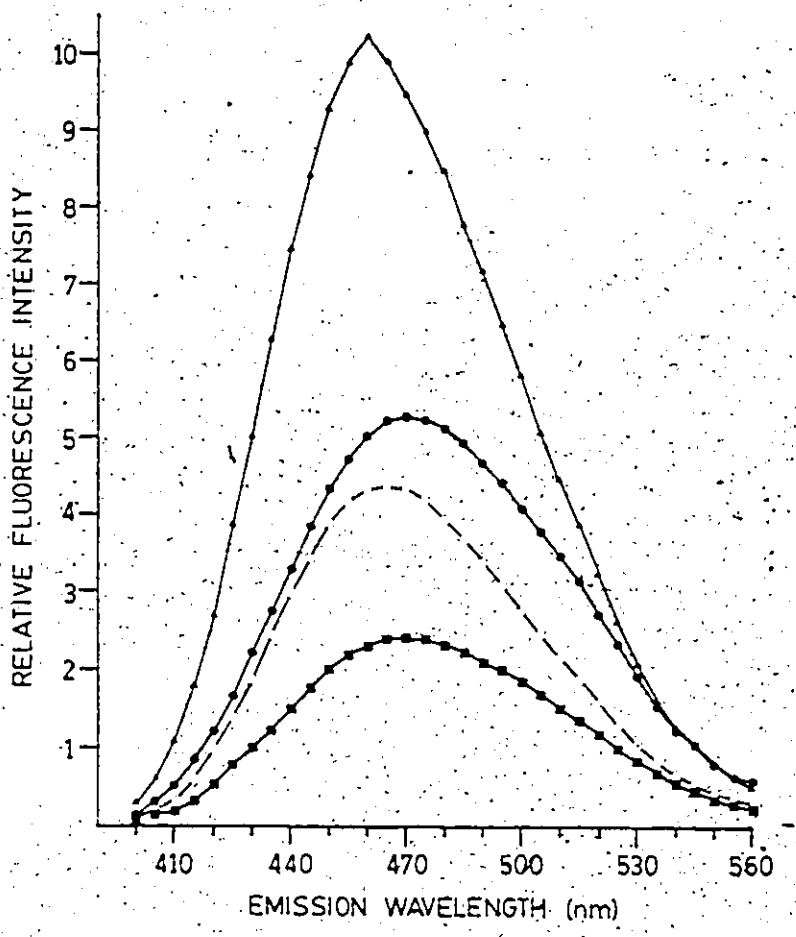


Fig. 17

Emission spectra of fluorescamine-imidazolylethylamine derivatives measured every 5 nm following excitation at 370 nm. Relative fluorescence intensity (machine units) was determined for histidine (●), methylhistidine (▲), histamine (■) and methylhistamine (□).

(Fig. 17) spectra of these derivatives were determined by plotting the relative fluorescence intensity (F_I) (in machine units) recorded every 5 nm. At almost all wavelengths investigated, the rank order of relative F_I was MeHis > His > MeHA > HA at equimolar concentrations. Since this approach did not yield histidine specific characteristics, the evaluation of the various FL solvent mediums was attempted. Since dioxane, acetone and acetonitrile are also water miscible solvents and reported to promote optimal reaction between fluorescamine and primary amines (Weigele et al., 1972), the above reagents were evaluated at various fluorescamine concentrations (Fig. 18). When fluorescamine was dissolved in dioxane the same rank order of relative F_I prevailed. However, the relative F_I was less than a third of that observed with acetonitrile and there was a profound dependence of F_I on the FL concentration. When FL was introduced in an acetone medium, the relative F_I was much higher but dependent on the FL concentration. However, using acetonitrile as the organic solvent, the relative F_I was greater and nearly independent of the fluorescamine concentration (Fig. 18). Although a histidine specific characteristic was not evident, several important aspects concerning the choice of an organic solvent are highly relevant. Since FL reacts simultaneously with all possible primary amines at various rates of reaction, the concentration of FL at any single moment would be in a dynamic state. Therefore, conditions where the ultimate F_I (which depends on the number of FL-imidazole derivatives formed) is independent of the changing FL concentration would be the most desirable. This difficulty is exacerbated by the rapid hydrolysis of FL in aqueous media. A solution to this problem is to use acetonitrile. In addition, acetonitrile has the advantage that it retains a liquid phase at temperatures below 0°C whereas dioxane solutions freeze upon refrigeration.

Altering several of the chemical and spectral parameters of this procedure failed to show any properties selective for His. However, examination of the concentrations of potential interfering substances present in mammalian brain

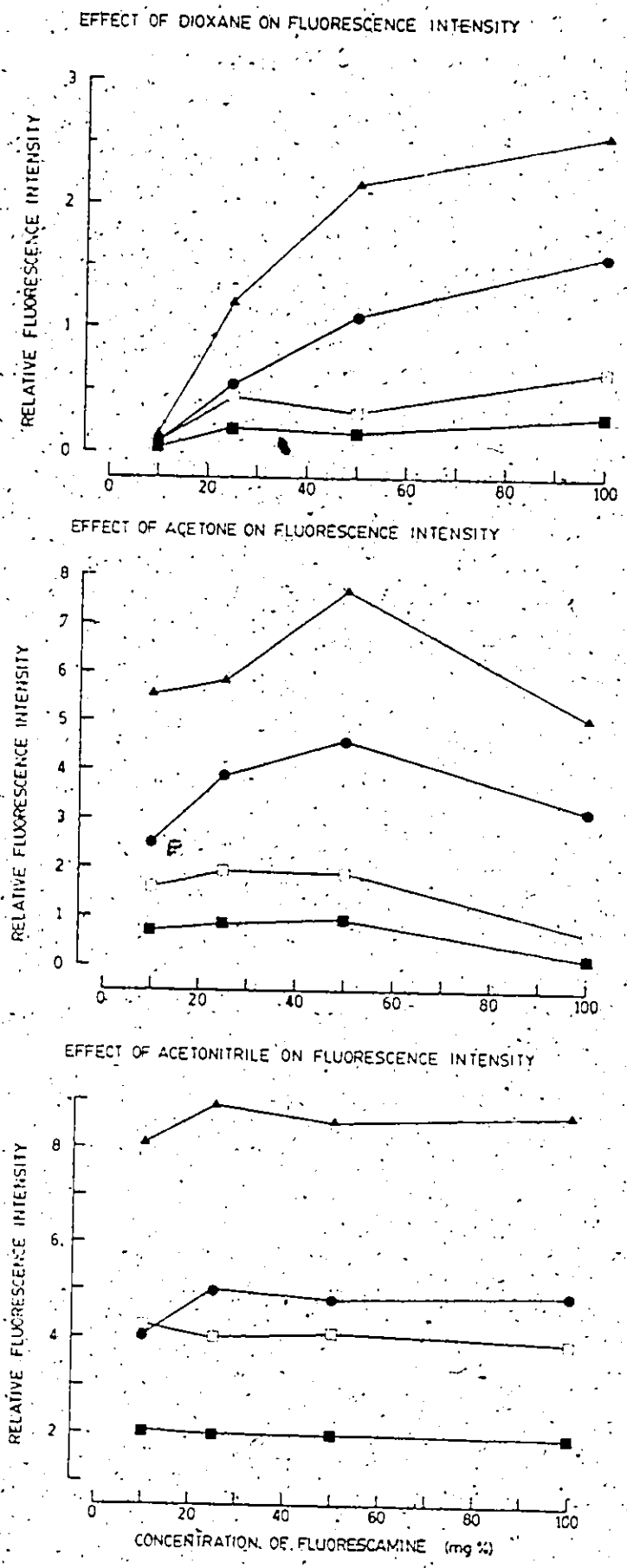


Fig. 18

Relative fluorescence intensity (machine units) dependence on various concentrations of fluorescamine dissolved in dioxane, acetone or acetonitrile. Fluorescamine concentrations of 10, 25, 50 and 100 mg% were added to buffered solutions containing 10 nanomoles of histidine (●), methylhistidine (▲), histamine (■) or methylhistamine (□).

led to the conclusion that His is approximately 100-500 times more abundant than the most likely substances capable of fluorescence under these conditions (Table4). In the hypothalamus, the region of highest HA and MeHA concentrations, the His/HA and His/MeHA ratios are about 100. In the cortex, these ratios are

Table 4

Comparison of Brain Histidine, Histamine and Their Methyl Derivatives

Tissue	Substance	(μg/g)	Ratio
Hypothalamus	Histidine	18-25 (1)	-
	Histamine	0.19 - 0.21 (1)	His/HA 86-132
	Methylhistamine	0.18 (2)	His/MeHA 100-139
	Methylhistidine	? (3)	?
Cortex	Histidine	7.9 - 9.4 (1)	-
	Histamine	0.027-0.029 (1)	His/HA 203-348
	Methylhistamine	Approx. 0.032 (2)	His/MeHA 247-294
	Methylhistidine	? (3)	?

1) Taylor and Snyder (1972a)
2) Hough and Domino (1979)
3) undetermined

even greater. Moreover, the relative F_I's of HA and MeHA at equimolar concentrations are only 40-80% that of His. Therefore, under the current tissue preparation procedure and assay conditions, these amines would be diluted to such an extent that no fluorescence could be detected from their FL adducts. Free MeHis is virtually absent from mammalian brain (Young and Munro, 1978) although this assertion has been recently challenged (and shall be dealt with later).

The claim for FL-imidazolylethylamine selective fluorescence after heating in acid was further corroborated by examining the relative F_I of several groups of

agents and comparing them individually to that of equimolar histidine (Tables 5a-c). Consistent with the structural requirements outlined by Nakamura (1977), π-methylhistidine and π-methylhistamine had negligible F_I while that of τ-methylhistidine nearly doubled the relative F_I of His. D-Histidine had only 70% the relative F_I of the L-isomer, a repeated and interesting finding which remains unexplained. Of all the amino acids investigated, L-tryptophan had the highest relative F_I although only 1.2% that of His (Table 5a). The level of brain tryptophan is important not only as a precursor for serotonin and melatonin, but also for its possible interference with histidine uptake (see section VII). However, this small analytical interference is further dwarfed by two other factors. First, much of the thermally-labile tryptophan is destroyed by the near boiling of homogenates prior to centrifugation. Second, in most brain regions, the free His content exceeds that of tryptophan. The substances which cause profound interference with the OPT analysis of brain HA or His, i.e. spermine and spermidine do not fluoresce under these conditions.

L-Histidine methylester- and L-histidinol-FL adducts yield substantial fluorescence while α-methylhistidine, N^{α}-methylhistamine, imidazoleacetic acid, its methylderivative and representative histidine and methylhistidine containing dipeptides of neuropharmacological interest show negligible F_I. Almost all of the agents interacting at HA receptors or with the enzyme histidine decarboxylase fail to fluoresce with the exception of α-hydrazinohistidine (Fig. 5b). Evaluation of some central neurotransmitters, their precursors, metabolites and related-agents also indicated negligible fluorescence. An examination of several anti-depressant agents (many generously donated by Dr. P. Hrdina, Department of Pharmacology, University of Ottawa) showed insignificant fluorescence with the exception of trazodone which had about 5% the relative F_I of equimolar His (Table 5c). Several psychoactive drugs, many related to drug abuse studies, also failed to exhibit fluorescence.

Table 5a

Relative Fluorescence Intensity of Various Amines, Compounds and Drugs of Biochemical, Neurochemical or Pharmacological Interest Compared to Histidine (set at 100 relative units) at an Equimolar Concentration of 100 μM Prior to Addition of HCl. Values of 0 signify that the F_I was indistinguishable from that of blanks.

Substance Analyzed	F_I Compared to Histidine
L-Histidine-HCl-H_2O	100
D-Histidine HCl	69.1
τ-Methylhistidine	184.7
Π-Methylhistidine	<0.1
Histamine $(HCl)_2$	39.6
τ-Methylhistamine $(HCl)_2$	79.6
Π-Methylhistamine $(HCl)_2$	1.3

L-Alanine	0.6	L-Serine	0
L-Arginine	0	L-Threonine	0
L-Asparagine	0	L-Tryptophan	1.2
L-Aspartic Acid	0	L-Tyrosine	0
L-Cysteine	0	L-Valine	0
L-Glutamic Acid	0		
L-Glutamine	0	Spermine	0
Glycine	0	Spermidine	0
L-Histidine	100	Putrescine	0
L-Isoleucine	0	Creatinine	0
L-Leucine	0	Taurine	0
L-Lysine	0	Imidazole	0
L-Methionine	<0.1		
L-Phenylalanine	0		
L-Proline	0		

Table 5b

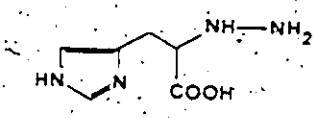
Substance Analyzed		Substance Analyzed	
	F_I Compared to Histidine		F_I Compared to Histidine
α-Methyl-DL-histidine $(HCl)_2$	0.7	Tyramine	0
L-Histidine methylester $(HCl)_2$	58.1	Octopamine	0
L-Histidinol $(HCl)_2$	77.8	γ-Aminobutyric acid (GABA)	0
N^{α}-methylhistamine $(HCl)_2$	0	Serotonin	0
Imidazole-4-acetic acid HCl	0	α-Methyltryptophan	0.9
L-Methyl-4-imidazole acetic acid HCl	0	5-Hydroxyindoleacetic acid	0
		3-methoxytryptamine	<0.1
L-Carnosine	0.3	Melatonin	0
L-Anserine nitrate	0	Dopamine	0
L-Homocarnosine sulfate	0	Norepinephrine	<0.1
		Epinephrine	0
Mepyramine maleate	0	DOPAC	0
Diphenhydramine HCl	0	Metanephrine	0
Chlorpheniramine maleate	0	DOPA	<0.1
Tripelennamine HCl	0.2	Normetanephrine	0
Tripolidine HCl	0	Homovanillic acid	0
Metiamide	0	4-Hydroxy,3-methoxyphenol glycol	0
Burimamide	0.2	α-Methyl-p-tyrosine methyl ester	0
Cimetidine	0	α-Ketoglutarate	0
α-Hydrazinohistidine	3.0	Glutathione (reduced)	0.2
4-Thiazolylmethoxyamine $(HCl)_2$	0	Corticosterone	0
α-Methylhistidine	0	Met-enkephalin acetate	0
Aminoguanidine H_2CO_3	0	Leu-enkephalin acetate	0

Table 5c

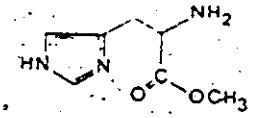
Substance Analyzed	F_I Compared to Histidine		
Amitryptyline HCl	0	Morphine sulfate	0
Chlorimipramine HCl	0	Methadone HCl	0
Cyclobenzaprine HCl	0	Pentazocine	0
Trans-demethyldoxepin HCl	0	Naloxone HCl	0
Desipramine HCl	0	Ethanol	0
N-desmethylclomipramine HCl	0	Acetaldehyde	0
Desmethylmaprotiline mesylate	<0.1	3-Carboxysalsolinol	0
Doxepin HCl	0	Haloperidol	<0.1
Fluoxetine HCl	0	Chlorpromazine	0
Imipramine HCl	0	Cocaine	0
Maprotiline HCl	0	d-Amphetamine sulfate	0
Mianserin HCl	0	l-Amphetamine sulfate	0
Nisoxetine HCl	0	Propranolol HCl	0
Nomifensin maleate	<0.1	Diazepam	0
Nortriptyline HCl	0		
Norzimelidine $(HCl)_2H_2O$	0		
Protriptyline HCl	0		
Trazodone HCl	5.1		
Trimipramine maleate	0		
Zimelidine $(HCl)_2H_2O$	0		

While most agents exhibited relative F_I indistinguishable from that of distilled water blanks, those agents which potentially could confound the alleged specificity of this technique required further scrutiny. The structures of these compounds (Fig. 19) generally fit the molecular characteristics outlined by Nakamura (1977) with the exception of trazodone. α-Hydrazinohistidine, a histidine decarboxylase inhibitor, has an amino group which departs from the "obligatory" primary amine structure. Yet this minor structural modification caused a 97% loss in fluorescence capacity. Methylester and methyl alcohol groups on the α-carbon conform to the structural requirements; however, like α-hydrazinohistidine, they are not endogenously present in mammalian brain. L-Histidine methylester is a synthetic compound often used or formed in order to render the carboxyl group inactive during biochemical manipulation. A literature survey failed to find any reference to L-histidinol's normal presence in mammalian tissue. In bacteria that synthesize histidine, L-histidinol is a precursor. The structure of trazodone clearly does not meet the above mentioned molecular requirements; however, the age of the drug (>9 years), the possibilities of impurities or ring-opening and/or rearrangement during the acid heating step may partially account for this F_I which may be up to 5% that of L-histidine. Repeating this experiment three times yielded the same results. Since the nature of the molecular events occurring with heating in acid are unknown, this trazodone-FL-induced fluorescence cannot presently be explained.

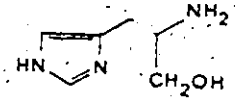
Fluorescamine derivatization coupled with heating in acid would, theoretically be selective for histidine when studied in the brain. The comparison of histidine's relative F_I with several other agents evaluated individually is consistent with this selective process. This conclusion was strengthened further after similar analysis of histidine alone and in a standard mixture of several other amino acids (Table 6). When His was studied at a concentration equimolar to histidine alone, similar relative intensities resulted. The claim that ammonium ions may



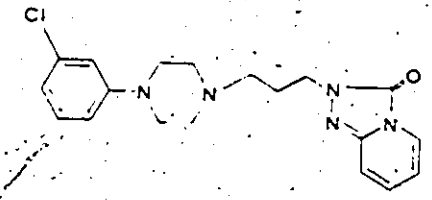
α-HYDRAZINO-
HISTIDINE



L-HISTIDINE
METHYLESTER



L-HISTIDINOL



TRAZODONE

Fig. 19

Structures of α-hydrazinohistidine, L-histidine methylester, L-histidinol and trazodone.

Table 6

Relative Fluorescence Intensity (Machine Units) of Histidine Alone and In Combination with Other Amino Acids and Ammonium Ions

Values represent mean ± S.E.M. of eight determinations.

Substance Analyzed	Concentration	Relative F_I
Histidine-HCl-H_2O	100 µM	4.44±0.04
*Amino acid mixture plus NH_4Cl	100 µM (each)	4.64±0.10
$(NH_4)_2SO_4$	50 µM	0
Histidine-HCl-H_2O	10 µM	0.51±0.01
*Amino acid mixture plus NH_4Cl	10 µM (each)	0.51±0.01
$(NH_4)_2SO_4$	5 µM	0

*Amino acid Standard Solution (Sigma Chemicals) contains 17 amino acids and ammonium chloride in 0.1 N HCl. Asparagine, glutamine and tryptophan were not included.

interfere with this fluorescamine fluorescence method (Murray et al., 1981) were not confirmed when studied individually (as the sulfate salt) or in a mixture (as the chloride salt), as NH_4^+ failed to fluoresce under these conditions. These results were observed at two different concentrations.

Effect of Heating on Fluorescamine-Derivative Fluorescence Intensity

Fluorescamine derivatives of several primary amines, amino acids, peptides and related substances all generate substantial fluorescence when formed in basic media. It is the acid heating step that confers selectivity to several compounds of the imidazolylethylamine class. The effect of changing the duration of heating was studied to examine its influence on the final relative F_I of His, MeHis, HA and MeHA and, of a potential interfering substance, tryptophan (Fig. 20). Prior to heating the relative F_I of an equimolar tryptophan concentration more than doubled that of most of the other agents; but, continued heating had two

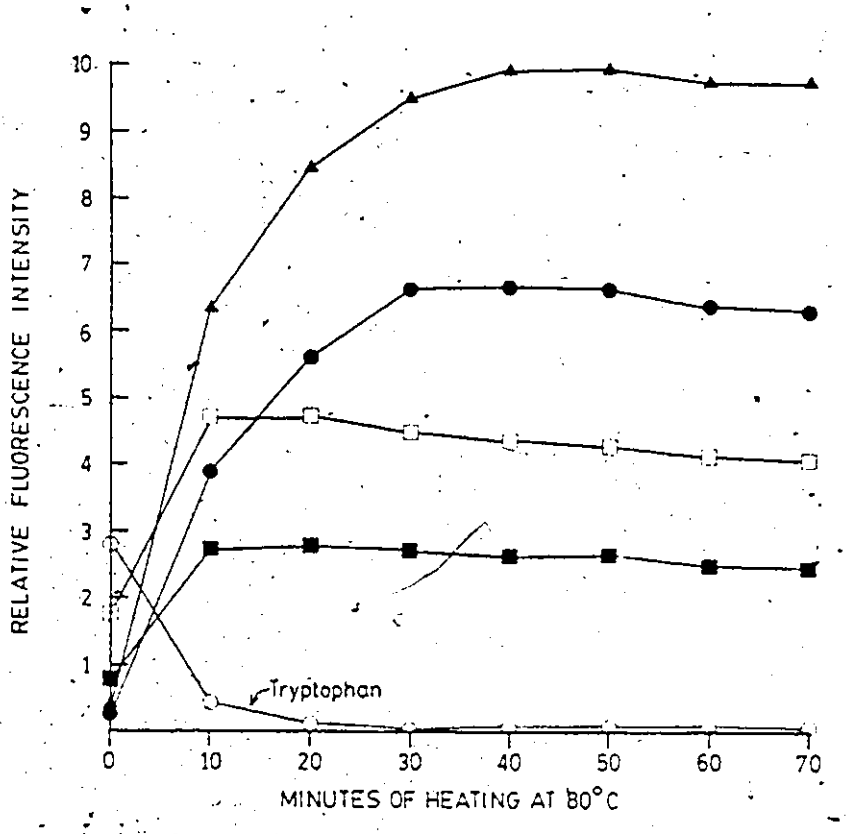


Fig. 20

Effect of heating time on the relative fluorescence intensity (machine units) of fluorescamine-primary amine derivatives. Symbols represent histidine (●), methylhistidine (▲), histamine (■), methylhistamine (□) and tryptophan (○).

main effects. First, tryptophan-FL fluorescence almost disappeared. This is consistent with the original observations of Nakamura and Pisano (1976) and Nakamura (1977). Second, and more importantly, the relative F_I of these agents which were selectively spared this acid and heat sensitive procedure, increased up to 25 times. The methylated agents attained maximal intensities at or before the earliest times measured whereas their non-methylated parent compounds reached maximal levels after about 30-40 minutes. It was apparent that with continued heating there was a progressive loss of intensity. This was attributed to evaporation of a mixture under pressure since sealing the capped tubes with parafilm abolished this slow fluorescence decay. Since pure acetonitrile-water solutions form an azeotropic mixture which boils at 76.5°C, heating to temperatures far above 80°C could cause boiling, rupture of the reaction tubes and/or significant sample loss. Thus, this temperature was not exceeded.

Absence of Fluorescamine Quenching of FL-Histidine Generated Fluorescence

Excessive FL has been alleged to quench the F_I of FL-amino acid adducts in general (DeBernardo et al., 1974; Chen et al., 1978) and more recently, FL-histidine derivatives in particular (Moriyama and Watanabe, 1982). For FL to be useful as a tool in the analysis of biological samples, it must be in excess to allow reactions to proceed to completion. In the case of histidine, FL must react with all potential substrates in addition to histidine prior to selective quenching in an acidic medium. Therefore, FL-induced quenching would invalidate its use for sensitive analytical studies. This issue was studied in two ways. First, fixed amounts of His, MeHis, HA and MeHA were subjected to reaction with fixed volumes of solutions of increasing amounts of FL in acetonitrile up to final concentration of 1.8 mM (nearly twenty times the concentration of the adduct product) (Fig. 21). The relative F_I increased until stoichiometric equivalence was attained for all four agents, thereafter it remained maximal throughout the FL concentration range. Second, in an experiment analogous to that of Chen et al.

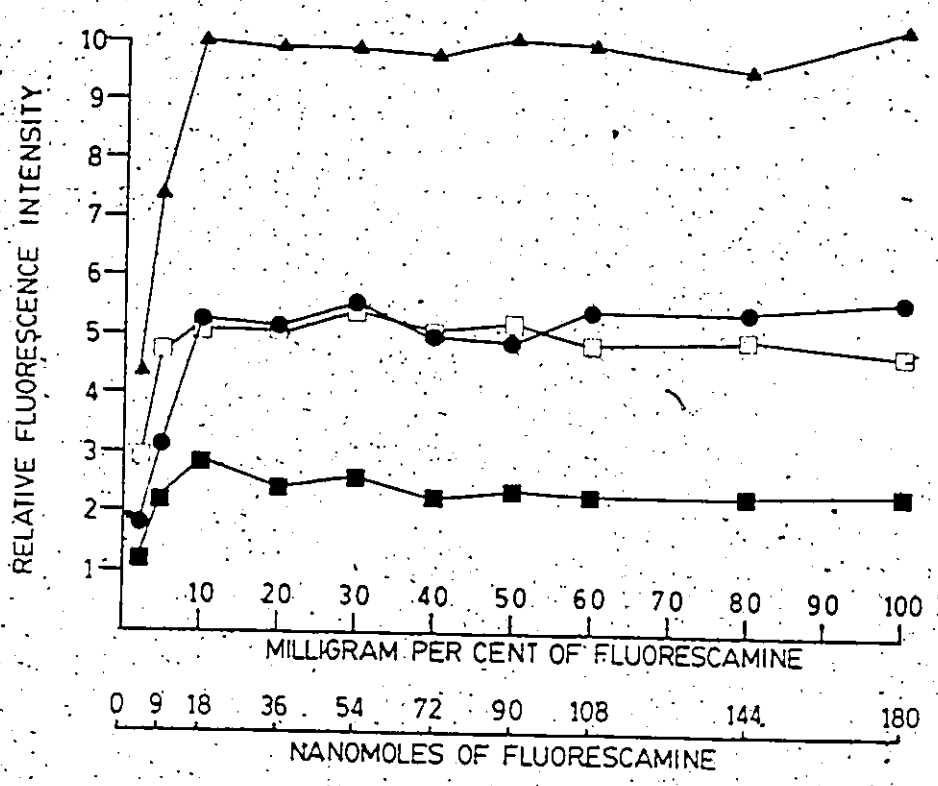


Fig. 21

Effect of several fluorescamine concentrations (below and above stoichiometric equivalence) on the relative fluorescence intensity (machine units) of fluorescamine-imidazolylethylamine derivatives. Abscissa is plotted as mg% and nanomoles of fluorescamine added to 10 nanomoles of substrate histidine (●), methylhistidine (▲), histamine (■) or methylhistamine (□).

