A FLUORIMETRIC METHOD FOR THE ESTIMATION OF HISTIDINE AND OF HOMOCARNOSINE IN BRAIN

By

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DEDICATION

TO MY MOTHER

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SUMMARY

SUMMARY

1. The aim of the research was to obtain a method for the estimation of histidine in small samples of brain tissue which would allow an investigation eventually to be made of a possible dependence of the histamine levels and turnover in various regions of the brain on the local availability of its amino acid precursor.

2. In the introductory section of this thesis the available information on the occurrence of histidine in brain and its principal metabolic pathways has been reviewed. Reported methods for the estimation of the amino acid and certain peptide derivatives such as homocarnosine in simple solution and tissue extracts are also considered and their limitations discussed.

3. A sensitive fluorimetric method for the estimation of histidine based on the production of a fluorophor by interaction with o-phthalaldehyde has been developed. By this method it is possible to measure the amino acid in simple solution in an amount as low as 0.02 µg in the sample for fluorimetry.

Homocarnosine(γ-aminobutyrylhistidine) was found to react similarly, the fluorescence intensity of the fluorescent derivative being such that an amount of homocarnosine as low as 0.2 ug

in the sample for fluorimetry could be measured. This finding suggested the possibility of estimating both substances in the same brain sample.

These developed fluorimetric methods for the estimation of histidine and homocarnosine are more sensitive than any other known method for the measurement of these substances.

4. A study was made of the behaviour under the conditions of fluorophor production from histidine and homocarnosine, of several substances likely to be present in brain tissue in order to assess their potential to interfere with the measurements.

5. A method for the separation and purification of histidine and homocarnosine in tissue extracts to an extent suitable for their fluorimetric measurement has been elaborated. The method involves a combination of ion-exchange and thin-layer chromatography.

Basic interfering substances, specifically histamine, agmatine and arginine, are removed from a trichloroacetic acid extract of the tissue by adsorption on a column of a weak cation exchanger 'Amberlite CG-50' buffered to pH 8. Histidine and homocarnosine in the effluent are adsorbed on a column of the same resin buffered to pH 5.75 and are thus separated from the acidic and most of the neutral amino acids. Histidine and homocarnosine in the eluate are separated by thin-layer

chromatography on cellulose. The compounds are eluted from the appropriate portions of the developed chromatogram and their content in the eluates measured fluorimetrically.

Specificity is conferred on the estimates by the comparable behaviours of the substances so estimated and the authentic compounds on the ion-exchange columns and thin-layer chromatogram and by the demonstration that the fluorescence characteristics of the estimated fluorophors are identical with those derived from the authentic substances.

6. In control analyses from simple buffer solutions the mean recovery of histidine, in 0.5 to 2.0 µg amounts, through the analytical procedure was 66% ± 4.3% (S.E., 18 experiments) and that of homocarnosine, from 5.0 to 10.0 µg amounts, was 55% ± 3.6% (S.E., 10 experiments). The mean recoveries of 1.0-2.0 µg histidine and of 5.0 µg homocarnosine added to brain samples in three experiments were 92% (range 71-122%) and 64% (range 50-86%) respectively.

7. The method has been used to estimate histidine and homocarnosine in small (< 100 mg) samples of brain tissue. Specifically, it has been applied to estimations in two gross anatomical regions of mouse brain, one caudal to

(the 'pons-medulla' region) and the other rostral to (the 'cerebral hemispheres' region) the upper boundary of the pons.

In the 'pons-medulla' the estimated mean concentration of histidine per g wet weight of tissue was 9.7 ug ± 1.1 ug (S.E., 7 estimations) and that of homocarnosine 32 µg ± 1.76 µg (S.E., 3 estimations); in the 'cerebral hemispheres' the respective estimates were 10.5 µg ± 0.91 µg (S.E., 12 estimations) and 19.0 µg ± 2.41 µg (S.E., 7 estimations). These estimates have not been corrected for recoveries.

The concentrations of histidine in the two regions did not differ significantly ($P > 0.5$) but there appeared to be a significant ($P < 0.005$) difference in the homocarnosine concentrations.

8. A preliminary study has been made of the effect of histidine administration on the concentrations of histidine and homocarnosine in mouse brain (the 'cerebral hemispheres' region). Histidine in doses in the range 15 µg/g to 120 µg/g body weight was injected intraperitoneally 15 min before killing the animals.

The brain histidine concentration was found to increase with all dose levels but the increase was limited, the maximum rise (more than 100%) following a dose equal to, or in excess of, 30 µg/g.

It is suggested that the observed limited rise in concentration would be explicable in terms of saturation at the higher dose levels, of an active mechanism concerned with the uptake of histidine into the brain.

No alteration in the homocarnosine concentration was detected. Possible implications of this observation are discussed.

9. In an extensive search of the literature no method suitable for measuring histidine and/or homocarnosine in small samples of brain tissue was found to be reported.

With the methodology which has been developed for the estimation of histidine and homocarnosine and by the incorporation, which it readily permits, of the method of Adam (1961) for the estimation of histamine, it is now possible to measure the concentrations of the amino acid histidine, its decarboxylation product histamine, and one of its dipeptide derivatives homocarnosine in the same small (< 100 mg) sample of brain tissue. It is considered that the method opens the way to the investigation of the possible interrelations of these three substances in the normal, pathological and drug-altered metabolism in defined regions of the brain of laboratory animals.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

When L-histidine is given by slow intravenous infusion in the conscious rabbit, the concentration of histamine rises in the brain and, within a limited range, the increase is related to the total dose injected (Abou, 1968). This finding suggested that the relative concentrations of histidine in blood and brain might be important in controlling the concentration of histamine in brain.

The object of the present work was to devise a sensitive method for the estimation of histidine which could be applicable to small samples of brain, blood and cerebrospinal fluid. The method was developed as an extension of that previously described for histamine (Adam, 1961), so as to allow measurement of the two substances in a single sample. In the course of the work, it was found that the method could also be used to estimate, if only semi-quantitatively, the histidine containing dipeptide homocarnosine.

Both histidine and homocarnosine are measured by spectrophotofluorimetric assay. The method makes it possible to estimate histidine in the range of 0.02 to 0.6 µg and homocarnosine in the range of 0.2 to 3.0 µg. Application of the methodology to a study of the effect of peripheral administration of histidine on the histidine and homocarnosine contents of mouse brain is reported.

The review of the literature is divided into two parts. The first deals with products of histidine metabolism, i.e. imidazoles and dipeptides, some of which, having similar structural formula to that of histidine, might interfere with its fluorimetric assay. This information is intended as a background to the methods that have been employed for the separation of histidine. The second part is a survey of the reported methods for the estimation of histidine in tissues and body fluids.

CHAPTER I

INTRODUCTION

PART I - HISTIDINE AND ITS METABOLISM

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HISTIDINE AND ITS METABOLITES IN BRAIN

Although histidine is known to be a normal constituent of mammalian brain (Schurr, Thompson, Henderson and Elvehjem, 1950; Williams, Schurr and Elvehjem, 1950; Tallan, Moore and Stein, 1954; Clouet, Gaitonde and Richter, 1957; Yockey and Marshall, 1969), little is known about its metabolism in the central nervous system (CNS). A minor but important route is the decarboxylation of histidine with the formation of histamine. The regional distribution of the amine (Harris, Jacobsohn and Kahlson, 1952; Adam, 1961; Adam and Hye, 1966) corresponds roughly with that of histidine decarboxylating enzyme (White, 1959).

Since histamine does not pass readily from blood into brain (Halpern, Neveu and Wilson, 1959; Adam, Hye and Waton, 1964; Snyder, Axelrod and Bauer, 1964; Snyder and Axelrod, 1965) its concentration in brain must depend, among other factors, on the local supply and uptake of histidine. The concentration of histidine in brain greatly exceeds that of histamine, and there is evidence that its concentration varies in different regions of the brain (Kandera, Levi and Lajtha, 1968; Battistin, Grynbaum and Lajtha, 1969).

Histidine also enters into the formation of dipeptides which have been identified in brain, namely, carnosine (Hosein and Smart, 1960; Abraham, Pisano and Udenfriend, 1962; Yockey and Marshall, 1969), homocarnosine (Pisano, Wilson, Cohen, Abraham and Udenfriend, 1961; Abraham et al., 1962; Levi, Kandera and Lajtha, 1967; Yockey and Marshall, 1969), anserine (Hosein and Smart, 1960; Tsunoo, Horisaka, Kawasumi, Aso and Tokue, 1963) and homoanserine (Nakajima, Wolfgram and Clark, 1967) (see section on dipeptides, p 23).

The betaine of thiolhistidine, ergothioneine (Crossland, Mitchell and Woodruff, 1966) and the 1- and 3-methyl derivatives of histidine (Tallan et al., 1954) are also present in the brain. The significance of these imidazole derivatives in brain, as of histamine itself, is not yet understood.

Estimates of brain histidine as the free amino acid, have been collected from the literature and are presented in Table 3 (p68). In the brain, as elsewhere in the body, histidine is believed to derive entirely from the diet. In the intestine histidine may be converted to histamine (Waton, 1956; Telford and West, 1961; Schayer, 1962) or destroyed in other ways through bacterial action (Raistrick, 1917). Knowledge of its metabolism has been obtained mainly from studies on microorganisms and on mammalian tissues other than brain.

In the dog and rat, but apparently not in man, deprivation of histidine leads eventually to loss of weight and negative nitrogen balance (Meister, 1965a). The effect of histidine deficiency on the structure and functions of the CNS, including the formation of biogenic amines, does not appear to have been investigated.

METABOLISM OF HISTIDINE IN ANIMALS

In animals the metabolism of histidine follows several pathways, as outlined in Fig 1 (p 11). These include incorporation into protein, catabolism and conjugation. The evidence for each of the pathways is reviewed briefly.

Incorporation of histidine into protein. When rats were fed with diets containing L-histidine labelled with ^{15}N in the imidazole ring, the replacement of histidine in protein by the labelled amino acid was found to be 28% in the liver, 8% in the carcass and 3% in the erythrocytes (Tesar and Rittenburg, 1947). These results were later confirmed by Borsook, Desay, Haagen-Smit, Keighley and Lowy (1950), who injected ^{14}C-labelled histidine into mice and guinea-pigs. In further experiments (Borsook, Deasy, Haagen-Smit, Keighley and Lowy, 1952) these authors also demonstrated the incorporation of carboxyl-labelled histidine into the rabbit reticulocytes in vitro.

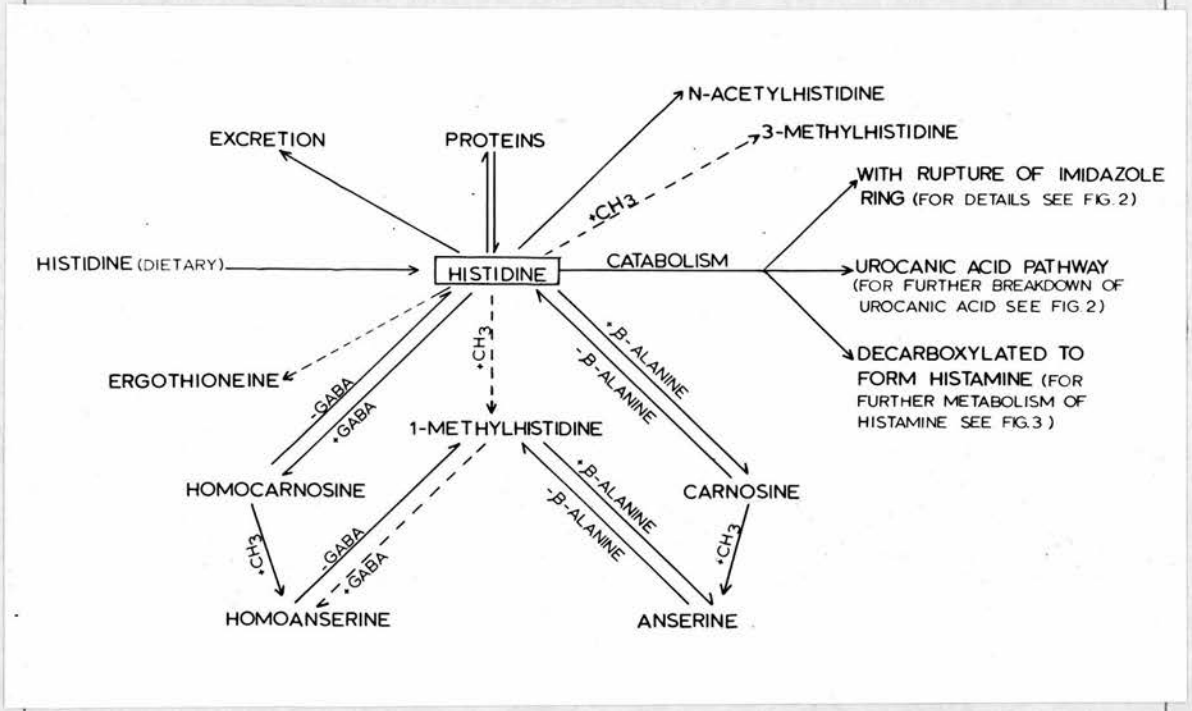


Fig 1. Summary of the metabolic pathways of histidine.

Broken lines indicate pathways not known to occur in animal tissues.

The incorporation of isotopically labelled histidine into proteins of the CNS does not appear to have been studied.

Histidine catabolism.

The pathways are those for the formation of urocanic acid and histamine and the subsequent destruction of these metabolites.

Urocanic acid formation. Raistrick (1917; 1919) was the first to demonstrate *in vitro* the bacterial conversion of histidine to imidazole acrylic acid which later became known as urocanic acid. In experiments on the dog, Kotake and Konishi (1922) gave histidine by mouth and by subcutaneous injection and isolated urocanic acid from the urine. On the basis of this finding they postulated the conversion *in vivo* of histidine into urocanic acid.

In 1926, Edlbacher reported that mammalian liver mince decomposed histidine with the liberation of ammonia. Györgyi and Röthler (1926) independently obtained a similar result which was later confirmed by others (Kauffman and Mislowitzer, 1930; Abderhalden and Buadze, 1931). These findings led to the tentative conclusion that opening of the imidazole ring represented the first step in the degradation of histidine *in vivo* (Fig 2, p 14). The later work of Edlbacher and his colleagues supported this conclusion since they were unable to demonstrate

urocanic acid in the urine after giving histidine to dogs, rabbits and guinea pigs (Edlbacher, Baur and Staehelin, 1941; Edlbacher and von Bidder, 1942; Edlbacher, 1943).

The conversion of histidine to urocanic acid *in vitro* with a purified liver enzyme was first described by Sera and Yada (1939) and later confirmed by Hall (1952) and Mehler and Tabor (1953). According to Tabor (1954) the failure to demonstrate urocanic acid in the earlier experiments was probably due to the presence of urocanic acid-splitting enzyme in the crude liver preparations.

The degradation *in vitro* of L-histidine through the urocanic acid pathway has since been reported for the livers of rat, rabbit, dog (Hall, 1952), cat (Hall, 1952; Borek and Waelsh, 1953) and guinea-pig (Mehler and Tabor, 1953). The enzyme catalyzing the conversion of histidine to urocanic acid is commonly known as "histidase", a term first used by Edlbacher (Edlbacher, 1926); it has also been named "histidine-deaminase", "histidine- -desaminase", "histidine- -deaminase" and "desamino-histidase" (Tabor, 1954).

Destruction of urocanic acid. An enzyme postulated in this reaction is urocanase (urocanicase) (Tabor, 1954). When histidine or urocanic acid is degraded *in vitro* by liver (Borek and Welsch, 1953, 1953a; Mehler and Tabor, 1954) or by bacterial preparations (Tabor and Hayaishi, 1952;

Fig 2. Degradation of histidine through urocanic acid pathway or through initial opening up of the imidazole ring. (Reproduced from Tabor, 1954).

Pathway (A) according to Edlbacher (1943);

(B) " " Walker and Schmidt (1944);

(C) " " Sera (1951);

(D) " " Oyamada (1944) and Akamatsu (1943);

(E) " " Suda, Miyahara, Tomihata and Kato (1952); Suda, Tomihata, Nakaya and Kato (1953); Suda, Nakaya, Hara, Kato and Ikenaka (1953); Tabor and Mehler (1954; 1954a) and

(F) according to Ichihara, Uchida, Itagaki and Matsuda (cited by Suda, Nakaya, Hara and Kato (1953)).

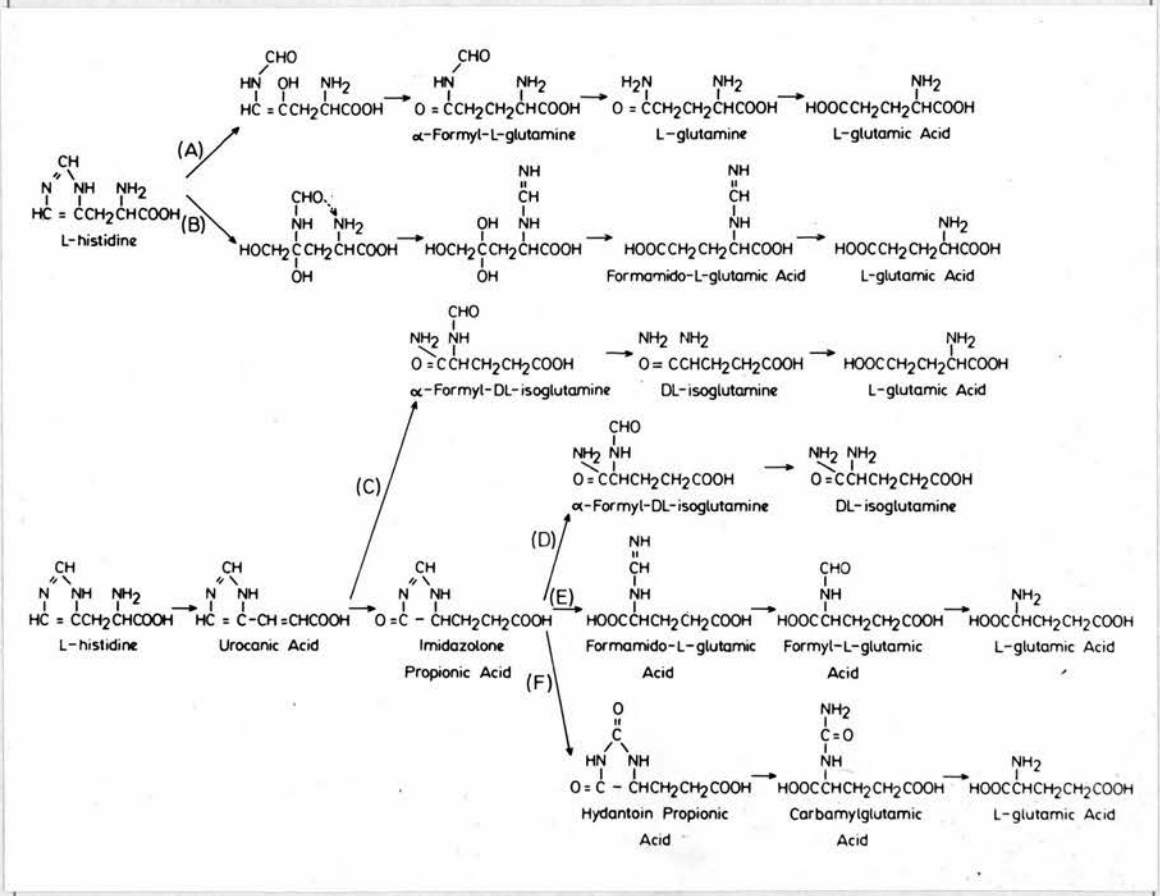


Fig 2. Degradation of histidine through urocanic acid pathway or through initial opening up of the imidazole ring.

Tabor, Mehler, Hayaishi and White, 1952; Tabor, Silverman, Mehler, Daft and Bauer, 1953; Tabor and Mehler, 1954a) a product is obtained which on alkaline hydrolysis yields ammonia, L-glutamic and formic acids (Oyamada, 1944a; Sera, 1951; Borek and Welsch, 1953, 1953a; Tabor and Mehler, 1954, 1954a). Although these workers were agreed that ammonia, L-glutamic and formic acids were final products, they differed in their conclusions regarding intermediates formed in the course of the reaction (Fig 2 p 14). Thus, Sera (1951) claimed that formyl-DL-isoglutamine was the first intermediate of urocanic degradation; others maintained that the intermediate was imidazolone propionic acid (Oyamada, 1944a; Akamatsu, 1943; Suda, Nakaya, Hara, Kato and Ikenaka, 1953; Tabor and Mehler, 1954; Ichiara et al. (quoted by Suda et al., 1953)). According to Ravel and Magasanic (1958), working with Aerobacter aerogenes, imidazolone propionic acid is the precursor of N-formiminoglutamic acid in the metabolic pathway.

A scheme of pathways proposed by various authors is shown in Fig 2, p 14 .

Metabolism of histidine through the urocanic acid pathway

These findings and arguments have been summarised because of their possible implications for the CNS. There appears to have been very little work on the formation and degradation of urocanic acid as a possible metabolic pathway of histidine in the brain.

Formation of histamine from histidine.

Histidine is a specific substrate for a decarboxylase in mast cells, foetal liver of rat and other actively growing mammalian tissues (Kahlson and Rosengreen, 1968). In brain, however, there is a decarboxylating enzyme with a low affinity for histidine and which is not specific since it also decarboxylates precursors of the monoamines, such as tyrosine, 5-hydroxytryptophan, DOPA and other amino acids (Lovenberg, Weissbach and Udenfriend, 1962; Shepherd and Mackay, 1967).

White (1960) demonstrated the formation of ^{14}C-histamine in cat's brain during perfusion of the cerebral ventricles with ^{14}C-histidine; ^{14}C-histamine appeared in the perfusate and was also extractable from the grey matter of the third ventricle. When histidine is infused intravenously over a period of 2 hours in the conscious rabbit, the brain histamine rises in proportion to the dose of amino acid until a limiting value is reached (Abou, Adam and Stephen, unpublished).

Catabolism of histamine.

Two major pathways appear to exist for the degradation of histamine in the tissues of man and other animals (Fig 3, p 17). One of these pathways involves oxidative deamination catalysed by the enzyme histaminase (diamine oxidase) (Zeller, 1951; Schayer,

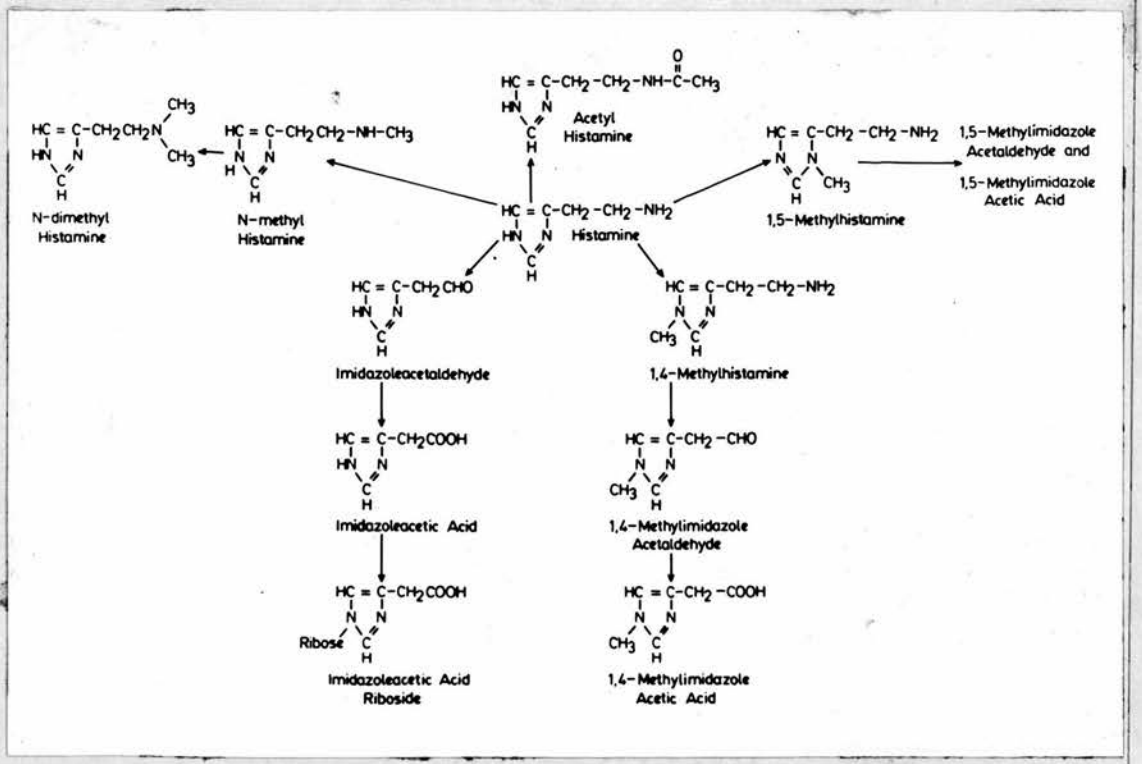


Fig 3. Various pathways of histamine catabolism (modified from Schayer, 1959). For details see text, p 16.