(1978), a comparison was made of the final F_I of FL-histidine and FL-methyl-histidine derivatives after 50 µl of FL (100 mg%) was added at once or in several smaller volumes with 5 min elapsing between additions. Fluorescamine was also added in volumes of 100 and 200 µl; however, due to the decreased fluorophor concentrations, correction for the effects of dilution were made (Table 7). Batch or series addition of FL up to a concentration (1.8 mM), already shown to be without a quenching effect (Fig. 21) had resulted in nearly identical ultimate

Table 7

Comparison of Single and Multiple Additions of 100 mg% Fluorescamine and the Final F_I Derived from the Histidine- and Methylhistidine-FL Derivatives

Volume additions (µl)	Fluorescence Intensity	
	Histidine	Methylhistidine
50 (at once)	4.8	9.0
10, 40	4.4	8.6
10, 10, 30	4.7	8.9
10, 10, 10, 20	4.7	8.9
10 x 5	4.5	9.1
100 (a)	4.7	8.7
200 (b)	4.6	9.6

Final volume increased by (a) 1.33 and (b) 2.33.
Values represent the average of two experiments using two samples each.

relative F_I. A single addition of 200 µl of the 100 mg% FL solution which brought the final FL concentration up to 7.2 mM was also without major effect. Although Chen et al. (1978) reported that sequential additions of FL to aspartate and asparagine containing solutions boosted fluorescence about 130% and 160%, respectively, more than equimolar batch introduction, they did not evaluate histidine fluorescamine fluorescence. Moreover, they showed that the relative F_I following sequential reagent addition to alanine was elevated no more than 6%. Therefore, the fluorescamine-induced fluorescence quenching may be an observation which has been over-extended to erroneously include FL-histidine derivatives (in preparation).

Linearity and Sensitivity of Histidine Analysis Using Fluorescamine

A plot of the relative F_I generated by standard amounts of His base is shown in Figure 22. This linear relationship ($r>0.99$) extended up to 6 nmoles of

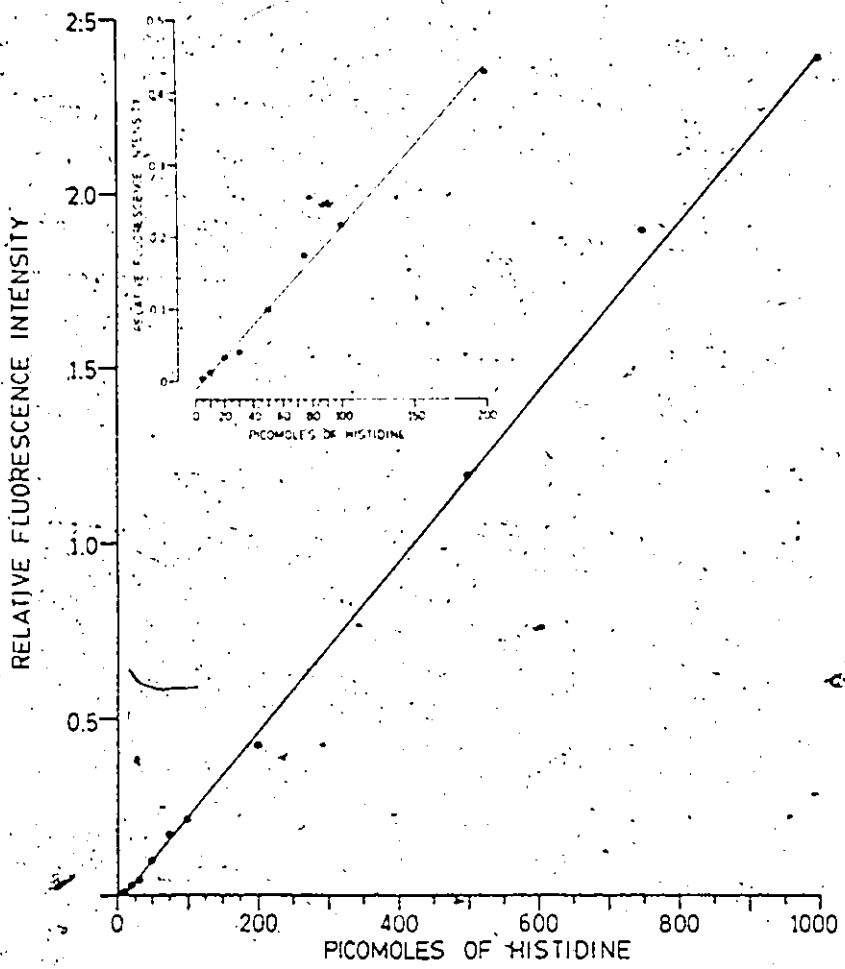


Fig. 22

Linearity and sensitivity of the assay for histidine using fluorescamine. (Insert enhances the plot of relative fluorescence intensity versus histidine below 200 picomoles).

His (not shown). Three picomoles could be detected and 10-12 picomoles gave fluorescence values twice those of buffer blanks. For amounts less than 200 picomoles (see figure insert), each point is the mean of 5 determinations; however, with the exception of 30 picomoles His, the S.E.M.'s are smaller than the points used to graphically represent their means. This linear relationship in the presence of overwhelming excess FL also argues against FL "self-quenching" in this concentration range.

Application of This Fluorescamine Method to Rat Brain Tissue

Early in this study, high concentrations of protein (>200 mg%, bovine albumin, fraction V) were found to generate fluorescence and thus interfere with this proposed fluorescamine method in brain supernatants. This was confirmed in experiments using unboiled supernatant samples from rat hypothalamic and cortical regions. These samples generated relative F_I values nearly three times that of boiled tissues. Even after homogenate boiling, the rank order of F_I changed with the speed of centrifugation (for 20 min) such that 50,000 x g > 70,000 x g > 100,000 x g. Therefore, protein was suspected to be an interfering constituent. While different methods for protein precipitation exist, several constraints had to be borne in mind. The supernatants from brain, already somewhat dilute, could not be diluted several-fold further in the process. Precipitation with ethanol had been used in urine (Nakamura and Pisano, 1976), but required five-fold dilution. The pH of this system could not be altered severely as this method is pH sensitive. Thus, procedures which utilize acids were not considered as a primary choice. Due to these limitations, the methods used for glucose analysis in plasma were attempted (Oser, 1965).

The $Ba(OH)_2/ZnSO_4$ method of Somogyi (1945) appeared attractive since both agents could be introduced, each in a small volume stoichiometrically balanced, and nearly totally removed from the supernatant upon centrifugation. Following this general procedure, protein could not be detected in the filtrate. However,

while $Ba(OH)_2$ and $ZnSO_4$ individually had no effect on the relative F_I of standard amounts of His, MeHis, HA and MeHA, when the latter were combined, the ultimate intensities decreased 30-50%. Several modifications proved fruitless. A more extensive literature survey uncovered the fact that barium and zinc salts may precipitate several amino acids, among them, His (Neuberg et al., 1950).

An earlier method of Somogyi (1930) was far more successful. In this technique, $Zn(OH)_2$ is the actual precipitating agent, prepared from a solution of $ZnSO_4$ mixed with an equal volume of NaOH at twice the $ZnSO_4$ concentration. The following reaction occurs:

$$ZnSO_4 + 2\ NaOH \longrightarrow Zn(OH)_2\ (s) + Na_2SO_4$$

Neither $ZnSO_4$, NaOH, $Zn(OH)_2$ (once formed) nor Na_2SO_4 interfered with the ultimate F_I of the imidazole derivatives. Nevertheless, the amounts of $ZnSO_4$ and NaOH were titrated so that NaOH would not be far in excess and eventually alter the pH of the filtrate.

The Issue of Tele-Methylhistidine in Rat Brain: A Potential Source of Interference?

The above arguments for a highly selective method to measure brain His based on fluorescamine derivatization are moot if one of the principal requirements is not true. That is, the assumption that no other substances are present in the brain in sufficient quantities to significantly interfere with His measurements. While the concentrations of HA and MeHA have been studied in brain regions of the rat, MeHis has received little attention. Several attempts to detect free MeHis in mammalian brain were unsuccessful (Tallan et al., 1954; Haverberg et al., 1975). Nakajima et al. (1967) were able to extract up to 4 mg of MeHis from a total of 45 bovine whole brains (and presumably from the blood that bathed them).

Perhaps the most comprehensive analysis of MeHis and related amino acids present in mammalian brain is that of Nakamura et al. (1979) who studied whole guinea pig brain using high performance liquid chromatography with OPT. While

standard curves for several brain amines (authentic compounds dissolved in lithium citrate buffer) including MeHis show clear linearity and peak area ratios (amine/standard) of as little as 10 picomoles of amine, the graphical reproduction of fluorescence intensity following injection of deproteinized guinea pig whole brain extract was far less impressive. Confirmation of the peak identities by thin layer chromatography and further characterization with OPT and fluorescamine leave little doubt that τ-methylhistidine was indeed present in the extracts. However, separate isolation and quantification of MeHis were not reported. Back calculation of data supplied in the table and figure (Nakamura et al., 1979) leads to the conclusion that an approximate mean of 36±9 nanomoles of MeHis per gram of whole guinea pig brain exists which corresponds to as much as 6 nanograms of this amine per gram tissue. Unfortunately, in this report the presence of whole blood, mesenchyme and related tissues as a contaminant of the whole guinea pig brain preparation is clearly evident from the methodology presented. Thus, the exact source of the MeHis or the relative contribution from each tissue (brain vs. blood) cannot be precisely ascertained. Since in the cat, levels of MeHis from plasma extracts could be detected while those of the brain could not (Tallan et al., 1954), one cannot presently attribute all the MeHis to cerebral tissue alone although species differences could, at least partially, account for the discrepancy.

This prompted a specific search for MeHis in the rat brain. Unfortunately, an amino acid analyzer and HPLC equipment available for analysis did not have sufficient sensitivity to detect brain His nor MeHis, under the present conditions of tissue preparation and methodology. Therefore, qualitative analysis using thin-layer chromatography became the most obvious approach. Rat hypothalamic and cortical tissues were prepared and derivatized with FL as in the brain His procedure described in the methods. However, instead of measuring the resulting F_I, the mixture was applied to silica gel TLC plates and eluted on two solvent systems along with authentic His, MeHis, HA and MeHA. The solvent systems:

n-butanol-acetic acid-water (80:20:100 - upper phase) and isopropanol-acetic acid-water (120:40:40) were among those suggested by Nakamura (1977). Only one fluorescence positive spot, that corresponding to the His-FL adduct, was visible for either hypothalamic or cortical tissues in each solvent system. Adding large aliquots to the TLC plates served only to intensify the FL-His fluorescence while no other fluorescence positive regions were apparent. In a second experiment, MeHis (prepared in borate buffer) was added to the supernatant filtrates in increasing amounts. Various known amounts of MeHis were run on separate channels of the same plates. The "minimum visually detectable threshold" of MeHis, i.e. the least amount of MeHis which must be present in order to be seen with the unaided eye under UV lighting was approximately 30 picomoles. In the hypothalamic and cortical samples, only in the samples to which 30 picomoles or more MeHis were added could a fluorescence positive region be observed. Unfortunately, this method is not reliably quantitative. Yet, it can be inferred that the exogenous MeHis could only have added to endogenous stores that were either meager or non-existent.

Comparison With Published Data

Following the development of this methodology using fluorescamine, the histidine levels of the two brain regions whose fluorescence specificity had already been characterized, were determined. The results are summarized in Table 8 and compared with the range of values reported by Taylor and Snyder (1972a) using the bacterial HD method of histidine determination.

Table 8

Rat Brain Histidine Content (µg/g)

Tissue	Taylor & Snyder (1972a)	Fluorescamine Method*
Hypothalamus	18-25	20.6±4.1
Cortex	7.9-9.4	9.2±2.2

*Mean of 6 determinations

Limitation to the Fluorescamine Method for Histidine Analysis

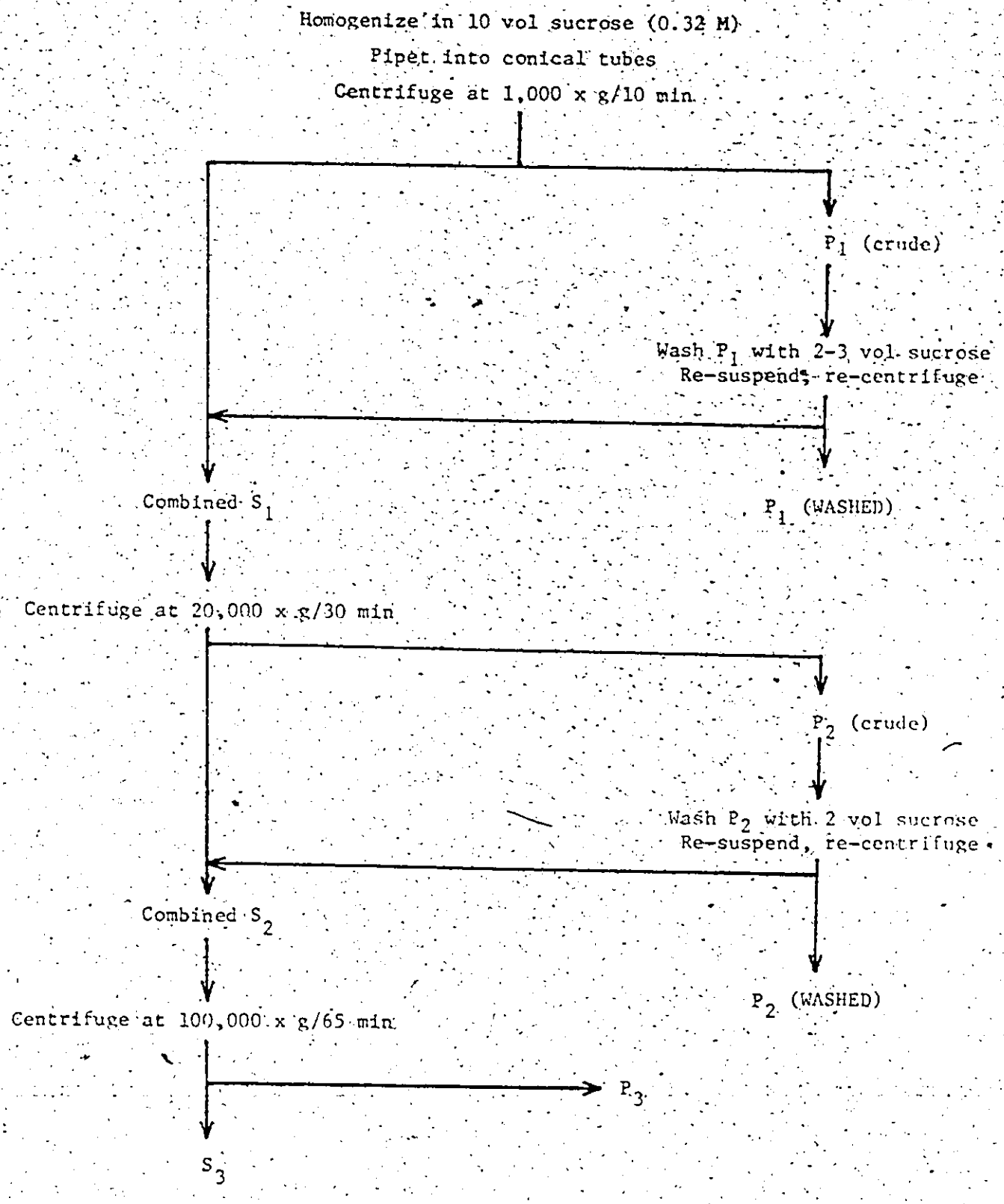
While brain His analysis with fluorescamine is a highly selective, sensitive, inexpensive and rapid procedure that utilizes comparatively unsophisticated laboratory equipment, extending this method to other tissues or biological samples may not be appropriate. Prior or subsequent separation of potential substrates (i.e. those compounds which derivatize with LF and exhibit F_I after heating in acid) for this method is required unless prior knowledge of their concentrations justifies omission of this procedure. This is the case for rat brain. Adult rat (>250 g body weight) plasma and urine may possibly be evaluated by this method because MeHis, present in substantial amounts in the plasma of younger rats, is N^{α}-acetylated in the adult prior to kidney clearance (Young et al., 1972). This method clearly is not appropriate for human urine due to substantial presence of MeHis (Prell, in preparation). Protein removal is obligatory and the tissue must be well "cleaned" before homogenization to remove any potential interference from other substances present, e.g. plasma or connective tissue.

8. Preparation of tissue and general procedure for subcellular fractionation of brain tissue

Rats were sacrificed between 09:00-12:00 by decapitation and the brains were rapidly removed then dissected on ice according to the method of Glowinski and Iversen (1966). The hypothalamus was homogenized in 500 µl whereas the midbrain and right cerebral cortex regions were homogenized in 10 volumes of 0.32 M sucrose. Homogenization was accomplished with 10 strokes using a model AA Potter-Elvehjem homogenizer fitted with a Teflon pestle (0.25 mm clearance) driven at 750 r.p.m. Aliquots for histamine determination and protein analysis (50 µl and 25 µl, respectively) were retained. The remainder was subjected to multiple centrifugations by a modification of the procedure outlined by Gray and Whittaker (1962) leading to recovery of various fractions, as depicted in Fig. 12. The homogenates were transferred to Sybron/Nalge 17 x 119 mm polypropylene conical tubes and centrifuged at 1,000 x g for 10 minutes in a Beckman J2-21M centrifuge to generate the crude P_1 pellet. The supernatants (S_1) were decanted into other 17 x 119 mm conical tubes while the crude pellet was washed with 2-3 vol. of 0.32 M sucrose, resuspended and centrifuged again at 1,000 x g for 10 min. The resulting supernatants from this procedure were combined with the primary supernatants and centrifuged at 20,000 x g for 30 min forming the crude P_2 pellet. These S_2 supernatants were decanted into 10 ml Sybron/Nalge Oak Ridge

Fig. 23

General Scheme for Subcellular Fractionation of Brain Tissue Into Nuclear (P_1), Mitochondrial (P_2), Microsomal (P_3) and Supernatant (S_3) Fractions



type polycarbonate high speed centrifuge tubes while the crude P_2 pellet was washed with 2 volumes of 0.32 M sucrose, resuspended and recentrifuged. The secondary and primary S_2 supernatants were combined and centrifuged at 100,000 x g for 65 min in a Beckman L8-70 preparative ultracentrifuge. The resulting high speed supernatants (S_3) were removed from the P_3 pellets. Due to the technical difficulties of isolating the paper thin, crumbling hypothalamic P_3 pellets in an uncontaminated form, this precipitate was not retained. All pellets ans supernatants were maintained on ice baths and centrifuged at 4°C.

RATIONALE FOR THE PROCEDURE FOR SUBCELLULAR FRACTIONATION OF BRAIN TISSUE

In a survey of the literature on the preparation of various subcellular fractions from brain tissue and the HA levels in each fraction, a clear, well established technique failed to emerge (see Table 9). While almost all of the studies generated P_1 and P_3 fractions at 10^4 and 10^6 g-min, respectively, a wide spectrum of gravitational forces and centrifugational times (ranging from 3.4 - 10.2 x 10^5 g-min) were reported for formation of the mitochondrial (P_2) fraction. Since this fraction is presumed to contain the bulk of the synaptosome population and the highest histamine concentration (in most studies), its precise preparation is vital for proper analysis. Therefore, in this study, the arithmetic average of the various published methods was used to form the P_2 fraction, i.e. 6.1 x 10^5 g-min or approximately 20,000 x g for 30 min; nearly the same conditions used by Kataoka and DeRobertis (1967), Young et al. (1971), Snyder et al. (1974) and Schwartz et al. (1979a). This decision was supported by the fact that in two of these studies (Kataoka and DeRobertis, 1967; Snyder et al., 1974), electron microscopy, neurochemical markers and analysis of synaptosome abundance using discontinuous sedimentation techniques supported the methodology applied for fraction preparation. These latter studies not only verified the unequivocal presence of HA in the P_2 fraction, but also localized much of it to the synaptosomal subfraction. However, in the absence of subfractionation analysis, changes in fractional histamine content, especially in the P_2 fraction, do not prove conclusively that the alterations occur in the synaptosomal nor even the neuronal stores. However, changes occurring in the neuronal stores of histamine would almost certainly by expected to appear in the mitochondrial (P_2) fraction.

Table 9

Histamine Content (Per Cent of Total) in Various Subcellular Fractions of Rat or Guinea Pig Brains

Authors	Species	Method of Analysis	Fraction			
			P_1	P_2	P_3	S_3
Michaelson and Whittaker, 1962	guinea pig	fluorometric	60	>30	← <10 →	
Carlini and Green, 1963	rat	bioassay & fluorometric	17.6	62.5	← 19.9 →	
Michaelson and Dowe, 1963	guinea pig	fluorometric				
Michaelson and Coffman, 1967	guinea pig	fluorometric	40	← 60 →		
Kataoka and DeRobertis, 1967	rat	bioassay	7.7	21	51.4	19.9
Kuhar et al., 1971b	rat	enzymatic-isotopic	20	60	← <20 →	
Young et al., 1971	rat	enzymatic - isotopic	23	30	3	44
Snyder and Taylor, 1972	rat	enzymatic-isotopic	21	22.5	5	51.5
Snyder et al., 1974	rat	enzymatic-isotopic	20.2	46.4	15.6	17.7
Dismukes et al., 1974	rat	enzymatic-isotopic	17.9	52.0	← 30.0 →	
Schwartz et al., 1979a	rat	fluorometric	36	38	14	12

9. Determination of brain histamine concentration in subcellular fractionation studies: single labelled method

Due to the reduced HA content in each sample, a more sensitive single isotope method and the highly active rat kidney HMT as the exogenous methylating enzyme were applied to analyze histamine content of the homogenates and various fractions. The enzyme was prepared by a modification of the method of Shaff and Beaven (1979) and was a generous gift from Dr. R. K. Harding of the Department of National Defense, Canada. The single isotope method used was a combination of the single isotopic techniques of Taylor and Snyder (1972a) and Beaven et al. (1972) and incorporated several modifications suggested by Beaven and Horakova (1978).

Homogenate samples (see above) were diluted with 2 volumes of sodium phosphate buffer (50 mM, pH 7.9) and vortexed. The pellets were resuspended in 2-5 volumes of the same buffer and vortexed, 50 µl aliquots of these samples and the S_3 samples were retained for protein analysis. Protein concentrations were determined by a modification of the method of Lowry et al. (1951) as described earlier. The remaining resuspended material and S_3 fraction were heated in a boiling water bath for 10 min, then centrifuged at 50,000 x g for 20 min in a Beckman L5-50 preparative ultracentrifuge. Fifty microliter supernatant aliquots were retained for HA analysis in polycarbonate culture tubes and frozen at -80°C until the time of analysis. The supernatant samples (once

thawed) and assay blanks were buffered with 50 µl of sodium phosphate buffer (50 mM, pH 7.9) and thereafter constantly maintained in an ice bath. Blanks were composed of 5 mM phosphate buffer (pH 7.9). To each sample and blank, 50 µl of an "activation cocktail" were added. This consisted of 1.2 ml of the exogenous enzyme buffered with addition of 1.8 ml of phosphate buffer (50 mM, pH 7.9) and 3 ml of a similarly buffered S-adenosyl-L-(methyl-^{3}H)methionine (SAM) solution. The SAM concentration of the final mixture was 3.0 µM with a volumetric specific activity of 1.39 nCi/µl. The mixture was vortexed and incubated for 90 minutes at 37°C.

Following incubation, the reaction was terminated by addition of 1.2 N perchloric acid to yield a concentration of 0.67 mM. This mixture was vortexed, immediately placed in an ice bath and rendered alkaline by the addition of 200 µl of 10 N NaOH. To this mixture, 3.0 ml of chloroform (Baker Resi-Analyzed, J.T. Baker Chemical Co.) was added, then vigorously vortexed and centrifuged for 3 min at 1,000 x g. The aqueous layer was removed by aspiration. The remaining organic extract was again washed with the NaOH solution as above and the aspiration of the aqueous layer was repeated. Two milliliters of the residual organic phase were transferred to polyethylene liquid scintillation counting vials and evaporated to dryness under a nitrogen gas stream. The residue was dissolved in ethanol and shaken for 10 min prior to the addition of 10 ml of Econofluor (NEN). The tritium was counted to within a two sigma error of 2% in a Beckman model 9000 liquid scintillation spectrometer. The standard curves relating the disintegrations per minute as a function of the nanograms of incubated histamine were obtained by substituting 0.02-3.0 ng base of buffered histamine $(HCl)_2$ (in a volume of 50 µl) for the supernatant samples. In each assay of tissue histamine concentration, standard HA solutions of 0.2, 0.5 and 0.8 ng were run in parallel for comparison. Histamine dihydrochloride was purchased from Baker Chemicals.

Commentary on the Single Isotope Method

The single labelled method used in this study borrows from the techniques outlined by Taylor and Snyder (1972a) and Beaven et al. (1972) with additional modifications and adjustments based largely on experience with the double labelled method and HMT assay procedures. For the subcellular fractionation studies in particular, the problems of sensitivity and precision were the most serious issues with which to contend. The anticipated reduction of total HA concentration in brain tissues from ethanol-treated and withdrawn rats, the reduced tissue content per fraction and the progressive dilution due to multiple washing steps necessitated a procedure by which one could consistently determine HA levels below 0.5 ng. This reduced level would also have to be determined in volumes which would not introduce marked errors in pipetting (<25 µl) nor require further excessive dilution of samples already diluted in the fractionation procedure. Thus, 50 µl amounts of supernatants of boiled homogenates, resuspended pellets or high speed supernatants seemed an appropriate compromise. The homogenization of brain tissue in 10 volumes of buffer obviated the potential "volume of homogenate-histamine level" effect postulated by Orr and Eichelman (1979). Homogenization of the hypothalamus (20-35 mg of tissue) in 500 µl of buffer did not excessively dilute this tissue while providing sufficient volume for further concomitant analyses of the same homogenate sample.

The destruction of SAM and the denaturation of most enzymes which occurs by boiling tissue appears to be obligatory. However, results comparable to literature values (e.g. Taylor and Snyder, 1972; Schwartz et al., 1979a) can be obtained by subjecting the samples to 80°C temperatures for 10 minutes. This reduces losses due to evaporation which has the effect of concentrating the remaining material in a smaller volume possibly leading to higher HA values.

Except for this step, conditions for centrifugation and subsequent freezing of supernatants were not altered.

Some modifications were introduced concerning the composition of the mixture added for incubation. Tritiated SAM appears to be stabilized in the presence of HMT (Beaven and Horakova, 1978). The double labelled HA method utilizes a sequential addition of HMT and ^{14}C-SAM which causes the cpm of blanks (and presumably tissue samples) to increase in proportion to the time that elapses before addition of guinea pig brain HMT and ^{14}C-SAM. Therefore, an "activation cocktail" composed of ^{3}H-SAM and guinea pig brain HMT buffered at pH 7.9 was added before the incubation process. In this way, the blanks remained nearly constant throughout the cocktail introduction period which lasted up to 80 minutes when large numbers of samples were evaluated. Batch addition tends to overcome this potential artifact.

The termination of the incubation reaction with perchloric acid followed by a strong NaOH solution is a marked departure from the Taylor and Snyder (1972a) methodology while the multiple washing and aspiration steps depart from that of Beaven et al. (1972) and Beaven and Horakova (1978). Chloroform was used as the extraction solvent largely due to its volatility, although other solvents such as toluene-isoamyl alcohol may be suitable under conditions of the single labelled technique. Following evaporation, elution was done with ethanol and Econofluor (NEN) was added and counted as described earlier.

Using the above methodology, blank values obtained were about 800-1,000 cpm; 0.1 ng of histamine could be consistently measured with confidence while 0.5 ng were about 3 times higher than blank values. All HA standards were analysed in triplicate and triplicate blanks were distributed between every 9 tubes containing standards. HA levels as low as 0.06 ng could be detected; however, throughout an experiment containing up to 15 buffer blanks, the cpm values of some of the latter occasionally exceeded those of this small HA standard. An enlargement of

the lower portion of a HA standard curve (linear over the range from 0.06 - 3.0 ng) is shown in Fig. 24.

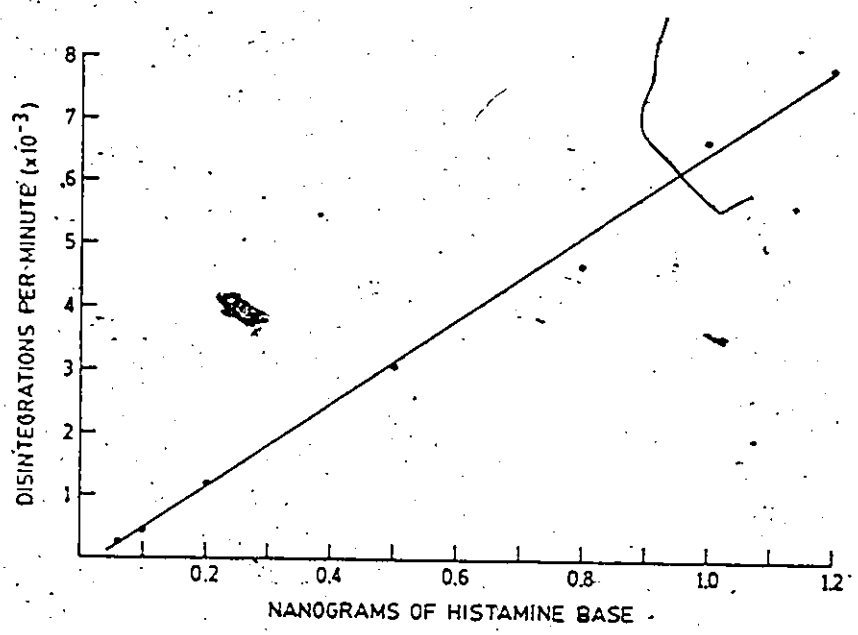


Fig. 24

The sensitivity and linearity of the histamine assay using the single labelled method.

Single Labelled Method Using Rat Kidney HMT

The use of rat kidney HMT as the exogenous methylating enzyme (Shaff and Beaven, 1979) had marked advantages because it improved the sensitivity of the assay and gave lower values for (assay) blanks than those obtained in a similar method in which guinea pig brain HMT was used. With rat kidney HMT the blanks were in the range of 300-400 cpm and 0.02 ng HA was detectable and 0.2 ng HA

gave approximately twice the blank values. However, the dpm/ng HA ratio was generally reduced to about half that observed with the guinea pig enzyme. In addition, the individual samples of the triplicate set showed less variation when using the rat kidney source.

Using this more active methylating enzyme, it could be shown that in the presence of 2.0 ng HA the methylating process was already maximal by 30 minutes. However, after 90 min of incubation, the recovered radioactivity had increased by about 6% over this amount. This may be largely due to non-specific reactions with the labelled SAM which generates products that are chloroform extractable. Unfortunately, due to the large number of samples which are analyzed, an incubation period of at least 60 min was required. If this highly likely, non-specific reaction occurs in all the samples to approximately the same extent, then this issue has little impact on the results. Since this general method has profound utility in the brain histamine field, the nature of this non-specific increase in radioactivity with respect to time shall be investigated further.

B. Protocol for Ethanol Administration

1. Schedule for acute studies

Male Sprague-Dawley rats (200-250 g) obtained from Bio-Breeding Laboratories (Ottawa, Ontario) were housed 2 or 3 per cage maintained in a 07:00-19:00 light cycle and fed Purina Rat Chow (#5012). Water was continuously available but food was withdrawn at 07:00 the day of the experiment. Rats were administered, by gastric intubation, either 1 or 2 g/kg of a 25% (w/v) solution of ethyl alcohol (obtained from the Liquor Control Board of Ontario) in tap water. Rats serving as controls received isovolumetric saline to account for the stress of intubation and gastric distention.

Tissues were prepared by the procedure outlined in the double labelled histamine method. However, since HD is labile, the 300 µl homogenate aliquots for HD activity determination were immediately buffered and maintained in an ice bath. They were then given to Dr. Bozena Bielkiewicz for initiation of the first steps of the analysis while the remaining homogenates were prepared for other biochemical analyses.

2. Schedule used for chronic studies (doses up to 6 g/kg)

Male Sprague-Dawley rats (190-210 g) obtained from Bio-Breeding Laboratories (Ottawa, Ontario) were housed 3 per cage with a light cycle of 07:00-19:00 at a room temperature of 22°C ± 1°C. The ethanol treated animals received 25% (w/v) ethanol in tap water via gastric intubation in doses ranging from 1 g/kg increasing 1 g/kg on days 3, 6, 19, 13 and 17 to 6 g/kg according to a modified schedule of LeBlanc et al. (1969;1973). Rats serving as controls received the caloric equivalent of ethanol as a 43.8% (w/v) sucrose solution in tap water. In order to account for the stress of intubation and gastric distention, a second group of control rats were administered saline isovolumetric to the ethanol and sucrose treated animals. Animals (n=9 each) were treated between 07:00-10:00 with the dosing staggered such that 3 rats from each group were sacrificed on day 23.

Almost all authoritative sources agree that no single animal analogue meets the requirements necessary to mimic human alcoholism (Lester and Freed, 1973; Mello, 1973; Cicero, 1979). Of the methods available to circumvent selected aspects of these difficulties, the "forced" methods of ethanol administration produce dependence and tolerance but unfortunately remove the volitional aspects associated with human alcohol seeking behavior. This approach has important pharmacological utility, i.e. more effective control of dose and attainment of high blood ethanol concentrations. The technique with the greatest pharmacological advantage is the intubation method since high blood ethanol levels can be attained, precise quantities can be administered, large numbers of animals can be treated, no special equipment is required and marked tolerance and dependence can be produced. However, the obvious disadvantages of this technique are that the method may be stressful, high concentrations may lead to gastric disorders such as ulcers and the combined GIT irritation and intoxicated state may lead to reduced food consumption.

This leads to the problem that chronic administration of ethanol to any species, including man, is associated with a unique difficulty not posed by any other psychoactive drug. Chronic alcohol ingestion is associated with considerable caloric intake, i.e. about 7 kilocalories per Kg of ethanol. Therefore, chronic ethanol administration in increasing doses will necessarily displace a significant fraction of other sources of calories from the diet. When calories from ethanol represent a large portion of the total caloric intake, it fails to support growth or the maintenance of weight as well as an isocaloric amount of rat (about 9 cal/g) or carbohydrate or protein (each about 4 cal/g) (Orten and Neuhaus, 1975). This has been a serious problem with chronic ethanol studies and the subject of several critical reviews (Westerfeld and

Schulman, 1959; Lester and Freed, 1973; Mello, 1973; Cicero, 1979; Goldstein, 1983).

The present method was adopted so as to exploit the above advantages mentioned earlier using the equipment available. Sucrose was used as the nutritional control and administered in quantities isocaloric to the doses of ethanol (LeBlanc et al., 1969; 1973; Kalant, personal communication). However, since some investigators had not utilized nutritional controls in their methodologies (especially those investigating ethanol and brain histamine interactions, e.g. Subramanian et al., 1978) and as there was no information concerning the effects of sucrose on brain HA, saline controls were also used. These would also serve as a control for the daily stress of intubation.

3. Schedule used for chronic studies (doses up to 8 g/kg)

Male Sprague-Dawley rats obtained from Bio-Breeding Laboratories (Ottawa, Ontario) were housed 3 per cage under conditions described earlier. They were administered 25% ethanol (w/v) in tap water via gastric intubation in doses ranging from 1 g/kg increasing to 8 g/kg by day 26. The dosage was increased at 1 g/kg increments every day until day 22 and maintained at 8 g/kg thereafter. Control rats received the caloric equivalent of ethanol as a sucrose solution (43.8%, w/v) in tap water. An ethanol withdrawal group was given sucrose as a substitute for ethanol 2 days before the time of sacrifice. The animals were treated after 17:00 until day 23 thereafter they were dosed before noon. Rats given ethanol, sucrose or withdrawn were housed with similarly treated animals. The dosing schedule was staggered such that on the day of sacrifice, 3 or 4 animals from each group were sacrificed between 09:00-12:30.

C. Behavioral Evaluation by the Use of the Tilting Plane Test

The tilting plane test was performed by a modification of the method of Arvola et al. (1958) and Wallgren et al. (1960) as suggested by Dr. Harold Kalant (Personal Communication). In this method, a plane (60 cm x 35 cm) covered with 80-mesh netting was tilted at a constant rate from 0 to 90° in about 5 seconds. The angle at which the rat would slide down the plane was measured and reflected the state of intoxication, the decrease in performance being proportional to the alcohol dosage. On day 18 of ethanol administration, rats which were then receiving 5 g/kg and naive (untreated) rats of comparable weight were tested on the tilting plane. This performance was then compared to that observed after oral administration of a 2 g/kg dose of 25% (w/v) ethanol. The test was performed every 30 minutes for a period of 3 hours for both groups consisting of eight animals each. Each rat was tested at least 3 times.

D. Evaluation of Rat Body Temperature

The rat body temperatures were determined by a rectal probe using a model 43T Tele-Thermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio)

graded to ± 0.05°C. The temperatures were recorded every 2 hours for 12 hours starting at 10:00 on days 20 and 21 when 6 g/kg ethanol was being administered.

E. Statistical Evaluation of the Data

Statistical evaluation of the present experimental data began with application of the Q test (Dean and Dixon, 1951) to determine the suitability of eliminating potential extraneous values from further analysis. Thereafter, mean values from control and experimental groups and their respective standard errors were determined and compared using a "two-tailed" Student's t-test. Differences were considered statistically significantly different if $p < 0.05$. In cases where multiple controls were utilized, the data was evaluated using analysis of variance (ANOVA). If differences were considered statistically significantly different ($p < 0.05$), a further test, the Duncan multiple range test, was used so as to classify the data into groups significantly different from one another.

RESULTS

Effects of Ethanol, Acetaldehyde, Morphine and Naloxone on Histamine N-Methyl-Transferase Activity in vitro

The arithmetic mean (± S.E.M.) of control rat brain histamine N-methyltransferase (HMT) activities of the six investigations were 2.41±0.10, 2.05±0.07 and 1.77±0.08 μmoles of methylhistamine produced per hour per gram of supernatant protein for the hypothalamus, thalamus-midbrain and cerebral cortex, respectively. These control values are graphically depicted by dotted lines in Figures 25-28 for the sake of comparison. In all cases, the rank order of tissue HMT activity was hypothalamus > midbrain > cortex.

No marked changes in HMT activity were observed in these regions when incubated in the presence of either ethanol or acetaldehyde (Fig. 25). At ethanol concentrations of 1, 3 and 10 mM, the mean HMT activity also failed to deviate more than 10% from control HMT activities in the three brain regions. In contrast, HMT activity showed a marked departure from control values when incubated in the presence of large concentrations of morphine sulfate or naloxone (Fig. 26). Morphine concentrations above 10 μM tended to elevate HMT activity and profoundly inhibited it above 100 μM. Naloxone induced an increase in enzyme activity above 10 μM while at lower concentrations, both it and morphine showed little effect on HMT activity in each of the brain regions studied. The effects of various ethanol concentrations on HMT activity remained essentially unaltered in the presence of 1 μM morphine sulfate (Fig. 27). At a concentration of 1 μM, naloxone had no influence on the effects various morphine concentrations had on HMT activity (Fig. 28). The individual values of the triplicate set never varied more than 12% from the mean value of that triplicate except for 10 mM ethanol in which case some values varied up to 10% from the mean.

Effect of Acute Ethanol Administration (1 and 2 g/kg) on Regional Brain Histamine Concentrations and on Histidine Decarboxylase and Histamine N-Methyltransferase Activities

Figure 29 indicates the time-course of ethanol-induced changes in regional

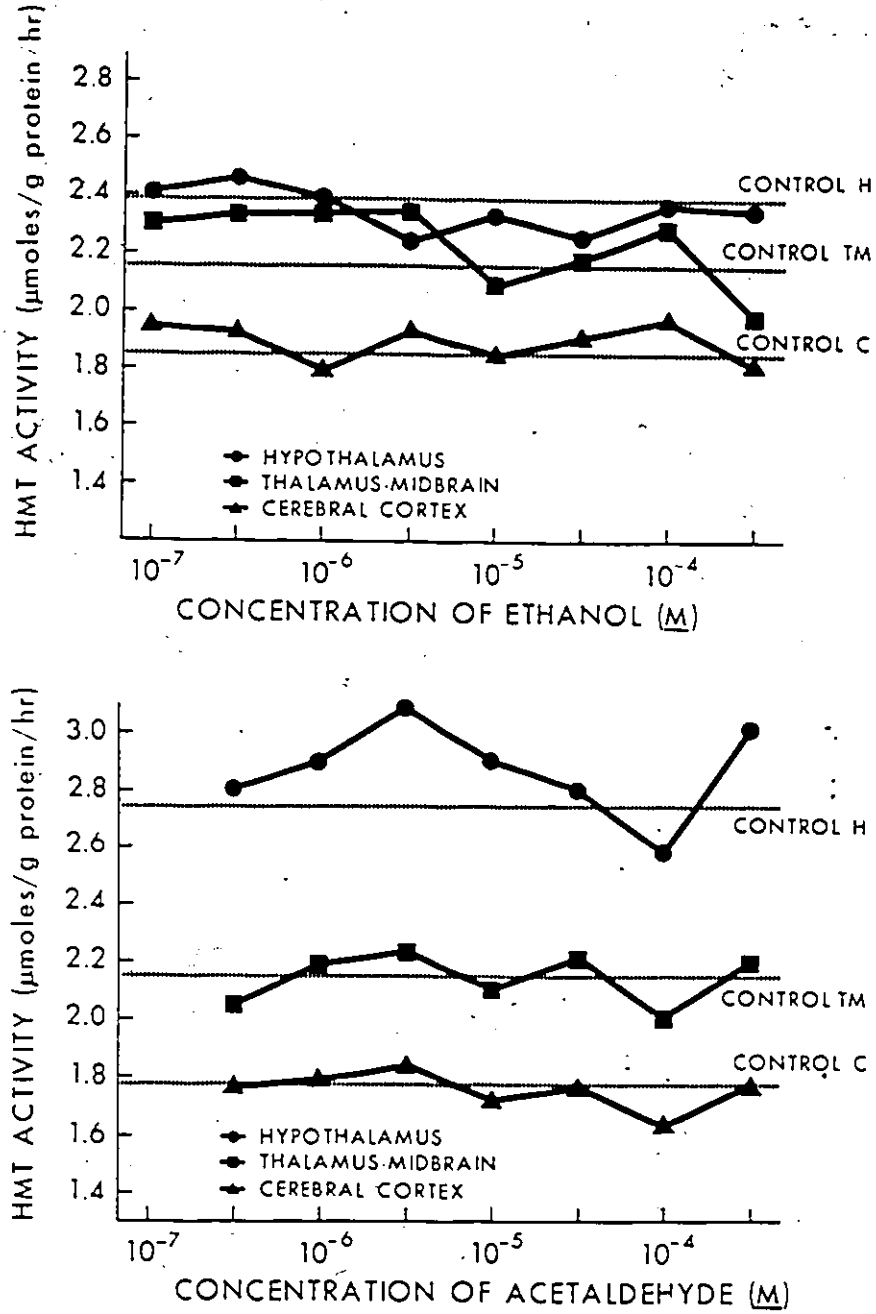


Fig. 25

Effects of various concentrations of ethanol (upper graph) and acetaldehyde (lower graph) on regional brain histamine N-methyltransferase activity. Each point represents the mean of two independent determinations. Experiments were performed in triplicate.

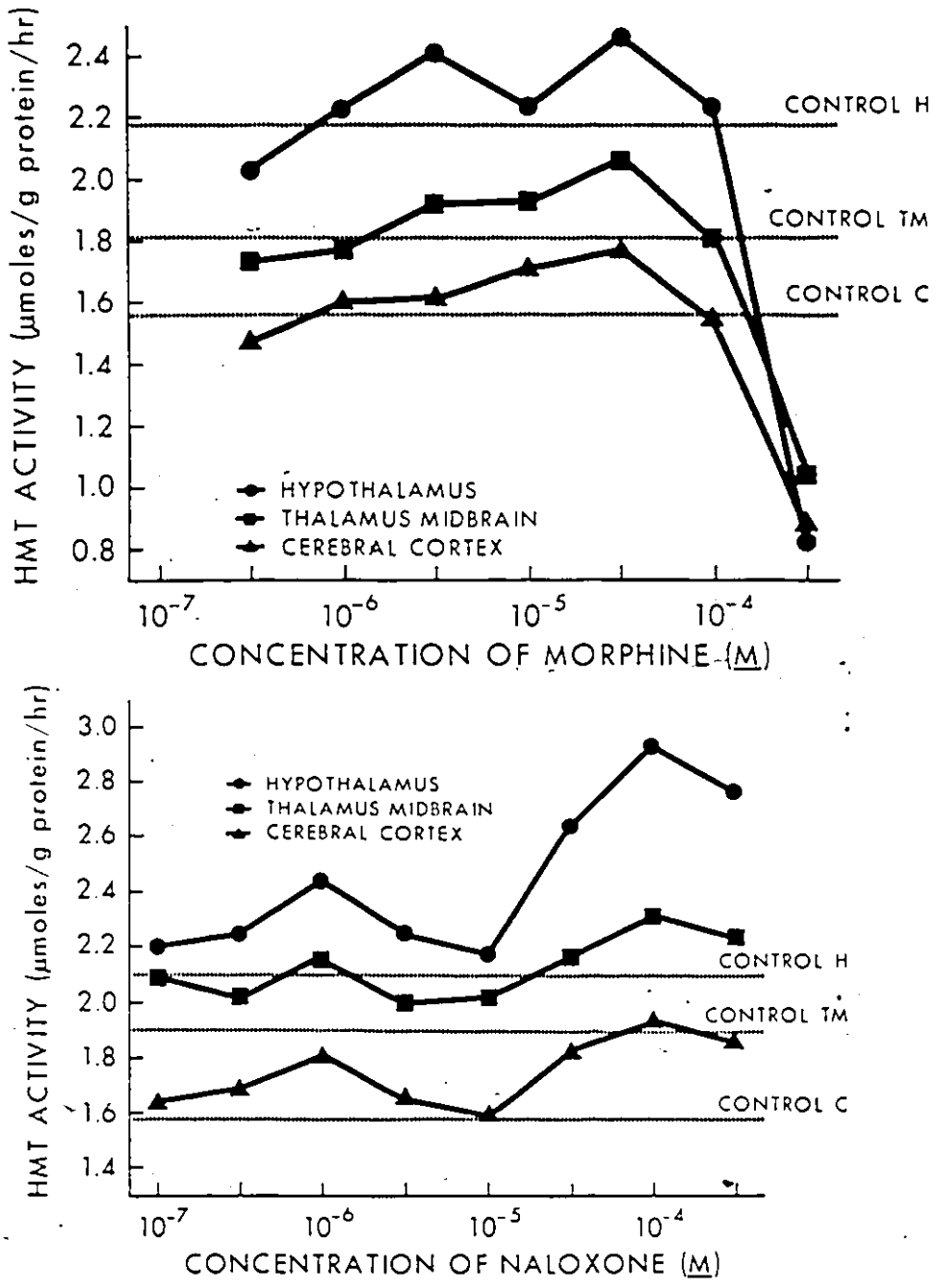


Fig. 26

Effect of various concentrations of morphine (upper graph) and naloxone (lower graph) on regional brain histamine N-methyltransferase activity. Each point represents the mean of three independent determinations. Experiments were performed in triplicate.

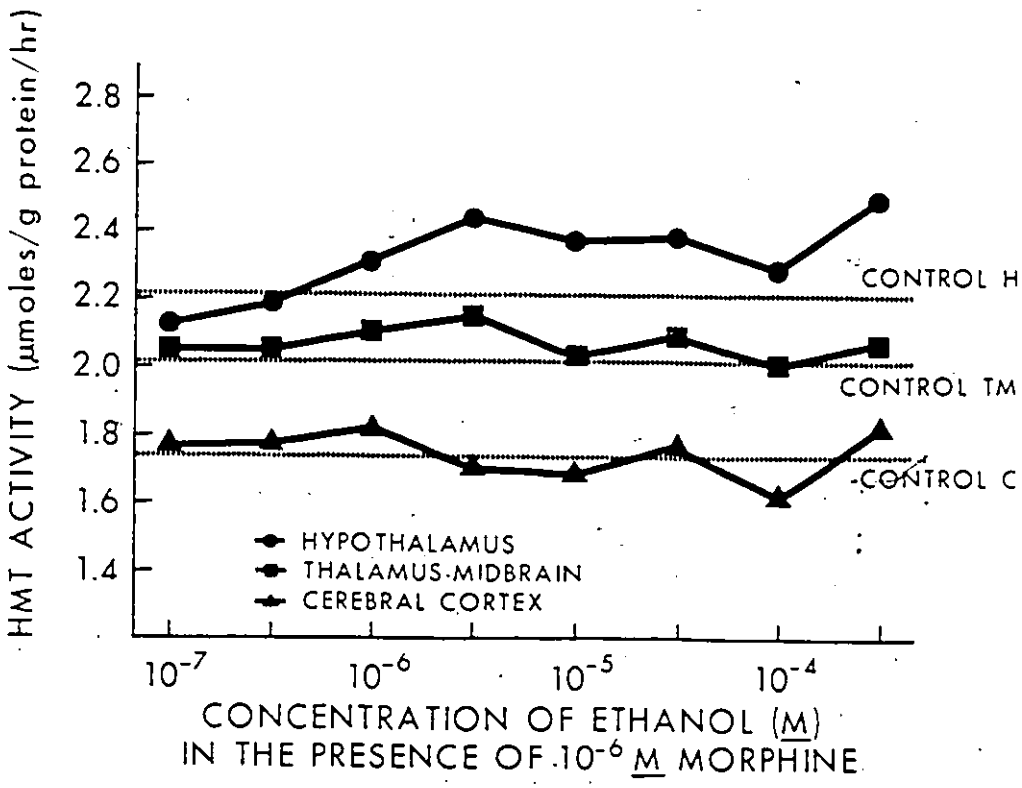


Fig. 27

Effect of various ethanol concentrations on regional brain HMT activity in the presence of 1 μM morphine. Each point represents the mean of two independent determinations. Experiments were performed in triplicate.