1952; Mehler, Tabor and Bauer, 1952) which yields imidazoleacetaldehyde (Zeller, 1951). This appears in the urine as its further oxidation product imidazoleacetic acid partly in free state (Schayer, 1952; Mehler et al., 1952) and partly as a conjugate with ribose (Karjala, 1955; Tabor and Hayaishi, 1955; Karjala, Turnquest and Schayer, 1956). The second pathway of histamine catabolism involves methylation of imidazole ring (see below) to yield 1, 4-methylhistamine; a portion of this may be excreted unchanged in the urine but most is oxidatively deaminated by monoamine oxidase to 1-methylimidazole-4-acetic acid (Schayer and Karjala, 1956). The probable intermediate is 1-methylimidazole-4-acetaldehyde. When large amounts of histamine are injected, some abnormal isomers, 1, 5-methylhistamine and 1, 5-methylimidazoleacetic acid, may be formed (Karjala and Turnquest, 1955). The evidence for the participation of diamine oxidase in the catabolism of physiological quantities of histamine was the demonstration in rats (Schayer, 1953) that inhibitors of diamine oxidase, isoniazid (Zeller, Barsky, Fouts, Kirchheimer and van Orden, 1952) and aminoguanidine (Schuler, 1952) prevented the formation of a major metabolite which was later identified by Karjala et al., (1956), as 1-ribosylimidazole-4-acetic acid. Similarly, pretreatment of animals with iproniazid or isobutylnicotinylhydrazine which blocked monoamine oxidase, prevented

destruction of 1, 4-methylhistamine (Rothschild and Schayer, 1958; White, 1960).

The relative importance of the metabolic pathways varies from species to species (Adam, 1970).

The brain, unlike other tissues of the body, appears to be deficient in the enzyme histaminase (Zeller, Birkhauser, Mislin and Wenk, 1939; Birkhauser, 1940; Cotzias and Dole, 1952). The first and principal step in the catabolism of histamine is methylation in the imidazole ring (N-1) (Schayer, 1956; Schayer and Karjala, 1956; Schayer, 1959; Brown, Tomchick and Axelrod, 1959). In vitro, the enzyme imidazole-N-methyltransferase catalyses the reaction in the presence of Mg^{++} and S-adenosylmethionine as a methyl donor (Brown, Tomchick and Axelrod, 1959; Lindahl, 1960). Although this enzyme is widely distributed in the brain (Brown, Tomchick and Axelrod, 1959; Axelrod, MacLean, Albers and Weissbach, 1961), the distribution of methylhistamine (White, 1966) in brain follows closely the restricted distribution of histamine (Adam and Hye, 1966; White, 1966). To a small extent, methyl-histamine in brain is converted via the aldehyde, to methylimidazole acetic acid (White, 1959; White, 1960; Jonson and White, 1964).

The methylation of amino group of the side chain is evidenced by the presence of N-mono- and -dimethyl-histamine in human urine (Kapeller-Adler and Iggo, 1957). So far, there is no report that in the brain histamine

undergoes methylation of the terminal amino group of the side chain to form N-mono- or -dimethylhistamine nor that the group undergoes acetylation to form N-acetylhistamine which is mainly synthesised by intestinal bacteria after oral administration (Urbach, 1949).

After intraperitoneal injection of labelled histidine, the presence of imidazoleacetic acid riboside and ribotide representing small fractions of the total tissue radioactivity was detected in rat brain (Robinson and Green, 1964).

HISTIDINE DERIVATIVES IN BRAIN

Methyl derivatives of histidine.

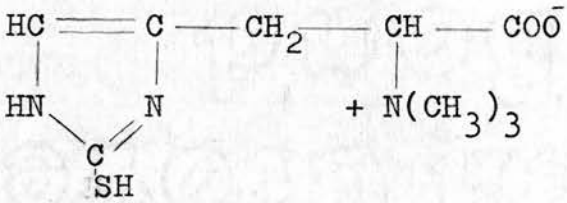
1- and 3-methylhistidine were first detected in urine of man and cat (Searle and Westall, 1951; Datta, 1953; Stein, 1953; Tallan, 1953) and later in cat brain (Tallan, Moore and Stein, 1954). The concentrations of these amino acids in cat brain are shown in Table 3 (p 68).

Like histidine these methyl derivatives are believed to derive from the diet; so far there is no evidence to suggest that they are formed independently in the body through the methylation of histidine. It is of interest in this connection that there is evidence (see below) of ring methylation of histidine when the amino acid is in a conjugated dipeptide form.

According to White (1959), 1-methylhistidine is

unlikely to be the source of 1, 4-methylhistamine in cat brain. Nevertheless, the amino acids may take part in the formation of dipeptides present in the brain and elsewhere. For example, 1-methylhistidine is a constituent of anserine (Tabor, 1954), and in the rat, carnosine (β -alanyl-histidine), undergoes methylation in N-1 of the imidazole ring to form anserine (McManus, 1956).

Thiol derivative of histidine.



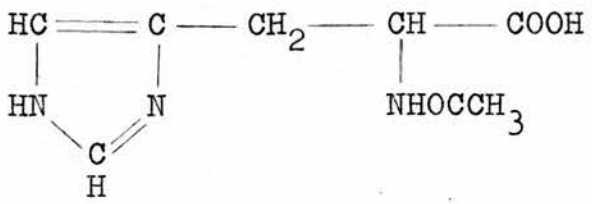
Ergothioneine is the betaine of thiolhistidine and was first isolated from ergot (Tanret, 1909). It has been detected in many animal tissues, including the brain in some species. In the rat, ergothioneine has been found in the liver, blood cells, kidneys, lungs, spleen, skeletal muscle, stomach and seminal vesicles but not in blood plasma or brain (Melville, Horner and Lubschez, 1954). The concentration found in the tissues appeared to be related to the quantity of ergothioneine consumed in the diet.

Crossland, Mitchell, and Woodruff (1966) claim to have demonstrated the presence of ergothioneine in the cat and guinea pig brain, where the concentration was

highest in the cerebellum (3.0 to 10 µg/g fresh tissue) (Table 3, p 68). They advanced the hypothesis that ergothioneine might be identical with the "cerebellar factor", a substance extractable from cerebellum which on injection into the anaesthetized cat excites electrical activity in the cerebellum (Crossland, 1970).

The biosynthesis of ergothioneine has been followed in Claviceps purpurea (Heath and Wildy, 1956) and in Neurospora Crassa (Melville, Genghof, Inamine and Kovalenko, 1956). Studies with ^{35}S-labelled sulphate and ^{14}C-labelled methionine led to the conclusion that the sulphur atom attached to the imidazole ring probably derives from cysteine and that the methyl groups on the side chain derive from methionine (Melville, Eich and Ludwig, 1957). Ergothioneine is not known to be formed in mammals and is regarded as "strictly exogenous" (Cantarow and Schepartz, 1967).

N-acetylhistidine



So far the presence of N-acetylhistidine has not been reported in the mammalian brain. According to Baslow, Turlapaty and Lenney (1969) the compound occurs in tissues of bony fish, amphibians and reptiles only.

It is present in various tissues including brain of frog (Anatasi, Correale and Erspamer, 1964; Erspamer, Roseghini and Anatasi, 1965) and fish (Baslow et al., 1969). The concentration varies from 240 - 600 µg/g in frog brain (Anatasi et al., 1964; Erspamer et al., 1965) and 520 - 1,140 µg/g in fish brain (Baslow et al., 1969) (Table 3, p 68).

Baslow et al., (1969) observed formation of labelled N-acetylhistidine in brain, heart and lens of the eye after intraperitoneal injection of ^{14}C-histidine in gold fish, Carassius auratus.

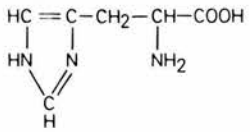
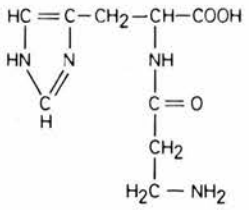
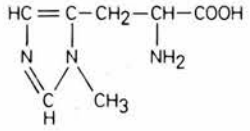
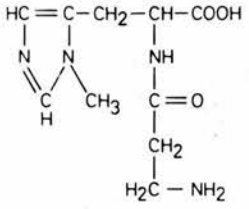
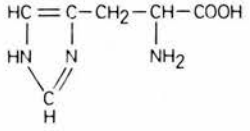
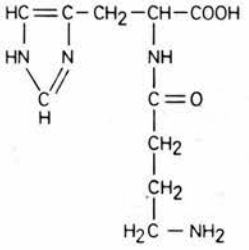
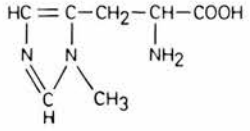
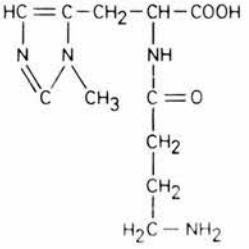
HISTIDINE CONTAINING DIPEPTIDES

Carnosine

Carnosine is the dipeptide of L-histidine and β-alanine (β-alanyl-L-histidine) (Table 1, p 24). It is found principally in skeletal muscle of vertebrates (Cantarow and Schepartz, 1967), the concentration being highest in the pectoral muscles of chicken (Davey, 1957). Hosein and Smart (1960), using rats, were the first to detect carnosine in mammalian brain. Later its concentration was estimated (Table 3 , p 68) in other mammalian brains where the values varied from traces to 10.6 µg/g (Abraham et al., 1962; Yockey and Marshall, 1969). The highest concentration (340 µg/g) has been encountered in frog brain (Yockey and Marshall, 1969) (Table 3, p 68).

Table 1

Structural formulae of the histidine containing dipeptides and their constituent amino acids.

DIPEPTIDES	COMPONENTS		STRUCTURAL FORMULAE
CARNOSINE	$H_2N-CH_2-CH_2-COOH$ β-ALAMINE	 HISTIDINE	
ANSERINE	$H_2N-CH_2-CH_2-COOH$ β-ALAMINE	 1-METHYLHISTIDINE	
HOMOCARNOSINE	$H_2N-CH_2-CH_2-CH_2-COOH$ γ-AMINOBUTYRIC ACID	 HISTIDINE	
HOMOANSERINE	$H_2N-CH_2-CH_2-CH_2-COOH$ γ-AMINOBUTYRIC ACID	 1-METHYLHISTIDINE	

Carnosine may be synthesized from its constituent amino acids in the presence of a purified enzyme from chick pectoral muscles (Kalyanker and Meister, 1959, 1959a; Winnick and Winnick, 1959) which normally contain a high concentration of the dipeptide (Davey, 1957). Conversely, it is hydrolysed into L-histidine and β-alanine by the enzyme carnosinase from hog kidney (Hanson and Smith, 1949).

Anserine

Anserine is the dipeptide of 1-methylhistidine and β-alanine (Table1 , p.24) and occurs in skeletal muscles of different species (Davey, 1957). Its concentration like that of carnosine, is highest in chick pectoral muscle (Davey, 1957). Anserine has been found in rat brain (Hosein and Smart, 1960) and in chicken brain (Tsunoo, Horisaka, Kawasumi, Aso and Tokue, 1963). Anserine from rat brain was characterised by identification of the products after hydrolysis with concentrated hydrochloric acid (Hosein and Smart, 1960).

Homocarnosine

Homocarnosine, γ-aminobutyryl-L-histidine (Table 1, p.24) was first detected in bovine brain, and identified by acid hydrolysis of the dipeptide into its constituent amino acids, histidine and γ-aminobutyric acid (Pisano *et al.*, 1961). Later, Kanazawa, Kakimoto, Miyamoto and Sano (1965) isolated the compound in

crystalline form from brain of the same source.

According to Abraham et al., (1962) homocarnosine is found only in the central nervous system; it was not detectable in skeletal muscle and other tissues of the rat; nor was it found in the sciatic nerve of rabbit. In human brain, the higher concentration of homocarnosine in the white matter of the frontal cortex of the cerebrum observed by Abraham et al., (1962) was not confirmed by Kanazawa and Sano (1967) who did not find significant difference in the homocarnosine levels of white and grey matter of the cerebral cortex. Kanazawa and Sano (1967) did not find the dipeptide in the intestine, kidney, liver, lung, spleen or muscles of the rat and guinea pig, but they did find 1-2 µg/g in rabbit liver. This latter finding is in contrast to the previous report (Abraham et al., 1962), and if confirmed, is the first demonstration of homocarnosine in tissue other than the central nervous system. Excretion of homocarnosine in the range of 480-2352 µg/day in the urine of normal individuals and of patients with a variety of disorder has also been reported (Abraham et al., 1962). Homocarnosine is also present in small amounts (0.24 µg/ml) in human cerebro-spinal fluid (Abraham et al., 1962).

Homoanserine

Homoanserine is the dipeptide of l-methylhistidine and γ-aminobutyric acid (GABA) (Table 1, p. 24).

The presence of homoanserine in bovine brain was mentioned briefly by Kanazawa et al., (1965) who, however, did not provide evidence for its identification. Nakajima et al., (1967) have since reported the chemical isolation of homoanserine from bovine brain, in a yield of about 1 mg/kg brain (wet weight). Analysis of acid hydrolysate of the isolated homoanserine showed that the compound was composed of 1-methylhistidine and γ-aminobutyric acid (Nakajima et al., 1967). There are no reports of its presence outside the central nervous system.

Formation of histidine dipeptides in the central nervous system.

Whether the dipeptides of histidine or its ring methylated derivative are taken up into brain from the blood, is not yet known. It is possible that histidine in brain is utilised to form carnosine and homocarnosine; similarly that 1-methylhistidine is a source for anserine and homoanserine. Yockey and Marshall (1969) have demonstrated using labelled histidine, that frog brain synthesizes carnosine and homocarnosine both in vivo and in vitro. McManus (1962) observed methylation of homocarnosine to give homoanserine in the presence of extracts of rat and guinea pig brain. Unequivocal evidence of the synthesis of homocarnosine, carnosine or anserine by mammalian brain tissue either in vivo or in vitro has

not been reported to date. The somewhat restricted distribution of homocarnosine, and possibly also of homoanserine, to the central nervous system probably reflects the similarly restricted distribution of the precursor γ-aminobutyric acid (Abraham et al., 1962). It may therefore be argued that the synthesis of the dipeptide most probably occurs in the central nervous system.

Studies on the biosynthesis of carnosine in vivo (Harms and Winnick, 1954; Yockey and Marshall, 1969) and in vitro (Kalyankar and Meister, 1959, 1959a; Winnick and Winnick, 1959; Yockey and Marshall, 1969) suggest that the peptide is synthesized from its constituent amino acids. In the presence of adenosine triphosphate, magnesium ions and an enzyme preparation obtained from chick pectoral muscle, carnosine is synthesized from its constituent amino acids, β-alanine and histidine (Kalyankar and Meister, 1959, 1959a; Winnick and Winnick, 1959).

There is some evidence that in the synthesis of anserine the nature of the 'histidine' precursor may be species specific. Cowgill and Freedburg (1957) detected in rabbit, chicken and frog the formation of labelled anserine after injection of $^{14}CH_3$-1-methyl-histidine. In the rat, however, no such formation was observed, a finding confirming and consistent with the earlier observations of Harms and Winnick (1954) and

McManus (1956). Harms and Winnick (1954) found that after administration of labelled 1-methylhistidine that there was no labelling of the anserine in the skeletal muscle of the rat but there was in the chicken. McManus (1956) fed $^{14}CH_3$-methionine to rats; radioactive anserine, but not labelled 1-methylhistidine was detected in skeletal muscle. It would appear from the available evidence that, in the rat at least, anserine formation takes place only by methylation of carnosine.

N-ring methylation of carnosine does not, however, appear species specific. Using S-adenosylmethionine-methyl-^{14}C as methyl donor McManus (1962) obtained *in vitro* evidence of carnosine N-methyl-transferase activity in many tissues (brain, skeletal muscle, heart, liver, kidney, spleen and lung) from a variety of species including rat, rabbit, guinea-pig and chicken.

It would seem that in some species at least anserine formation may take place from histidine through carnosine or from preformed 1-methylhistidine.

On reviewing the previous literature it would seem that the syntheses of the dipeptides have not yet been the subject of extensive study and that little is known of their origin in the mammalian brain.

The available information obtained from studies of other tissues has been presented in relation to its possible implication for the central nervous system,

because the data usefully serve as pointers to the planning of further investigation of brain tissue.

UPTAKE OF HISTIDINE BY BRAIN TISSUE

There is no evidence that the central nervous system can synthesize histidine and it would therefore appear that the amino acid must be acquired from the blood stream.

The uptake of histidine by brain tissues has been observed both *in vivo* and *in vitro* studies. Kamin and Handler (1951) gave an intravenous infusion in the dog and observed that the concentration rose in the brain. Nakamura (1963) found that after intraperitoneal injection into mice, L-histidine entered the brain more rapidly than D-histidine in spite of a higher concentration of the latter in the plasma. Abou, Adam and Stephen (unpublished results) have shown that the histamine concentration rises in certain areas of rabbit brain following intravenous infusion of histidine; this rise could be attributed to the probable passage of histidine from blood into brain, and may be considered as an indirect evidence of uptake of histidine by brain tissue *in vivo*.

In vitro studies of the uptake of histidine into brain have been made using brain slices. Neame (1961) examined the uptake of amino acids by slices of mouse brain and found that several (histidine, lysine, proline, methionine, ornithinine and arginine) were

taken up against a concentration gradient. The concentration ratio between tissue and medium was highest for histidine and was influenced by duration of incubation, temperature, pH and the concentration of amino acid in the suspending medium; anaerobic conditions or cyanide inhibited the uptake. In later experiments (Neame, 1964) he showed that the relative uptake of various imidazole compounds in comparison with L-histidine was as follows: L-histidine, 100; D-histidine, 51; dihydro-urocanic acid, 28; imidazole acetic acid, 11 and imidazole, 4. The presence of either the D- or L-isomers of acidic amino acids reduced the uptake of histidine by slices of rat brain, but did not alter the uptake by intestinal mucosa, testis or spleen (Neame, 1965).

Regional variations in uptake of amino acids by slices of guinea pig brain were observed by Nakamura and Nagayuma (1966). The uptake of L-histidine, L-glumatic acid, L-aspartate or DL-tyrosine was higher in cerebral cortex than in diencephalon, mesencephalon and subcortical white matter. In cat brain the uptake was highest in slices from caudate nucleus and thalamus, and lowest in slices from the corpus callosum, optic chiasma and spinal cord (Battistin, Gryanbaum and Lajtha, 1969).

All these findings in <u>in vivo</u> and <u>in vitro</u> studies suggest that uptake of histidine by brain tissue is an

active process and is mediated by some transport mechanism, favouring the naturally occurring L-isomers.

CHAPTER I

INTRODUCTION

PART II - A SURVEY OF THE METHODS OF SEPARATION AND ESTIMATION OF HISTIDINE AND HOMOCARNOSINE

INTRODUCTION

PART II - A SURVEY OF THE METHODS OF SEPARATION AND ESTIMATION OF HISTIDINE AND HOMOCARNOSINE

This part of the thesis is devoted to the review of various methods employed for the separation of histidine and homocarnosine and for the estimation of these compounds. Techniques for the separation of histidine from biological materials are described followed by a review of methods which have been used for the estimation of histidine in pure solution and in biological materials.

Since reported data on the separation and estimation of homocarnosine are comparatively limited, both these aspects have been discussed together.

Separation of histidine

The methods that have been commonly applied for the separation and/or purification of histidine are based on:

1. Selective precipitation
2. Solvent extraction
3. Electrodialysis
4. Ion exchange column chromatography
5. Paper chromatography
6. Thin-layer chromatography
7. Paper electrophoresis

These methods are briefly described below.

1. Selective precipitation. Using phosphotungstic acid Dreschel (1896, quoted by Van Slyke, 1911) separated mixtures of amino acids into two fractions - the basic amino acids which were precipitated and other acids which were not. Soon afterwards Hedin (1896) employing this technique isolated histidine. To a solution of protein hydrolysate, Hedin first added phosphotungstic acid to precipitate the bases, and then separated histidine from the base fraction by treatment with silver nitrate. In the same year Kossel (1896) simultaneously and independently isolated histidine by precipitating it with mercuric chloride from an alkaline solution containing the products of hydrolysis of sturin, a protamine.

Kossel and Kutscher (1900) observed that when a solution containing histidine and arginine was treated with barium hydroxide in the presence of excess silver salt, histidine silver precipitated first and arginine silver began to precipitate on the addition of excess alkali.

Hanke and Koessler (1920) isolated histidine from a hydrolysate of red blood cells by first precipitating with mercuric chloride. The precipitate was dissolved in hydrochloric acid and the solution was saturated with hydrogen sulphide to

remove the mercury. Vickery and Leavenworth (1927) precipitated histidine and other substances in protein hydrolysate with an excess of silver salt. The solution was brought close to pH 7.0 with barium hydroxide. Histidine was further purified by precipitating with mercuric sulphate and treating the precipitate with hydrogen sulphide (Vickery and Leavenworth, 1928).

2. Solvent Extraction. The separation of a weak acid or a weak base by solvent extraction depends on the distribution of the molecules between two solvent systems i.e. water (polar) and an immiscible non-aqueous phase (less polar). The ratio of solubility in the two solvent systems at equilibrium is called the partition coefficient and is influenced by the pK_a of the solute and pH of the aqueous phase. Thus at high pH, a weak organic base will be present mainly in the unionised form and, as such, it dissolves more freely in solvents of low polarity.

Koessler and Hanke (1919a) made use of this principle in separating histidine from histamine in a protein hydrolysate. An aliquot of a solution containing these and other substances was treated with sodium hydroxide. Histamine was thus converted to the free base and histidine to anionic form. The solution was extracted with amyl alcohol. Histamine

being unionised passed readily into organic solvent while histidine which was ionised, remained in the aqueous phase.

Shore, Burkhalter and Cohn (1959) separated histamine and histidine on the same principle. A perchloric acid extract of brain tissue was made alkaline with NaOH and the histamine was extracted with butyl alcohol, histidine remaining in the aqueous phase. Separation was assisted by saturating the aqueous phase with solid sodium chloride i.e., by salting out.

3. Electrodialysis. When a protein hydrolysate is placed in the centre of a cell divided into three compartments by semi-permeable membranes such as cellophane, and a direct current is passed through the solution, from electrodes in the outer compartments, the amino acids may be separated into three fractions. Under suitable conditions of pH, amino acids which are predominantly acid, including aspartic acid and glutamic acid, move towards the anode; basic amino acids which include arginine, histidine and lysine move towards the cathode; the remaining neutral amino acids remain in the central compartment. Ikeda and Suzuki (1912) first introduced this method for the separation of amino acids. Foster and Schmidt (1921-22, 1923) demonstrated that by adjustment of the pH of the hydrolysate, histidine could be separated

from other two basic amino acids. At pH 5.5 the three basic amino acids moved towards the cathode, but at pH close to 7.6 arginine and lysine migrated towards the cathode, and histidine stayed in the central compartment. Histidine was then isolated and purified from the solution by the method of Hanke and Koessler (1920). Albanese (1940) performed the electrodialysis of protein hydrolysate at acid pH and then separated histidine from the cathode fraction by fractional precipitation with mercuric chloride.

Separation by ion-exchange resins.

Amino acids behave as anions or cations or as uncharged particles (zwitterions) depending on the pH of the solution. This phenomenon has been made use of to separate individual amino acids by means of ion-exchange materials.

Whitehorn in 1923 showed that the artificial zeolite, Permutite, adsorbed histidine and lysine in exchange for sodium (Na^+).

Block (1942) described the separation of basic amino acids by the use of synthetic organic ion-exchange resins, Amberlite IR-4 and IR-100. A protein hydrolysate was stirred with acid binding resin IR-4 until the reaction of the solution was approximately pH 6. The resin was removed and washed thoroughly. The treated solution and the wash fluid containing

the unadsorbed basic amino acids was then passed through a column of the cationic exchanger IR-100 (H^+ form). The adsorbed amino acids were subsequently eluted with 2N HCl and histidine, arginine and lysine isolated from the eluate by selective precipitation. The procedure was later modified by Block (1946, 1946a); amino acids adsorbed on the column were eluted selectively by varying the concentration and/or composition of the eluting agents.

Winters and Kunin (1949) reported on the amino acid exchange properties of two new resins, Amberlite IRC-50 (R-COOH) and Amberlite IRA-400 ($R-NH_3Cl$), and proposed a scheme by which histidine, arginine and lysine could be separated from each other and from other amino acids by the use of multiple columns at different pH values.

Stein and Moore (1949) were the first to show separation of individual components of a mixture containing 17 amino acids including histidine by analysing the eluate from a single column (0.9 x 55 cm) of Dowex-50 in hydrogen form ($R-SO_3H$). The amino acids were eluted successively with increasing concentrations of HCl (1.5 N, 2.5 N and 4.0 N HCl). The serine-threonine resolution was, however, only partial.