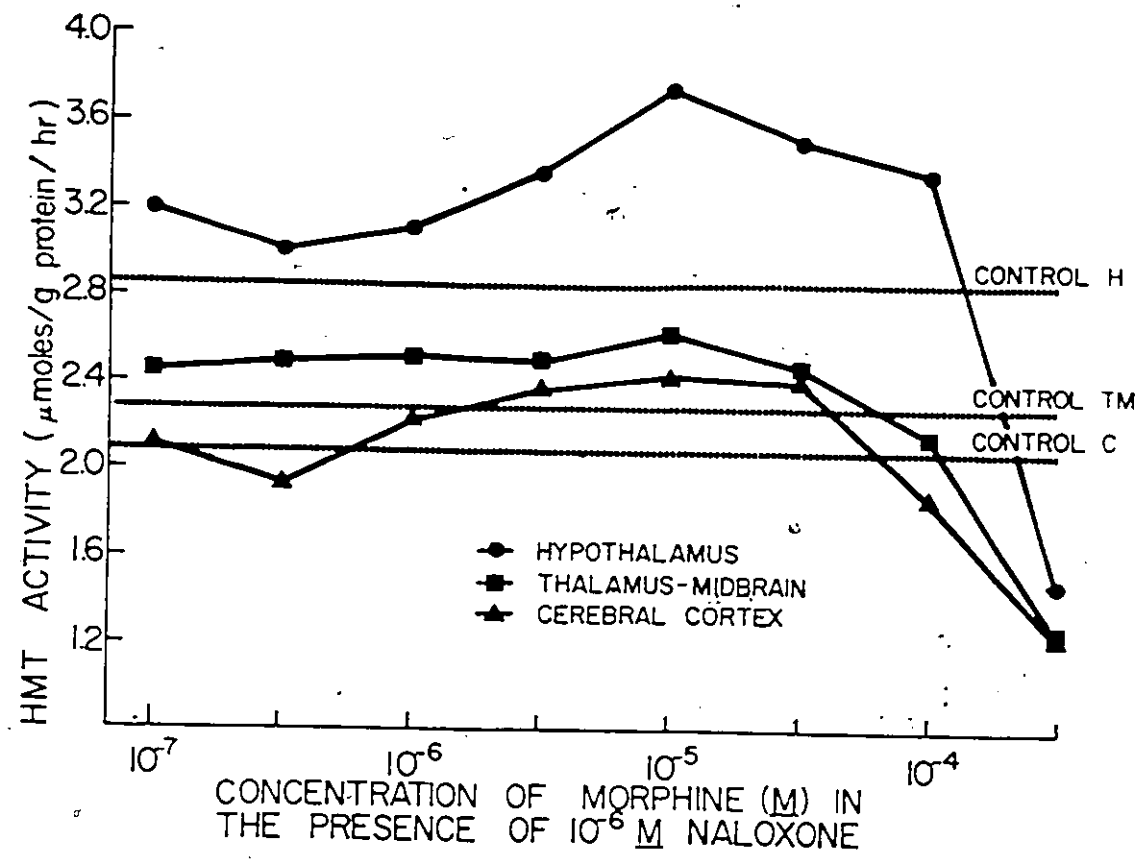


Fig. 28

Effect of various morphine concentrations on regional brain HMT activity in the presence of 1 μM naloxone. Each point represents the mean of two independent determinations. Experiments were performed in triplicate.

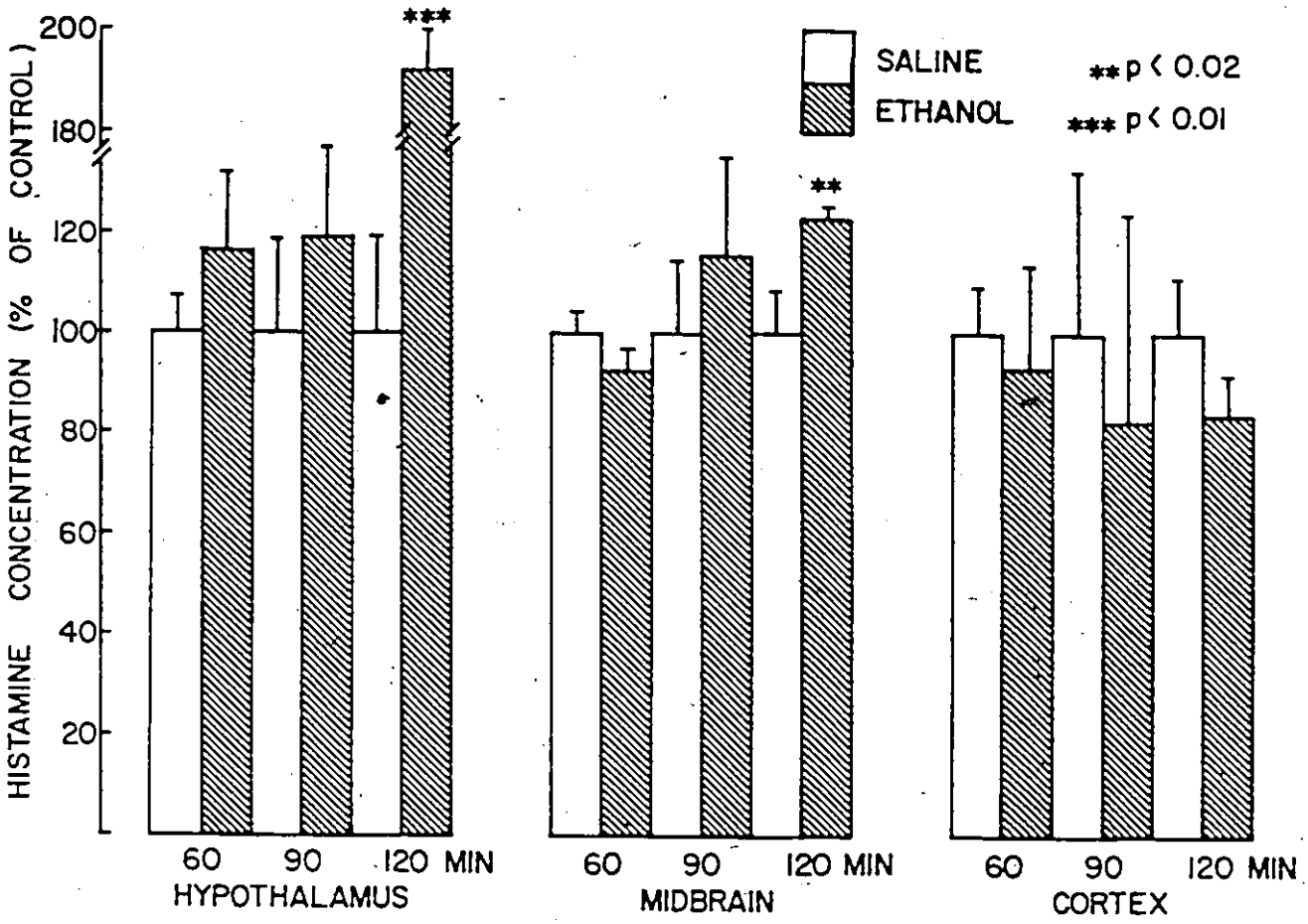


Fig. 29

Effect of 1 g/kg ethanol on regional brain histamine concentrations determined 60, 90 and 120 min after administration.

brain histamine (HA) content 60, 90 and 120 min after drug treatment. Both hypothalamic and midbrain HA levels were significantly elevated (+92% and +23% of control values, respectively) 120 min after 1 g/kg ethanol (25%, w/v) was administered by intragastric intubation. At 60 and 90 min, hypothalamic and midbrain HA levels were not significantly changed. Mean cortical HA levels tended to decrease at all three times but failed to attain statistical significance. Control HA levels in the hypothalamus, midbrain and cortex were 178.7±13.0, 58.6±2.5 and 29.6±2.7 nanograms/gram wet tissue weight, respectively.

Histidine decarboxylase (HD) activities were significantly increased in the hypothalamus (+88% and +28% of control values following ethanol administration, respectively)(Fig. 30). While midbrain and cortical HD activities were not significantly altered during the same time period, the former tended to increase while the latter tended to decrease compared to saline-treated controls. The control values for HD activity were 2.25±0.26, 0.37±0.04 and 0.43±0.07 nanomoles of HA formed per 90 min per gram tissue in the hypothalamus, midbrain and cortex, respectively. Histamine N-methyltransferase activity was not significantly changed in any brain region at the time intervals studied following 1 g/kg ethanol administration (Fig. 31). Control HMT activities were 2.28±0.11, 1.76±0.05 and 1.42±0.04 μmoles of methylhistamine formed per gram supernatant protein per hour in the hypothalamus, midbrain and cortex, respectively.

In contrast to the lower dose of ethanol, a twice higher dose (2 g/kg) induced a significant decrease (-42% of control values) in hypothalamic HA concentration 90 min after ethanol administration (Fig. 32) while the reduced mean hypothalamic HA levels at 60 and 120 min failed to reach statistical significance. Midbrain and cortical HA concentrations were not significantly affected at any time investigated. Histidine decarboxylase activity was altered in all 3 brain regions. HD activity was significantly increased (+57%, +59% and +31%) in the hypothalamus, midbrain and cortex 60 min after ethanol administration

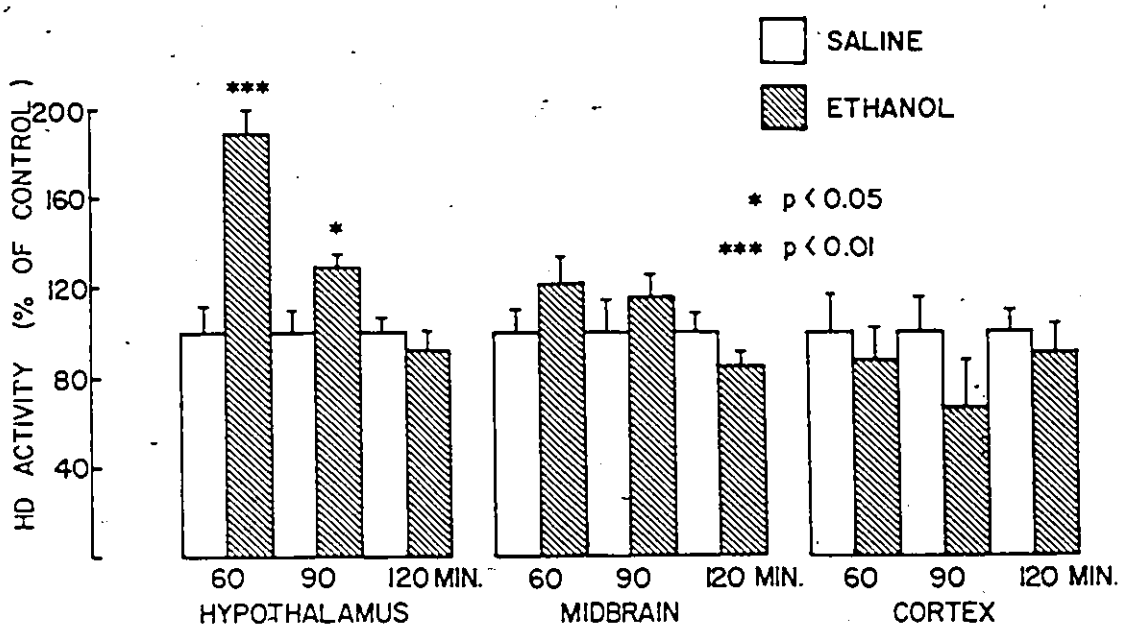


Fig. 30

Effect of 1 g/kg ethanol on regional brain histidine decarboxylase activity determined 60, 90 and 120 min after administration.

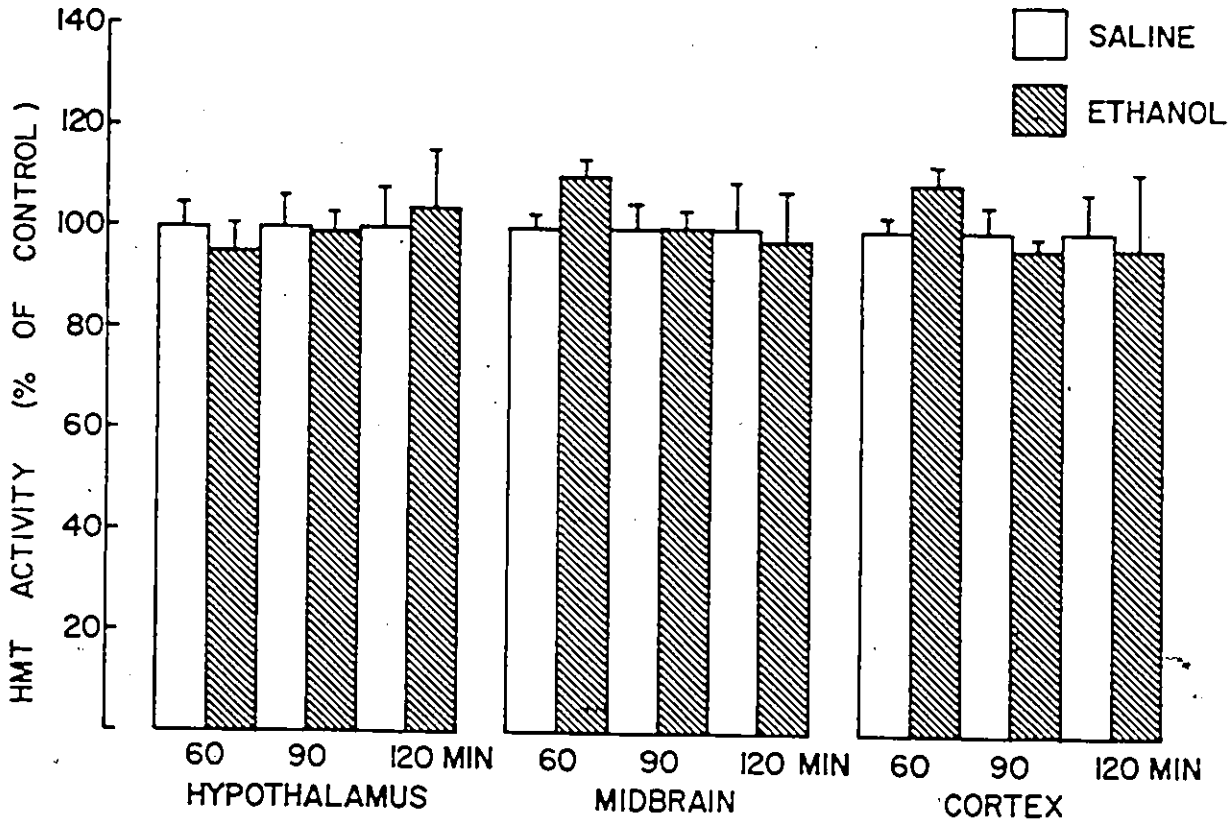


Fig. 31

Effect of 1 g/kg ethanol on regional brain histamine N-methyltransferase activity determined 60, 90 and 120 min after administration.

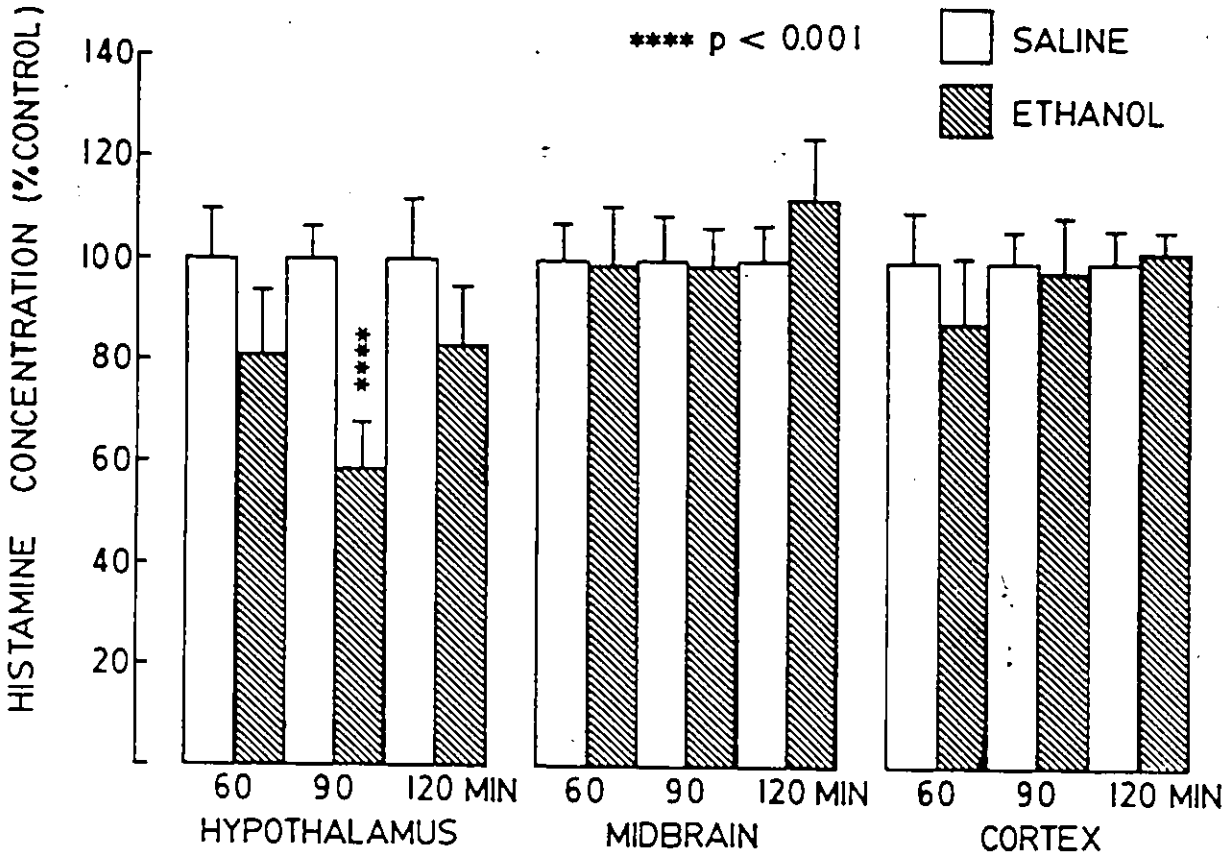


Fig. 32

Effect of 2 g/kg ethanol on regional brain histamine concentrations determined 60, 90 and 120 min after administration.

and (+68%) in the hypothalamus after 90 minutes (Fig. 33). At this ethanol dose, regional HMT activity remained unaltered at the three times examined (Fig. 34).

Plasma corticosterone levels showed a tendency to increase following a 1 g/kg dose of ethanol, but these changes were not statistically significant (Fig. 35). The larger dose of ethanol produced a similar tendency to increase after 60 and 90 min, but plasma corticosterone levels were significantly increased (+44%) only after 120 minutes. Plasma ethanol levels were determined at 60, 90 and 120 min following each ethanol dose. The mean plasma ethanol concentration 60 min after a 2 g/kg dose was approximately double that observed after 1 g/kg at the same time interval (Table 10) while 90 and 120 min after 2 g/kg ethanol administration, the plasma levels were approximately four times higher than those reached after the 1 g/kg dose.

Table 10

Plasma Ethanol Concentrations (mg%) Following Acute Oral Administration

Dose	Time After Administration (minutes)		
	60	90	120
1 g/kg	41.9±4.7	24.8±6.8	20.1±7.1
2 g/kg	90.6±8.2	96.8±12.4	91.3±9.0

Data represent the mean ± S.E.M. of 8 ethanol-treated rats.

Effect of Acute Ethanol Administration (1 and 2 g/kg) on Regional Brain Histidine Concentrations

Rat brain histidine concentration was determined 60 min after ethanol (25%, w/v) administration in the hypothalamus, midbrain and cortex. The results following intragastric intubation of 1 and 2 g/kg ethanol are shown in Table 11.

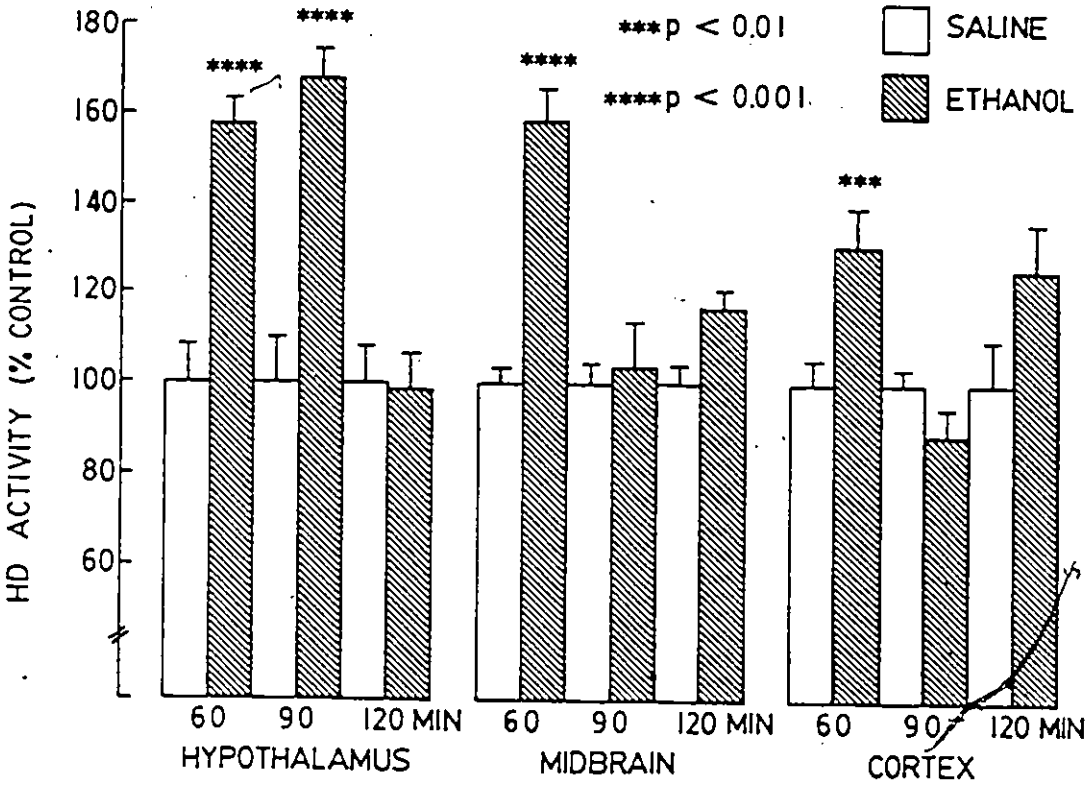


Fig. 33

Effect of 2 g/kg ethanol on regional brain histidine decarboxylase activity determined 60, 90 and 120 min after administration.