In later years they reported various modifications of the technique designed to improve the separation,

such modifications including for example preliminary removal of acidic amino acids on a strong anion exchange resin, Dowex 1, before adsorbing on and fractionally eluting the remaining amino acids from a strong cation exchange resin column. The effects of modifications of the degree of cross linkage of the adsorbing resins, pH and ionic strength of the eluting fluids were studied (Moore and Stein, 1951; Moore and Stein, 1954; Hirs, Moore and Stein, 1954; Moore, Spackman and Stein, 1958). McManus (1960) separated histidine and 1-methylhistidine from the dipeptides carnosine and anserine in a muscle extract by passing it through a column of Dowex 50 column buffered with 2,6-lutidine followed by elution with 0.1M 2,6-lutidine. An acid hydrolysate of the fraction containing the peptides was applied to a column of Dowex 50 buffered with α-picoline and the adsorbed amino acids were fractionally eluted with 0.1 M -picoline. This column also separated 3-methylhistidine as well as -alanine, histidine and 1-methylhistidine, the constituent amino acids of the dipeptide.

Pisano et al., (1961) separated histidine, carnosine and homocarnosine from a brain extract by adsorbing the compounds on a column of Dowex-50 x 4, buffered to pH 4.70 and fractionally eluting with 0.2 M ammonium acetate, pH 4.70.

Abraham et al., (1962) and Yockey and Marshall (1969)

also used a Dowex 50 column but buffered with 2,6-lutidine for the separation of the same substances in the brain extracts.

Kahlson, Rosengren and Thunberg (1963) separated ^{14}C-histamine from a commercial sample of ^{14}C-histidine by passing a solution through a column of 200 mg of Dowex 50-W x 4 (100 - 200 mesh), buffered at pH 6.0. The ^{14}C-histidine, in an amount not exceeding 4 mg in 1 ml of phosphate buffer, pH 6.5, was applied to the column followed by wash with 4 ml of the same buffer. The ^{14}C-histidine appeared in the effluent while the contaminating ^{14}C-histamine was retained on the column. Abou (1968) also showed separation of histidine from histamine on a 0.7 x 2.0 cm column composed of a mixture of 50 mg of the weakly acidic (R-COOH) cation exchange resin Amberlite CG-50 (100 - 200 mesh) and 300 mg cellulose powder (Adam, 1961). The column was found to be suitable for the treatment of extracts from small amounts of brain tissues (even less than 100 mg).

5. Separation by paper chromatography. This method is basically a partition chromatography and, in a given solvent system, the separation of individual components of a mixture applied to the paper depends on their individual partition coefficients between the static and the mobile phases of the solvent system. The distance travelled by a compound from the origin bears

a constant ratio to the distance travelled by the solvent system. This ratio is known as the Rf value of the compound in that solvent system. With a suitable solvent system the Rf value of a compound may differ sufficiently from that of even closely related substances to allow its separation (and identification) from other components in a mixture.

Dent (1948) separated histidine from other amino acids and a number of ninhydrin reacting substances by a two dimensional paper chromatography using first phenol-water and then "lutidine-collidine"-water solvent systems. Histidine could be separated by a single run from other amino acids in a number of solvent systems including n-butanol-acetic acid-water, n-butanol-pyridine-water, methanol-pyridine-water solvent systems. Because of streaking, its separation in phenol-water and phenol-ammonia-water solvent is not satisfactory. Addition of ethyl alcohol to a phenol-ammonia solvent system prevents streaking but depress the Rf value of histidine and of other amino acids (Smith, 1969).

Ames and Mitchell (1952) studied the separation of imidazole compounds including histidine by paper chromatography using propyl alcohol-ammonia, and propyl alcohol-acetic acid solvent systems.

Smith (1969) stated that a satisfactory separation of histidine from anserine, carnosine, and 1-methyl-

histidine could not be achieved in n-butanol-acetic acid-water, n-butanol-pyridine-water, or isopropanol-ammonia-water solvent systems. For this separation phenol-water, phenol-hydrochloric acid, phenol-acetic acid, and phenol-ammonia solvents were recommended (Smith, 1969).

Recently Nakajima et al., (1967) have used an isopropanol-hydrochloric acid system for the separation of histidine from homocarnosine by paper chromatography.

6. Thin-layer chromatography. The underlying mechanism of separation of amino acids by thin-layer chromatography largely depends on the materials of the layers. On a cellulose thin layer, separation of the compounds is effected by the partitioning of the moving compounds between two liquid phases - static and mobile, but their movement is also affected by non-specific adsorption to the cellulose powder composing the layer. On a layer prepared with silica gel or with an adsorbent containing impurities which behave as ion-exchangers, the separation is influenced by heteropolar chemical bonds formed reversibly between the moving compounds and the adsorbent. In thin-layer chromatography in practice, a combination of adsorption, ion-exchange and partition chromatography is usually involved, with one of these factors predominating (Randerath, 1963). Ahrland, Grenthe and Noren (1960, 1960a) observed that the

titration curve of silica gel was similar to that of a weakly acidic ion-exchange resin. Brenner, Niederwiser and Pataki (1965) observed that in neutral solvent systems such as ethanol- or n-propanol-water mixtures, the acidic amino acids travel on silica gel layers faster than the basic amino acids, lysine and arginine; and these workers suggested that the difference might be due to cation exchange, which would have the affect of adsorbing and retarding the basic amino acids.

The chromatographic characteristics of histidine have been compared with a number of amino acids, amines and imidazole compounds on thin layers of silica gel (Carisano, 1964; Brenner et al., 1965), of silica gel-cellulose mixture (Aures, Fleming and Hakanson, 1968) and of cellulose (Smith, 1969) in various solvent systems.

7. Paper electrophoresis. This method is based on the movement of a charged molecule in an electrical field, and the underlying principle is the same as that of electrodialysis (p. 36) from which it differs in instrumentation. In paper electrophoresis, the separation of individual molecules is effected on a piece of filter paper carrying a suitable buffer system through which a current of electricity is passed. The speed of migration of a substance from its point

of application depends on the amount of ionisation and nature of charge it carries, under the condition of operation.

Since the amino acids are ampholytes, they may carry a positive or a negative charge or may behave as uncharged molecules depending on the pH of the buffer solution. Thus in an electrical field an amino acid may migrate towards anode or cathode depending on the nature of the charge it carries or may stay at the origin if the pH of the buffer solution corresponds to its iso-electric point or close to it.

The rate of migration of a charged particle will be influenced by the intensity of the electric field applied, higher voltages giving faster and sharper separation of the components of a mixture of a charged molecules. Practical difficulties such as heating of the paper and the safety factor impose limitations to the voltage. In practice, techniques of paper electrophoresis are usually described, somewhat arbitrarily, as low voltage electrophoresis and high voltage electrophoresis.

In this review experiments with potential drop of less than 30 v/cm have been discussed, arbitrarily, as low voltage electrophoresis, and the experiments with a potential drop of 30 v/cm or more as high voltage electrophoresis.

Low voltage paper electrophoresis. Durrum (1950) applied low voltage electrophoresis for the separation

of amino acids and demonstrated that a number of the acids could be separated by this technique. Similar results were reported by other workers (Harris and Warren, 1954; Runeckles and Krotov, 1957; Whitehead, 1958). A later systematic investigation by Evered (1959) of the problem of separation of amino acids by low voltage paper electrophoresis showed that the method is particularly useful for separating acidic, basic, β-amino acids and cystine. The α-monoamino monocarboxylic acids not included in the above groups cannot be separated from one another by this method when all are present in a mixture. With electrophoresis in a pH 6.0 buffer the α- and β-monoamino monocarboxylic acids (mostly neutral) move only slightly towards cathode. Histidine, carnosine, anserine and 1-methylhistidine distribute themselves on the paper in that order from the origin towards the cathode while lysine, arginine and ornithine along with the bases histamine and ethanolamine move rapidly off the paper into the cathode vessel and acidic amino acids migrate towards anode (Evered, 1959). Abraham et al., (1962) applied this technique for the separation of histidine from homocarnosine and carnosine by electrophoresis at pH 10.0.

High voltage paper electrophoresis. Considerable improvements in the separation of amino acids by paper electrophoresis have arisen from the use of apparatus

allowing the application of higher voltages. By this technique the separation is achieved in a shorter time and spots obtained are more compact than with low voltage electrophoresis (Estoe, 1966) and prior desalting of the specimens is not necessary (Efron, 1960).

Atfield and Morris (1961) isolated histidine from other amino acids by high voltage paper electrophoresis at pH 5.2. Using electrophoresis at pH 2.0 Mabry and Todd (1963) separated the free amino acids including histidine in urine by this technique. Kanazawa and Sano (1967) separated histidine, homocarnosine and carnosine in a tissue extract by electrophoresis at pH 10.0 after prior separation of the basic compounds from acidic and neutral amino compounds by adsorption on a short column of a strong cation exchange resin, Amberlite IR-120.

THE ESTIMATION OF HISTIDINE

Various methods for the estimation of histidine in pure solutions as well as in biological samples have been proposed and are reviewed briefly in this section.

Pauly (1904) introduced the first qualitative test for histidine and other imidazoles in pure solution. Since then various quantitative methods have been developed which can be classified as photometric, microbiological, enzymatic, isotopic. Each is described briefly.

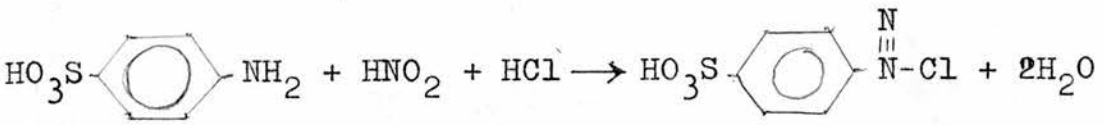
Photometric methods.

These are colorimetric, spectrophotometric and spectrophotofluorimetric.

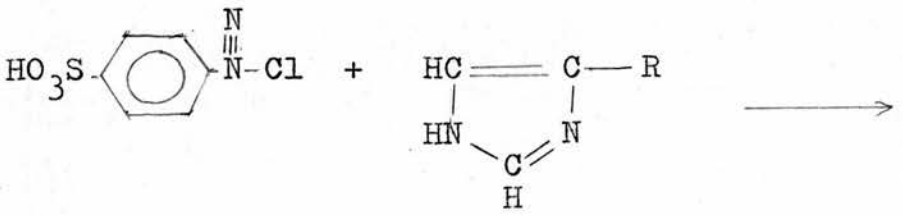
Colorimetric and spectrophotometric methods are based on reactions described by Pauly (1904) and Knoop (1908) and have undergone a number of modifications.

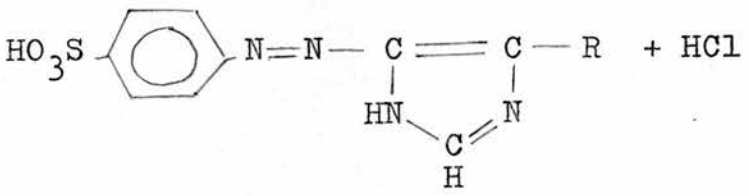
Methods based on the Pauly reaction. Pauly (1904) demonstrated that histidine and other imidazole compounds, in the presence of sodium carbonate, reacts with p-diazobenzene sulphonic acid to give a cherry red colouration which changes to orange on the addition of acid. Steps in the reaction are:

1. sulphanilic acid is diazotized with nitrous acid in the presence of hydrochloric acid (diazo reagent)



2. The reagent couples in the position contiguous to the imino group of the imidazole ring.





The reaction is carried out at 0^{o} and colour develops to maximum intensity in the course of 5 min.

The first attempt at a quantitative method for histidine was based on this reaction (Weisz and Ssobolew, 1914). To the histidine containing solution was added the diazo reagent and the mixture made alkaline by the addition of sodium carbonate solution. The colour developed from a standard solution of histidine was then compared with those from a series of dilutions of the unknown at between 5 and 10 min after adding the alkali. The result was interpolated from the dilutions giving colour intensities most closely matching the standard solution. The main difficulty with this method lay in the instability of the colour

developed in the reaction.

Koessler and Hanke (1919) provided the first detailed analysis of the chemical changes involved in the colorimetric reaction. They not only increased the sensitivity of the method of Weisz and Ssobolew, 1914 but extended its use to other imidazoles. They introduced stable standards by mixing suitable dyes (Congo Red and Methyl Orange) and calibrating them in a Dubosq colorimeter against standard solutions of histidine in the range of 1 to 50 µg. From their study of the various stages of the reaction they concluded that the imidazole solution should be added to the diazo reagent only after the later had been made alkaline. This procedure, they argued, not only neutralized free nitrous acid before it could attack aliphatic amino groups but also increased the concentration of the coupling compound.

Further modifications followed (Jorpes 1932, Yokoyama 1936, quoted by Macpherson 1942) and a major advance was made by Macpherson(1942, 1946) who showed that the final colour would be stabilised by the addition of ethanol. This obviated the need for an artificial standard. He also carried out the diazotisation and coupling with histidine at room temperature. Using a Pulfrich photometer, standard curves for histidine were found to be linear in the range of 20 - 100 µg histidine.

Relatively recent work using Macpherson's (1946) method for histidine estimation is that of Neame (1961) who measured the absorbance of the solutions in a spectrophotometer (Unicam, Cambridge).

Abraham et al., (1962) introduced a further modification when they employed a stabilized diazonium salt, Fast Red Salt TRN (General Aniline and Film corporation, Dyestuff and Chemical Division, New York, N.Y.) instead of diazotized sulphanilic acid for the estimation of imidazole compounds.

Methods based on Knoop's reaction. Knoop (1908) observed that when bromine water was added to a solution of histidine, a brownish-red colour developed on heating. Hunter (1922) made the further observation that colour intensity varied with the quantity of bromine added to histidine solutions of the same concentration. Maximal intensity was obtained in the presence of excess bromine and it was calculated that each molecule of histidine had reacted with 3 atoms of bromine.

A quantitative colorimetric method based on Knoop's reaction for the estimation of histidine was first developed by Kapeller-Adler (1933). Bromine in acetic acid was added to the histidine containing solution. After 10 min. a mixture of ammonia and ammonium carbonate was added and the solution heated. A deep violet-blue colour developed which was stabilized by

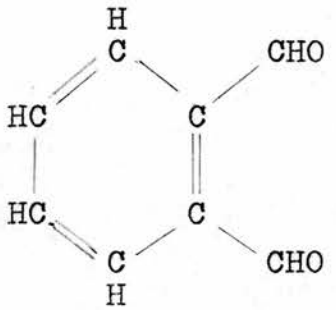
the addition of ethanol. The sensitivity of the method based on this reaction was 20 µg/ml histidine. Whereas the Pauly reaction involves the imidazole ring of the histidine molecule, the Knoop reaction depends on the integrity of the alanyl side chain. Thus, imidazole acetic and other similar acids do not give the reaction, nor does the isomer of methylhistidine in which the NH_2- of the side chain is methylated. The reaction is also negative for carnosine, but histidine methylated in the ring (N at position 1) and histamine both produce a weak colour reaction.

Modifications to the method have been introduced for the removal of excess bromine (Conrad and Berg, 1937; Chattaway, 1947) and for conducting the reaction at room temperature (Oyamada, 1944; Hunter, 1952). The method has been used relatively recently for the estimation of histidine in urine (Hunter, 1965). Compared with the method based on the Pauly reaction it has the merit of being more nearly specific for histidine.

Fluorimetric method

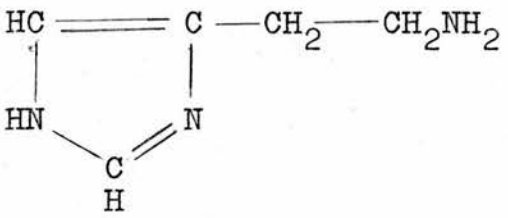
Shore, Burkhalter and Cohn, (1959) observed that histamine reacts with o-phthalaldehyde under strongly alkaline conditions to form a fluorescent product which remains stable under acid conditions for about 30 min. They suggested that a condensation product as shown

below might have been formed by the reaction of histamine with o-phthalaldehyde.

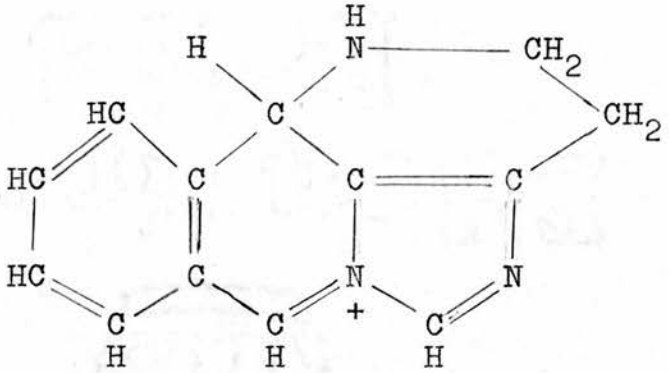


o-Phthalaldehyde

+



Histamine



Condensation product

The intensity of fluorescence emitted by the product was found to be proportional to the histamine concentration between 0.005 and 0.5 µg/ml, a 2 ml sample being required under their experimental conditions.

Pisano et al., (1961) described a similar method for the estimation of histidine in a hydrolysate of bovine brain extract. To an aliquot containing about

1.5 to 4.5 µg histidine, they added 1 ml of 0.02N NaOH followed by 0.1 ml of 1% (w/v) o-phthalaldehyde in methanol. After 4 min. the fluorescent reaction product was stabilized by the addition of 0.1 ml of 3 N HCl. Fluorescence was measured at 450 nm on activation at 360 nm these wavelengths being the maxima, characteristic of the fluorophor (see appendix, p. 262)

Recently, Ambrose, Crimm, Burton, Paulin and Ross (1969) described a method for the fluorimetric estimation of histidine in blood. They added, to a 0.5 ml portion of protein-free serum or heparinised plasma, 2 ml of N NaOH followed by 1 ml of alcoholic o-phthalaldehyde solution (50 mg in 100 ml of 95% ethanol) and allowed the reaction to continue at 30°C for 15 min., after which 1 ml of phosphoric acid reagent was added to the mixture. Then the reaction was allowed to proceed at 30°C for another 15 min. at the end of which the fluorescence was measured at 436 nm on activation and at 360 nm. An amount as low as 0.5 µg of histidine in a sample could be measured by this method. The method was thus more sensitive than that of Pisano et al., (1961) probably because the histidine was allowed to react with o-phthalaldehyde under alkaline conditions for a longer time (for 15 min. instead of 4 min. as in the method of Pisano et al., (1961)). The fluorophor formed was claimed to be

stable for several days. The method was not specific to histidine as histamine, lysine, asparagine, kynurenine, arginine and γ-amino-n-butyric acid also produced detectable fluorescence under the conditions of the experiments.

Microbiological method for estimation of histidine

The development of methods for studying bacterial growth in chemically defined media made it possible to define the synthetic abilities of many microorganisms (Stephenson, 1944).

Although some bacteria can synthesize their own amino acids, others do not grow without an external source. Appropriate microorganisms that are dependent on a supply of one or more amino acids may be used as a specific test in the identification and measurement of those same amino acids in unknown solutions (Snell, 1945). When a particular amino acid on which the organism is dependent is omitted from the culture medium, growth does not occur; addition of the missing amino acid restores growth in proportion to its concentration in the medium. The growth response is measured by counting the microorganisms, or by turbidimetry, or by determination of some constant product of their metabolism, for example, an organic acid or hydrogen ions (Snell, 1945).

It was on such principles that a microbiological

method was originally devised for the estimation of histidine and other amino acids in biological fluids, proteins, foodstuffs and tissues including the brain (Snell, 1945; Hier and Bergeim, 1946; Henderson and Snell, 1948; Horn, Jones and Blum, 1948; Schurr, Thompson, Henderson and Elvehjem, 1950). The method depended on the observation that certain microorganisms which ferment glucose produce lactic acid in amounts that can be related to their rate of growth (Snell, 1945). In the actual assay, the quantity of acid formed during the exponential growth in a fixed interval of time, is determined by titration and plotted against the concentration of amino acid added to the medium. Values for unknowns are obtained by comparison with a standard curve (Snell, 1945; Henderson and Snell, 1948; Horn et al., 1948).

Leuconostoc mesenteroides, Streptocooccus faecalis and various lactobacilli have been used to assay from one to several amino acids in such tests (Henderson and Snell, 1948), Schurr et al., (1950) have used such a microbiological technique for the estimation of histidine in brain. Cerebral hemispheres of rats were homogenized, and an aliquot of the homogenate was taken and protein removed by the addition of tungstic acid. The protein free extract was added to a medium which had been inoculated with Leuconostoc mesenteroides or Streptococcus faecalis, and then incubated for 60 to 72 hours at 37°C. Histidine, in the range, of 0.1 to

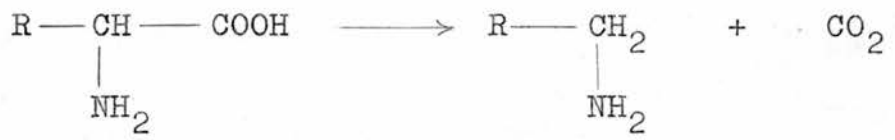
1.0 µg, was added control inoculated media. Lactic acid produced was titrated electrometrically in the assay tubes with 0.04 N NaOH delivered from a microburette. The volume of alkali required for neutralisation was taken as a measure of growth and of the concentration of histidine in the extract. Values obtained by this method are in the range of 8.7 to 17.4 µg/g (Schurr et al., 1950; Schurr, Thompson, Henderson, Williams, Jr., and Elvehjem, 1950; Thompson, Schurr, Henderson and Elvehjem, 1950).

Enzymatic Assay of histidine

The methods depend on one or other of two reactions; decarboxylation or oxidation.

Decarboxylation. Many bacteria produce pharmacologically active amines when grown in the presence of amino acids. The amines are formed through the action of specific amino acid decarboxylases. These enzymes are synthesized within the bacterial cells in response to amino acid substrates in the medium (Gale, 1946). Six decarboxylases have been identified, including one which is specific for L(-)-histidine. The enzymes can be extracted in soluble form from bacterial cell cultures and used to determine the concentration of their specific amino acid substrates in solution (Gale, 1957).

The decarboxylase attacks a specific amino acid according to the equation



The quantity of amino acid can be obtained by measuring the amount of CO_2 or amine produced in the reaction. Certain conditions, however, require to be met: first, the specific decarboxylase preparation should be free of other enzymes which liberate CO_2 and of enzymes which destroy or inactivate the amine by oxidation or in other ways; second, the forward reaction should run to completion or to a constant proportion of the theoretical yield.

In the method described by Gale (1957) for histidine, the enzyme is prepared from cultures of Cl welchii BW 21 (N.C.T.C. No. 6785). The cells are treated several times with acetone, which removes glutamase activity, and are finally dried with ether. The dried powder is standardized to contain 0.07 units of activity per mg, where the unit is equivalent to the release of 1 µmol CO_2 per minute at 37° from L(-)-histidine in a concentration of 0.083 M. The sensitivity of the assay using a gasometric (Warburg) technique is in the range 5 to 20 µmol (775 to 3100 µg) histidine (Gale, 1957).

Various authors have developed methods based on measuring the quantity of histamine formed in the reaction. Hutzler, Odievre, and Dancis (1967) estimated histamine spectrophotometrically after coupling with dinitrofluorobenzene (Dickerman and Carter, 1962). The lower limit of measurement by this method is approximately 10 ug/ml.

The sensitivity of the method can be increased by more than 10,000-fold when the enzyme is applied to ^{14}C-histidine and ^{14}C-histamine is measured by isotope dilution assay (Reilly and Schayer, 1968).

Oxidation. The enzyme used is a snake venom L-amino acid oxidase (Baldridge and Greenberg, 1963). At pH 7.8 and in the presence of borate ions, the enzyme preparation reacts with aromatic amino acids and histidine to yield enol-borate complexes of the corresponding keto acids formed by oxidative deamination. At pH 6.5 only the aromatic amino acids are oxidised and histidine is virtually excluded from the reaction. Thus a deproteinised serum filtrate may be assayed for aromatic amino acids at pH 6.5 by measuring the optical density at 292 nm. In the second assay the reaction is carried out at pH 7.8 the optical density then being measured at the same wavelength as before. The value obtained at pH 7.8 is the sum of the absorbancies due to enol-borate

complexes formed from the aromatic amino acids plus histidine, and the difference between the optical densities obtained at pH 7.8 and at pH 6.5 represents the amount of histidine. A portion of 2 ml of serum or plasma was used for the assay and the standard histidine solution contained 125 ug L-histidine-monohydrochloride monohydrate/ml.

So far the method has been applied only to the estimation of histidine in plasma and in 20 adult subjects the concentration was in the range of 0.3 to 2.6 mg per 100 ml of plasma.

1- methyl and 3-methylhistidine are also converted into the corresponding enol-borate complexes at both pH 7.8 and 6.5 and so do not interfere with the assay of histidine. Carnosine, anserine and imidazole acetic acid do not react under the conditions of the assay (Baldridge and Greenberg, 1963).

Histidine estimation by use of isotopes.

Keston, Udenfriend and Cannan (1946) devised a technique for the estimation of amino acids by isotope labelling. The method was based on the quantitative reaction of the amino groups of the acids with radioactive p-iodophenylsulphonyl chloride (pipsyl chloride) labelled either with ^{131}I or ^{35}S. This labelled derivative formed was then mixed with a known amount, constituting an overwhelming excess, of

unlabelled derivative (the carrier). The pipsyl derivative was isolated and purified, finally by repeated recrystallizations to a constant specific activity. The object of the use of large excess of non-isotopic derivative was to allow effective purification by crystallizations, distribution between solvents and selective adsorptions. From the specific activity of the purified sample and the known amount of added carrier, the quantity of the amino acid in the original sample for assay could be calculated. Keston, Udenfriend and Levy (1950) abandoned the carrier technique for determination of amino acids owing to the difficulties encountered in removing radioactive impurities and purified the labelled derivative by paper chromatography.

Considering the small amount of material recovered in a pure form, they observed that paper chromatography was a more efficient method of separating and purifying substances than was recrystallization. They converted the amino acids to be analysed into the pipsyl derivatives labelled with ^{131}I, and added to the preparation known amounts of the appropriate pipsyl derivatives labelled with ^{35}S (the "indicators"). The function of indicators was to monitor the fraction of unknown recovered through the purification processes. Although the methods described have been used for a number of amino acids (Keston *et al.*, 1946;

Keston, Udenfriend and Cannan, 1949; Keston et al., 1950) histidine was not included among them.

Schayer, Kobayashi and Smiley (1955) observed that carrier technique was very satisfactory for quantitative analysis of histamine and it was much easier to purify pipsylhistamine carrier than pipsyl-amino acids, since the former could be recrystallized from several organic solvents while pipsylamino acids were not readily soluble and must be precipitated by addition of acid to an aqueous ammoniacal solution. On the basis this observation Reilly and Schayer (1968) measured ^{14}C-histidine indirectly by converting it to ^{14}C-histamine in the presence of decarboxylating enzyme (Gale, 1957). Histamine was then converted into pipsylhistamine which was measured by carrier technique.

Since this method is based on the quantitative conversion of labelled histidine into labelled histamine by a decarboxylating enzyme and the labelled histamine formed was the measure of the labelled histidine the method may be called an "enzymatic-isotopic method". Presumably the basic principle of the Reilly and Schayer method could be used to estimate unlabelled histidine if the histamine formed by decarboxylation were coupled with labelled pipsyl chloride.

The review of the above methods for the estimation of histidine records the endeavour made by various

Table 2

Summary of various methods for the estimation of Histidine

Method of estimation	Sensitivity (lowest amount measured) µg	Reference
Photometric (based on Pauly reaction)	1000	Weisz and Ssobolew (1914)
"	1.0	Koessler and Hanke (1919)
"	5.0	Jorpes (1932)
"	20	Macpherson (1946)
Photometric (based on Knoop reaction)	1000	Kapeller-Adler (1932)
"	800	Chattaway (1947)
"	40.0	Hunter (1965)
Microbiological	0.1	Henderson and Snell (1948)
"	2.0	Horn et al., (1948)
Enzymatic	775	Gale (1957)
"	1000	Hutzler et al., (1967)
Fluorimetric	1.5	Pisano et al., (1961)
"	0.5	Ambrose et al., (1969)
Enzymatic isotopic	0.014	Reilly and Schayer (1968)

workers to improve the methods at successive stages. Methods most commonly used in recent years are the colorimetric methods based on the Pauly reaction or the Knoop reaction, or the fluorimetric method based on reaction with o-phthalaldehyde. Of these, the fluorimetric methods as described by Pisano *et al.*, (1961) and Ambrose *et al.*, (1969) are more sensitive than the colorimetric methods (Table 2), and an amount of histidine as low as 0.5 µg can be measured (Ambrose *et al.*, 1969). Since small amounts of histidine are expected to be obtained from brain samples of the order of 100 mg tissue and some of which is likely to be lost in the process of separation, attempts have been made in the present work to make further improvements of the fluorimetric assay methods which appeared to be the most readily applicable to the proposed study.

The enzymatic isotopic method (Reilly and Schayer, 1968) (p 58), although highly sensitive, is not applicable as it stands, to estimation of endogenous histidine.

Amino acid autoanalysers are automated systems now commonly used for the rapid assay of the amounts of individual amino acids in complex mixtures. They depend on the fractional elution of each amino acid adsorbed on an ion-exchange resin column assembly under controlled conditions and are based fundamentally on the work of Moore and Stein and their collaborators referred to earlier. Such methods have been used by

several workers for the estimation of histidine and homocarnosine and various other amino acids and derivatives in extracts of brain (Levi et al., 1967, Kandera et al., 1968, Battistin et al., 1969). Each amino acid as it is eluted is quantified spectrophotometrically after reaction with ninhydrin (Shaw and Heine, 1965; Laboratory Manual, Amino acid Analyzer, Technicon Instrument Co. Ltd.). The sensitivity is such, however, that the measurement of the 0.5 µg- 1µg amount of histidine which, it was considered likely, would be required to be measured in the proposed work would not be possible with this technique.

Estimation of homocarnosine in brain

So far only one method, that is the method of Abraham et al., (1962), for estimation of homocarnosine has been reported in the literature. Later, Kanazawa and Sano (1967) and Kockey and Marshall (1969) measured homocarnosine concentration in a number of species including fish and frog using this method with some modification.

Abraham et al., (1962) homogenised brain tissue (5 to 20 gm) in a Waring blender with 2 volumes of water containing a known amount of tritiated homocarnosine. The object of addition of tritiated homocarnosine to the samples was to measure the loss of

homocarnosine in the process of separation and purification. The homogenate was then mixed with 8 volumes of absolute alcohol to precipitate proteins. After centrifugation, the supernatant was treated with a mixture of isoamyl alcohol and hexane (1:1, by vol) to dissolve lipoidal material. Upon the addition of one-fifth volume of water, two phases formed and the lower aqueous phase was removed and evaporated to dryness. The dried residue was redissolved in a small volume of water; and an aliquot containing not less than 12.0 ug homocarnosine was placed on a 1.2 x 15 cm Dowex 50-x-4-2, 6-lutidine column. Histidine, homocarnosine and carnosine were separated by fractional elution with 0.1 M lutidine. Homocarnosine was measured colorimetrically after coupling with diazonium salt, Fast Red Salt TRN (General Aniline and Film Corporation, Dyestuff and Chemical Division, New York, N.Y.). The reddish colour developed was stable for 60 min and the extinction as measured at 500 nm was linear over in the range 0.02-0.15 umol (4.8 - 36 μg) homocarnosine. The amount of tritiated homocarnosine present in the sample was measured in a Packard Tri-Carb liquid scintillation counter and the estimate for the endogenous homocarnosine corrected accordingly. Yockey and Marshall (1969) used this method of Abraham et al., (1962) for the estimation of homocarnosine in various mammalian and

and frog brains.

The method of homocarnosine estimation employed by Kanazawa and Sano (1967) was also basically dependent on methodology described by Abraham et al., (1962). It involves separation of basic amino compounds from acidic and neutral amino acids by the passage of the extract through a short column of Amberlite IR-120x8. The basic amino compounds eluted from the column with 3 M NH_4OH were separated by a paper electrophoretic technique which was a modification of that of Abraham et al., (1962) and employed by them for the identification of the histidine-containing dipeptides isolated from brain. Homocarnosine eluted from the paper was measured colorimetrically after reacting with diazotised sulphanilic acid (Pauly reaction). The sensitivity of the absorptiometry was such that 4 µg of homocarnosine gave an extinction about double that of a corresponding water blank.

When the homocarnosine concentration of the tissue was less than 3.6 mg/100 g, the solution containing basic amino compounds was again evaporated, the residue was dissolved in a volume of water so that the homocarnosine concentration was between 4.8 to 24 µg per 10 µl, and a 10 µl portion of solution was employed for analysis.

Some of the methods discussed in this part of the thesis have been employed for the estimation of the concentrations of histidine and histidine derivatives in the brain of various species. The reported values are quoted in Table 3, p 68 . In each case the source reference of the estimate is given together with an indication of the basic method employed for the measurement.

The cited figures indicate a considerable species variation in the brain concentrations of histidine and of its derivatives, homocarnosine, carnosine and ergothioneine. It will be evident however that, in those cases where measurements from the same species have been made by several groups of workers, there may be considerable differences between the estimates. In addition to such factors as strain differences or environmental differences such as diet, it might be suspected that such discrepancies may be related to the use of different methods of estimation some of which may be insufficiently specific.

Table 3

Estimates reported in the literature, of the concentrations of histidine, of dipeptides and derivatives of histidine in the brains of various species.

Mean values (µg/g). S.E.M. in parenthesis. Number of estimates //.
References are listed on facing page together with method employed for the estimations.

Species	Histidine	Ref.	Homocarnosine	Ref.	Carnosine	Ref.	Ergothioneine Ref. e Cerebrum *Estimate on pooled brains	Cerebellum
Mammals								
Cat	9.0	/1/ k	1.2 <1.2	c/1/, j/1/ a/1/, f/5/	-		0.1* (2)	3.2* (2)
Dog	-		1.2 <1.2	/1/ j /1/ a, f	-		-	-
Guinea-pig	10.4 (2.5) 12.0 (0.54)	/6/ p /3/ g	25.2 (1.9) 50.6 (3.0) 14.2 (2.1) 40.8 43.9	/6/ p /3/ g /4/ f /1/ a /2/ j	3.8 (0.9)	/6/ p	1.6 (0.1) /3/	9.53 (1.3) /3/
Man	-		79.2 112.8	c /1/ j			-	-
Monkey	-		31.2	/1/ j			-	-
Mouse	12.6 (0.44) 17.9 (1.1)	/3/ g /5/ p	58.1 (1.8) 20.4	/3/ g /2/ p	10.6	/2/ p	not* detected /1/	5.0 /1/
Ox	-		48.0	/1/ j			-	-
Pig	-		1.2 1.2	/1/ j /1/ a, c			-	-
Rabbit	-		27.4 (2.3) 43.0 48.0	/4/ f /3/ a /1/ j			1.2 /5/	5.0* (0.3) /5/
Rat	5.8 (0.31) 7.2 (0.99) 8.7 17.3 (0.69) 26.6 (1.14)	/9/ h /3/ g /1/ m /6/ n /6/ d	11.3 (0.5) 31.0 (5.5) 13.4 (2.1) 13.9 16.8	/9/ h /3/ g /3/ f /5/ a /1/ j	3.4 (0.45)	/9/ h	0.38*(0.18) /4/	4.2 (1.1) /4/
Sheep	-		-		-		0.6* /1/	3.0* /1/
Birds								
Chicken	9.3 (0.72)	/3/ g	4.1 (0.8) 7.2 7.7 (1.0)	/3/ g /1/ a /3/ f	-		-	-
Duck	-		1.2	/2/ a	-		-	-
Amphibia								
Frog	25.7 (1.17) 46.7 (1.71)	/3/ g /5/ p	681 (14) 456 (72) 240	/3/ g /5/ p /1/ c	341 (25)	/5/ p	-	-

Other histidine derivatives

1- and 3-methylhistidine, cat, 3.2 µg/g /1/ k
N-acetylhistidine, frog, 240-600 µg/g, b

References cited in Table 3

Reference	Method of estimation
(a) Abraham et al., (1962)	Photometric (Pauly Reaction)
(b) Anatasi et al.,(1964)	" "
(c) Anatasi and Erspamer (1964)	" "
(d) Clouet et al., (1957)	" "
(e) Crossland et al., (1966)	" "
(f) Kanazawa and Sano (1967)	" "
(g) Levi et al., (1967)	Autoanalyzer (Ninhydrin Reaction)
(h) Marshall and Yockey (1968)	Photometric (Pauly Reaction)
(j) Pisano, Abraham and Udenfriend (1963)	" "
(k) Tallan et al., (1954)	Ninhydrin Reaction
(m) Schurr et al., (1950)	Microbiological Assay
(n) Williams et al., (1950)	" "
(p) Yockey and Marshall (1969)	Photometric (Pauly Reaction)

GENERAL METHODS

Reagents

To avoid tedious repetition in the various parts of the experimental work described in this thesis, details of the source and grade of purity of various chemicals which were used, and of any further purification procedures considered necessary are given in an appendix (Appendix 2 p. 278). Also included in this appendix are details of the method of preparation and storage of various reagent solutions employed.

THE DEVELOPMENT AND APPLICATION OF METHODS FOR THE EXTRACTION AND FLUORIMETRIC ESTIMATION OF HISTIDINE AND HOMOCARNOSINE IN BRAIN TISSUE

From the survey of the reported methods for the estimation of histidine it seemed that none was directly applicable to the estimation of the amino acid in small samples of brain tissue.

The fluorimetric method based on fluorophor production by interaction with o-phthalaldehyde seemed to offer the most promising lead because of its potentially higher sensitivity. As already described in the introductory section of this thesis such a method has been applied by Pisano *et al.*, (1961) to the

estimation of histidine in bovine brain and more recently, with various modifications of the experimental conditions by Ambrose et al., (1969) to the estimation of histidine in blood. Under the operating conditions described by these workers amounts of histidine in excess of 0.5 μg were required for estimation.

In the present work the characteristics of the reaction were examined in an attempt to find conditions allowing the estimation of the submicrogram quantities of histidine which might be expected to be present in small amounts of brain tissue. When this aim had been achieved, there remained the necessity to develop a separative technique which would purify the histidine-containing extract from brain tissue to a degree sufficient to make the assay specific to that substance. Concurrent studies of the quantitative fluorophor production from other substances likely to be present in brain extracts possibly also reacting with o-phthalaldehyde and thus interfering with the histidine estimation demonstrated the importance of developing an adequate separation technique.

The data obtained in the development of the fluorimetric method and the separation technique indicated that concurrent estimation of the histidine-containing dipeptide, homocarnosine, in the same brain

sample would also be possible.

The following section of the thesis reports the experimental investigations of the present work. The first part deals with the development of the methodology from model experiments carried out with authentic substances. The second part deals with the application of the methodology to the estimation of histidine and homocarnosine in mouse brain tissue.

Fluorimetry

The fluorescence intensities of solutions were measured in a Zeiss spectrophotofluorimeter coupled to a potentiometric recorder to allow automatic recording of activation fluorescence spectra. A short description of this instrument together with a brief discussion of certain essential general considerations in the practice of fluorimetry is given in an Appendix (p.254).

Measurements of fluorescence intensity were made at the wavelengths showing the maximal excitation and fluorescence appropriate to the fluorophor being studied. The monochromator slit widths were set at 0.5 mm for fluorescent light and at 1.0 mm for activating light which gave a band pass of 2.5 nanometer (nm) through the "fluorescence" grating-type monochromator and of 10 - 14 nanometer (nm) through the "activating" prism-type monochromator. Because of the

light dispersal characteristics of the latter monochromator, the exact width of the band pass varied with wavelength and was determined in any series of measurement by the maximum activation wavelength for the particular fluorophor being assayed.

For routine measurements, the fluorescence monochromator was set at the wavelength appropriate to the maximal fluorescence and the excitation spectrum of each sample was recorded from 300 nm. The relative intensities of fluorescence of solutions containing varying amounts of fluorophor were measured from the heights of potentiometric recorder tracing at the excitation wavelength characteristically showing maximal fluorescence to the fluorophor.

Sensitivity of a fluorimetric method for the estimation of a compound, in the present work, has been arbitrarily defined as the lowest amount of the compound that would produce fluorescence intensity equal to that of the "blank" and the limit of detection as the amount that would produce fluorescence the intensity of which was one-half of that of the "blank".

To obviate errors due to short term changes in the intensity of the activating light source, the spectrum was recorded in duplicate at least, for each sample. The mean values for the peak relative fluorescence were

used in any subsequent calculation.

All wavelengths quoted are uncorrected instrumental readings.

Glassware

The measurement of small quantities of substances by fluorimetry demands a high standard of cleanliness of the glassware used in order to avoid the appearance of spurious fluorescence or other types of interference by contamination of the solutions or extracts.

All glassware used in this work was therefore subjected to a rigid cleansing regimen as described in the Appendix (p. 266).

EXPERIMENTAL INVESTIGATIONS

Fluorophor formation from various substances by interaction with o-phthalaldehyde.

Appropriate volumes of a standard solution of the substance were transferred to glass-stoppered 10 ml test tubes and each diluted to 1.00 ml with water. For the determination of reagent "blank" fluorescence (see Appendix, p. 267), water was substituted for the solution of the compound under study.

Each solution was made alkaline by the addition of 0.2 ml M NaOH and 0.05 ml of o-phthalaldehyde solution (0.5% w/v in methanol) was added. In some experiments

described later (p. 77) the concentration of the o-phthalaldehyde solution was altered in order to investigate the effect of such alteration on the production of fluorophor.

After mixing the solutions, the reaction was allowed to proceed in the stoppered tube at room temperature (21°C) for the time appropriate to the aim of the experiment before determining the fluorescence intensity.

EXPERIMENTS TO DETERMINE THE OPTIMUM EXPERIMENTAL CONDITIONS FOR THE FLUORIMETRIC MEASUREMENT OF HISTIDINE

Characteristics of the histidine fluorophor.

Fig. 4 (p. 75) shows the activation and fluorescence spectra of a solution containing histidine-o-phthalaldehyde reaction product(s). The intensity of the fluorescence was found to be maximal when the solution was irradiated with light of 375 nm; the fluorescence showed a maximum at 435 - 440 nm. Routinely measurements of fluorescence were therefore made at 375 nm (activation) and at 437 nm (fluorescence).

Rate of formation of fluorophor and its stability.

Samples containing 0.0, 0.1, 0.2 and 0.3 µg histidine were allowed to react with o-phthalaldehyde

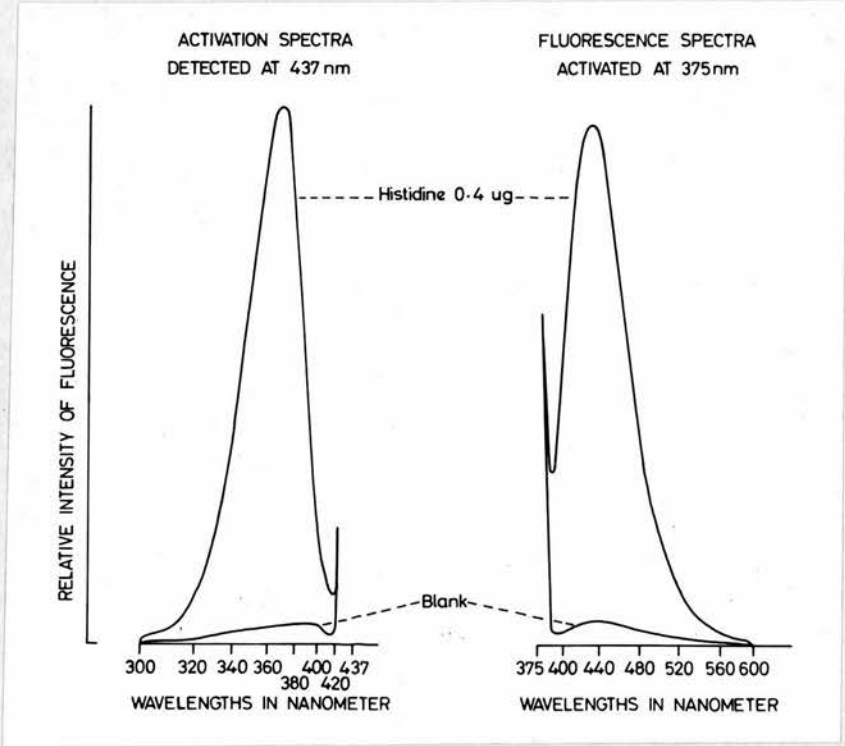


Fig 4. Activation and fluorescence spectra of histidine-o-phthalaldehyde fluorophor.

Peak fluorescence intensity 375 nm (activation) and 437 nm (fluorescence). The spectra from the reagents alone ("blank" fluorescence) are also shown.

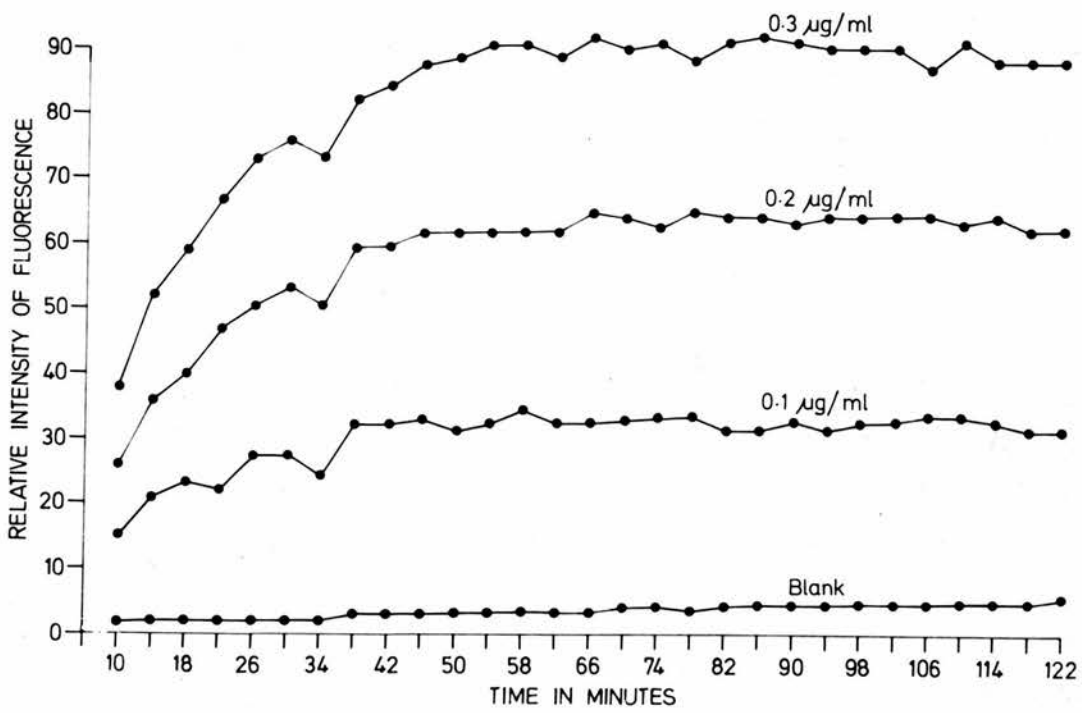


Fig 5. Rate of formation and stability of histidine-o-phthalaldehyde fluorophor.

Reaction mixture - histidine solution (1 ml) (concentrations of histidine standard solutions used shown on the graph), 0.2 ml N NaOH and 0.05 ml 0.5% o-phthalaldehyde in methanol (w/v).

and the fluorescence intensity of each sample was determined every 4th min. starting 10 min. after the addition of the aldehyde.

The results (Fig. 5, p. 76) showed that under these conditions, the fluorescence takes about 60 min. to reach maximum intensity and remains stable for at least another 60 min.

In the light of these findings, the reaction was allowed to proceed for 60 min. before measuring the fluorescence of 'unknown' solutions.

Influence of o-phthalaldehyde concentrations on the fluorescence derived from interaction with histidine.