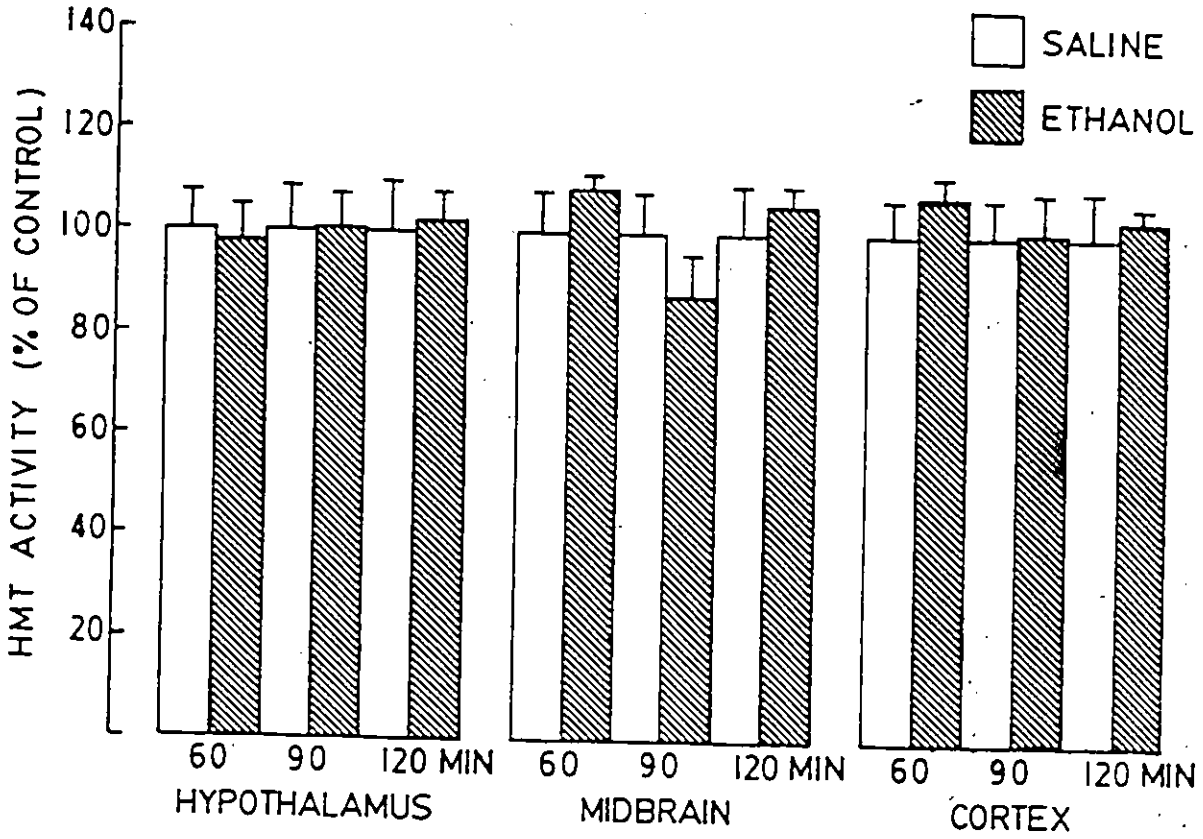


Fig. 34

Effect of 2 g/kg ethanol on regional brain histamine N-methyltransferase activity determined 60, 90 and 120 min after administration.

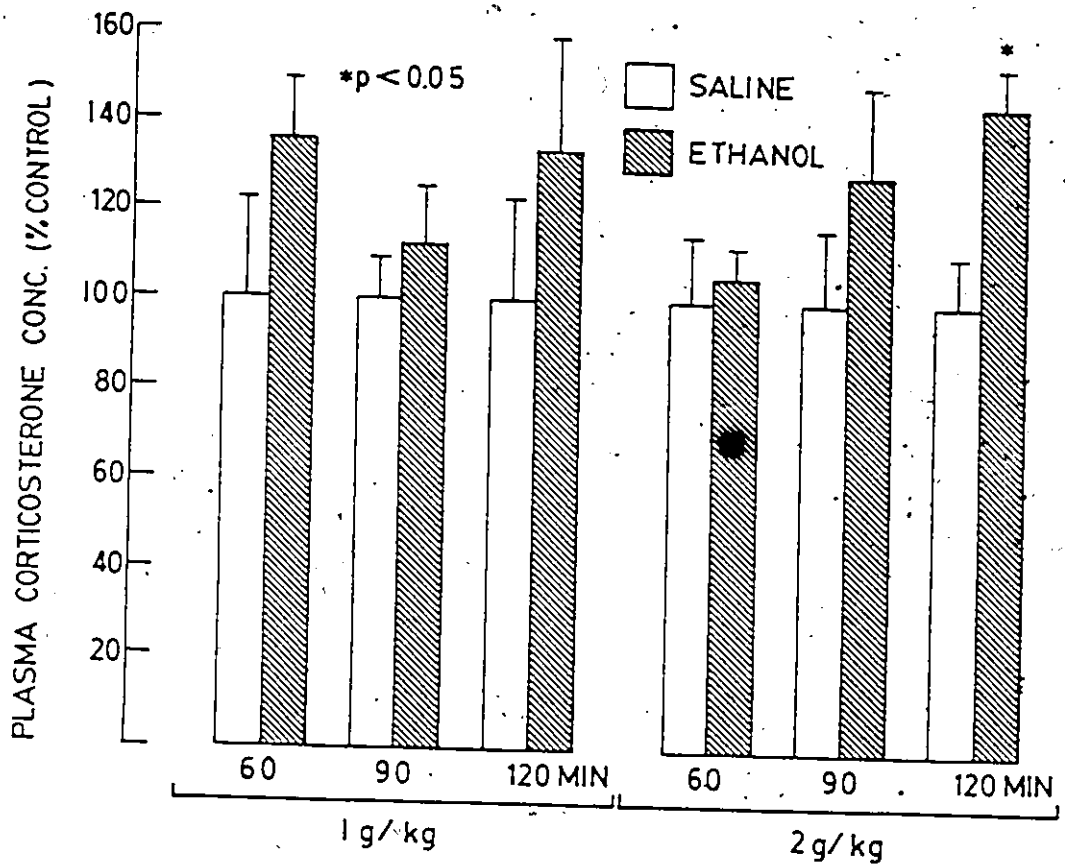


Fig. 35

Effect of 1 or 2 g/kg ethanol on plasma corticosterone concentrations. Corticosterone levels were determined 60, 90 and 120 min after administration.

Table 11

Effect of Acute Ethanol Administration (1 and 2 g/kg) on Regional Brain Histidine Concentration Determined 60 min After Drug Treatment

expressed as µg/g tissue

1 g/kg

	Saline	Ethanol
Hypothalamus	24.7±2.9	23.6±2.1
Midbrain	11.2±0.9	12.9±1.1
Cortex	10.1±1.1	10.1±1.2

2 g/kg

	Saline	Ethanol
Hypothalamus	22.9±1.9	24.1±1.7
Midbrain	13.0±1.2	13.1±1.2
Cortex	11.4±0.5	10.7±0.6

Effect of Chronic Ethanol Administration (up to 6 g/kg) on Regional Brain Histamine Concentration and Behavior

Figure 36 indicates that chronic treatment with ethanol resulted in a statistically significant reduction (-15.4%) in the hypothalamic HA concentration compared to saline control values. No significant changes were observed in the HA content of the midbrain (-12.0%) nor the cortex (-20.9%) regions of ethanol treated rats although their mean values were lower than those of saline or sucrose controls. Rats treated with sucrose isocaloric to ethanol failed to show marked alteration in histamine levels of any region investigated when compared to saline controls. The histamine concentrations (ng/g wet tissue weight) of saline controls were 413.8±24.3 in the hypothalamus, 32.9±4.9 in the midbrain and 18.2±1.8 in the cerebral cortex.

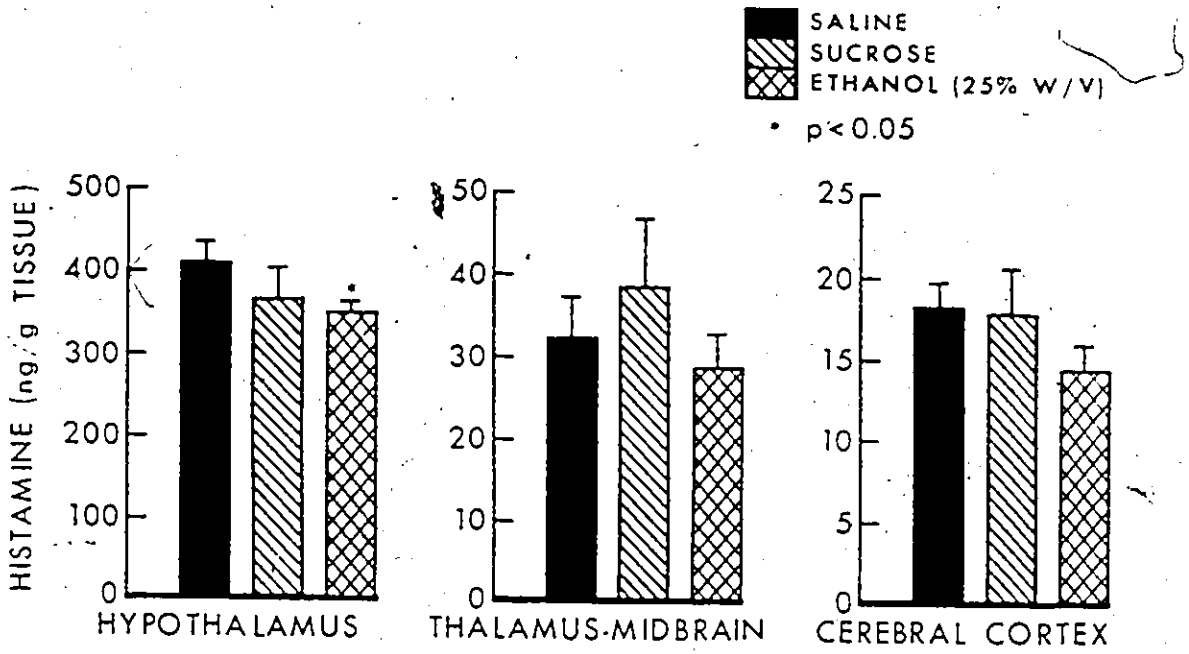


Fig. 36

Brain histamine concentrations of rats chronically treated with ethanol. Rats were administered increasing doses of ethanol (up to 6 g/kg) by gastric intubation for 22 days. Controls received isocaloric sucrose or 0.9% saline for the same period. Animals were sacrificed about 24 hr after the last treatment. The data represents the mean ± S.E.M. from 9 rats in each group. Statistical significance refers to saline injected controls.

The body weights of the saline or sucrose treated rats increased throughout the experiment and were not significantly different from one another (Fig. 37). However, the ethanol treated rats showed significantly lower weights when compared to sucrose controls on and after day 3 of the treatment period and lower than saline controls on and after day 8. Experimental rats gained significantly less weight throughout the drug administration period.

The performance of chronically treated rats on the tilting plane, which were then receiving an ethanol dose of 5 g/kg 24 hours before testing, did not differ significantly from that of naive rats. Yet, rats administered ethanol for 17 days performed better than naive rats when both groups were challenged with a 2 g/kg dose of 25% (w/v) ethanol by gastric intubation (Fig. 38). In chronically treated animals, a significant impairment compared to pretreatment performance occurred at a single time, 120 min after ethanol (2 g/kg) administration (-10.0%, $p < 0.01$). In contrast, naive rats displayed significant decreases in performance at all times of the testing period after the same dose.

There were no significant differences in the body temperatures of these groups of rats prior to saline, sucrose or ethanol administration on day 19. However, 6 g/kg ethanol administration induced a significant hypothermia which was observed 2 hours post-treatment (Fig. 39). The body temperature remained significantly depressed for up to 8 hours compared to both saline and sucrose controls. With the approach and onset of the dark cycle the rectal body temperatures rose. Ethanol treated rats experienced the greatest rate of rise. Body temperatures of sucrose treated rats were significantly elevated (+ 1% , $p < 0.05$) compared to saline treated rats only at 4 hours after sucrose administration.

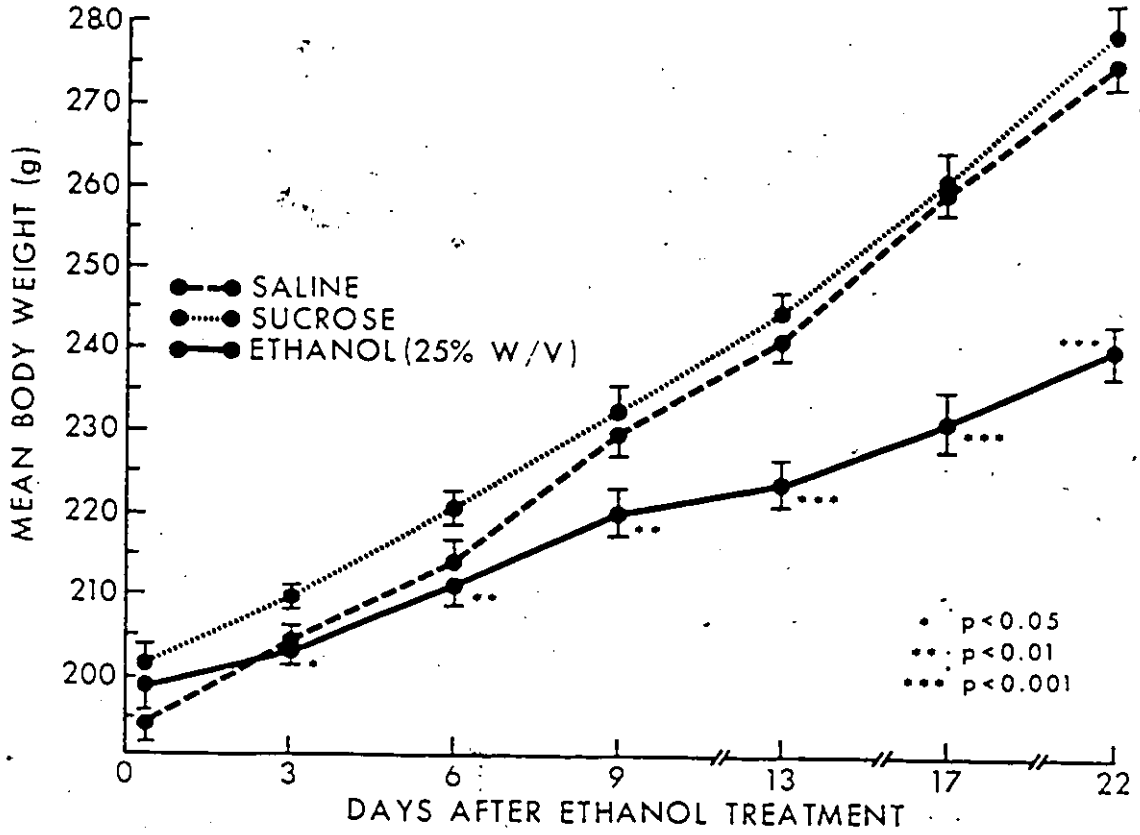


Fig. 37

Body weights of rats chronically treated with increasing doses of ethanol (up to 6 g/kg). Mean body weights (g) of saline, sucrose or ethanol treated rats after the last day of each incremental drug administration (g/kg) starting with 1 g/kg up to 6 g/kg (see methods). The data represent mean ± S.E.M. of 9 rats in each group. (*) Statistical significance refers to differences from sucrose controls on days 3 and 6 of the experiment, and to saline controls on day 9 and the following days.

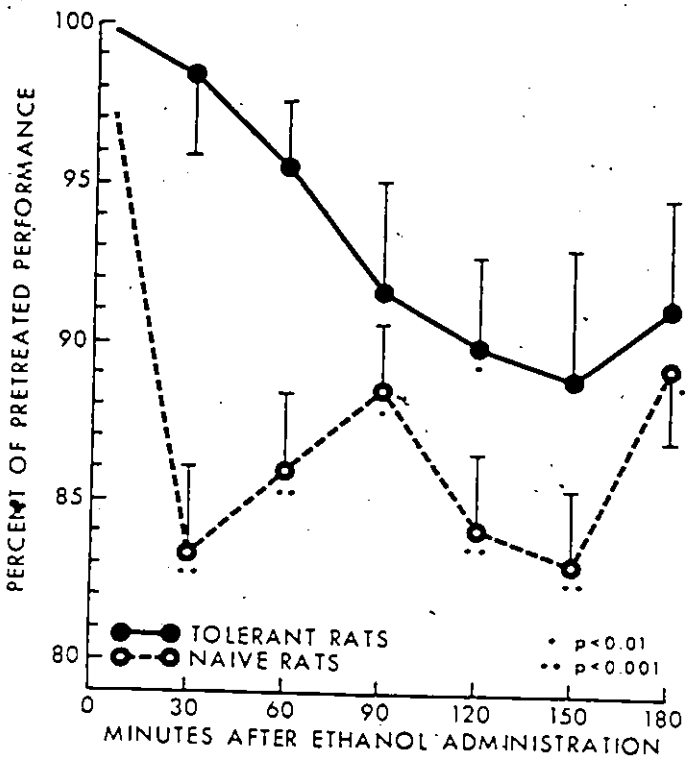


Fig. 38

Tilting plane performance of chronically treated and untreated rats following a 2 g/kg dose of ethanol. Rats chronically treated for 17 days with up to 5 g/kg of ethanol and naive rats were both tested on the tilting plane following a 2 g/kg dose of ethanol via gastric intubation. Behavior was measured by comparing performance following drug treatment to that 30 min prior to ethanol administration. Each rat served as its own control. Testing was done at 30 min intervals up to 180 min with 8 rats in each group. The data represents the mean ± S.E.M. of the per cent decline in pretreatment performance and was the basis for determination of statistically significant differences.

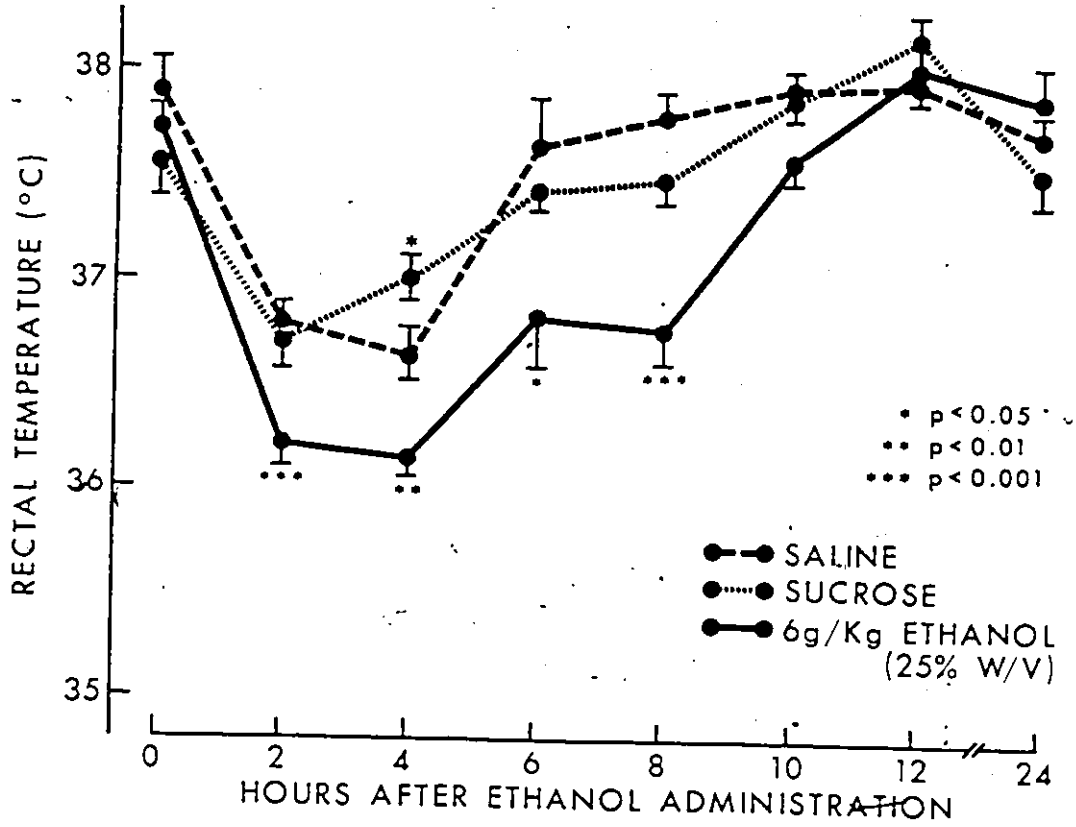


Fig. 39

Body temperatures of rats chronically treated with ethanol, sucrose or saline. Rectal body temperatures of rats chronically treated for 19 days and currently receiving 6 g/kg ethanol (25%, w/v), isocaloric sucrose or isovolumetric saline were determined every 2 hours after gastric intubation at about 08:00 (time 0). The data represent the mean ± S.E.M. of 9 rats in each group. Statistical significance compared to saline injected controls.

Effect of Chronic Ethanol Administration (up to 8 g/kg) and Withdrawal from Ethanol on Regional Brain Histamine Concentration and Histidine Decarboxylase and Histamine N-Methyltransferase Activities

In order to be more statistically accurate and be able to compare our results with other reports, this data was analyzed by both Student's t-test and analysis of variance (ANOVA). Figure 40 indicates that chronic ethanol administration and subsequent withdrawal for 2 days resulted in a significant decrease (-37% and -36% compared to saline controls, respectively) in hypothalamic HA concentration (when subjected to Student's t-test and ANOVA) compared to both saline and sucrose treated (96% of saline controls) rats. Hypothalamic-saline control values were 178.0±24.0 ng HA/g wet tissue weight. Histamine levels in the midbrains of both chronically treated and withdrawn rats decreased significantly (-26% and -28% of saline controls, respectively) compared to sucrose treated rats (+14% of saline) when evaluated by ANOVA. However, only in withdrawn rats was midbrain HA content significantly reduced when compared to saline control levels (37.2±4.2 ng/g tissue). Cortical HA levels when subjected to ANOVA indicated that HA levels of chronically treated and withdrawn groups had only a tendency to decrease ($p < 0.10$); yet, a significant decrease in cortical HA of ethanol treated (-31% of saline controls) or withdrawn rats (-30% of saline controls) was evident when compared to saline or sucrose treated rats using Student's t-test. The cortical HA concentrations of saline treated rats was 28.9±3.3 ng/g tissue.

Hypothalamic HD activity increased (Student's t-test and ANOVA) only in withdrawn rats (+50% of the mean saline control value which was 1.31±0.11 nanomoles of HA formed per gram tissue per 90 minutes) (Fig. 41). A significant reduction in midbrain HD activity occurred in ethanol treated (-29%) and withdrawn groups (-32%) compared to the saline and sucrose treated groups when evaluated by Student's t-test; however, ANOVA and the multiple range test of Duncan indicated that significance occurred only when compared to saline controls (0.24±0.01 nanomoles per g tissue per 90 min) but not to sucrose treated rats.

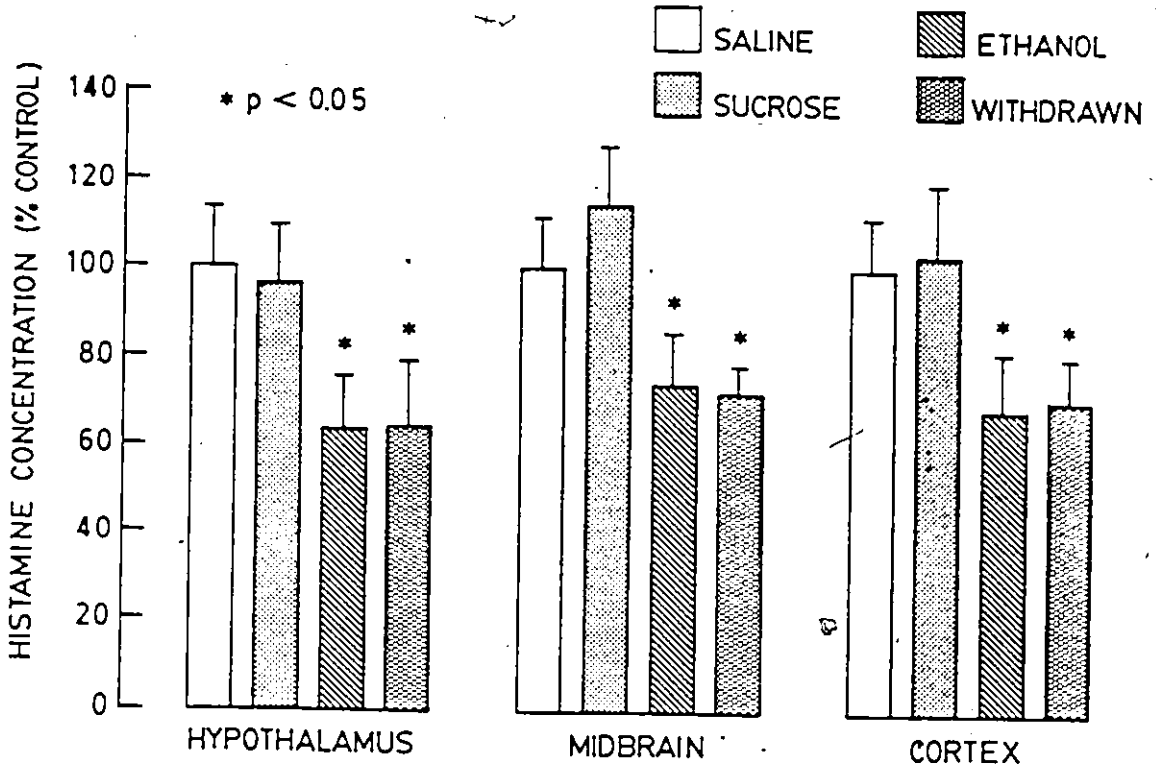


Fig. 40

The effect of chronic ethanol treatment and withdrawal on brain histamine levels in rats. Data represent mean ± S.E.M. of 9 experiments in each group. Saline and sucrose treated rats were used as controls. (*) indicates statistical significance (Student's t-test) compared with saline or sucrose treated rats (see Results).