Samples, 1.0 ml, containing 0.0, 0.1, 0.2 and 0.3 µg histidine were reacted with 0.2 ml of N NaOH solution and 0.05 ml of a 0.1%, 0.2%, 0.5% or 1.0% o-phthalaldehyde solutions in methanol for 60 min. and the relative fluorescence intensities measured. Satisfactory linear relations of fluorescence intensity to histidine content were obtained using both 0.5% and 1.0% o-phthalaldehyde solution in the reaction (Fig. 6, p. 78). If due allowance is made for the higher "blank" with the larger amount of aldehyde, the fluorescence intensity of the fluorophor obtained from a given amount of histidine was the same indicating that the lower amount of o-phthalaldehyde was adequate

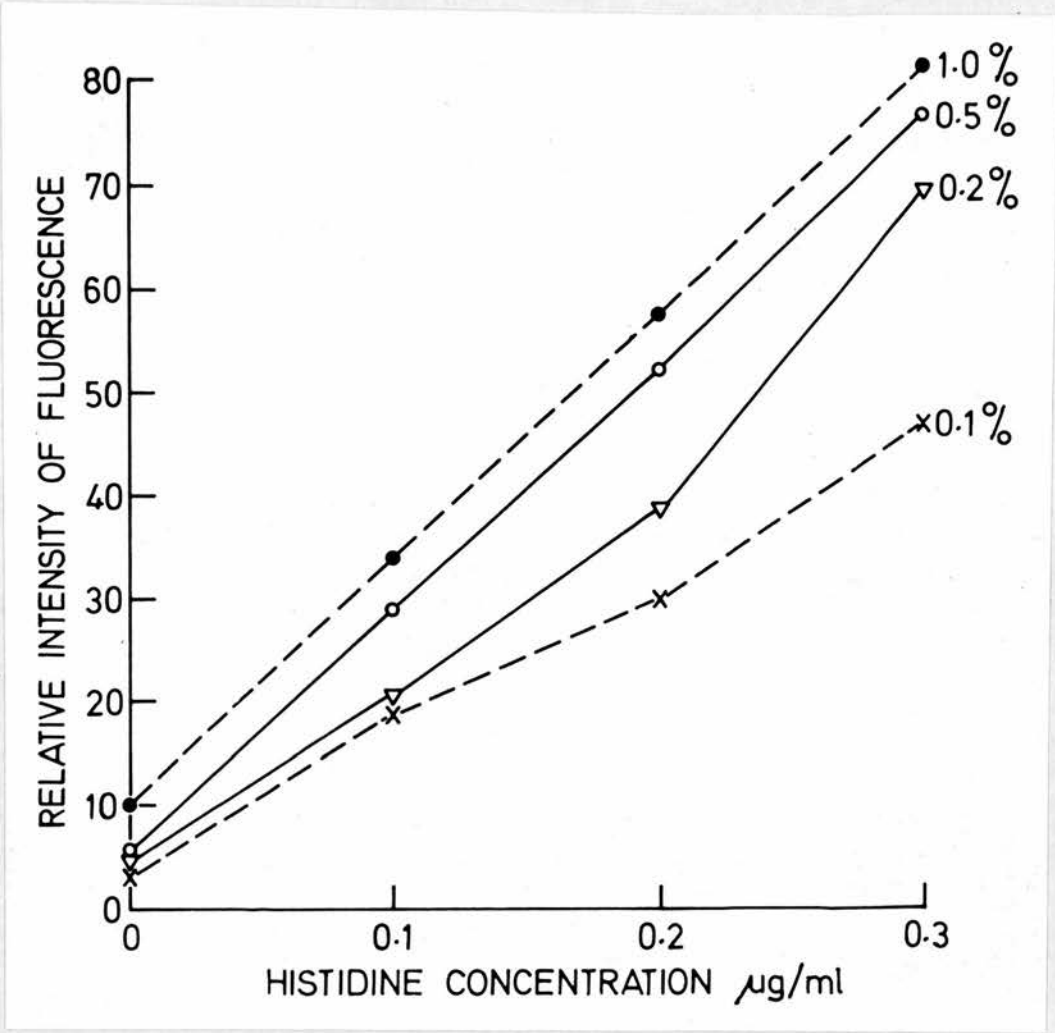


Fig 6. Influence of o-phthalaldehyde concentration in reaction mixture on the fluorophor production from histidine.

Aqueous samples, each of 1 ml containing 0.0, 0.1 µg, 0.2 µg or 0.3 µg histidine, were made alkaline by addition of 0.2 ml N NaOH. o-Phthalaldehyde solution, 0.05 ml of one of several concentrations (w/v as shown on the graphs) in methanol was added to a sample. Fluorescence at 375 nm (activation) and 437 nm (fluorescence) measured after 60 min.

to produce maximal fluorophor formation at least with the quantities of histidine tested. The lower amount was therefore used routinely because of the added advantage of yielding a lower reagent "blank".

Relation between amount of histidine and the fluorescence intensity of the derived fluorophor.

Fluorescence was measured after a reaction period of 60 min. A linear relation was observed between the fluorescence intensity and the amount of histidine present over a range 0.02 to 0.6 µg/ml of the amino acid (See Fig. 7, p. 80 and Fig. 8, p.81). At higher concentrations the intensity of the fluorescence was relatively lower.

The reagent "blank" was equivalent to about 0.02 ug histidine so that the sensitivity of the method, arbitrarily defined as the amount of substance giving rise to a fluorescence intensity equal to that of the reagent "blank" (see Appendix, p.267)was 0.02 µg histidine in pure solution.

Effect of pH of the reaction solution on the formation of histidine o-phthalaldehyde fluorophor.

Samples containing 0.2 µg histidine in 1.0 ml water were reacted with 0.05 ml o-phthalaldehyde solution (0.5% w/v in methanol) in presence of 0.2 ml of N HCl. In another experiment the samples were reacted with the o-phthalaldehyde solution without pretreatment with

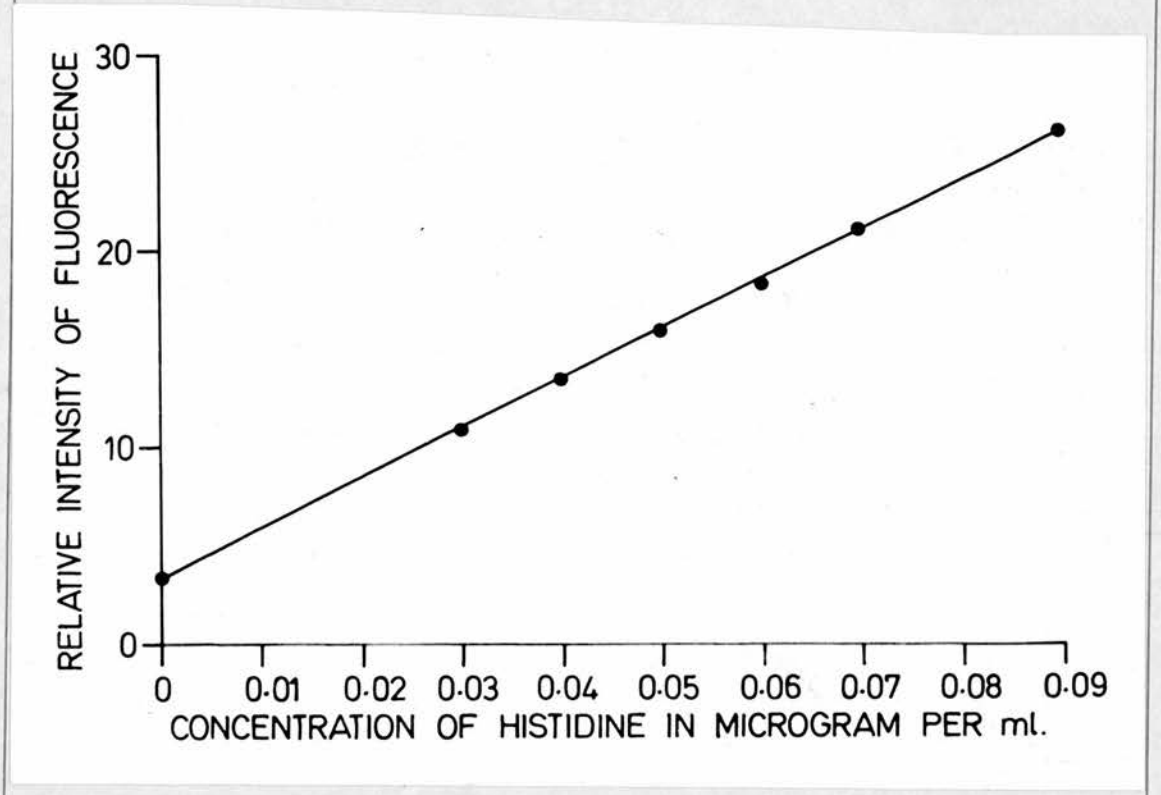


Fig 7. **Histidine calibration curve for amounts less than 0.1 ug.**

Aqueous samples, 1 ml, containing known amounts of histidine were allowed to react with 0.05 ml 0.5% o-phthalaldehyde (w/v) in methanol in the presence of 0.2 ml N NaOH. The reaction was allowed to proceed for 60 min at room temperature (21°C) before measuring the fluorescence at 375 nm (activation) and 437 nm (fluorescence).

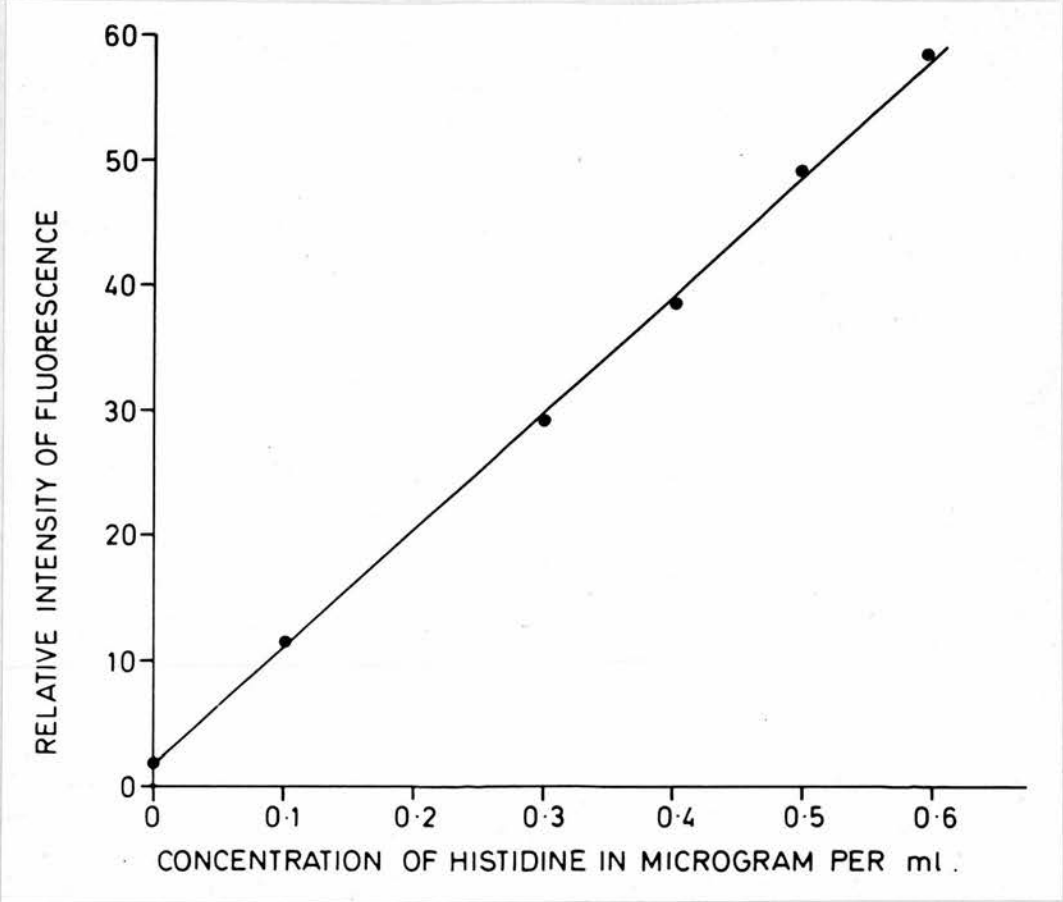


Fig 8. Histidine calibration curve for amounts greater than 0.09 µg.

Aqueous samples, 1 ml, containing known amounts of histidine were allowed to react with 0.05 ml 0.5% o-phthalaldehyde solution (w/v) in methanol in the presence of 0.2 ml N NaOH for 60 min at room temperature (21 °C) before measuring the fluorescence at 375 nm (activation) and 437 nm (fluorescence).

alkali or acid. In neither experiment was a measurable amount of fluorescence obtained from the samples indicating that histidine reacts with o-phthalaldehyde to produce a fluorescent product only in alkaline solution.

Effect of acidification on the fluorescence intensity of the histidine-o-phthalaldehyde fluorophor.

Samples containing 0.0, 0.1, 0.2 and 0.3 µg of histidine in 1.0 ml water were allowed to react with 0.2 ml N NaOH and 0.05 ml 0.5% o-phthalaldehyde (w/v in methanol) solution for 60 min. before measuring the intensity of fluorescence of the fluorophor formed. The samples were then acidified by the addition of 0.05 ml 6 N HCl and the fluorescence of the samples remeasured within 3 min. after acidification.

Acidification of the medium caused marked reduction of the intensities of the fluorescence of the samples and increased the size of the "blank" (Table 4). A shift of both the activation and fluorescence maxima of about 20 nm towards the longer wavelength was observed.

Table 4

Effect of acidification on the intensity of fluorescence of histidine-o-phthalaldehyde fluorophor. The samples were reacted with o-phthalaldehyde for 60 min. before acidification.

Samples	Relative Intensity of fluorescence	
	Before acidification	After acidification
Blank	9	17
0.1 µg histidine	34	23
0.2 µg histidine	61	34
0.3 µg histidine	81	44

In the o-phthalaldehyde method for the estimation of histamine described by Shore et al., (1959), the alkaline reaction solution is acidified before reading the fluorescence. The purpose of this acidification was stated to be the stabilisation of the fluorophor. It was of interest in view of the above results with histidine to see if acidification would produce a quantitative alteration in the fluorescence intensity of the histamine fluorophor. Histamine 0.2 µg in 1.0 ml aqueous solution to which 0.2 ml N NaOH was added was reacted with 0.05 ml 0.5% o-phthalaldehyde in methanol for 90 min. The fluorescence of the solution was measured at 365 nm (activation) and 430 nm (fluorescence) and then the solution was acidified by the addition of 0.05 ml 6 N HCl and the fluorescence redetermined. A 2.5 fold increase in the fluorescence was observed as a result of the acidification and this is to be contrasted with the decrease observed with the histidine fluorophor under similar circumstances (Table 4, p 83).

METHOD FOR THE FLUORIMETRIC ESTIMATION OF HISTIDINE FINALLY ADOPTED

Based on the experimental findings cited above the method for the fluorimetric estimation of histidine finally adopted was as follows.

Development of histidine fluorophor. A 1.0 ml sample of the solution containing 0.02 to 0.6 µg histidine was placed in a glass-stoppered 10 ml test-tube. To each sample was added 0.2 ml N NaOH, followed by 0.05 ml of 0.5% o-phthalaldehyde solution (0.5% w/v in methanol). The contents of the tube was mixed and the reaction was allowed to proceed at room temperature (21°C) for 60 min. before measuring the fluorescence intensity within the following 60 min.

Measurement of the histidine fluorescence. The fluorescence monochromator was set at 437 nm and the activation spectrum was recorded from 300 nm. The relative fluorescence intensity of each sample was measured at 375 nm (activation wavelength) and 437 nm (fluorescence wavelength), these being the maxima characteristic of the histidine fluorophor. The histidine content of a sample was, usually, obtained by reference to a calibration curve prepared from concurrent measurements of the fluorescence derived from 0.1, 0.2 and 0.3 µg histidine in aqueous solution.

When assaying extracts from tissue samples, an internal standard of 0.2 µg histidine was generally added to a portion of the sample. The observed increment in fluorescence measured that derived from the added standard and was used to calculate, from the fluorescence intensity of the sample alone, the amount

of histidine in the sample.

A measure of the reagent "blank" fluorescence (see Appendix, p. 267) was obtained by measuring of the fluorescence of 1.0 ml water treated with the reagents.

INVESTIGATION OF OPTIMUM EXPERIMENTAL CONDITIONS FOR THE MEASUREMENT OF HOMOCARNOSINE FLUORESCENCE.

Like histidine, homocarnosine reacts with o-phthalaldehyde only under alkaline conditions to form a fluorophor. The characteristics of the fluorescence, the rate of homocarnosine-o-phthalaldehyde reaction and the molecular yield of fluorescence were however, found to differ from those of histidine.

Fluorescence characteristics of the homocarnosine fluorophor. The wavelengths of maximum fluorescence of the homocarnosine-o-phthalaldehyde fluorophor were determined by recording the activation and fluorescence spectra (Fig. 9, p. 87). Maximum intensity of fluorescence was observed at 410 nm when the solution was activated by light of 340 nm.

Rate of formation of homocarnosine-o-phthalaldehyde fluorophor and its stability. Samples, 1.0 ml, containing different quantities of homocarnosine were treated with 0.2 ml N NaOH and 0.05 ml 0.5%

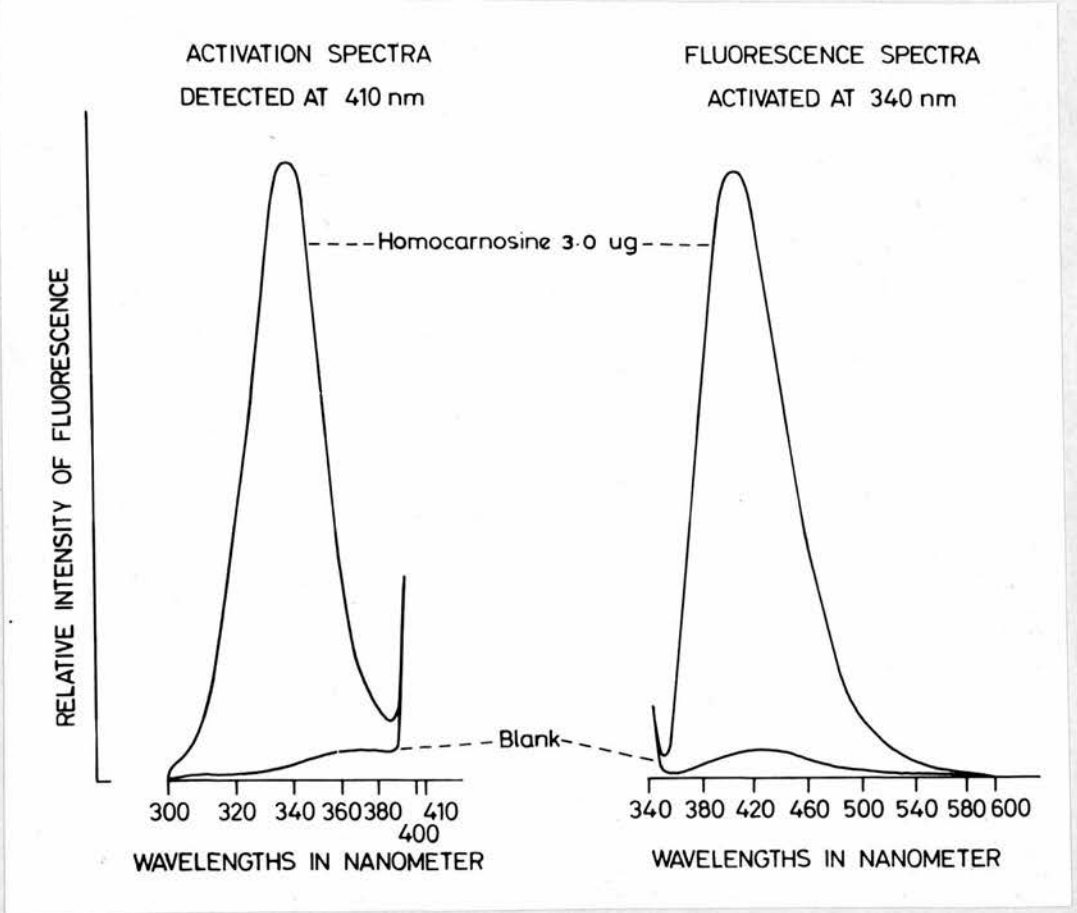


Fig 9. **Activation and fluorescence spectra of** the **homocarnosine-o-phthalaldehyde fluorophor.**

Peak fluorescence intensity at 340 nm (activation)and 410 nm (fluorescence). The spectra from the reagents alone ("blank" fluorescence) are also shown.

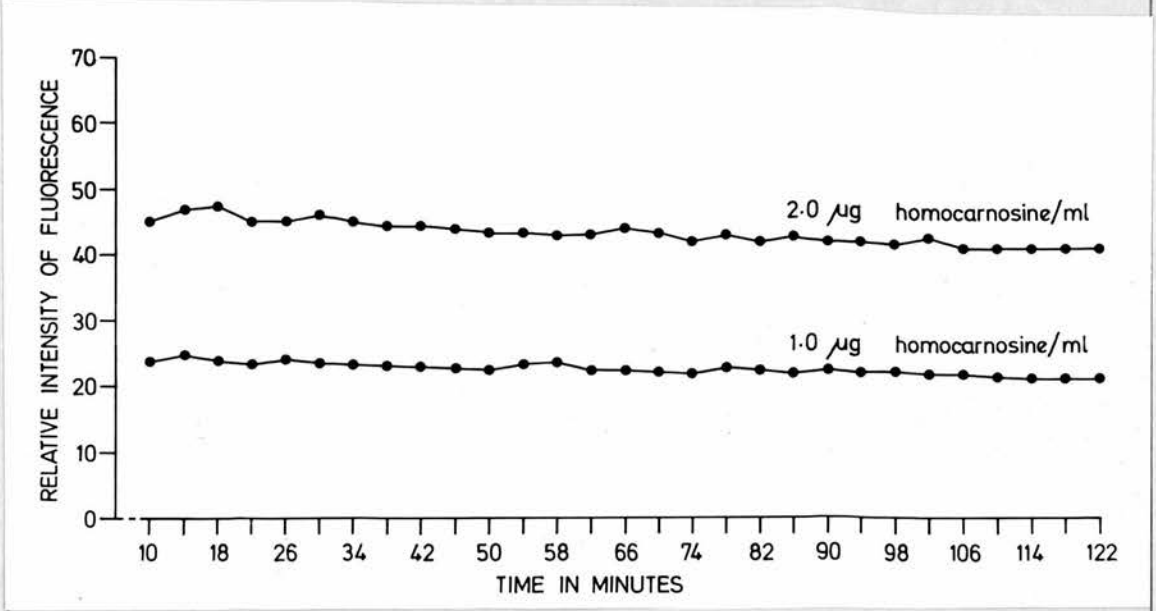


Fig 10. Rate of formation and stability of the homocarnosine-o-phthalaldehyde fluorophor.

Reaction mixture - homocarnosine solution (1 ml, concentrations shown on the graphs), 0.2 ml N NaOH and 0.05 ml 0.5% o-phthalaldehyde solution in methanol (w/v).

o-phthalaldehyde solution in methanol. The intensity of fluorescence of each sample was measured every 4th min. starting 10 min. after the addition of the aldehyde.

Fig. 10 (p. 88) shows that the fluorescence reached a maximinium within less than 10 min. and the fluorophor remains stable for at least 2 hr. after the addition of o-phthalaldehyde.

Relation between amount of homocarnosine and the fluorescence intensity of the fluorophor. As with the histidine fluorophor, the homocarnosine fluorescence show a good linear relationship when the dipeptide was reacted with 0.5% o-phthalaldehyde solution under alkaline conditions. This linear relationship between the amount of homocarnosine and the intensity of the fluorescence was observed over the range of concentration from 0.2 to 3.0 µg per ml (Fig. 11, p. 90 and Fig. 12, p. 91). The lower figure of the range was the limit of sensitivity of the method in pure solution since it gave a fluorescence intensity equal to that of the 'reagent' blank. Amounts in excess of the upper limit showed departure from linearity, a reading smaller than expected being obtained, possibly due to self-quenching.

Effect of pH of the reaction solution on the formation of homocarnosine-o-phthalaldehyde fluorophor.

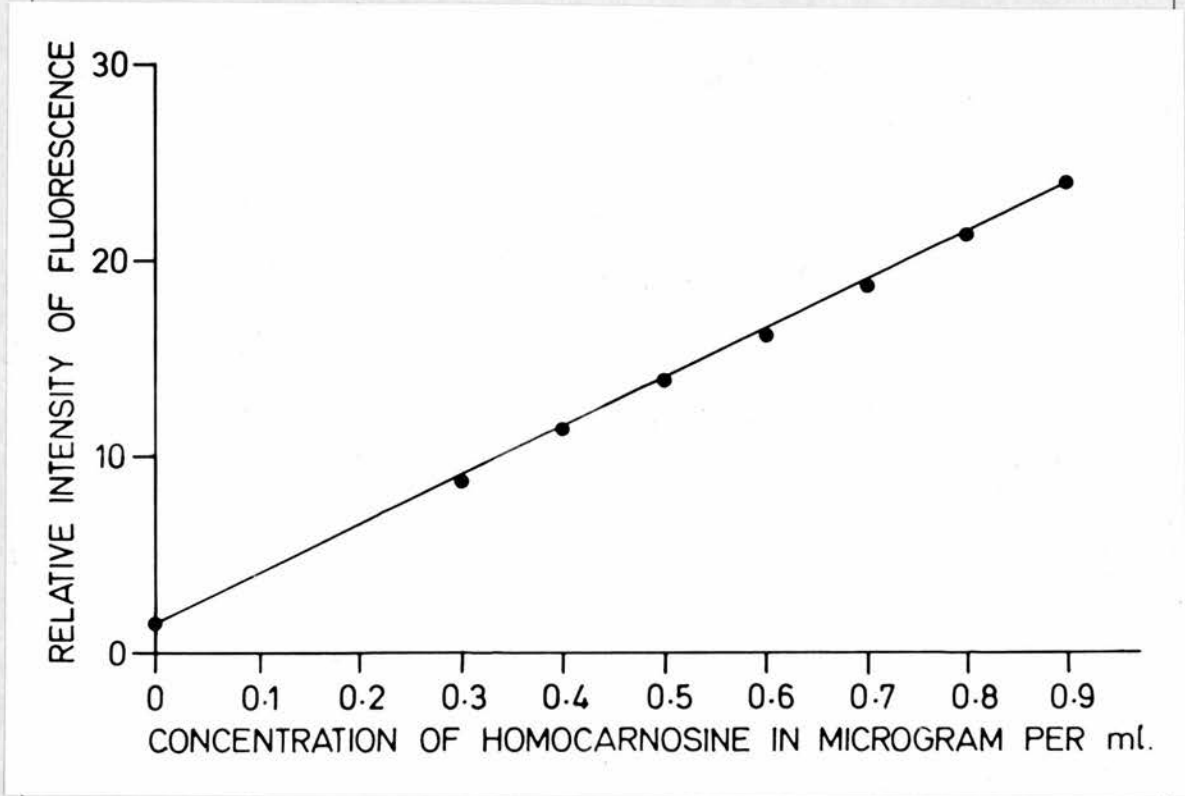


Fig 11. **Homocarnosine calibration curve for amounts less than 1.0 µg.**

Aqueous samples, 1 ml, containing known amounts of homocarnosine were allowed to react with 0.2 ml N NaOH and 0.05 ml 0.5% o-phthalaldehyde solution in methanol for 10 min at room temperature (21°C) before measuring the fluorescence at 340 nm (activation) and 410 nm (fluorescence).

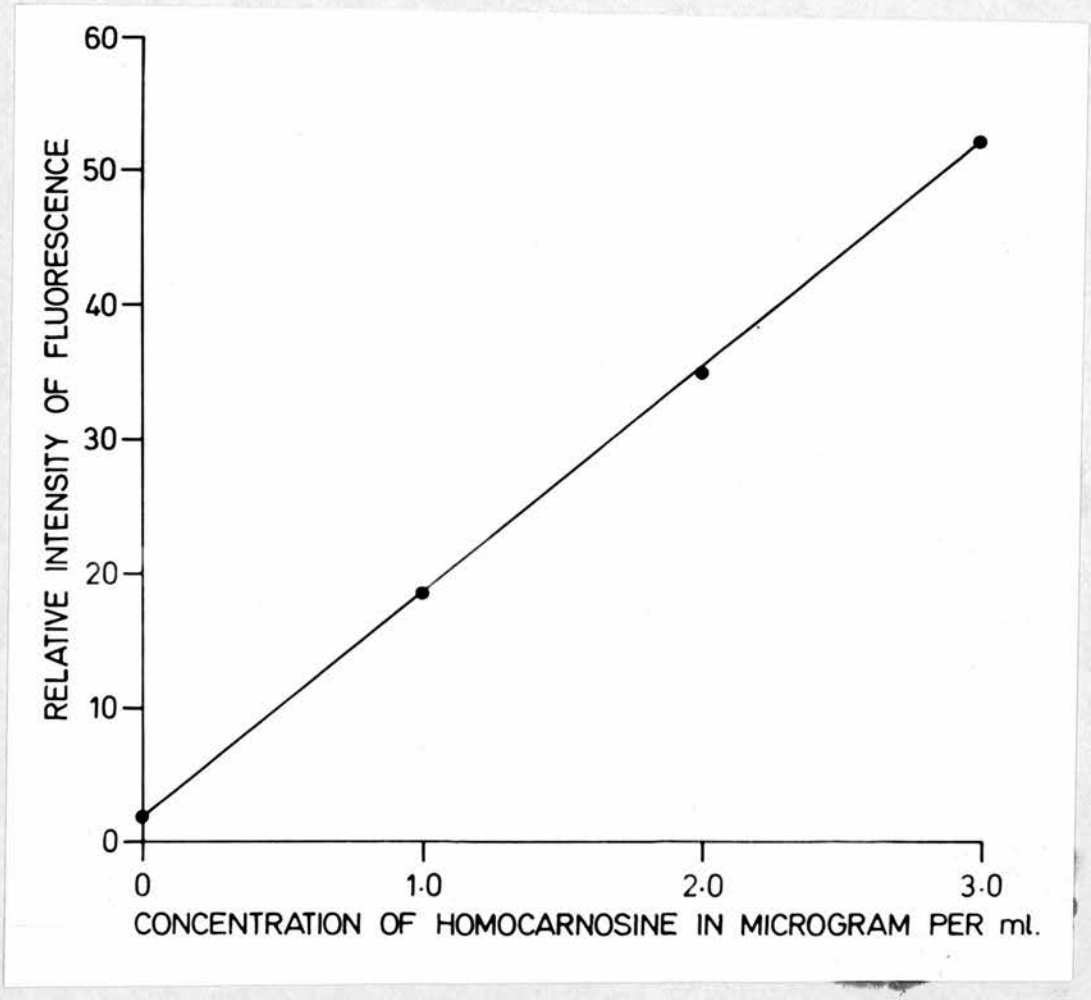


Fig 12. **Homocarnosine calibration curve for amounts greater than 0.9 µg.**

Aqueous samples, 1 ml, containing known amounts of homocarnosine were allowed to react with 0.05 ml 0.5% o-phthalaldehyde solution in methanol in the presence of 0.2 ml N NaOH for 10 min at room temperature (21°C) before measuring the fluorescence at 340 nm (activation) and 410 nm (fluorescence).

These experiments were carried out in a similar way to those described for histidine (p 82). Aqueous samples, 1.0 ml, containing 2.0 µg homocarnosine were treated with 0.2 ml of N HCl followed by 0.05 ml o-phthalaldehyde solution (0.5% w/v in methanol). In another experiment the samples were reacted with o-phthalaldehyde solution without pretreatment with alkali or acid. In neither experiment was a measurable amount of fluorescence obtained from the samples. These findings indicate that, like histidine, homocarnosine reacts with o-phthalaldehyde to give a fluorescent product only in alkaline solution.

Effect of acidification on the intensity of fluorescence of homocarnosine-o-phthalaldehyde fluorophor.

Samples containing 0.0 and 0.2 µg homocarnosine in 1.0 ml water were allowed to react with 0.05 ml o-phthalaldehyde solution (0.5% w/v in methanol) in presence of 0.2 ml N NaOH for 10 min before measuring the intensity of fluorescence of the fluorophor formed. The samples were then treated with 0.05 ml 6N HCl and the fluorescence of the samples were measured 3 min after acidification. The relative intensities of fluorescence of the sample containing 0.2 µg homocarnosine recorded before and after acidification were 45 and 5 while those of the blank were 3 and 6 respectively, indicating that

the fluorescence given by homocarnosine-o-phthalaldehyde reaction is not detectable in acid medium under the conditions of the experiment.

METHOD FOR THE FLUORIMETRIC ESTIMATION OF HOMOCARNOSINE FINALLY ADOPTED.

On the basis of the experimental findings detailed above, the following method was finally adopted for the fluorimetric estimation of homocarnosine.

Development of homocarnosine fluorophor. A 1.0 ml sample of the solution containing 0.2 to 3.0 µg homocarnosine was placed in a glass-stoppered 10 ml test-tube. To the sample was added 0.2 ml N NaOH followed by 0.05 ml of 0.5% o-phthalaldehyde solution in methanol. The contents of the tube were mixed and the reaction was allowed to proceed at room temperature (21°C) for at least 10 min before measuring the fluorescence intensity within the following 110 min.

Measurement of fluorescence. The fluorescence monochromator was set at 410 nm and the activation spectrum recorded from 300 nm. The fluorescence of the sample was measured at 340 nm (activation) and 410 nm (fluorescence), these wavelengths being the maxima characteristic of the homocarnosine fluorophor.

The homocarnosine content of a sample, as for

histidine content (p 85), was usually obtained by reference to a calibration curve prepared from concurrent measurements of the fluorescence derived from varying amounts of homocarnosine in aqueous solution.

When assaying extract from tissue samples, an internal standard of 1.0 µg was generally added to a portion of the sample. The observed increment in fluorescence measured that derived from the added standard and was used to calculate, from the fluorescence intensity of the sample alone, the amount of homocarnosine in the sample.

Reagent "blank" fluorescence. Fluorescence obtained from 1.0 of water similarly treated with reagents was a measure of the reagent "blank" fluorescence (see Appendix, p 267).

OTHER SUBSTANCES WHICH PRODUCE FLUORESCENCE UNDER THE EXPERIMENTAL CONDITIONS OF HISTIDINE AND HOMOCARNOSINE ESTIMATION.

A number of compounds have been reported to react with o-phthalaldehyde in alkaline media. These include arginine and agmatine (Cohn and Shore, 1961), 1-methylhistidine and N-acetylhistidine (Aures et al., 1968). Besides these, carnosine, which like homocarnosine contains an unsubstituted imidazole ring and a free NH_2 group on a side chain, and 3-methylhistidine which is an isomer of 1-methylhistidine were also tested for their ability to produce fluorescence with o-phthalaldehyde.

In addition, histamine and spermidine whose concentration in brain have been measured fluorimetrically (Shore et al., 1959; Kremzner and Pfeiffer, 1966; Kremzner, 1966; Shaw, 1968) following interaction with o-phthalaldehyde under conditions slightly different from those used in the present work, were also included. Of the amines known to occur in animal tissue adrenaline, noradrenaline, dopamine and 5-hydroxytryptamine were also tested under these conditions.

Procedure

Development of fluorescence. Samples, 1.0 ml, containing different quantities of one of the above substances in water were placed in glass-stoppered

Table 5

Production and characteristics of fluorescence derived from various compounds by interaction with o-phthalaldehyde in alkaline solution

Compounds	Fluorescence Characteristics		Fluorescence Maximum		Calculated quantity giving fluorescence intensity equal to that from 0.1 ug histidine	
	Activation Maximum nm	Fluorescence Maximum nm	Reached at min*	Maintained for min	at the characteristic wavelengths of the individual fluorophor µg	at the characteristic wavelengths of histidine fluorophor µg
Histidine	375	437	60	>60	0.1	0.1
Homocarnosine	340	410	<10	>110	1.0	18.0
Carnosine	340	410	20	>100	23.0	204.0
1-methylhistidine	-	-	-	-	>10.0	>10.0
3-methylhistidine	-	-	-	-	>10.0	>10.0
N-acetylhistidine	375	435	65	>65	230	230
Arginine	355	480	40	20	0.6	3.5
Agmatine	355	480	20	25	0.3	0.9
Histamine	365	430	90	>60	0.2	0.23
Spermidine	-	-	-	-	>25	>25
Adrenaline	360	445	60	90	200	225
Noradrenaline	-	-	-	-	>5	>5
Dopamine	-	-	-	-	>5	>5
5-HT	-	-	-	-	>5	>5

* After addition of o-phthalaldehyde solution

10 ml test-tubes. To each sample was added 0.2 ml n NaOH and 0.05 ml 0.5% o-phthalaldehyde solution (w/v, in methanol) and mixed.

Measurement of fluorescence. The reaction solution was examined for fluorescence at short intervals over a period of 120 min. or more starting 10 min. after the addition of o-phthalaldehyde. If fluorescence was detected, its characteristics were determined and readings of the intensity made at these wavelengths. The time required for the fluorescence intensity to reach a maximum was noted. After 60 min. reaction time the intensity of fluorescence was also determined at the wavelengths producing maximum fluorescence from the histidine fluorophor.

Results.

The observations made in these experiments are shown in Table 5, p. 96 . The rate of reaction of o-phthalaldehyde with the compounds tested and consequently the time taken by individual compounds to produce peak fluorescence and the stability of the fluorophors formed were found to vary. For comparison purposes the amount of each substance producing a fluorescence intensity the same as that from 0.1 µg of histidine under the same reaction conditions has been calculated and is cited together with the fluorescence characteristics of the derived fluorophor.

Two types of comparison have been made. In the first, the amounts of each substance yielding the same fluorescence intensity at the wavelengths characteristic of the fluorophor derived from that compound are compared; this gives a measure of the relative fluorescence yield from each substance and is, for example, indicative of the potential of the reaction under these specific experimental conditions to serve as a basis for the fluorimetric assay of the compounds. In the second, which indicates the potential of each substance to interfere with the assay of histidine if it were also present in a histidine-containing solution, the amount of each substance yielding the same intensity of fluorescence at the wavelengths characteristic of the histidine fluorophor are compared, the comparison being made after a reaction time of 60 min which is required for histidine to produce maximal fluorescence. In some instances the fluorescence derived from a compound had, in fact, decreased from its maximum at this time; compounds showing this phenomenon are indicated in the table of results (Table 5, p 96).

With the compounds tested, the homocarnosine-o-phthalaldehyde reaction was complete in the shortest time, less than 10 min, and the fluorophor formed remained stable for more than 120 min after the addition of o-phthalaldehyde; that between histamine and o-phthalaldehyde

took the longest time, about 90 min, and remained stable for at least one hour thereafter. In this connection it is interesting to note that Shore et al., (1959) in their method of histamine estimation only allowed the histamine-o-phthalaldehyde reaction to proceed for 4 min; according to present observations the fluorescence intensity continues to increase for 90 min and at 10 min it is less than half the maximum. Weight for weight, the fluorescence yield from homocarnosine was about one-tenth and that from histamine was about one-half of that from the same amount of histidine.

Carnosine reacted with o-phthalaldehyde to give a fluorescent product with characteristics the same as that from homocarnosine; the time taken for the fluorescence to attain its peak was longer some 20 min being required. The fluorescence yield was comparatively poor, about 10 µg being required to give a measurable fluorescence in the sample compared to 0.2 µg of homocarnosine.

Arginine and its decarboxylated product, agmatine, also reacted with o-phthalaldehyde to produce fluorescences which on a weight basis of the fluorophor precursor, were more intense than those from homocarnosine and carnosine but less than that from histidine. The time taken for arginine-o-phthalaldehyde fluorescence to reach maximum intensity was about 40 min and the

fluorophor formed remained stable for a further 20 min. agmatine-o-phthalaldehyde reaction was complete in a shorter time, 20 min, as observed by the maximum fluorescence attained and the fluorophor derived from the reaction was stable for 25 min.

The methyl derivatives of histidine, 1- and 3-methylhistidine, and the N-acetyl derivative of the amino acid in a concentration of 10 µg/ml did not give detectable fluorescence. N-acetylhistidine in a concentration of about 230 µg/ml gave a fluorescence of intensity equal to that derived from 0.1 µg histidine. This fluorescence was not due to the native fluorescence of the compound, as it was not present in the absence of o-phthalaldehyde.

Spermidine under these reaction conditions did not give rise to detectable fluorescence even in a concentration of 25 µg/ml. This is to be contrasted with the measurable fluorescence reported by Kremzner (1966) from a concentration as low as 1.0 µg/ml. That author reacted spermidine with o-phthalaldehyde in alkaline solution in the presence of ethylene diaminetetraacetic acid for 6.5 min before acidifying with phosphoric acid. The fluorophor showed maxima at 350 nm (activation) and 400 nm (fluorescence).

The other cerebral biogenic amines noradrenaline,

dopamine and 5-hydroxytryptamine did not produce measurable amounts of fluorescence in a concentration as high as 5.0 µg per ml. These amines were not examined in higher concentrations because the amounts tested were already considerably in excess of those to be expected (see for example Adam, 1968) to be obtained from 100 mg brain, the upper limit of the amount of tissue which it was planned to use for histidine assays. Adrenaline was also examined and, in a concentration of 5 µg/ml, was found to produce fluorescence of a very low intensity with maxima at approximately 360 nm (activation) and 445 nm (fluorescence). It was calculated that the amount of adrenaline which would give a fluorescence equal in intensity to that from 0.1 µg histidine would be of the order of 200 µg.

Discussion

Like histamine, histidine and homocarnosine reacted with o-phthalaldehyde in an alkaline medium to produce fluorescent derivatives. The nature of the derived fluorophors is not known; perhaps condensation products similar to that suggested for histamine by Shore *et al.*, (1959) (see p. 52) may be produced.

The rate of reaction between histidine and o-phthalaldehyde is much slower than that between homocarnosine and o-phthalaldehyde. As judged from the production of maximum fluorescence intensity it takes

about 60 min for the reaction between histidine and o-phthalaldehyde to be completed while that between homocarnosine and o-phthalaldehyde takes less than 10 min. Under the same experimental conditions the reaction between histamine and o-phthalaldehyde requires some 90 min for maximum production of the fluorophor.

After the completion of the reaction, the histidine-o-phthalaldehyde fluorophor thus formed remains stable in the alkaline medium for at least another 60 min and the homocarnosine-o-phthalaldehyde fluorophor is stable under the same conditions for not less than 2 hr after the addition of o-phthalaldehyde solution to the sample. The fluorescence derived from the interaction of histamine and o-phthalaldehyde remains constant in the alkaline reaction medium for at least 60 min after reaching its maximum. Using what appears from the present work to be a sub-optimal time of 4 min for the reaction, Shore et al., 1959 found that acidification of the medium at this time stabilised the fluorescence of the sample which then remained unchanged in intensity for 30 min thereafter.

Cohn and Shore (1961) observed that agmatine reacts with o-phthalaldehyde in alkaline media

and the fluorescence reaches a maximum in 10 min. The fluorophors derived from the reaction remain stable under alkaline conditions for another 15 min.

No explanation for these differences in the rate of reaction and stability of the various fluorescent derivatives can be offered at present. As Cohn and Shore, 1961 have implied it may be that in some cases the reaction stops at the formation of a simple Schiffs base type of compound while in others the initial reaction product undergoes a further but slower reaction.

On acidification of the reacted solution the intensity of histidine fluorescence is markedly reduced. Cohn and Shore (1961) observed that on changing the reaction of the medium from alkaline to acidic, the fluorescence of the agmatine-o-phthalaldehyde fluorophor is completely destroyed in contrast to that of the product derived from histamine.

Pisano et al., (1961) measured histidine in bovine brain hydrolysate by a fluorimetric method. Histidine present in the hydrolysate was allowed to react with o-phthalaldehyde in alkaline medium for 4 min at room temperature. Acid was added to the reaction solution and the fluorescence

was measured under acid conditions. Aliquots containing approximately 1.5 to 4.5 µg histidine were required for measurement. Ambrose et al., (1969) modified the Pisano method by using an elevated temperature (30°C) and a longer time (15 min) for the reaction of histidine with the o-phthalaldehyde in alkaline medium before acidifying and determining the fluorescence intensity. The minimum amount of histidine which could be measured was 1 µg.

The sensitivity of these two methods are much less than that of the present method. It would appear from the present work that this is because the reaction with the aldehyde was allowed to continue for only 4 min in alkaline medium (Pisano et al., 1961) or 15 min (Ambrose et al., 1969) and the measurement of the fluorescence was conducted in acid conditions in which the fluorescence intensity is much less than in alkali.

Our findings with histidine in the present work confirms the earlier observations of Aures *et al.*, (1968). They observed that histidine-o-phthalaldehyde fluorophor formation is favoured by an alkaline pH of the reaction solution. But we failed to confirm their findings with 1-methylhistidine and N-acetylhistidine. On reacting with o-phthalaldehyde in alkaline conditions, they obtained fluorescent derivatives from histidine, 1-methylhistidine and N-acetylhistidine. Fluorescence derived from these compounds showed their peaks at the same characteristic wavelengths i.e. at 450 nm for the fluorescence and 350 nm for activation. Unfortunately Aures *et al.*, (1968) do not give the concentrations of the histidine derivatives at which they observed this fluorescence production. I did not detect fluorescence from 1-methylhistidine, N-acetylhistidine and 3-methylhistidine in a concentration as high as 10 µg/ml although histidine gave a detectable fluorescence in a concentration of 0.02 µg/ml. Evidence was obtained that a detectable fluorescence could be produced from N-acetylhistidine but the concentration required was of the order of 40 µg/ml. In view of these findings it seems possible that Aures *et al.*, (1968) may have used relatively large amounts of these compounds in their experiments.

Adrenaline produced a just measurable amount of

fluorescence when the amount of the compound in 1.0 ml aqueous sample was raised to 5.0 µg, while dopamine, noradrenaline and 5-HT did not produce fluorescence even at that amount which, as already pointed out (p 100), is in large excess of that expected to be obtained from 100 mg of brain sample. It is of interest that reaction with o-phthalaldehyde forms the basis of a sensitive method of assay of 5HT and some of its biogenic derivatives and metabolites (Maickel and Miller, 1966) but fluorophor production takes place only on heating in strongly acidic solution.

Spermidine did not produce fluorescence in an amount as high as 25 µg in the 1.0 ml sample while its concentration in brain varies from 20 to 111 µg/g (Shaw, 1968). The values of the concentrations of 1- and 3-methyl-histidine and N-acetylhistidine in brain of various species have been given in Table 3 (p 68).

In the present work it was found carnosine produces measurable fluorescence with o-phthalaldehyde in alkaline medium when the concentration in the solution was 10 µg/ml. This could not be due to contamination with histidine because the fluorescence characteristics of the fluorophors differed. Aures et al., (1968) however failed to obtain fluorescence from carnosine under similar experimental conditions in the test-tube, but observed fluorescence originating

from carnosine when adsorbed on a thin-layer of cellulose-silica gel and sprayed with o-phthalaldehyde.

In the present work, ergothioneine (betaine of thiolhistidine) (p. 21) was unfortunately not available for testing its ability to produce fluorescence with o-phthalaldehyde under alkaline conditions. Since its amino ($-NH_2$) group in the side chain is not unsubstituted and three methyl groups ($-CH_3$) are attached to it, it would be expected (see below) to require an amount in large excess of that expected to be obtained from 100 mg brain sample, the normal level of the compound being from a trace to 10.0 µg/g (Crossland et al., 1966).

Like histidine, histamine reacts with o-phthalaldehyde under alkaline conditions (Shore et al., 1959; and present work) but the molecular yield of fluorescence is only about one-half of that of histidine (Table 5, p. 96). In contrast to the decrease in fluorescence from histidine which is observed if the reacted solution is then made acidic (Table 4 , p. 83) the fluorescence from histamine shows a marked increase (p. 84). If however, the reaction solution is acidified before the addition of o-phthalaldehyde histamine does not yield any fluorescence (Shore et al., 1959).

Spermidine would appear to behave similarly to

histamine. Under alkaline conditions after reaction with o-phthalaldehyde spermidine does not emit a detectable fluorescence in a concentration even as high as 25 µg/ml (Table 5, p. 96) but fluoresces when the reacted solution is made acidic (Kremzner, 1966; Kremzner and Pfiffer, 1966; Shaw, 1968).

From my observations, it appears that it is essential that the ionisation of NH_2 and/or NH group of the molecule be supressed for the reaction with o-phthalaldehyde to take place for the production of fluorescence. And for small amounts of imidazole compounds it is essential that the ring nitrogens should be unsubstituted and there should be at least one free NH_2 group in the side chain. According to Aures et al., (1968) o-phthalaldehyde reacts with all compounds having an amino group, but products capable of emitting fluorescence are formed only by a limited number of these substances, and then only under particular conditions.

A STUDY OF METHODS FOR THE SEPARATION OF HISTIDINE PRIOR TO FLUORIMETRIC ASSAY

The purification of histidine is an essential prerequisite for its fluorimetric assay. Substances in tissue extracts which may interfere with the assay include histamine (Shore *et al.*, 1959; present work), arginine and agmatine (Cohn and Shore, 1961; present work) and homocarnosine (present work).

SEPARATION OF HISTIDINE BY ION EXCHANGE CHROMATOGRAPHY

Model experiments based on the use of cation exchange resins were undertaken to study the separation of histidine from the interfering substances mentioned above and other amino acids. It was decided to study, first, the adsorption of histidine on ion exchange resin columns over a range of pH values and second, the adsorption of the other substances at selected pH values where their separation from histidine was likely to be greatest.

Experimental

Adsorption of histidine on cation exchange resin columns in relation to pH.

The object was to measure the adsorption of histidine on cation exchange resin columns equilibrated at different pH values, so that suitable parameters could be selected for the separation of histidine from other substances. The experiments were based on the following considerations. A typical amino acid contains at least two ionisable groups, an amino ($-NH_2$) group and a carboxyl (-COOH) group. When the amino ($-NH_2$) group is ionised the molecule behaves as a cation; when the carboxyl (-COOH) group is ionised it behaves as an anion. The net charge of the molecule whether positive or negative, depends on the ionisation of these two opposing groups. The pH at which the amino acid molecule behaves as an uncharged molecule is known as the iso-electric point. Since the pK_a values of the groups are different for different amino acids, their iso-electric points are also different (Table 6, p 111). At low pH values, the ionisation of the amino ($-NH_2$) group is favoured and that of the carboxyl (-COOH) is depressed; an amino acid therefore behaves as a cation at pH values lower than its iso-electric point. At a higher pH the opposite obtains and an amino acid

Table 6

The iso-electric points of amino acids commonly occurring in animal tissues (Biology Data Book, 1964; * Handbook of Chemistry and Physics 1970-1971).