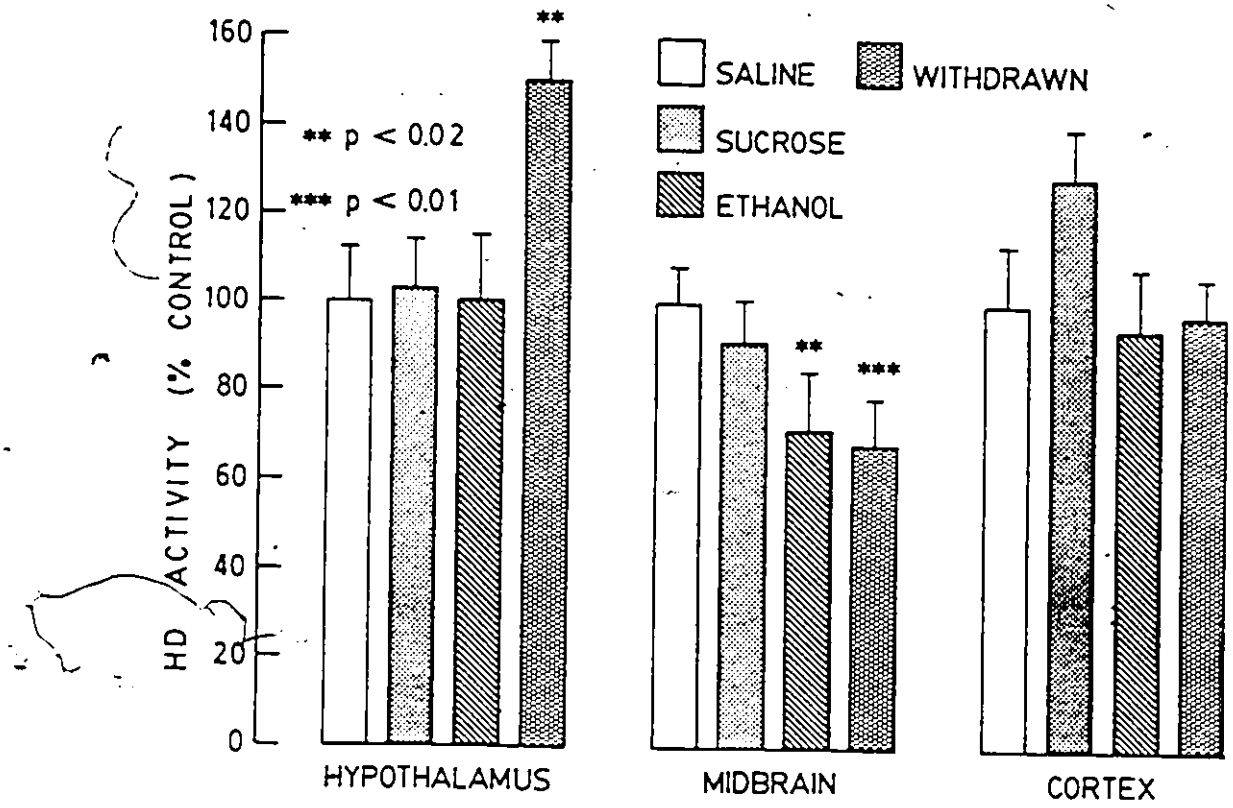


Fig. 41

The effect of chronic ethanol treatment on histidine decarboxylase activity in ethanol treated and ethanol withdrawn rats. Data represent mean ± S.E.M. of 9 experiments in each group. Asterisks indicate statistically significant difference when compared with the control values.

No significant changes in cortical HD activity occurred in any group when compared to sucrose or saline (0.21±0.02 nanomoles/g tissue/90 minutes).

No significant alterations were observed in hypothalamic nor midbrain activities of HMT (Fig. 42) of ethanol treated or withdrawn rats. However, ethanol treated rats exhibited a significant elevation in cortical HMT activity (+18% of saline controls) when tested by ANOVA. Control saline values of HMT activity in the hypothalamus, midbrain and cortex regions were 2.25±0.09, 1.99±0.08 and 1.56±0.08 µmoles of methylhistamine formed per gram supernatant protein per hour, respectively.

In all brain regions studied there were no significant changes in protein content when compared to saline or sucrose treated animals following chronic ethanol administration or withdrawal (Fig. 43). Control saline values for the hypothalamic, midbrain or cortical regions were 2.30±0.07, 1.52±0.02 and 1.72±0.05 µg protein per mg wet tissue weight.

A highly significant increase in plasma corticosterone content was observed in ethanol treated (+199%) and withdrawn rats (+261%, compared to saline controls) (Fig. 44). The plasma corticosterone concentration of sucrose treated rats (-20% of saline controls) was not significantly altered. Analysis of variance and Duncan's test revealed that no significant difference in corticosterone levels existed between ethanol treated and withdrawn rats.

Effect of Chronic Ethanol Administration (up to 8 g/kg) on Rat Brain Histidine Concentration

Rat brain histidine concentration was determined after chronic ethanol administration and in animals withdrawn from ethanol for 2 days in the hypothalamus, midbrain and cortex regions. Isocaloric sucrose-treated rats served as controls. The results are shown in Table 12.

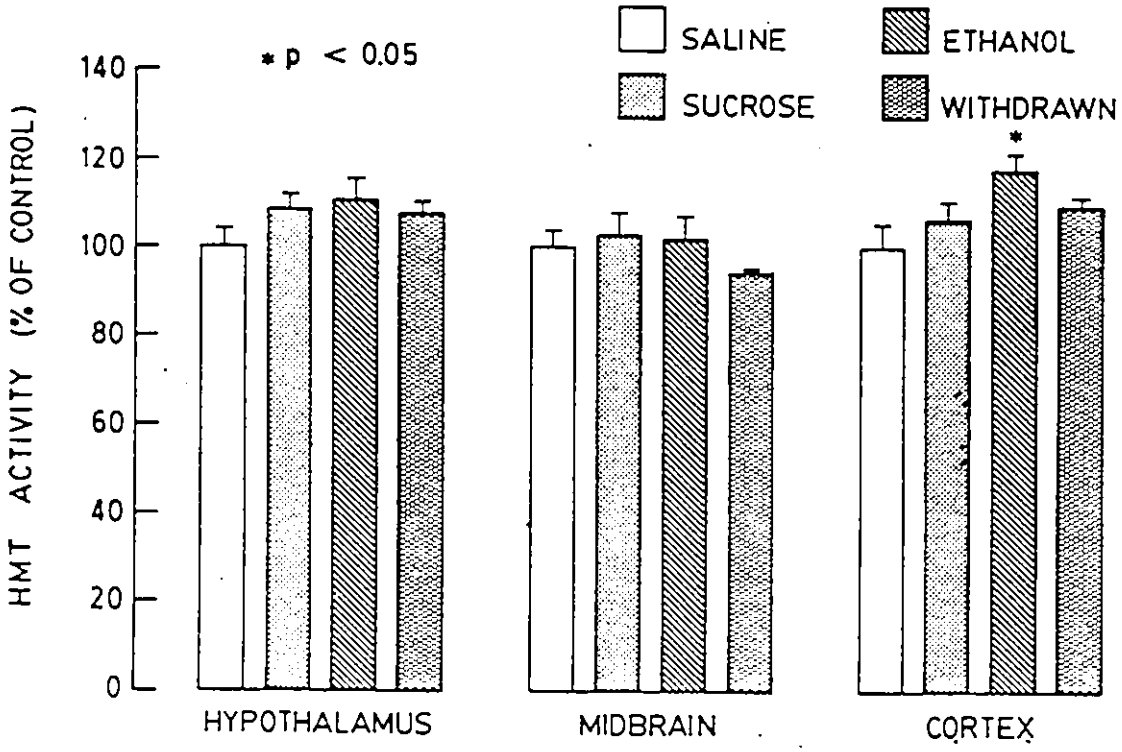


Fig. 42

Histamine methyltransferase activity in ethanol treated and ethanol withdrawn rats. Data represent mean ± S.E.M. of 9 experiments in each group. Asterisks indicate statistically significant difference when compared with the control values.

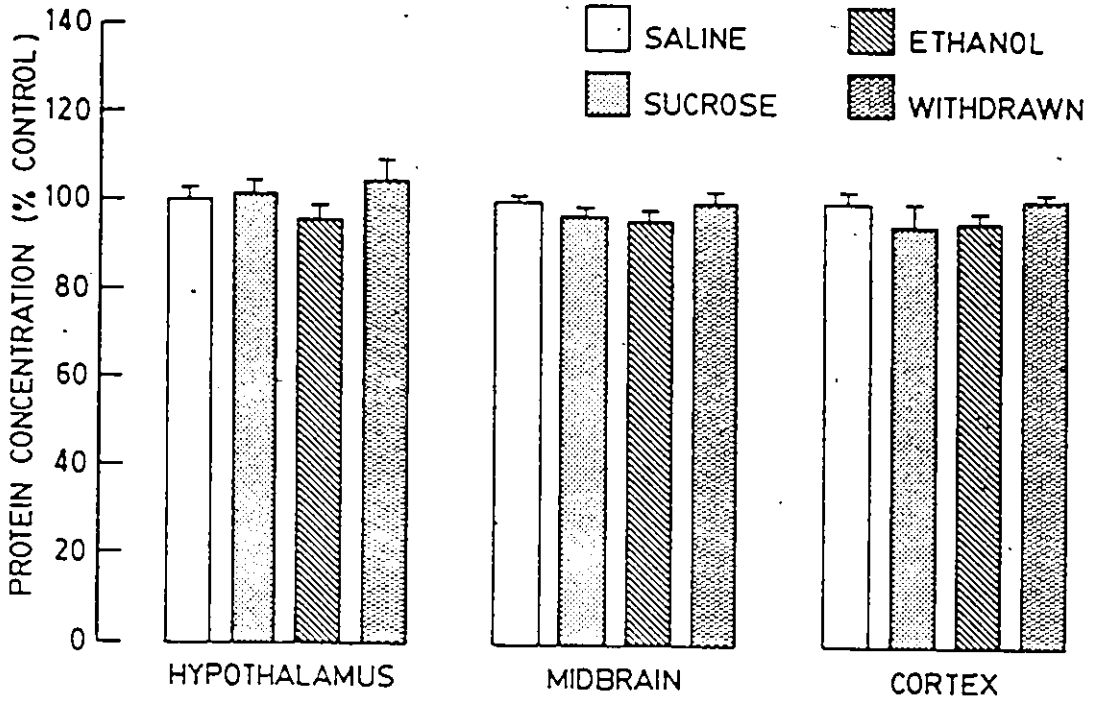


Fig. 43

Protein concentration in the brain supernatant obtained from ethanol treated and ethanol withdrawn rats. Protein content from saline or sucrose treated rats is also incicated. Data represent mean ± S.E.M. of 9 experiments in each group.

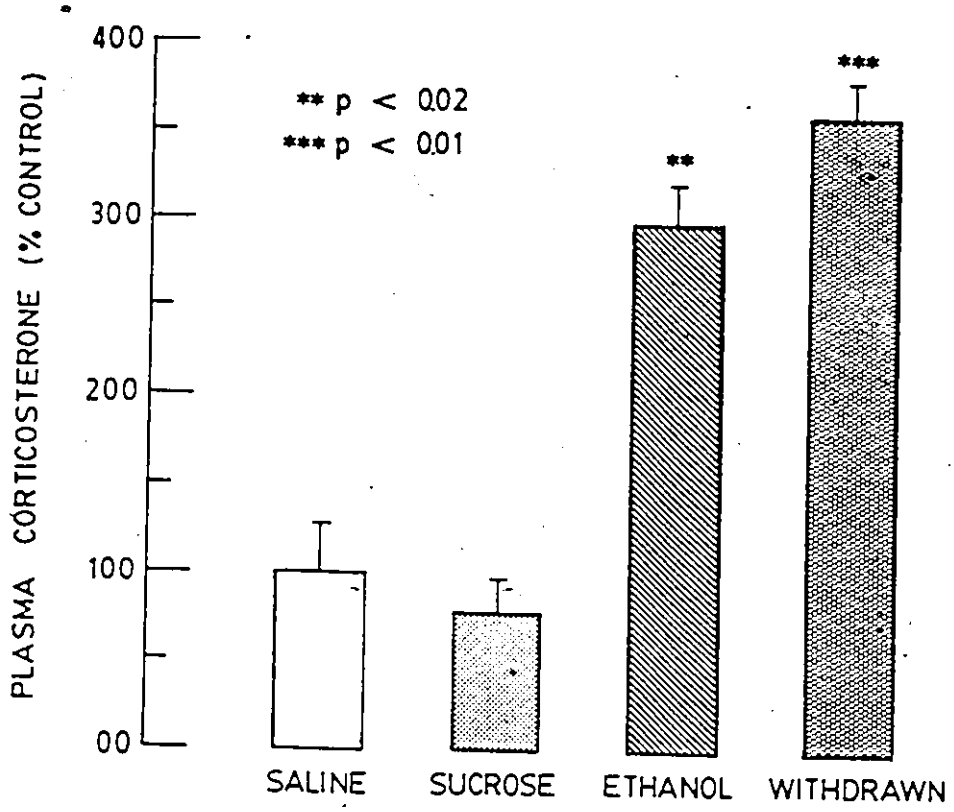


Fig. 44

Plasma corticosterone concentration of rats chronically treated with saline, sucrose, ethanol and withdrawn from ethanol treatment. Data indicates mean ± S.E.M. of 9 experiments in each group. Asterisks indicate statistically significant difference when compared with the control values.

Table 12

Effect of Chronic Ethanol Administration (up to 8 g/kg) on Rat Brain Histidine Concentration (ug/g wet tissue weight ± S.E.M.)

	Sucrose Controls	Ethanol Treated	Withdrawn From Ethanol
Hypothalamus	30.1±2.1	27.0±0.4	26.4±0.8
Midbrain	7.2±0.3	7.4±0.3	7.1±0.3
Cortex	10.3±0.3	11.1±0.4	9.3±0.8

Histamine Concentration in Subcellular Fractions Following Chronic Ethanol Administration (up to 8 g/kg)

In all three brain regions examined, i.e. hypothalamus, midbrain and cortex, the protein concentrations of the ethanol treated and withdrawn rats were not significantly different from those of sucrose treated controls. This was a consistent observation both in homogenates as well as in the subcellular fractions (Figure45 , Table13). The relative distribution of the recovered protein for all fractions was determined on a concentration (µg/µl) basis by evaluation of the protein content of the various fractions from sucrose treated rats (Table14 Since the hypothalamic P_3 protein concentration was not determined, the relative distribution of the remaining hypothalamic fractions must be regarded as maximal when compared to the content of all four fractions. In an attempt to circumvent this limitation, these values were recalculated with the hypothalamic P_3 component arbitrarily set at 10%.

In contrast, the histamine concentrations of both ethanol treated and withdrawn rats were significantly reduced in homogenates (-20 to -35%) of these three brain regions when compared to the HA content of sucrose-treated controls (Fig. 46). The mean hypothalamic, midbrain and cortical histamine control values (± S.E.M.) were 233.2±17.5, 92.5±9.7 and 58.6±6.7 ng HA/g tissue, respectively.

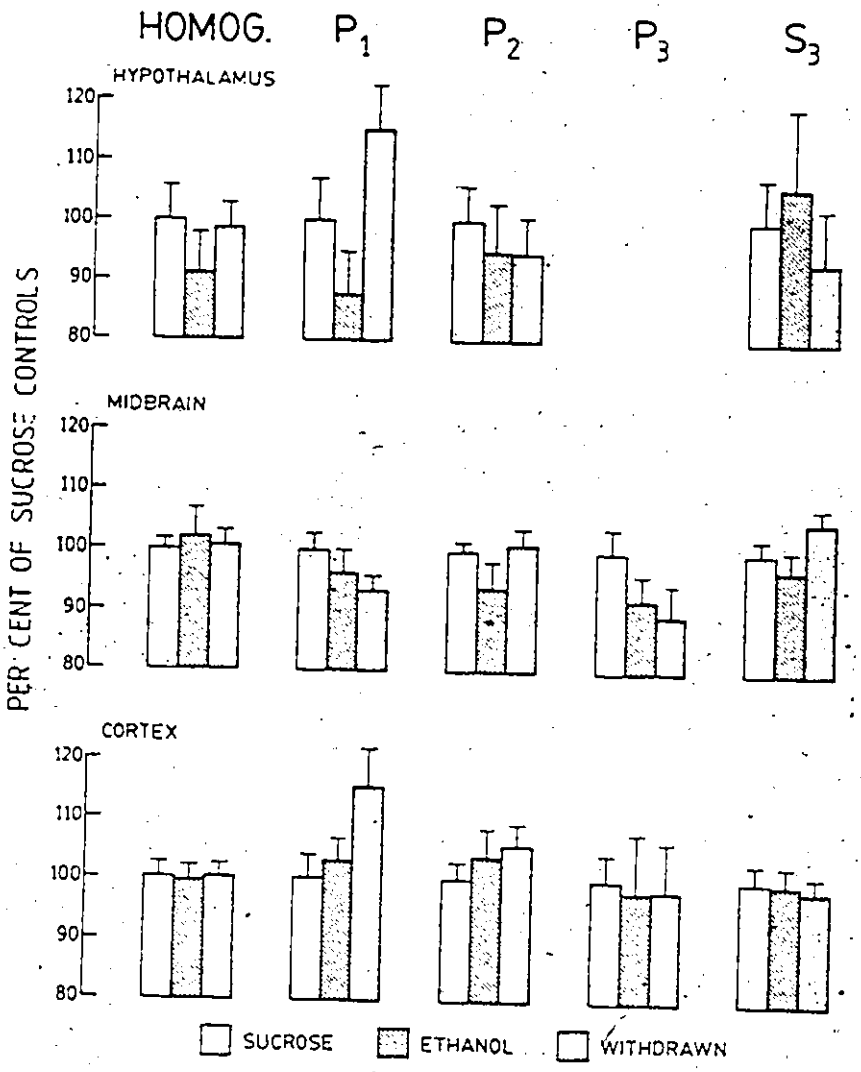


Fig. 45

Protein concentrations of the homogenate and subcellular fractions of the rat hypothalamus, midbrain and cortex following chronic ethanol administration and withdrawal. Values are expressed as a percentage of sucrose treated animals.

TABLE 13

Protein Concentration (ug/50 ul of resuspended fraction) of Various Brain Fractions After Chronic Ethanol Administration and Withdrawal

	Sucrose Controls	Ethanol	Withdrawn
Homogenates*			
HYPO	150.2±8.8	137.2±10.1	148.5±6.2
MB	366.1±6.2	374.0±17.7	369.0±8.7
CX	397.6±10.3	396.1±9.8	398.0±7.9
P1 Fraction			
HYPO	50.9±3.6	44.7±3.6	58.7±3.7
MB	488.2±13.0	470.8±18.7	456.4±12.4
CX	373.4±13.9	384.8±13.4	431.4±24.4
P2 Fraction			
HYPO	79.2±4.7	75.3±6.3	75.2±4.8
MB	843.4±14.0	793.8±36.6	854.7±22.3
CX	676.2±19.5	702.4±31.5	716.2±22.8
P3 Fraction			
HYPO	-	-	-
MB	286.7±12.0	264.6±11.8	257.1±15.0
CX	182.1±7.9	178.8±17.7	179.3±14.3
S3 Fraction			
HYPO	31.0±2.4	32.9±4.1	29.0±2.8
MB	106.3±2.8	103.7±3.5	112.3±2.2
CX	113.7±3.5	113.3±3.5	112.2±2.9

*Expressed as ug protein per 25 ul of sucrose homogenate

Abbreviations: HYPO, hypothalamus; MB, midbrain; CX, cortex

TABLE 14

Relative Distribution of Protein Concentrations Expressed as the Percentage Recovered From Various Fractions of Brain Tissue[a,b]

Fraction	Hypothalamus[c]	Midbrain	Cortex
P_1	<31.6 (28)	28.3	27.8
P_2	<49.2 (44)	48.9	50.3
P_3	- (10)	16.6	13.6
S_3	<19.2 (17)	6.2	8.5

a) Based on protein concentration (ug/50 ul) of sucrose control fractions

b) Values derived from the equation: $\frac{\text{fractional protein content}}{\text{protein content of } P_1+P_2+P_3+S_3} \times 100$

c) Units are expressed as ceiling values since the P_3 fraction was not evaluated. Units in parentheses are values recalculated with P_3 fractional content arbitrarily set at 10%.

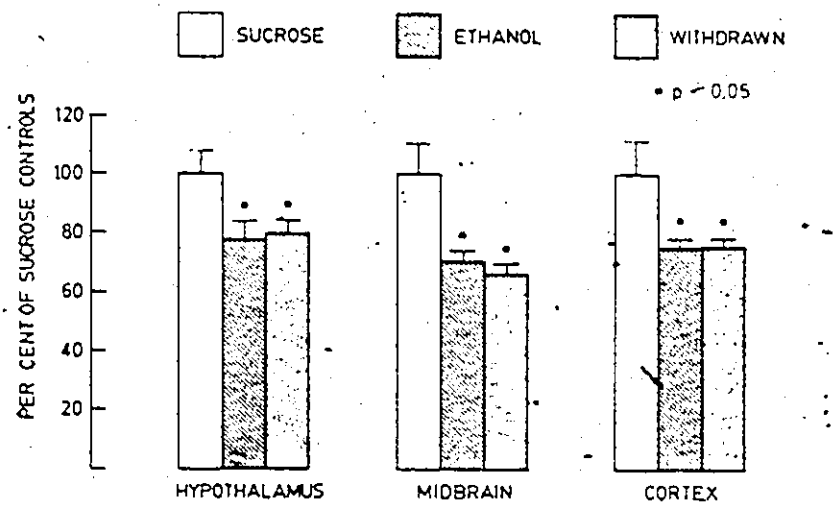


Fig. 46

The effect of chronic ethanol administration and withdrawal on regional brain histamine concentration. Values are expressed as a percentage of sucrose treated animals which were 233.2±17.5, 92.5±9.7 and 58.6±6.7 ng HA/g wet tissue weight in the hypothalamus, midbrain and cortex, respectively. Asterisks indicate statistically significant difference when compared with the control values.

However, when the homogenate HA content was expressed on a nanogram HA per mg protein basis, only the hypothalamic and midbrain histamine levels of both drug treated and withdrawn rats were significantly reduced, -20 to -25% (Fig. 47, Table 15). In the P_1 (nuclear) fraction, the HA content was significantly reduced in the hypothalamus (-13%) and cortex (-14%) of withdrawn rats only, while HA levels in the P_2 (mitochondrial) fraction of both drug treated and withdrawn rats were significantly reduced (-14 to -27%) in all three regions. Chronic ethanol administration and withdrawal failed to markedly alter P_3 (microsomal) fractional HA content. Cytoplasmic HA, approximated in the S_3 fraction, approached the limits of detection of the HA assay under the experimental conditions utilized. The resulting decrease in sample size and the reduced protein concentration (mathematically magnifying the ng HA/mg protein value) caused large standard errors. Due to the diminished number of detectable samples and the spuriously high HA values (which could not be rejected using the critical Q test), the mean hypothalamic values of ethanol treated rats (n=6 detectable) and midbrain values of withdrawn rats n=5 detectable) exceeded those of sucrose controls. Cortical HA content in ethanol treated rats was undetectable except in one sample (-57%) while only 4 could be measured in ethanol withdrawn (-37%) rats.

Since the decrease of P_1 fractional HA concentration of the hypothalamic ($p < 0.05$) and cortical ($p < 0.001$) regions of withdrawn rats was small (<15%) (Fig. 47) and the mean protein content was elevated in both cases (>15%, though not significantly) (Fig. 45), the HA content per 50 µl sample was evaluated in these regions and compared to controls. Control hypothalamic and cortical HA content (3.63 and 5.78 picomoles HA, respectively) were found not to differ significantly from those of the withdrawn rats (93% and 99%, respectively).

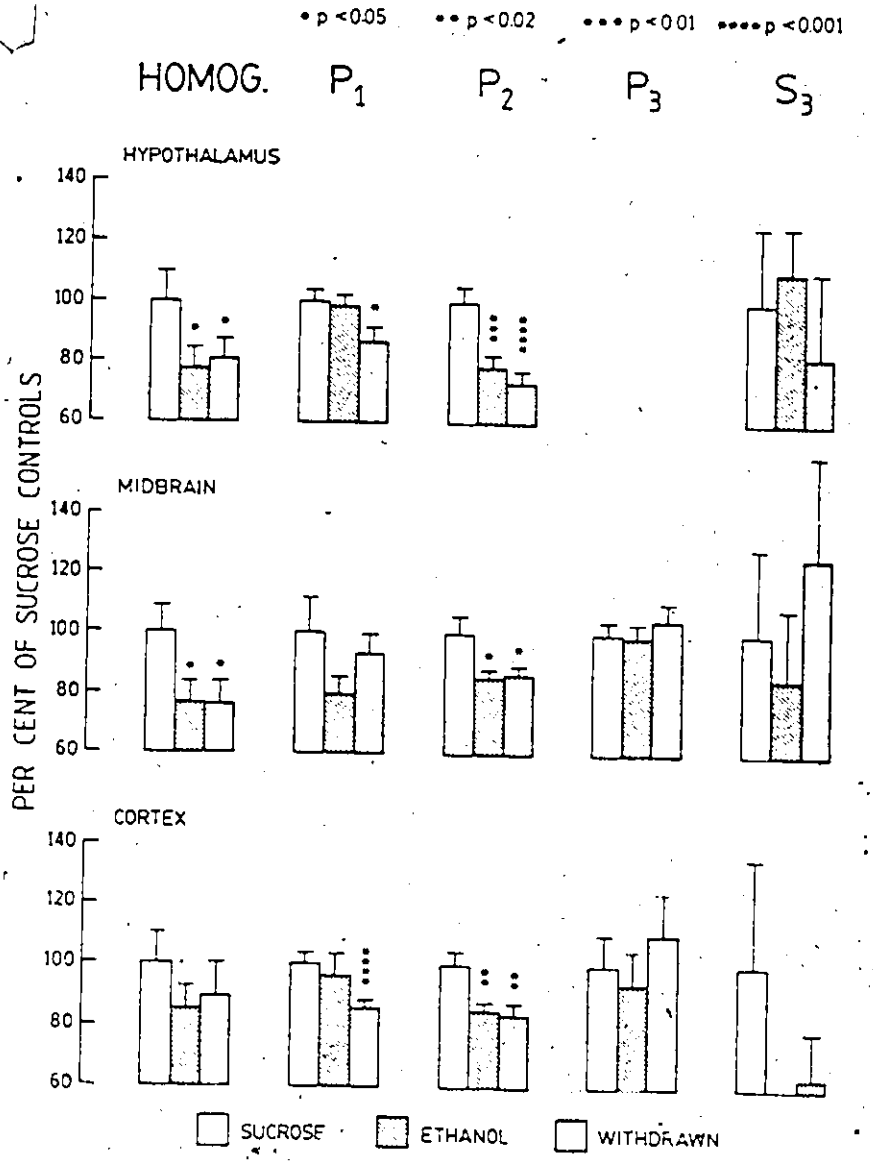


Fig. 47

The effect of chronic ethanol administration and withdrawal on histamine concentration (determined on a ng/mg protein basis) in homogenate and subcellular fractions of the rat hypothalamus, midbrain and cortex. Values are expressed as a percentage of sucrose treated animals. Asterisks indicate statistically significant difference when compared with the control values.

TABLE 15

Histamine Concentration (ng/mg protein) of Various Brain Fractions Observed After Chronic Ethanol Administration and Withdrawal

	Sucrose Controls	Ethanol	Withdrawn
Homogenates			
HYPO	2.04±0.20	1.58±0.15*	1.70±0.13*
MB	1.00±0.09	0.77±0.07*	0.77±0.07*
CX	0.56±0.06	0.48±0.04	0.50±0.06
P1 Fraction			
HYPO	7.54±0.30	7.54±0.28	6.54±0.30*
MB	3.47±0.41	2.77±0.19	3.24±0.22
CX	1.73±0.06	1.66±0.12	1.48±0.05****
P2 Fraction			
HYPO	8.06±0.41	6.34±0.33***	5.93±0.32****
MB	2.00±0.12	1.71±0.06*	1.74±0.06*
CX	1.47±0.07	1.25±0.04**	1.23±0.06**
P3 Fraction			
HYPO	-	-	-
MB	5.30±0.22	5.27±0.23	5.57±0.31
CX	5.48±0.56	5.15±0.61	6.03±0.77
S3 Fraction			
HYPO	1.83±0.47	2.03±0.27	1.50±0.52
MB	1.16±0.33	0.99±0.28	1.47±0.39
CX	0.64±0.23	0.28	0.41±0.10

*p <0.05 **p <0.02 ***p <0.01 ****p <0.001

Abbreviations: HYPO, hypothalamus; MB, midbrain; CX, cortex

DISCUSSION

XVI. DISCUSSION

In vitro Analysis of Histamine N-Methyltransferase Activity

Since HA methylation by HMT is the primary enzyme regulating the termination of HA action (once released) in the CNS, alteration of HMT activity may influence brain HA levels (section VIII.B). Agents which markedly inhibit HMT activity have been shown to elevate brain HA content (Duch et al., 1979). It was therefore of interest to investigate whether some of the changes induced in brain HA concentration following acute or chronic morphine (Mazurkiewicz-Kwilecki and Henwood, 1976; Henwood and Mazurkiewicz-Kwilecki, 1977) or ethanol (Prell and Mazurkiewicz-Kwilecki, 1980; Subramanian et al., 1978) administration could be linked with alterations in brain HMT activity. Therefore, a method for determination of histamine N-methyltransferase activity was developed.

After establishing near optimum conditions for this assay, it became apparent that several of the kinetic constants (K_M values) and other parameters of this method (e.g. substrate inhibition with HA) were similar to those of other reports (Brown et al., 1959; Taylor and Snyder, 1972c; Thithapandha and Cohn, 1978; Matuszewska and Borchardt, 1983). In addition, the rank order of regional brain tissue HMT activities was the same as that reported earlier (Taylor and Snyder, 1972a), i.e. hypothalamus > midbrain > cortex. However, rat brain HMT activity in unpurified brain supernatants (obtained after homogenate centrifugation at 50,000 x g for 20 min) using the present procedure resulted in enzyme activity approximately three times greater than that reported by Taylor and Snyder (1972a). This difference in the results obtained may be attributed mainly to two factors. First, the supernatant protein content was used to determine enzyme activity in this study; a value which is about one-third of that found in the homogenate,

the source of Taylor and Snyder's (1972a) protein values. Since the rate of formation of methylhistamine (MeHA) was estimated in supernatants (which were the preparations used for routine analysis), the protein content in this medium was considered to be appropriate for calculation of supernatant enzyme activity. Second, no correction was made for the efficiency of extraction of the newly formed MeHA into the toluene-isoamyl alcohol solution by Taylor and Snyder (1972a). This is noteworthy as Snyder and Axelrod (1965) observed that extraction was not quantitative. They reported an extraction efficiency of about 70% although their methodology was not elaborated. Synthesis of ^{14}C-MeHA and subsequent evaluation of fractional MeHA recovery in the present investigation indicated that about 80% of the total MeHA was extracted (XIV.A.2). In order to determine this value, ^{14}C-MeHA was added near the end of the incubation period. In this way, substantial enzymatic metabolism by monoamine oxidase (type B) or non-specific chemical reactions with the ^{14}C-MeHA would be less likely to occur. ^{14}C-Methylhistamine was prepared by the straightforward and relatively inexpensive technique outlined (see methods) which appeared more attractive than the expensive and complicated technique for synthesizing ^{3}H-MeHA (Schwartz et al., 1973a) or other lengthy chemical synthetic routes (Jones, 1966).

When HMT was incubated in the presence of increasing concentrations of ethanol, acetaldehyde, morphine or naloxone, alone or in combination, it was apparent that only large concentrations of morphine or naloxone had any appreciable effect on <u>in vitro</u> HMT activity (Figs. 25-28). A combination of ethanol and morphine failed to show any synergistic or antagonistic effects on HMT activity (Fig. 27). A representative compound of the tetrahydroisoquinoline group, 3-carboxysalsolinol, a generous gift from Dr. Maurice Hirst, also failed to alter HMT activity at concentrations up to 1 mM (Prell; unpublished). Morphine and naloxone in high concentrations had opposite effects as the former lowered and the latter increased HMT activity. The increased activity was most evident

in the hypothalamus. The biphasic effect of morphine alone, and in the presence of 1 μM naloxone, is similar to the profile of antihistamines, chlorpromazine, desmethylimipramine and haloperidol on mouse brain HMT activity (Taylor and Snyder, 1972c). However, similar to latter findings, only large in vitro concentrations of morphine caused strong inhibitory actions; concentrations which are clearly toxic to mammals (Lemberger and Rubin, 1976). The opposite effects of higher concentrations of morphine and naloxone on HMT activity (Fig.26) are interesting, but largely unexplained. The fact that morphine lowered HMT activity was initially attributed to non-specific interactions with the enzyme; however, the elevated activity due to naloxone was surprising. Nevertheless, the inability of naloxone (1 μM) to markedly alter morphine's action on HMT activity (Fig.28) may suggest that a stereospecific site-mediated effect on HMT is not involved. Therefore, it is unlikely that these findings have significant physiological relevance in altering brain HA levels in vivo through the induction of changes in HMT activity. A similar situation has been reported in which chlorpromazine (15 mg/kg) failed to alter whole brain histamine levels in mice (Taylor and Snyder, 1972b) and a 10 mg/kg dose had the same result in rats (Green and Erickson, 1964). But a 40 mg/kg dose caused rat brain HA to increase 43%; a dose at which even the authors questioned the specificity of chlorpromazine-induced effects (Green and Erickson, 1964). These results suggest that the reduction of brain HA levels due to chronic morphine administration (Mazurkiewicz-Kwilecki and Henwood, 1976; Henwood and Mazurkiewicz-Kwilecki, 1977) may not be due to alterations of HMT activity nor is it likely that the naloxone reversal of morphine-induced brain HA depletion is due to HMT inhibition. Present results are consistent with in vivo data that HA levels (Mazurkiewicz-Kwilecki and Henwood, 1976) and HMT activity (Henwood, 1977) were unaltered following acute morphine administration.

Even high in vitro levels of ethanol (10 mM) which are encountered in vivo (equivalent to plasma levels of about 45 mg%) failed to alter in vitro HMT

activity. This result is in line with the present in vivo acute ethanol studies which failed to show changes in regional brain HMT activity (Fig. 31 and 34) and in line with other reports (Subramanian et al., 1978; 1980a).

The observation that acetaldehyde had almost no effect on HMT activity (Fig. 25) is noteworthy since, as ethanol's primary metabolite, its levels might be expected to rise somewhat during chronic ethanol administration schedules in parallel to the increase in ethanol dosages over the course of the study. Indeed, HMT activities remained unaltered after chronic ethanol administration and withdrawal except for a small but significant increase (+18%) in the cortices of ethanol treated rats (Fig. 42 , see below).

The Action of Acute Ethanol Administration on the Central Histaminergic System

The present data indicate dose- and time-dependent changes in brain HA levels and in HD activity following acute ethanol administration. These changes were most pronounced in the hypothalamus, the brain region with the highest HA concentration (section VI) and most rapid turnover rate (section VIII.C). While the lower dose of ethanol (1 g/kg) increased hypothalamic and midbrain HA levels (Fig. 29), the larger dose (2 g/kg) significantly reduced the hypothalamic HA concentration (Fig. 32). However, cortical HA levels were not significantly altered with either dose. It is also noteworthy that these changes occurred at plasma ethanol levels which were not excessive (Table10). These observed changes may be primarily due to ethanol-induced alterations in histamine synthesis and/or release. The elevated hypothalamic HD activity preceeding the increase in hypothalamic histamine content is consistent with increased HA synthesis. The tendency for midbrain HD to rise and the small yet significant rise in midbrain HA content also tends to support this view. Although the assay used for the determination of HD activity has strong limitations (e.g. substrate dilutional effects, among others), the fact that brain histidine content did not change in any brain region investigated at either dose of ethanol administered (Table 11)is significant. It suggests

that this "apparent" rise in enzyme activity may reflect an actual increase in the number of active enzyme molecules capable of synthesizing HA. This lack of an ethanol-induced alteration in brain histidine concentration is consistent with a recent report indicating that 60 min after acute administration of a 2 g/kg dose of ethanol (20% w/v, i.p.) whole rat brain levels of several amino acids, including histidine, were unchanged compared to saline-treated rats (Eriksson et al., 1980).

Another explanation for these in vivo results is an alteration in HA release due to ethanol. The elevation of hypothalamic HD activity (Fig.33) and the simultaneous marked reduction of hypothalamic HA levels (Fig.32) following the 2 g/kg dose suggest that HA stores were being depleted, although its rate of synthesis had increased more than 60%. This might indicate that a compensatory mechanism which could lead to an increase in HD activity had been initiated. Such a mechanism was demonstrated by Verdiere et al. (1975; see section VIII.A) in slices of rat hypothalamus preincubated with ^{3}H-histidine. K^{+}-induced depolarization caused a depletion of ^{3}H-HA tissue stores which stimulated further synthesis of tissue ^{3}H-HA. It is possible that in the present study, the already increased hypothalamic HA synthesis could not keep pace with the postulated HA release; hence, the depleting effects may have become evident. An altered HA release rate may also explain why midbrain and cortical HA levels were unaltered while HD activity was elevated 59 and 31%, respectively.

Histamine N-methyltransferase activity remained statistically unaltered following both doses at all times, in all regions examined (Figs. 31 and 34). This is consistent with in vitro data which indicated no alteration in HMT activity in the presence of either ethanol or acetaldehyde over a wide concentration range (Fig. 25). It should be noted that the apparent paucity of HA present in the mammalian CNS is very likely overshadowed by the high activity of HMT. Therefore, large fluctuations in the amount of HA released (should they

occur) may not be accurately reflected in the present studies.

Saline treated control rats had elevated plasma corticosterone levels when compared to levels routinely obtained in control untreated rats in this laboratory (Mazurkiewicz-Kwilecki and Taub, 1978). This could be attributed to the stress of intubation. The failure of ethanol to significantly increase plasma corticosterone levels above saline controls at the earliest times at which measurements were made infer that ethanol alone rather than the stress by gastric intubation may have been responsible for the changes in brain HA. The increase of plasma corticosterone at 120 min following the 2 g/kg dose is consistent with the reported ability of ethanol to elevate plasma corticosterone levels in acutely treated rats (Cicero, 1981). Stress-induced changes would be expected to be greatest at earlier times after intubation. This observation may be particularly important for two reasons. First, brain histamine has been shown to be altered by stress (Mazurkiewicz-Kwilecki and Bielkiewicz, 1982). Second, the elevation of hypothalamic HA at 120 min, but not at 60 or 90 min following the 1 g/kg dose, and the decline after the 2 g/kg dose at 90 min argue against stress-induced changes in brain HA since stress rapidly elevates hypothalamic HA levels (Mazurkiewicz-Kwilecki and Taub, 1978; Mazurkiewicz-Kwilecki and Bielkiewicz, 1982).

The ethanol-induced biphasic effect on hypothalamic HA concentration, i.e. elevation with a lower dose and reduction after a higher dose, is in line with results of a simultaneous (though independent) study of ethanol-induced changes in whole brain HA levels in mice (Papanicolaou and Fennessy, 1980). In this study, 0.18, 0.9 and 1.8 g/kg of ethanol (10 µl/g body weight, i.p.) caused a 68% increase and reductions of 40% and 65% in brain HA concentration, respectively. Also, at the highest dose tested, a time-dependent reduction of brain HA was observed. The amounts of HA present (approx. per cent of saline control HA levels) were 50, 35, 45 and 80% at 15, 30, 60 and 120 min after ethanol injection.

Unfortunately, a regional analysis of brain HA levels, enzyme activities or plasma ethanol levels were not reported. While the results of Papanicolaou and Fennessy (1980) are not readily comparable with the present data due to differences in species, doses and methods of administration, a biphasic dose-dependent alteration in rat brain HA content is evident (Figs. 29 and 32). In addition, at the higher dose in the present study, the hypothalamic HA levels display a similar time-dependent nadir (Fig. 32).

These results are also consonant with many of the observations of Subramanian et al. (1978). In their study, intragastric administration of ethanol caused a dose-dependent elevation of hypothalamic HA concentration 60 min after a 0.8, 1.2 or 1.6 g/kg dose (concentration of ethanol unreported). Similarly to the present results, HD activity was also shown to be elevated while HMT activity remained unchanged. Subramanian et al. (1978) also reported that both basal and K^+- stimulated efflux of radiolabelled HA from rat hypothalamic slices were inhibited in the presence of ethanol (see section XII.C). Based on these observations, the elevated HA levels in the hypothalamus were attributed to both inhibition of HA release and to increased synthesis of HA (Subramanian et al., 1978). The authors also speculated that the increase in hypothalamic HA concentration may not totally represent the neuronal HA content since levels of plasma HA (presumably derived from mast cell HA) showed a dose-related increase, i.e. +61% and +92% at the lowest and highest ethanol doses, respectively.

The hypothesis of enhanced brain HA synthesis in the presence of ethanol could partially account for the present results and those of Subramanian et al. (1978). However, the conclusion that HA release is impaired due to ethanol (at concentrations attained in the latter study) may not be as well supported. First, this method suffers from the limitations shared by ^{3}H-HA release studies based on ^{3}H-HA uptake into brain tissue slices (previously discussed in section VIII.A). Second, in a later study, it was revealed subsequently that as much as 10% of the

tritium recovered was due to ^{3}H-methylhistamine while 75% may have been due to ^{3}H-HA (Mulder et al., 1983). Third, the tritium recovered in the superfusion medium was not used as the index of release, but rather the per cent initial radioactivity remaining in the slices, i.e. the radioactivity that was not released was the criterion upon which release rates were determined. Lastly, the ethanol concentrations required to significantly inhibit basal tritium release (80 and 160 mM, equivalent to plasma levels of about 370 and 740 mg%, respectively) were far above concentrations encountered in their study. The lower ethanol concentrations which were evaluated (20 and 40 mM, equivalent to plasma ethanol concentrations of 92 and 184 mg%, respectively) had no significant effect on basal efflux. This is noteworthy as Subramanian et al. (1978) asserted that the 0.8 g/kg dose resulted in brain ethanol concentrations of about 20 mM. The K^{+}-stimulated tritium release was inhibited when measured in the presence of 100 mM ethanol. Studies using other concentrations were not described. Thus, these studies of ^{3}H-HA release, even if carried out under physiologically ideal conditions (which are presently in doubt), do not support the idea of ethanol-induced inhibition of HA release in the present study nor in the acute ethanol study of Subramanian et al. (1978).

The present results are at variance with those of Subramanian et al. (1980a) in which their previous procedure (Subramanian et al., 1978) was repeated. A dose-related elevation of cortical HA concentration was noted together with a significant reduction in HD activity. HMT activity was unchanged. It was also shown that ^{3}H-HA efflux from cortical slices was inhibited in the presence of 200 mM ethanol, but not a 10 mM concentration. The elevated cortical HA content was postulated to be due to reduced HA release while the reduction of HD activity was asserted to "represent a feedback product inhibition due to increased levels of histamine" (Subramanian et al., 1980a). However, this suggestion is highly improbable as HA has been shown not to inhibit its own synthesis (see section VII, Schwartz et al., 1979a).

Subramanian et al. (1980a) concluded that the changes in hypothalamic HA content, HD activity, or both observed in the earlier report (Subramanian et al., 1978) were probably due to changes occurring in mast cells. This was similar to the conclusion reached by Rawat (1980) based on his studies in adult rats acutely administered 2 g/kg ethanol or 78 mg/kg acetaldehyde (intragastrically, ethanol concentrations were unreported) which had elevated whole brain histamine levels. HA levels were determined using a modified fluorescence technique with o-phthalaldehyde. Rawat (1980) also determined plasma HA levels which were elevated +99% and +130% by ethanol or acetaldehyde administration, respectively compared to saline treated controls. Based on increased HA levels in brain and plasma, Rawat (1980) concluded that the elevation of brain HA levels was a direct consequence of the local release of brain HA due to ethanol or acetaldehyde metabolism in the body.

The results of these two investigations and the conclusions based on them are noteworthy. Employing two different techniques for determining plasma HA levels, i.e. Subramanian et al. (1978; 1980a) using the method of Taylor and Snyder (1972a) and Rawat (1980) using a modified Shore et al. (1959) method, both groups of authors obtained control plasma HA levels which were at least four to five times higher than those routinely found in the literature (Beaven and Horakova, 1978). However, both Subramanian et al. (1978) and Rawat (1980) stated that the elevation of plasma HA levels following oral administration of ethanol was in accordance with the literature; both reports citing the review of Ivy and Bachrach (1966). This is important as Ivy and Bachrach (1966) in their review, contrary to the above assertions, clearly stated an absence of plasma HA changes due to ethanol and cited several reports in support of their conclusions. Irvine et al. (1961) failed to find a detectable increase of HA in gastric venous blood after i.v. infusion of 300 ml of 10% (v/v) ethanol in chloralose-anesthetized dogs. Further, when dogs were administered ethanol (7%) by i.v. infusion or by stomach tube,

no increase in urinary HA or HA metabolite concentrations were observed (Irvine et al., 1960). In guinea pig and rat lung preparations sensitized to antigen, ethanol concentrations as low as 2% were reported to inhibit compound 48/80- or antigen-induced HA release in vitro (Chakravarty, 1960). Further study suggested that in the presence of ethanol, rat peritoneal mast cells were desensitized at a stage in the HA release mechanism which was distal to the antigen-antibody interaction process (Johansen and Chakravarty, 1975). Therefore, the literature is not consonant with an ethanol-induced increase in plasma HA concentration due to HA released from mast cells or any other source.

Subramanian et al. (1978) suggested that some of the hypothalamic HA elevation is due to inhibition of neuronal release while both Subramanian et al. (1978; 1980a) and Rawat (1980) postulated that HA levels are elevated due to HA release from non-neuronal stores. While the inhibition of neuronally released HA (if indeed neuronal) is of questionable physiological relevance at the doses encountered in their studies, the idea of HA elevation due to increased local HA release is even less tenable. The rapid metabolism by HMT which is well established (section VIII.B) would preclude this possibility. Since ethanol does not inhibit HMT in vitro (Fig. 25) nor in vivo (Figs. 31 and 34 ; Subramanian et al., 1978; 1980a), it is unlikely that HA would accumulate if released. Unfortunately, the analytical limitations of methods for determining regional endogenous methylhistamine in mammalian brain precluded its analysis in the present investigations.

The present data demonstrates that ethanol-induces a biphasic effect on hypothalamic HA levels. However, it is difficult to demonstrate conclusively in the absence of further data that altered HA release is involved since a quantitative measure of methylhistamine concentration could not be determined. Unfortunately, this laboratory is not presently equipped for routine measurement of brain HA metabolites. Nevertheless, this tentative conclusion that this biphasic effect is due to altered HA synthesis and release, although incompletely supported, currently agrees with the data available.

Effect of Chronic Ethanol Administration (up to 6 g/kg) on the Central Histaminergic System and Behavior

The methodology used for chronic ethanol administration and some of the advantages and limitations accompanying this approach have been discussed (see XIII. B and XIV. B. 2). While several different methods exist they have generally been developed to address a specific aspect associated with chronic ethanol intake. Such studies have dealt with a specific behavioral impairment, an examination of drinking patterns, the onset or severity of withdrawal, alterations of organ function, among others (Cicero, 1978; 1979). Since the study of chronic ethanol-induced neurochemical changes is not associated with a well accepted treatment protocol, the more direct method outlined earlier was adopted.

In the initial study, rats were administered increasing doses of ethanol (up to 6 g/kg). Under these conditions, hypothalamic, midbrain and cortical HA concentrations were reduced in ethanol-treated rats; only hypothalamic levels were significantly decreased compared to saline treated rats (Fig. 36). (Note: Since this data was obtained by a method which was later modified, the values are different from those obtained in acute or subsequent chronic experiments.) Since no other techniques were then available to further explore the neurochemical mechanisms responsible for this regional brain HA reduction, this study remained only a preliminary observation. However, it indicated that chronic ethanol administration affects the central histaminergic system.

The significant reduction in the rate of body weight gain in ethanol-treated rats compared to the saline and sucrose controls (Fig. 37) is in agreement with other reports describing this phenomenon (Wang et al., 1976; Friedman and Lester, 1977). This confirms the concept that when alcohol derived calories represent a major portion of the total caloric intake, it proves to be a poorer source of calories than carbohydrate, protein or fat (Westerfeld and Schulman, 1959). This phenomenon of "caloric wasting" has been clearly demonstrated even in

pair-feeding paradigms (Banks et al., 1970; Wang et al., 1976; Cicero and Badger, 1977).

Development of behavioral tolerance during the present ethanol dosing schedule was evident from data on the tilting plane test (Fig. 38). The choice of a 2 g/kg challenge dose of ethanol (25%, w/v) to naive (untreated) and tolerant rats was made so as to more readily compare the present results to those of Arvola et al. (1958). However, in their study, a similar dose was administered to naive rats only. In the present study, the latter demonstrated 83.4% of their pretreated performance when evaluated 30 min after ethanol administration. This is nearly identical to the 84% pretreatment performance reported by Arvola et al. (1958). Naive rats in the present investigation were significantly below ($p<0.01$) their pretreatment performance at all times investigated up to 3 hours. In chronically treated rats, the decrement in performance was significant only at 120 min (probably due to the small standard error) although the mean decrease in performance at 150 min was lower (Fig. 38). Even though the performance of chronically treated rats was superior to that of naive rats given the same dose, these values were not significantly different from each other 90 min following ethanol administration or thereafter.

This method of testing measures a complex of functions composed of vestibular and "grasping" reflexes with a strong cortical component (Arvola et al., 1958). When challenged with a 2 g/kg ethanol dose, the chronically treated rats exhibited a behavioral adaptation sufficient enough to overcome the deterioration of performance observed in naive rats receiving the same dose. Thus, by the 18th day of the experiment, the rats receiving ethanol displayed a tolerance to the deterioration of equilibrium and motor coordination common to ethanol intoxication.

The ethanol-induced fall in rectal body temperature noted in the present investigation is in agreement with other reports describing similar changes

(Freund, 1973; 1979; Ritzmann and Tabakoff, 1976). However, the decrease noted in this study is of less magnitude, possibly due to the development of tolerance to ethanol-induced hypothermia. The body temperature elevation of sucrose treated rats may possibly be due to the more rapid carbohydrate absorption and utilization. However, this possibility requires further clarification. It is noteworthy that ethanol administration by gastric intubation has been shown to produce less hypothermia than when given by intraperitoneal injection (Freund, 1973; 1979).