Amino acid	Iso-electric points
1. L-Alanine	6.11
2. β-Alanine	6.90
3. Arginine	10.76
4. Asparagine	5.41
5. Aspartic acid	2.98
6. Carnosine	8.17
7. Citrulline	5.92
8. Cysteic acid	1.60
9. Cysteine	5.07
10. Cystine	5.02
11. 3, 5-Diiodotyrosine	4.29
12. Glutamic acid	3.22; 3.08*
13. Glutamine	5.65
14. Glycine	6.2; 6.06*
15. Histidine	7.64
16. Hydroxylysine	9.15
17. Hydroxyproline	5.82
18. Isoleucine	6.04
19. Leucine	6.04
20. Lysine	9.47
21. Methionine	5.74
22. Ornithine	9.70
23. Phenylalanine	5.91
24. Proline	6.3
25. Sarcosine	6.12
26. Serine	5.68
27. Tryptophan	5.88
28. Tyrosine	5.63
29. Valine	6.00

molecule then behaves as an anion. In selecting the range of pH values for these experiments two factors were taken into consideration; first, the ionisation of histidine and nature of the charge, and second, the ionisation of the weak cation exchange resin Amberlite CG-50. This particular type of cation exchanger was selected for study primarily because of its use in this laboratory in a procedure for the estimation of histamine in tissue extracts (Adam, 1961) and consequently a considerable body of knowledge of its properties was available.

There are three ionisable groups in the histidine molecule, an amino group ($-NH_2$ group on the side chain, pK_a value 9.17), an imino group (-NH group on the imidazole ring, pK_a value 6.00) and a carboxyl group (-COOH group, terminal group on the side chain pK_a value 1.82) (Meister, 1965). The iso-electric point is at a pH close to 7.6 (Meister, 1965; see also Table 6, p111). Hence pH values lower than 7.6 should favour the adsorption of histidine on the cation exchange resin column. However, the adsorption capacity of the cation exchange resin Amberlite CG-50 falls with the pH (Adam, Hardwick and Spencer, 1957) because of the weakly acidic nature of its functional groups. Thus at pH 4.0 the adsorption capacity of the resin is 0.5 mEq of cation/g of resin (dry weight), but at a pH below 3.5 the

resin is mostly unionised and so loses its exchange capacity (BDH Catalogue, ion exchange resins, fifth ed. p 12, 1965). The experiments to study the adsorption of histidine on to columns of the cation exchange resin were therefore carried out within the range of pH from 3.75 to 8.0.

Procedure

Preparation of the ion exchange columns. The columns were prepared with a mixture of the ion-exchange resin and cellulose powder. The object of adding cellulose powder was to produce a column of reasonable length from the relatively small amount of ion-exchange resin to ensure adequate ion-exchange facilities at a flow rate that could be readily controlled. It also had the advantage of reducing the possibilities of the passage of the fluid through the column being blocked due to shrinkage of the resins, especially when adjusted to a low pH value. To keep the conditions comparable cellulose powder was also used in the columns equilibrated at higher pH values.

500 mg of the ion-exchange resin Amberlite CG 50, Type 1, 100-200 mesh cleared of the fine particles (see Appendix p 281) and 200 mg of cellulose powder were mixed in a 25 ml conical flask. The volume of

NaOH solution required to convert a portion of the resin into Na^+ form and so adjust it to near the desired pH was calculated from the adsorption/pH curve of the resin XE-64 for Na^+ (Adam, et al., 1957) (Fig 13, p 115) as the resin CG-50 was chemically similar to the resin XE-64, and added to the resin-cellulose mixture in the flask. The volume of the fluid was made up to 5 ml with water. After the contents had been mixed by gentle shaking, the flasks were sealed with parafilm and kept at $4^{\circ}C$ overnight.

The column was prepared in a glass tube 14 to 15 cm long with an internal diameter of 0.8 cm surmounted by a glass bulb of 25 ml capacity and provided with a tap at the bottom (Fig 14, p 116). The tap of the chromatography tube was lightly greased with soft paraffin and a small pad of glass-wool was packed at the bottom of the tube. The tube was rinsed first with distilled deionised water and then with buffer solution. Seven ml of the buffer solution of appropriate pH was added to the resin-cellulose suspension. The contents of the flask were mixed well and poured into the bulb of the tube with the tap fully open. The resin-cellulose mixture was allowed to form a column and when the fluid level reached the top of the resin column, the tap was closed.

Equilibration of the ion-exchange column. The ion-

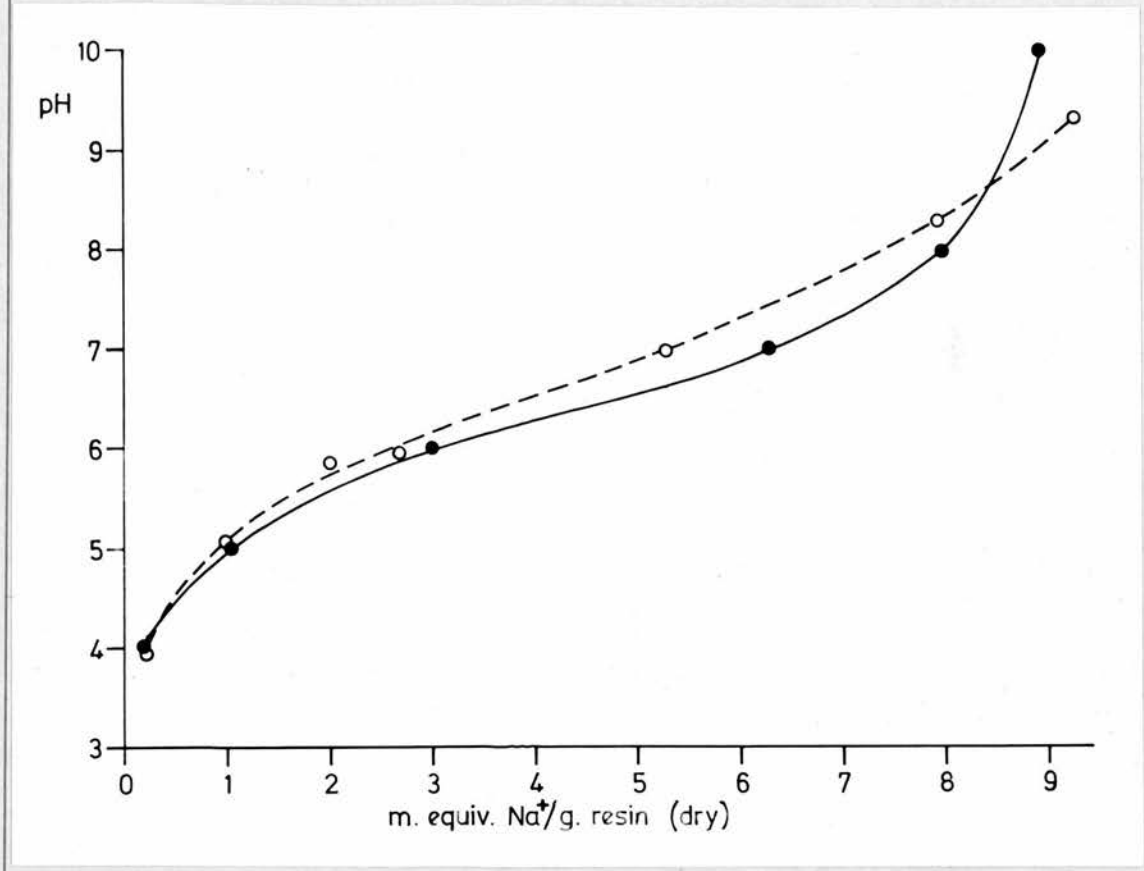


Fig 13. Adsorption/pH curves for sodium ion.

Adsorption on composite columns containing Amberlite XE-64 and cellulose (solid circles) and on columns of Amberlite IRC-50 (open circles) (reproduced from Adam et al., 1957).
The cation exchange resin CG-50 used in the present work is chemically similar to Amberlite XE-64.

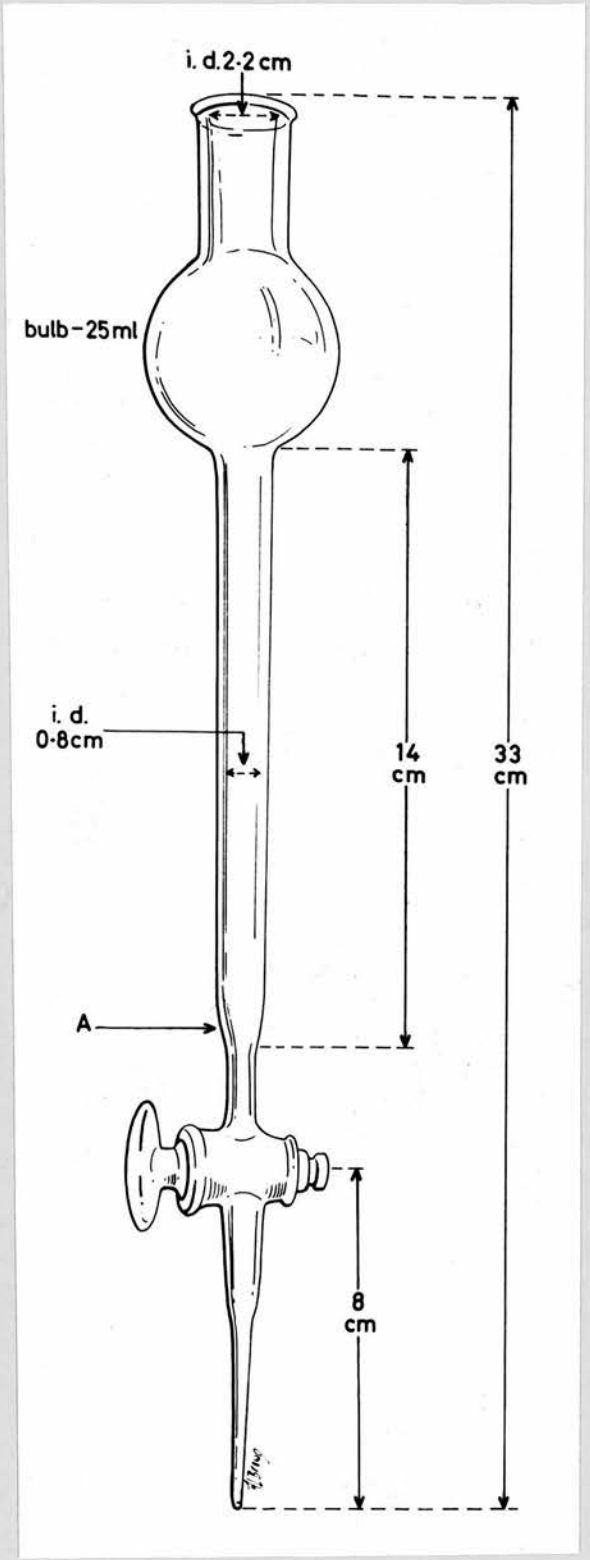


Fig 14. Glass column for chromatography (see text, p 114).

A - position of glass-wool plug.

exchange resin column was adjusted to the desired pH value by passage of a buffer solution of appropriate pH. The compositions of the buffer solutions used are shown in Table 7 , and detailed together with their preparation in Appendix 2 p 288. 20 ml of the buffer solution was applied to the column taking care to wash down the inner side of the bulb. The buffer solution was allowed to flow through the column at a rate of 0.3 ml per minute. After 1 hour, the pH of the effluent was tested (glass electrode) every 15 min. and additional buffer solution was allowed to percolate through the column until the pH of the effluent reached that of the influent ± 0.1 unit. After the column had been equilibrated, the tap was fully opened and the excess of the buffer was allowed to flow out rapidly. The column was then ready for use.

Preparation of solution for adsorption. Histidine stock solution (10 µg/ml), 0.5 ml, was placed in a glass-stoppered test-tube (capacity 50 ml) and 9.5 ml of buffer of the desired pH was added to it.

Adsorption on the column. The histidine solution was applied to a prepared resin-cellulose column, the test-tube being rinsed with 5.0 ml of the buffer solution which was added to the fluid above the column. A control column received 15 ml of the buffer solution.

The flow rate was adjusted to about 0.3 ml per minute. After the histidine solution had passed in, the column was washed with 5 ml of the same buffer. The effluent and wash fluid were collected in the same glass-stoppered test-tube.

Fluorimetric assay of histidine in the column effluent. Fluorescence produced by 1 ml of the effluent was measured as described before (p 85). The amount of histidine present in the aliquot was read, after deduction of the value of the "blank" (control column effluent) from the calibration curve obtained with histidine standard solutions immediately before measuring the fluorescence intensities of the sample.

Calculations. The total amount of histidine in the 15 ml of effluent was calculated and expressed as a percentage of the histidine applied to the column. The percentage of applied histidine retained by the column under the various conditions of hydrogen ion concentration during the chromatography was thus determined.

Results

The results are shown in Table 7 , p 119 , and a histogram depicting the relation of the percentage of

Table 7

Adsorption of histidine at various pH values on cation exchange resin (CG-50) columns composed of a mixture 500 mg resin and 200 mg cellulose powder. Amount of histidine applied to the columns was 5.0 µg. Buffers contained 100 mEq Na^+/litre.

pH	Volume of NaOH added for conversion of resin into Na^+ form	Buffers used for equilibration of resin and application of histidine to columns	Percentage of applied histidine recovered in effluent	Mean percentage recovery in effluent	Mean percentage of applied histidine adsorbed on column
3.75	2 ml of 0.1N NaOH	0.1 M acetate buffer	44 44 48	45	55
4.75	4 ml of 0.1N NaOH	0.2 M acetate buffer	Not detectable*	4	96
5.75	1.5 ml of N NaOH	0.1 M acetate buffer	Not detectable*	4	96
6.8	3 ml of N NaOH	0.066 M phosphate buffer	50 40 60	50	50
7.4	3.75 ml of N NaOH	0.055 M phosphate buffer	90 79	85	15
8.0	4 ml of N NaOH	0.05 M phosphate buffer	112 80 96	96	4

* Histidine content less than 0.01 µg/ml effluent.

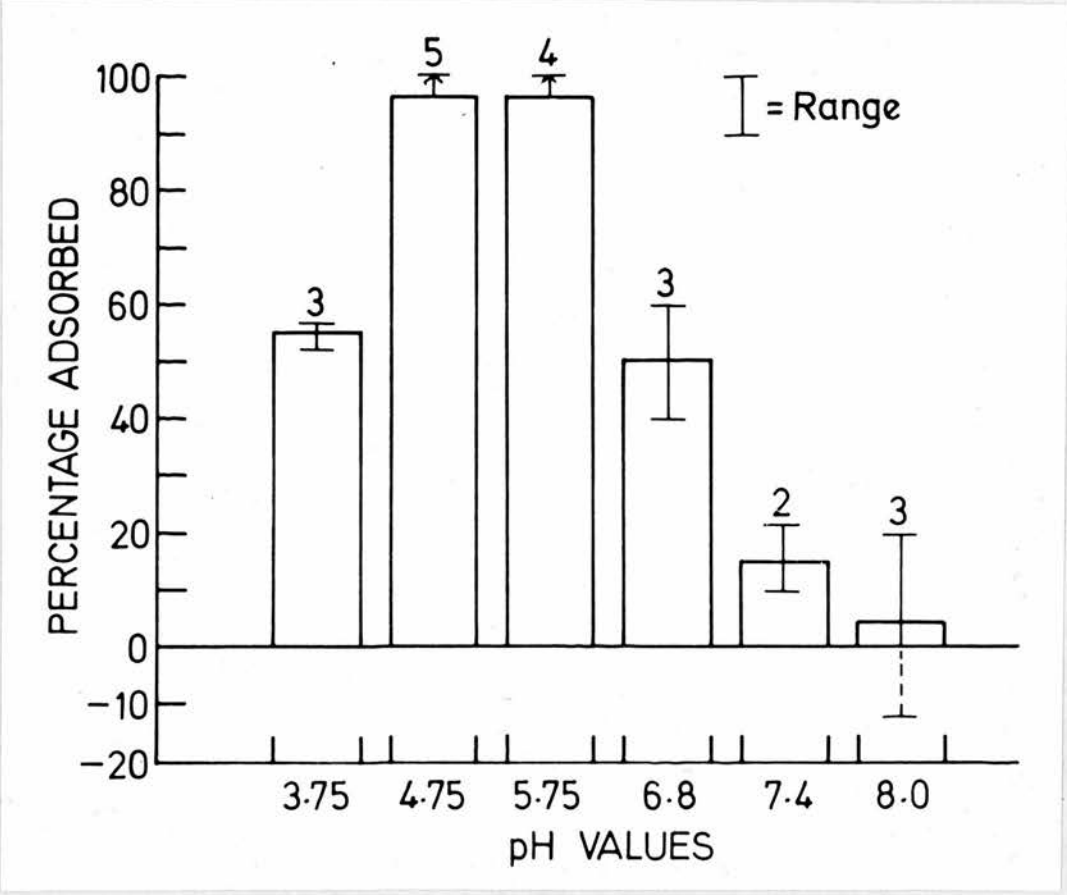


Fig 15. **Adsorption/pH histogram for histidine on cation exchange resin (Amberlite CG-50) - cellulose columns (for buffers used see Table 7 , p 119). I = range of values of observations; number of observations indicated at top.**

histidine adsorbed to the pH of the ion-exchange column and buffer solution appears in Fig 15, p 120. No histidine was detectable in the effluent from ion-exchange columns buffered to pH 4.75 and pH 5.75 suggesting that complete adsorption of the amino acid had occurred at these pH values. Lowering the pH value to 3.75 or raising it towards pH 8.0 resulted in decreasing adsorption; at pH 8.0 there was no significant removal of the histidine from the solution passing through the column.

OBSERVATIONS ON THE EXTENT OF PURIFICATION OF HISTIDINE BY THE USE OF A COMPOSITE COLUMN OF AMBERLITE CG-50 ION EXCHANGE RESIN AND CELLULOSE POWDER AT pH 8.0

Histamine is almost completely adsorbed on a column of cation exchange resin, Amberlite CG-50, at pH 8.0 (Adam *et al.*, 1957; Adam, 1961; Adam and Hye, 1966). Using this fact these workers employed columns composed of 50 mg Amberlite CG-50 admixed with 300 mg cellulose and buffered to pH 8 to purify histamine in tissue extracts prior to its assay. The adsorbed histamine was eluted off and assayed biologically.

The adsorption/pH histogram for histidine obtained in the present work, (Fig 15, p 120) shows that

particularly all the histidine applied to a CG-50 column at pH 8.0 appears in the effluent. This finding confirmed previous observations made by Abou (1968). He applied 20 to 400 ug of histidine at pH 8 to resin columns of the composition used for the adsorption and subsequent elution and estimation of histamine by Adam and his co-workers (see above) and was able to recover in the effluent 90%, on average, of the histidine applied to the column. These observations suggest that use of such a column could separate histidine from histamine and thus enable the estimation of histidine and histamine in the same tissue sample.

To be able to use the column, as described above, for the purification of histidine for its fluorimetric assay, it was essential to know the effectiveness of this column for the separation of histidine from interfering substances. Experiments were therefore performed to test the effectiveness of the column for the separation of histidine from homocarnosine, arginine and agmatine, compounds which were found to produce fluorescence to various degrees under the same experimental conditions as those used for the production of the histidine fluorophor.

Since the biogenic amines commonly occurring in the animal tissues would be mainly in the cationic

form (see Table 8, p 124) the amines therefore would be to a significant extent adsorbed on the cation exchange resin CG-50-cellulose column adjusted to pH 8.0.

The column, however, would not be expected to separate histidine from acidic and neutral amino acids because their iso-electric points are lower than pH 8.0 (Table 6, p 111).

Procedure.

Preparation of the ion exchange column. The column of the absorbing material was prepared with 50 mg resin and 300 mg cellulose powder the mixture being treated overnight with 4.0 ml of 0.1 M-NaOH solution for the conversion of a portion of the resin into Na^+ form in order to bring its pH close to 8.0 (Hye, 1964). The columns were prepared as described on p 113 .

Equilibration of the column. The column was adjusted to pH 8.0 by the passage of 20 ml of the buffer solution (0.05 M phosphate buffer, pH 8.0, 100 mEq Na^+/litre) through the column at 0.3 ml/minute. It took about 1 hour for the column to be equilibrated in this way as shown by the unchanging pH (glass electrode) of the buffer solution issuing from the column on passage of further amounts of the buffer.

Table 8

pK values, and the degree of ionisation at pH 8.0, of a number of biogenic amines.

Compounds	pK_1 (OH)	pK_2 (NH or NH_2)	Ionisation at pH 8.0 Percentage* as			
			Cation	Anion	Zwitterion	Base
Dopamine	8.9	10.63	88.8	0.1	11.2	-
Noradrenaline	8.82	9.78	86.6	0.2	13.1	-
Adrenaline	8.8	9.9	86.2	0.2	13.65	
5-HT	11.1	9.8	99.9	0.1	0.1	
Tyramine	9.65	10.78	97.8	0.1	2.2	-
Tryptamine**	-	10.2	99.4	-	-	0.6
Histamine**	5.78 (imidazole NH)	9.76	98.3	-	-	1.7

* Calculated from pK values of the amine

** Quoted from 'Data for Biochemical Research' (1969); other pK values from Lewis (1954).

Adsorption on the ion-exchange column. The adsorption of histidine, arginine, agmatine and homocarnosine on such prepared columns were studied in the following way. A known quantity of one of the substances in 8.0 ml of 0.05 M phosphate buffer, pH 8.0, 100 mEq Na^+/litre, was applied to a column. The test-tube which had contained the solution was rinsed with 2.0 ml of the same buffer solution and this was also applied to the column. For subsequent determination of the 'reagent blank', 10 ml of the buffer solution (0.05 M phosphate buffer, pH 8.0, 100 mEq Na^+/l) containing no additive was placed on a control column. The flow rate was maintained at 0.3 ml per min and after all the solution had passed through, the column was washed with 5 ml of the above buffer solution at the same flow rate. The effluent and the wash fluid were collected in one test-tube. The total volume was 15 ml.

Estimation of amounts of the substances in effluent

Estimation of histidine. The amount of histidine present in a 1.00 ml aliquot of the effluent was measured fluorimetrically as described before (p 85).

Estimation of homocarnosine. A 1.00 aliquot of the

effluent was used for the fluorimetric measurement as described before (p 93) and the amount of homocarnosine present in the aliquot was read from a calibration curve obtained with standard homocarnosine solutions immediately before sample measurements were made.

Estimation of agmatine. Agmatine present in the effluent was measured fluorimetrically by the method of Cohn and Shore (1961). A 2.00 ml portion of the effluent was placed in a 10 ml glass-stoppered test-tube and followed by 0.4 ml of 1 N NaOH. o-Phthalaldehyde reagent (0.1 ml of a 10 mg/ml solution in methanol prepared fresh just before use) was added to the tube, and mixed thoroughly. The reaction was allowed to proceed for 20 min at room temperature and the fluorescence of the mixture was measured at 355 nm (peak activation wavelength) and 480 nm (peak fluorescence wavelength), uncorrected. Cohn and Shore (1961) quote 355 nm (activation peak) 470 nm (fluorescence peak) for the characteristics of the fluorophor from agmatine.

Fluorescence obtained from the effluent was as low as that from the effluent of the 'reagent blank', suggesting that a measurable amount of agmatine was not present.

Estimation of Arginine. Arginine was measured in the

Table 9

Adsorption of histidine, agmatine, arginine and homocarnosine on Amberlite CG-50-cellulose columns at pH 8.0.

Compounds	Amount applied to column	Recovery in effluent		Percentage adsorbed
	µg	µg	percentage	(mean)
Histidine	3.0	3.1	103	<4
	3.0	2.8	93	
	3.0	3.0	100	
	3.0	3.0	100	
Agmatine	5.0	n.d.*	9	>91
	10.0	n.d.	5	
Arginine	15.0	n.d.	10	>90
	30.0	n.d.	5	
Homocarnosine	30.0	26.25	87.5	14
	30.0	25.50	85.0	

*n.d., not detectable (see p 72)
Agmatine, less than 0.45 µg in total effluent
Arginine, less than 1.5 µg in total effluent.

same way as described for agmatine (Cohn and Shore, cited by Udenfriend, 1962).

A detectable amount of arginine was not found in the column effluent.

Results. (Table 9 , p 127).

Nearly all the histidine applied to the column was recovered in the effluent, confirming the results of earlier experiments (p 118). Arginine (iso-electric point 10.76) and agmatine were completely adsorbed at pH 8.0. A major portion of the homocarnosine applied to the column was present in the effluent. This shows that, although the column operated at pH 8.0 could separate histidine from arginine and agmatine, it would fail to separate it from homocarnosine.

MODEL EXPERIMENTS TO DETERMINE SUITABLE OPERATING CONDITIONS FOR THE PURIFICATION OF HISTIDINE USING A WEAK CATION EXCHANGE RESIN BUFFERED TO pH 5.75

From the iso-electric points of the amino acid (Table 6, p 111) it appears that the acidic and the basic amino acids which are anions at pH 8.0 would be present, along with histidine and homocarnosine, in the effluent of the composite CG-50 ion-exchange column operated at pH 8.0.

It was, therefore, necessary to find and standardise a condition where histidine would be

separated from all or most of these substances.