Effect of Chronic Ethanol Administration (up to 8 g/kg) on the Central Histaminergic System

Since results of the earlier chronic study suggested that chronic ethanol administration significantly reduced hypothalamic HA levels, another chronic experiment was initiated in order to determine the mechanisms responsible for these changes. The drug administration schedule was lengthened and the dosage increased up to 8 g/kg. This change in protocol was made in order to examine if the hypothalamic, midbrain and cortical HA levels would be significantly reduced. A study of the effects of ethanol withdrawal on brain HA content was also carried out. In addition, the time of drug administration was altered from 08:00 to after 16:00 so as to reduce the stomach distention which occurs with intubation. Since rats feed mainly during the dark period, their stomachs would contain more material in the early "lights-on" period than several hours later. Administration of drug later in the "lights-on" period also removes the immediate effect of mechanically altering the absorption of food already consumed (Cicero, 1979). This change in the time of administration resulted in an enhanced growth rate of ethanol-treated rats as compared to previous experiments. However, by the time the rats were receiving 6 g/kg ethanol, their weights were significantly lower than the weights of rats receiving saline or sucrose.

The present data demonstrates that chronic ethanol administration and ethanol withdrawal for 2 days induces a significant depletion in hypothalamic HA levels (Fig. 40) when evaluated using analysis of variance (ANOVA). Since ANOVA is a more precise method of statistical analysis, comparisons using this method are more likely to be statistically valid. Therefore, the use of sucrose- or saline-treated rats as controls had no impact on these results. However, this was not the case for the midbrain and cortical regions. Midbrain HA levels significantly decreased when compared only to sucrose-treated animals; cortical HA levels were statistically unchanged when ANOVA was applied. If saline-treated rats (which were not significantly different from sucrose controls) were removed from consideration, the brain HA levels in all three brain regions of ethanol-treated and ethanol-withdrawn rats would be significantly reduced (evaluated by ANOVA). The isocaloric sucrose-treated rats most closely account for the nutritional factors described earlier. Therefore, this group provided a fair control with which to compare the effects of chronic ethanol treatment and withdrawal. This seemed justified since in all three brain regions in which any parameter was determined, no significant difference could be found between saline-treated and sucrose-treated rats (Figs. 40-43). Also, the brain HA values of saline- and sucrose-treated rats were approximately those normally obtained for saline-treated control rats in previous experiments (e.g. acute ethanol experiments).

The present data suggests that the decline of brain HA levels is due to decreased synthesis, an increase in the rate of HA release or a mechanism which incorporates both processes. In the hypothalamus, the rate of HA synthesis did not appear to be depressed in ethanol-treated rats (Fig. 41). In contrast, the HD activity of withdrawn rats was markedly increased (+50% compared to saline controls). Midbrain and cortical HD activities were not significantly reduced compared to sucrose-treated rats (using ANOVA). The elevated HD activity in

withdrawn rats may represent a "rebound synthesis" of histidine decarboxylase molecules due to ethanol withdrawal. Such an increase in the rate of brain protein synthesis has been demonstrated in rats chronically treated with ethanol (8 months) and then withdrawn for 24 hours (Jarlstedt, 1972).

The absence of a significant change in hypothalamic HD activity of ethanol-treated rats is noteworthy. In the brains of rats and mice chronically treated with ethanol, protein synthesis is often depressed (for review see Tewari and Noble, 1979). However, it has been demonstrated that the rate of synthesis of enzymes responsible for neurotransmitter synthesis may be near normal or elevated during chronic ethanol administration. Kuriyama et al. (1971) demonstrated that the rate of serotonin synthesis could be elevated in the brains of mice chronically treated for 8 or 14 days with ethanol although serotonin levels remained unchanged. This suggests that enzymes responsible for transmitter synthesis may themselves be synthesized at higher rates in order to compensate for the general decline in brain protein synthesis and/or neurotransmitter level. The possibility that hypothalamic HD activity is elevated in order to compensate for the reduction of HA levels following the higher acute ethanol dose was previously discussed. The hypothalamus has the highest HD activity (section VII), the most rapid HA turnover (section VIII.C) and is the region which appears to show the greatest magnitude and duration of changes due to ethanol (Figs. 29, 30, 32 and 33). Therefore, the hypothalamus may be more sensitive to changes in HA content and have a greater potential for initiating changes which may be more rapid or larger in magnitude than any other rat brain region. This rank order of histaminergic activity may explain why midbrain HD activity of ethanol-treated and withdrawn rats exhibits a reduced sensitivity to changes and the cortex shows no changes whatsoever.

The observed alterations in brain HD activity and HA levels are not likely due to changes in regional brain histidine concentrations (Table 12).

The single change in HMT activity observed in the cortical region of ethanol-treated rats (Fig. 42) may have no physiological significance. Since this enzyme is most likely absent from regions of HA storage (section VIII.B), elevations in HMT activity would not be expected to have any consequences on endogenous HA levels. The neurochemical basis for this change in enzyme activity in this particular brain region remains unanswered. The presently observed lack of significant changes in HMT activity in the other brain regions of ethanol-treated and withdrawn rats is in agreement with the observations of Subramanian et al. (1978; 1980a). Therefore, as in the case of acute ethanol studies (Figs. 31 and 34), histamine methylation processes are not likely to play a significant role in the presently observed chronic ethanol-induced brain histamine alterations.

The regional protein concentrations in the brains of ethanol treated and withdrawn rats were not significantly altered compared to saline- or sucrose-treated rats (Fig. 43), in agreement with other investigations (Tewari and Noble, 1979). This indicates that the changes in protein synthesis (should they occur) are very small compared to the size of the total protein pool. Whole brain and regional levels of protein are usually unchanged by chronic ethanol administration (Tewari and Noble, 1979).

The elevation in plasma corticosterone content due to chronic ethanol administration and withdrawal (Fig. 44) is in agreement with similarly reported increases following other chronic ethanol studies (see review of Cicero, 1981). It also suggests that the intragastric administration of sucrose did not in itself prove highly stressful. The failure of plasma corticosterone levels in withdrawn rats to be significantly altered suggests that at the time of sacrifice the withdrawn group may not have been markedly sensitive to handling leading to obvious stress-induced artifactual results.

The presently observed reduction in brain HA levels of rats chronically treated with ethanol is contrary to the results of the chronic studies of

Subramanian et al. (1978; 1980a) and Rawat (1980). In the former studies, ethanol was administered as a 15% (v/v) solution in drinking water for 4 weeks. Control rats presumably received rat chow and water without restriction. Hypothalamic and cortical HA levels of ethanol-treated rats were significantly elevated (+50% and +17%, respectively compared to controls). HD activity remained unchanged in both regions. In the study of Rawat (1980), the whole brain HA content of rat fetuses or neonates of mothers receiving 6% (v/v) ethanol were significantly elevated (+22% and +36%, respectively compared to controls). Two weeks of prolonged ethanol consumption (presumably in drinking water after weaning from mothers consuming ethanol) resulted in a +38% elevation in brain HA content. In all three groups, HD activity was unaltered.

Some of the problems with the above methods (i.e. forced drinking of ethanol in drinking water) were described earlier with respect to the erratic plasma ethanol levels attained (section XII.C.2) and the nutritional artifacts that can occur (see XIV. B. 2). The forced drinking paradigm results in little and/or erratic fluid consumption and therefore less food consumption. Occasionally a rat will eat no food and consume less than 5 ml of fluid during a 24 hour period (Prell, unpublished). This leads to severe malnutrition which further exacerbates the ethanol-induced weight loss and altered nutritional state of the rat. Moreover, it confounds the changes that can be attributed to ethanol alone. This is of particular importance for studies with brain HA since malnutrition causes a marked elevation in brain histidine levels (often more than tripling them) (Enwonwu and Worthington, 1973; 1974; 1975). Since elevated brain histidine content leads to increased brain HA levels, it is likely that the elevated histamine levels in the chronic studies of Subramanian et al. (1978; 1980a) and Rawat (1980) could be due to malnutrition. Conversely, in the present studies, the fact that the growth rate of ethanol-treated rats was not severely retarded and the brain histidine levels of both ethanol-treated

and withdrawn rats were no greater than sucrose control levels infers that the nutritional state of the former groups were not severely compromised.

The Effect of Chronic Ethanol Administration (up to 8 g/kg) on the Brain Histamine Content of Subcellular Fractions

The reduced HA content of homogenates obtained from hypothalamic, midbrain and cortex regions following chronic ethanol administration and withdrawal (Fig. 46) is consistent with the results obtained earlier using the same chronic schedule (Fig. 40). While the hypothalamic HA values of sucrose treated control rats were nearly the same as those of other control experiments, the values for midbrain and cortical HA concentration were nearly double those routinely observed in this laboratory. However, they are nearer to those obtained by Taylor and Snyder (1972a). This may be due to the use of 10 volumes of isotonic sucrose as the homogenizing buffer and not 1 ml of sodium phosphate buffer (10 mM, pH 7.9). The former would most affect the HA values of the midbrain and cortical tissues as Orr and Eichelman (1979) demonstrated an increase in brain HA values when the tissue analyzed was progressively diluted by the homogenizing buffer. However, the neurochemical basis why such chronic ethanol treatment caused cortical HA levels to be significantly reduced when determined on a ng/g tissue basis but not on a ng/mg protein basis is not clear. The homogenate protein content (ug/ul) of brain regions of treated animals versus their respective sucrose controls are nearly the same (Fig. 45 , Table 13) and similar to the findings described earlier when expressed on a mg protein/g tissue basis (Fig. 43). Therefore, the lack of a significant decline of HA in the cortex regions of ethanol treated and withdrawn rats cannot be explained by anomolous protein concentrations in this region. However, this observation may be related to the tendency of cortical HA levels to be less sensitive to acute and chronic ethanol treatment and withdrawal as suggested in the previous discussion.

The reduced P_2 fractional HA content in all three brain regions of ethanol-treated and withdrawn rats (Fig. 47 , Table 15) is suggestive that the reduced

HA content observed in samples derived from brain homogenates is due to reduction from neuronal stores. The apparent reduction of P_1 fractional HA content in the hypothalami and cortices of withdrawn rats was better explained by the rise in protein content of these 2 groups than by an actual increase in HA per se. This is because the HA content (ng/µl) was unaltered compared to control values. It is of interest that this rise in protein content in withdrawn rats (although not statistically significant) is in line with the observed increase in brain protein synthesis following alcohol withdrawal (Tewari and Noble, 1979). The reason for midbrain P_1 fractional protein content failing to demonstrate this effect is not clear.

These results infer that the observed changes in brain HA content are neuronal rather than mast cell or mast cell-like in nature. This may also suggest that the changes in brain HD activity associated with chronic ethanol administration or withdrawal are also neuronal in nature. This would be consistent with the predominant neuronal presence of HD activity (section VII). The present results describing the relative fractional distribution of HA in sucrose controls (Fig. 47 , Table 15) are in line with other reports which showed that the brain HA subcellular concentration was more abundant in the combined P_2, P_3 and S_3 fractions than in the P_1 fraction (Table 9). They are also in line with the results of Young et al. (1971), Taylor and Snyder (1972b) and Schwartz et al. (1979a) in which the hypothalamic HA content in the P_2 fraction alone only marginally exceeded that of the P_1 fraction.

Unfortunately, the validity of this fractionation procedure cannot be unequivocally appraised. The use of electron microscopy to evaluate the crude fractions obtained in this study (without further subfractionation procedures) would not lead to unambiguous conclusions. The methodology and apparatus required in order to use neurochemical markers such as K^+ or monoamine oxidase activity is not presently available in this laboratory. However, three

observations give support to the view that the fractionation procedure used and the results derived from this procedure are valid. First, as described above, these results are in accord with most studies which reported the subcellular distribution of brain histamine (Table 9). Second, the values obtained for sucrose control hypothalamic HA in the present studies (Table 15), when expressed as units of ng HA/mg protein, are nearly the same as those values reported in the literature, i.e. 2-7 ng/mg protein (Correa and Saavedra, 1981; 1983). Third, the profile of relative brain protein distribution among the various fractions in the present study (Table 14) is close to that observed by other investigators (Garbarg et al., 1976). These investigators reported relative cortical protein concentrations (expressed as a percentage of total protein recovered) of 21, 48, 10 and 21 in the P_1, P_2, P_3 and S_3 fractions, respectively.

Conclusions

There is an abundance of literature in which the effects of ethanol on central neurotransmitters are described (see reviews: Tabakoff, 1977; Okamoto, 1978; Hunt and Majchrowicz, 1979; Goldstein, 1983). However, a clear, consistent pattern concerning the effects of neurotransmitter-ethanol interactions has yet to emerge. The present study and those of Subramanian et al. (1978; 1980a) and Papanicolaou and Fennessy (1980) also demonstrate that the central histaminergic system is altered following acute or chronic ethanol administration in vivo. Similarly to the established central neurotransmitters, conflicting data concerning interactions of ethanol with the histaminergic system has emerged. However, some of the acute ethanol effects on brain HA observed in this study and reported by others show a similar pattern.

Acute administration of lower doses of ethanol tend to elevate brain HA levels while higher doses tend to lower brain HA content, exhibiting a dose-related biphasic profile. Alterations in histamine N-methyltransferase activit

do not appear to occur, suggesting that altered HA methylation may not play a significant role in acute or chronic ethanol-induced alteration of brain HA content. In addition, acute or chronic ethanol treatment does not seem to alter brain histidine levels, suggestive that substrate availability is not a neurochemically important mechanism regulating the alteration of brain HA levels. However, the presently observed elevation of HD activity in response to acute ethanol administration is consistent with the report of Subramanian et al. (1978) but not those of Subramanian et al. (1980a) or Rawat (1980). The reasons for this discrepancy are not entirely clear. However, an elevation in HD activity has been shown during periods of brain HA depletion (Verdiere et al., 1975; Schwartz et al., 1979a). Therefore, an elevation in HD activity could be consistent with an elevation of brain HA content and also in conditions where HA content is reduced; possibly in an attempt to restore normal homeostatic levels.

Several common observations of changes of the histaminergic system are also apparent following chronic ethanol administration. As observed in acute studies neither HMT activity nor histidine levels were altered. However, the levels of HA were significantly reduced, in marked contrast to the data of Subramanian et al. (1978; 1980a) and Rawat (1980). The methodology of the latter investigators could have generated a severe state of malnutrition in the chronically treated rats, a condition known to elevate brain histidine, and as a consequence, brain histamine levels (Enwonwu and Worthington, 1973; 1974; 1975). The present method of chronic ethanol administration did not cause alterations in brain histidine levels, suggestive that the ethanol-treated and withdrawn rats were not markedly malnourished. The possibilities that HD activity is reduced or undergoes elevated rates of synthesis to overcome reduced protein synthesis and the possible "rebound" activity in the hypothalamic regions of withdrawn rats are consistent with the presently observed reduced levels of HA.

This is also consistent with the literature concerning protein synthesis during chronic ethanol administration and withdrawal (Tewari and Noble, 1979). The reduction in P_2 fractional brain HA concentration suggests that the HA stores that were depleted may have been neuronal. There was a lack of evidence to suggest that mast cell or mast-cell like HA stores were significantly affected.

The present data suggests that ethanol-induced changes in the brain histaminergic system are mostly related to alterations in HD activity and HA levels, both of which are portions sensitive to change along the histaminergic metabolic pathway. Also, the presently observed changes appear to exhibit a rank order of intensities and durations of ethanol-induced changes, of HD activity and HA content, i.e. hypothalamus > midbrain > cortex. This rank order is the same as that ascribed to histamine neuronal activity, i.e. HD activity, HA concentration and HA turnover (Taylor and Snyder, 1972a; Schwartz et al., 1979a). Therefore, the effects of ethanol on the histaminergic system may not be selective for any brain region. This infers that the effects of ethanol on this particular putative neurotransmitter may be non-specific.

The release of neuronal HA presently hypothesized to occur may not necessarily be identical to the release mechanism occurring with elevated neuronal activity. Since ethanol alters membrane biophysical properties such as fluidity, transport mechanisms and receptor-membrane bound enzyme interactions (Shanbour and Kuo, 1979; Sun, 1979), the depletion of HA due to release may be a consequence of these biophysical events. This remains to be examined. Yet, it suggests that further investigations with other agents which are believed to share several similar non-specific properties with ethanol (e.g. barbiturates and general anesthetics) (Okamoto, 1978) may yield similar findings.

Perspective and Areas for Further Investigation

In all scientific endeavors, the conclusions of one study generate further questions for which more answers are sought in another. Once answered, the

seeds of further investigation are planted anew with an endless cycle initiated, requiring only the inquisitive human mind for fuel. This study has also generated questions, many, such as the nature of neuronal HA release, are currently beyond the field to investigate further. Many questions are technically possible to approach but beyond the present resources of the author. Still other ideas for investigation have been suggested by the present results which lie in related areas, not germaine to this project. Most importantly, the development of methodology with a broader spectrum of application has opened possibilities for research in areas completely unrelated to the present study.

The development of reliable, sensitive and reproducible methodology which is validated after interlaboratory comparisons is of paramount importance in the brain HA research field. In particular, a brain histidine decarboxylase activity assay which meets these requirements is needed in the field. Once developed and accepted by critical investigators in the brain histamine field, its utility and applications would be boundless. The effects of drugs, physiological manipulation and alteration by lesion studies as a putative histaminergic neuronal marker are more obvious uses. Studying its activity changes with neuronal development and later, with aging, would also be of great utility. The development of methodology to investigate neuronal versus non-neuronal brain histamine stores and brain HA metabolites are in their infancies and require further study. Methodology to assess brain HA release in vivo or in reliable in vitro models are also needed in the brain HA research field.

While the present investigation yielded many interesting results and suggested many possible neurochemical mechanisms to account for these results, many of the conclusions derived from this research are hypotheses based on present results and the literature. However, these hypotheses, although highly suggestive, need experimental confirmation before their credibility and extensive acceptance can be attained.

The present results suggested that ethanol induced changes in HD activity. The effect of various psychoactive drugs on brain HD activity *in vitro* has received almost no attention. The *in vitro* analysis of this enzyme activity at drug concentrations encountered *in vivo* could suggest a locus at which to further investigate drug-induced HD activity changes should they occur. This was useful for studies of HMT enzyme activity in this investigation. Reports suggesting that ethanol induces brain mast cell HA release while simultaneously reducing neuronal HA release appear to be untenable (see Discussion). The present conclusion that HA is released (by whatever mechanism) appears to account for some of the present results and is consistent with the literature. The demonstration that the reduction of HA occurred in the P_2 fraction, suggestive of neuronal release, supports the concept of ethanol-induced brain HA release from a neuronal rather than a mast cell-like storage site. Yet, this hypothesis needs confirmation. If an elevation of HA metabolite concentration does occur, this may be helpful in demonstrating increased HA release but the source of this released HA would have to be confirmed.

Further analysis of the subcellular HA stores affected by ethanol would be most useful. Subfractionation, verified with electron microscopy and the appropriate distribution of neurochemical markers, could potentially show if the alteration of synaptosomal HA stores are responsible for these observed changes. Further development of a subfractionation method may show if the subcellular fractionation procedure per se is altered by the high tissue content of ethanol that may be obtained during acute experiments. If not, this improved method could be used reliably to evaluate brain HA changes in acute studies.

Lastly, once the appropriate methods have been properly developed for study of the brain histaminergic system, the methodology of ethanol administration for acute and chronic studies should be re-evaluated for its advantages and disadvantages. With well mastered, experimental experience in the brain HA

field, alterations of the central histaminergic system could more confidently be attributed to drug-induced changes (e.g. ethanol or any other psychoactive agent). The experimental artifacts which often occur with psychoactive drug research (e.g. the metabolic or nutritional aspects of chronic ethanol investigation, mast cell degranulation due to morphine or chlorpromazine inhibition of HMT) could then be more effectively accounted for, possibly with the design of more appropriate experimental controls.

SUMMARY

XVII. SUMMARY

1. The effect of acute and chronic ethanol administration on the rat brain histaminergic system was studied in vivo and in vitro. To effectively determine the neurochemical mechanisms responsible for the observed changes, several methods were developed.

2. An assay for histamine N-methyltransferase activity was developed. Its kinetic constants were in the range reported by other investigators. In order to more accurately quantify the observed enzyme activity, the fractional recovery of tele-methylhistamine formed during the assay was determined. This required the synthesis of ^{14}C-tele-methylhistamine which was carried out by a modification of the method of Taylor and Snyder (1972a). Following its synthesis, isolation and purification, introduction of this ^{14}C-tele-methylhistamine into the assay showed that about 80% was recovered.

3. The effects of ethanol, acetaldehyde, morphine and naloxone, alone and in combination, on HMT activity were studied at various concentrations in vitro. None of the agents studied had any marked effect on HMT activity at concentrations attained in vivo.

4. The effects of acute ethanol (1 or 2 g/kg of a 25% w/v solution) on the rat brain histaminergic system were determined 60, 90 and 120 min following intragastric administration. Histamine exhibited a biphasic effect, i.e. an elevation at the lower dose and a reduction following the higher dose. While HMT activity was unchanged, HD activity increased. The HD activity elevation appeared to be more prolonged when HA levels were most markedly depleted. This was possibly due to a compensatory mechanism in which the brain attempts to restore HA content to normal homeostatic levels. These effects occurred at plasma ethanol levels which are similar to those obtained clinically. Therefore, these results may have clinical significance.

5. Chronic administration of ethanol in increasing doses (up to 8 g/kg/day) for 26 days and withdrawal caused HA levels to be reduced. HMT activity remained unaltered. The HD activity in the hypothalamic and cortical regions of ethanol-

treated rats was unchanged. The midbrain HD activity of ethanol-treated and withdrawn rats tended to decrease compared to the HD activity of saline- or sucrose-treated controls. However, HD activity in the hypothalamic regions of withdrawn rats was increased. These findings suggest that the hypothalamic HD activity which might be expected to be reduced due to reduced brain protein synthesis (occurring with chronic ethanol administration) was not affected as it remained unchanged. Withdrawal resulted in elevation of HD activity still further, possibly due to a rebound-like increase in brain protein synthesis which often accompanies ethanol withdrawal.

6. Subcellular fractionation of rat brain tissue into nuclear (P_1), mitochondrial (P_2), microsomal (P_3) and supernatant (S_3) fractions was carried out. Subsequent HA determination in these fractions from rats chronically treated with ethanol and withdrawn showed that the mitochondrial (P_2) fraction was depleted of HA. This was suggestive that the stores of HA which were depleted were neuronal.
7. Since the HD activity was limited by the small amounts of substrate used in the assay, possible alteration of endogenous histidine levels due to ethanol could have occurred. Therefore, a method for brain histidine was developed. This method used fluorescamine as the fluorogenic agent, which when derivatized with histidine, exhibited fluorescence intensity detectable at 10 picomoles of histidine.
8. This new fluorescamine-histidine method for the determination of brain histidine following acute or chronic ethanol administration showed that brain histidine concentration was unchanged. Therefore, the alteration of brain HA levels following ethanol administration were not likely due to altered substrate availability.
9. Since the magnitude and duration of changes in the histaminergic system exhibit a regional rank order parallel to that of regional HD activity, HA levels and HA turnover, it is possible that the observed ethanol-induced changes are non-specific. The depletion of brain HA in chronic studies may be due to ethanol-induced alteration of membrane properties leading to HA release.

Major Original Findings

During the course of this study several discoveries were made which had not previously appeared in the literature. The major finding associated with the assay of histamine N-methyltransferase activity was that the recovery of newly synthesized ^{14}C-tele-methylhistamine was approximately 80%. In order to do this, ^{14}C-tele-methylhistamine had to be synthesized, isolated and purified by a new method developed by the author. To evaluate brain histidine levels a method utilizing the fluorogenic agent, fluorescamine, was developed. This required that specificity for histidine and sensitivity be demonstrated. Heating imidazolylethylamine-fluorescamine derivatives in an acidic medium was found to be specific for histidine if the supernatant samples derived from boiled brain tissue homogenates had been previously deproteinized. Substances found to exhibit fluorescence are either not present in rat brain or are present in amounts which are analytically insignificant. This specificity was verified by analysis with thin layer chromatography using two solvent systems. The method developed was sensitive enough to detect 10 picomoles of histidine. When this method was used to determine histidine content in rat brain regions the values obtained were consistent with other literature reports.

Several of the observed ethanol-induced changes of the brain histaminergic system were either previously unreported or at variance with published reports. The observed biphasic effect of acute ethanol administration on rat brain histamine content as a function of dose was contrary to previous reports as was the observed reduction of hypothalamic histamine levels at a higher acute ethanol dose. The lack of change in the levels of the histamine precursor, histidine, in rat brain regions following acute ethanol administration had not been previously documented. The depletion of regional brain histamine levels

following chronic ethanol administration and withdrawal was a novel observation. This depletion was further investigated in subcellular fractionation studies where the reduction in the histamine content of the mitochondrial fraction observed would be consistent with a reduction in the neuronal histamine pool. This would at the same time be inconsistent with a marked change in a mast cell or mast cell-like histamine storage capacity. This is the first time that such a method was used to delineate the neuronal-like versus the non-neuronal-like source of changes in brain histamine following psychoactive drug administration.

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