The adsorption/pH histogram for histidine (Fig 15, p 120) shows that histidine is almost completely adsorbed on a CG-50 ion-exchange column in the range of pH 4.75 and 5.75. Since all the acidic amino acids would be anions and a larger number of the neutral amino acids would be either anions or less extensively ionised as cations at pH 5.75 than at any other pH in the range, it was considered desirable to devise a cation exchange resin column of suitable capacity for operation at pH 5.75, which could be used in the method for the purification of histidine for its fluorimetric assay.

Procedure

Preparation of the columns. Columns were prepared with varying quantities of resin and cellulose powder. For conversion of the resin partly to Na^+ form the amount of alkali necessary (see Table 10, p 132) was calculated from adsorption/pH curve of the cation exchange resin XE-64 for Na^+ (Fig 13, p 115).

Adsorption of histidine on the column. As already indicated (p 121) a method developed in this laboratory for the estimation of histamine in tissue extracts involves the adsorption of the amine onto a

composite Amberlite CG-50-cellulose column at pH 8.0. It has been demonstrated (p 121) that histidine is not adsorbed under these conditions and appears in the column effluent. Thus the histamine and its amino acid precursor could be separated and estimation of both in the same sample of tissue seemed possible.

In practice in the methodology of histamine estimation (Adam, 1961) the effluent volume from the adsorption column is 15 ml. Therefore in the experiments presently under discussion a known amount of histidine in a volume of 0.1-0.2 ml aqueous solution was diluted to 15.0 ml with the buffer solution (0.1 M acetate buffer, pH 5.75, 90 mEq Na^+/litre) in a 50 ml test-tube and the mixture applied to the equilibrated ion-exchange column. The tube was rinsed with 5 ml of the same buffer solution and this too passed through the column. A control column received 20 ml of the same buffer solution containing no histidine. The flow rate was maintained at 0.3 ml per min by adjusting the tap. After the solution had passed through, the column was washed with 5 ml of the buffer solution.

Elution of adsorbed histidine. Histidine adsorbed on the column was eluted with 0.25 N HCl. The amount

of acid employed (Table 10, p 132) was a little more than the total adsorption capacity of the column (calculated from adsorption/pH curve for Na^+, see Fig 13, p115).

During elution the flow rate was reduced to 0.2 ml per min. Elution of the column was continued with a known volume of water (Table 10, p 132). The total eluate was collected as a single sample for subsequent analysis.

Results

Histidine recovered in the eluate was measured fluorimetrically (p 85) and expressed as a percentage of histidine applied to the column. The recovery of histidine in the eluate from a column composed of 300 mg of the cation exchange resin CG-50 and 200 mg of cellulose powder buffered to pH 5.75 was, on the average, 93%. The percentage recovery of histidine was 73% from a column composed of 200 mg of the resin and 150 mg of cellulose powder, and 38% from a column composed of 135 mg resin and 220 mg cellulose powder.

During adsorption, a total of 25 ml of 0.1 M acetate buffer, pH 5.75, 90 mEq/Na^+/l (15 ml histidine solution for adsorption + 5 ml wash of the tube which had contained the solution + 5 ml wash of the column) passed through the column. The low recoveries of

Table 10

Adsorption of histidine on Amberlite CG-50 ion-exchange columns of different sizes buffered at pH 5.75

Composition of the columns	Total cation adsorption capacity mEq Na^+	Vol. of 0.1N NaOH added to adjust resin pH ml	Vol. of buffer to equilibrate columns ml	Vol. of 0.25N HCl applied for elution ml	Vol. of water applied to wash column after elution ml	Total vol. of eluate ml	Quantity of histidine applied to columns µg	Percentage of histidine recovered in eluate	Mean Percentage recovery in eluate
*135 mg resin + 220 mg cellulose powder	0.4	4.0	25	2	3	5	1.0	40 35 40	38.3
200 mg resin + 150 mg cellulose powder	0.6	6.0	40	3	5	8	2.0	72 72 65 72 83	72.8
300 mg resin + 200 mg cellulose powder	0.9	9.0	55	4	6	10	2.0	92 81 110 92 90	93

* Adsorption capacity of 135 mg Amberlite CG-50 at pH 5.75 is about the same as that of 50 mg of the resin at pH 8.0.

histidine from the two smaller columns could be due to eluting effects of the comparatively large volume of the buffer solution used at the stage of adsorption.

Adsorption of homocarnosine on a ion-exchange resin (CG-50) column at pH 5.75.

The results presented in the Table 10, p 132, show that the recovery of histidine was satisfactory with a column composed of 300 mg of CG-50, and 200 mg of cellulose powder.

Of the substances which produce fluorescence under the same experimental conditions as histidine, arginine and agmatine would be separated from histidine by the column operated at pH 8.0. Since homocarnosine behaved similarly to histidine on the ion-exchange resin columns at pH 8.0, it was essential to study the adsorption of homocarnosine on the CG-50 column at pH 5.75 at which pH histidine is adsorbed.

MODEL EXPERIMENTS TO STUDY THE ADSORPTION CHARACTERISTICS OF HOMOCARNOSINE ON CATION EXCHANGE RESIN CG-50-CELLULOSE COLUMN ADJUSTED TO pH 5.75

Procedure.

Columns were prepared with 300 mg of the resin and 200 mg of cellulose powder, and equilibrated with 0.1 M acetate buffer pH 5.75, 90 mEq Na^+/1 as described before (see also appropriate data in Table 10, p 132). Homocarnosine, 15 µg, was added to 15 ml of 0.1 M acetate buffer, pH 5.75, 90 mEq Na^+/1, and the solution applied to the column. The test-tube was washed with 5 ml of the same buffer solution and this was also applied to the column. Adsorption and subsequent elution with 4 ml of 0.25 N HCl followed by 6 ml of water were carried out as in the experiments with histidine.

Homocarnosine present in the eluate was measured fluorimetrically as described before (p 93).

Results.

In two experiments it was found that 100% and 103% of the 15 µg homocarnosine applied to a column was present in the acid eluate. Homocarnosine thus behaved in a manner similar to histidine under the operating conditions of chromatography.

Discussion

The adsorption characteristics of histidine on

the weak cation exchange resin CG-50 at different pH values suggest that histidine could be separated from most of the substances interfering with its fluorimetric estimation by the use of suitable column chromatography. On the basis of these observations, experiments were carried out with columns equilibrated at pH 8.0 and pH 5.75, the results of which showed that separation of histidine from arginine and agmatine could be effected on an ion-exchange resin column at pH 8.0 at which pH no adsorption of histidine occurs. This finding indicates at this pH histidine would also be separated from histamine which is adsorbed on the weak cation exchange resin at pH 8.0 (Adam, 1961). At pH 5.75, histidine is adsorbed on the cation exchange resin CG-50 and thus would be separated from acidic and many neutral amino acids.

The results of the column chromatography experiments done at pH 8.0 and 5.75 further showed that the adsorption characteristics of homocarnosine closely resembled those of histidine. Thus it was not possible to separate histidine from homocarnosine by the use of ion-exchange resin columns, especially when small quantities were involved.

Pisano *et al.*, (1961) separated histidine from homocarnosine by fractional elution from an 0.9 x 100 cm column of Dowex 50- x 4 (R- SO_3 H) at pH 4.7. Abraham

et al., (1962) obtained separation between histidine and homocarnosine by fractional elution with 0.1 M lutidine from a 1.2 x 15 cm Dowex 50-x4 - 2, 6, - lutidine column. These columns would be suitable when larger quantities were dealt with.

According to Pisano et al., (1961) the iso-electric point of homocarnosine lies between pH 9 and 10, which suggests that homocarnosine would be adsorbed on a cation exchange resin column at pH 8.0. As the major part of homocarnosine applied to the column at pH 8 was found in the present experiments to pass into the effluent, it appears that the iso-electric point of homocarnosine may be at a pH value closer to pH 8.0.

The adsorption/pH histogram for histidine (Fig 15, p 120) is consistent with the ampholytic nature of histidine and the ionisation characteristics of the resin. Poor adsorption of histidine at pH 3.75 shows the lower exchange capacity of the resin at this pH. This finding confirms the earlier observation of Adam et al., (1957) who obtained results explicable in terms of a depression of the ionisation of the functional carboxylic acid (-COOH) groups of the ion-exchange resin at lower pH values while studying the adsorption of Na^+ by cation exchange resins. Complete adsorption of histidine at pH values between 4.75 and

5.75 shows that both histidine and the resin were sufficiently ionised. At pH values above 5.75 the cationic nature of histidine was gradually suppressed resulting in poorer adsorption. No uptake of histidine by the ion exchange resin at pH 8.0 suggests that histidine was, perhaps, present in the solution as an anion at that pH (iso-electric point of histidine 7.6). The low recovery of histidine from the two smaller columns operated at pH 5.75 could be due to an eluting effect of the buffer itself.

The amounts of histidine and homocarnosine employed in this phase of the investigations were so chosen that their concentrations in the effluent or eluate from the ion-exchange resin columns could be easily detected and conveniently measured.

DESALTING OF HISTIDINE SOLUTIONS

The results of the column chromatographic experiments showed that histidine could be partially purified by this technique. Under the experimental conditions employed, histidine and homocarnosine behaved alike on the cation exchange resin columns both at pH 8.0 and 5.75. It appeared likely that, even with modifications of the experimental conditions, the separation of histidine from homocarnosine would not be satisfactory on the ion exchange resin columns especially when very small quantities were involved. This led to a consideration of other methods such as thin-layer chromatography and paper chromatography for the separation of histidine and homocarnosine in the eluates from the ion exchange columns.

Since the total adsorption capacity of the column (composed of 300 mg resin and 200 mg cellulose powder) equilibrated at pH 5.75 is 0.9 mEq (as calculated from adsorption/pH curve of the cation exchange resin XE- 64 for Na^+ (Fig 13, p 115), the same quantity of Na^+, as sodium chloride, was expected to be present in the eluate. To make the sample suitable for application to a thin-layer plate or chromatography paper, it was

necessary to remove the sodium chloride from the eluate.

In planning model experiments, three points were taken into consideration:- (i) removal of sodium chloride, (ii) recovery of histidine and (iii) simplicity of the technique.

The possibility of adequate removal of the salt from an acidified aqueous solution containing histidine and sodium chloride by the addition of an organic solvent such as ethanol or acetone in which the salt is poorly soluble, was investigated.

Comparative study on the effectiveness of ethyl alcohol and acetone for the separation of histidine from sodium chloride in acidified aqueous solution

Procedure. 1.0 ug histidine in 1 ml water and 1 ml of sodium chloride stock solution containing 0.9 mEq Na^+ (52.6 mg sodium chloride) were placed in a glass stoppered test-tube of 50 ml capacity. The volume of the sample was made 10 ml by adding deionised distilled water, as this would likely simulate the volume of the eluate from the cation exchange resin column equilibrated at pH 5.75.

For estimation of reagent blanks of the analysis methods 10 ml of distilled deionised water was run in parallel with sample solutions.

Samples were evaporated to dryness at 55°C in a water bath under reduced pressure (20 to 30 mm Hg). The pressure was gradually increased while the residue was still moist in order to prevent possible losses of the dried residue by spurting when the vacuum was reduced. The dried residue in each tube was taken up in 0,5 ml 6 N HCl, care being taken to wet all parts of the inside of the tube with the extractant.

Precipitation of sodium chloride with ethanol.

To the mixture in the test-tube was added 4 ml of ethanol which had been twice distilled, and the solution mixed by gentle shaking. Solid material adhering to the wall of the tube was detached with a glass rod. The insoluble material was allowed to settle for some time by gravity and then the supernatant fluid transferred to a 10 ml conical centrifuge tube with the aid of a Pasteur pipette. The tube was sealed with parafilm and centrifuged at 2000 r.p.m. for 30 minutes. The clear supernatant fluid was transferred, very carefully so as not to disturb the precipitate,

to a 50 ml glass-stoppered test-tube with the aid of a Pasteur pipette. Some 0.2 to 0.3 ml of supernatant was left over the surface of the small amount of precipitate to minimise any possible transfer of salt. The original precipitate in the 50 ml test-tube was re-extracted by shaking with a further 4 ml portion of ethanol. After the insoluble matter had settled, the supernatant was transferred to the same 10 ml centrifuge tube which had contained the first extract. After centrifugation as before, the supernatant was added to the first extract, taking the same precautions in the transfer as mentioned above.

The ethanolic extract was evaporated to dryness in a water bath at 50°C under a reduced pressure of 20 to 30 mm Hg. The tube containing the extract was connected to a water pump through an adapter; in order to avoid loss by sudden bumping of the solution, the contents of the tube was kept continuously in motion by gently rotating the tube by hand. The dried residue was taken up in 5.0 ml water, and the content of histidine and sodium chloride in the solution estimated.

Precipitation of the sodium chloride with acetone.

The method employed was essentially similar

to that used when ethanol was the precipitant, with the following modifications.

A volume of 10 ml of the solvent was added in the first precipitation and one of 5 ml was used in the second extraction. The combined extracts were evaporated to dryness under reduced pressure (20 to 30 mm Hg) first at room temperature to remove the acetone and then at 50°C in a water bath to remove the remaining aqueous acid.

Estimation of histidine and sodium chloride in the final extract.

Histidine was estimated fluorimetrically by the method described on p 85 . The amount of sodium chloride in the extract was calculated from the Na^+ content measured by flame photometry using an EEL Model A flame photometer.

Results.

From the results of these experiments shown in Table 11 (p 143) it is evident that, under these operating conditions, precipitation by acetone proved more effective than that by alcohol in removing the salt from the histidine-sodium chloride solution. On average, less than 2% of

Table 11

Recovery of histidine and its separation from sodium chloride by precipitation of the latter with ethanol or acetone.

Experiment No	Composition of histidine-sodium chloride mixture		Histidine recovered in extract	Percentage of histidine recovered	Na^+ present in extract	Sodium chloride present in extract
	Histidine µg	NaCl mg	µg		µEq	mg
Ethanol as precipitant						
1	-	52.6	-	-	70	4.1
2	-	52.6	-	-	90	5.22
3	-	52.6	-	-	95	5.51
4	1.0	-	0.95	95	-	-
5	1.0	-	0.85	85	-	-
6	1.0	-	1.0	100	-	-
7	1.0	52.6	1.15	115	75	4.38
8	1.0	52.6	0.76	76	110	6.38
9	1.0	52.6	0.72	72	90	5.22
10	1.0	52.6	0.83	83	100	5.84
				Mean = 89% (range 72 - 115%)		Mean = 5.23
Acetone as precipitant						
11	-	52.6	-	-	15.0	0.87
12	-	52.6	-	-	13.12	0.76
13	-	52.6	-	-	11.80	0.62
14	-	52.6	-	-	16.87	0.97
15	1.0	-	0.90	90	-	-
16	1.0	-	0.80	80	-	-
17	1.0	52.6	0.75	75	11.80	0.68
18	1.0	52.6	0.82	82	44.37	2.57
19	1.0	52.6	0.95	95	11.80	0.68
20	1.0	52.6	0.98	98	13.12	0.76
				Mean = 87% (range 75 - 98%)		Mean = 1.0

the added sodium chloride appeared in the final extract when acetone was used as precipitant whereas some 10% was present when ethanol was employed.

With both solvents about 88% of added histidine was found on average in the final extract. Some of the variability to be seen in the histidine recovery results may well have stemmed from some loss arising from the difficulty in transferring quantitatively with a Pasteur pipette the supernatant above the salt deposit while attempting to avoid at all cost simultaneous transfer of any of the precipitate which did not pack down really tightly on centrifugation. It was for this reason that this somewhat stressful step in the technique was eliminated by the modification described below.

Another possible source of loss of histidine was considered. The taking up of the dry histidine-sodium chloride mixture in 6 M hydrochloride acid does not produce solution of the residue since some at least of the sodium chloride remains undissolved due to the common ion effect. It was conceivable that a variable portion of the histidine might be occluded within the sodium chloride aggregates formed on evaporation of the

aqueous mixture of histidine and salt and that such occluded histidine might be discarded within the sodium chloride precipitate. This step in the procedure was therefore modified to minimize this possible source of loss.

Modifications to the method of salt removal by acetone precipitation.

The dried residue obtained from the evaporation of the aqueous histidine-sodium chloride mixture was taken up in 0.24 ml water and 0.26 ml conc. hydrochloric acid added to give a mixture 6 M with respect to hydrochloric acid, as in the original procedure. The addition of the acid caused formation of a precipitate presumably of sodium chloride but in the strongly acid conditions it seemed unlikely that any histidine would be co-precipitated.

Acetone, 10 ml, was added and the contents of the tube were thoroughly mixed by inversion several times. The precipitate was removed, not by centrifugation as previously, but by filtration through a glass wool filter. A small pad of glass wool soaked in acetone was tightly packed at the lower end of a glass tube (11 - 12 cm x 0.8 cm

Fig 16. A glass-tube used for filtration through glass-wool packed at the taper of the tube, of the HCl-acetone extract of dried residue of eluate from cation exchange resin CG-50-cellulose column equilibrated at pH 5.75 (see text, p 145).

A = position of glass-wool plug.

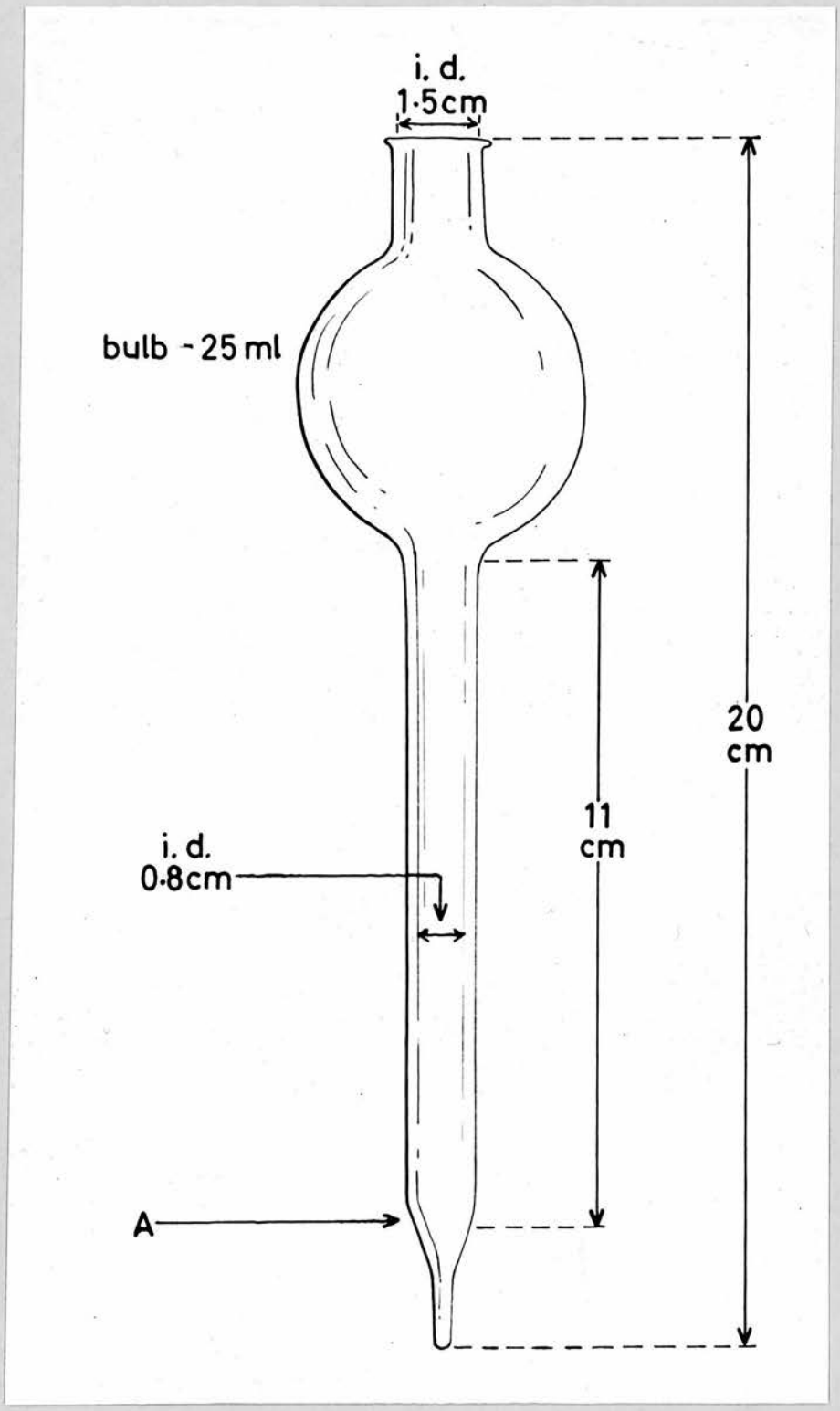


Fig 16. **See opposite page.**

internal diameter, surmounted by a wide mouth glass bulb 25 to 30 ml capacity (see Fig 16, p146). The suspension in acetone was poured into the bulb of the tube, the filtrate being collected in a 50 ml glass-stoppered test-tube. The tube which had contained the acetone suspension was rinsed with a further 5 ml acetone and this rinse also passed through the filter.

The filtrate was evaporated under a reduced pressure of 20 to 30 mm Hg first at room temperature and then, after reduction of the volume to about 0.5 ml, at 70°C in a water bath to remove the acid and produce a dry residue. Portions of the solution of the residue dissolved in 5 ml water were assayed for histidine and sodium chloride.

Results and discussion

Results of model experiments using this modified technique of salt removal are given in Table 12.

On average only 1.3% (range 1.1 to 1.6%, 9 experiments) of the 52 mg sodium chloride present in the original solution appeared in the final extract. This extent of desalting was considered satisfactory particularly since it was associated with a mean recovery of 94.5% (range 91% to 100%, 5 experiments) from 1 µg of histidine present in the original solution.

Table 12

Recovery and separation of histidine from sodium chloride by extraction with HCl-acetone mixture followed by filtration of the extract through packed glass wool.

Sample No.	Composition of histidine-sodium chloride mixture		Histidine in extract	Percentage of histidine recovered	Na^+ present in extract	NaCl present in extract
	Histidine	NaCl				
	µg	mg	µg		µEq	mg
1	-	52.6	-	-	13.12	0.76
2	-	52.6	-	-	13.12	0.76
3	-	52.6	-	-	15.0	0.87
4	-	52.6	-	-	13.12	0.76
5	-	52.6	-	-	11.87	0.69
6	1.0	52.6	0.92	92	11.87	0.69
7	1.0	52.6	0.97	97	11.87	0.69
8	1.0	52.6	0.91	91	10.0	0.58
9	1.0	52.6	0.92	92	10.0	0.58
10	1.0	-	1.00	100	-	
				Mean = 94.5 % (range 91 - 100%)		Mean = 0.71 (1.3% range 1.1 - 1.6%)

One stage of the desalting procedure involves evaporation of a solution containing a relatively high concentration of hydrochloric acid. It seemed possible that if the solution contained homocarnosine or carnosine or N-acetyl histidine some hydrolysis of these substances might occur and lead to erroneously high estimates for the histidine content. In model experiments in which solutions of 0.5 to 10.0 ug amounts of the above compounds in 0.5 ml 6 N HCl were evaporated to dryness and the residue put through the method for the fluorimetric estimation of histidine, no detectable (< 0.01 µg) amount of histidine was found, the fluorescence produced with o-phthalaldehyde being of the same intensity as that from a simple aqueous solution containing the same amount of the histidine derivative (p 199). Any significant hydrolysis occurring during the evaporation of the acidic solution would have led to a considerable increase in the fluorescence after reaction with o-phthalaldehyde because of the high fluorescence yield from histidine.

INVESTIGATION OF METHODS FOR THE SEPARATION OF HISTIDINE FROM HOMOCARNOSINE

Under the operating conditions which had been devised for the partial purification of histidine in biological extracts using columns of the ion-exchange resin CG-50, the separation of histidine from homocarnosine did not take place. Since the latter also produces a fluorophor by interaction with o-phthalaldehyde it was necessary to find a method of separating the compounds before fluorimetry. A number of preliminary tests were carried out with ion-exchange paper chromatography, partition paper chromatography and thin-layer chromatography in an endeavour to find a suitable technique and the results of these are described briefly here. The behaviour of the closely related dipeptide, carnosine was also examined in these experiments.

The positions of the compounds in the developed chromatograms were located by spraying the dried chromatograms with 0.2% ninhydrin in acetone and heating at 100°C for 30 minutes in an oven. Histidine gave a greyish brown, homo-carnosine a greyish pink and carnosine a greyish

green colour with the reagent.

Ion-exchange paper chromatography. Complete separation of histidine from homocarnosine was obtained in an overnight descending run on cellulose phosphate cation-exchange paper (Whatman), the chromatogram being developed with 0.1 M sodium acetate buffer pH 5.75. The migration distance from the origin was 15 cm for histidine and 26 cm for homocarnosine. Unfortunately the method proved unsuitable for quantitative work since eluates in 0.1 N hydrochloric acid gave unacceptably high blanks on reaction with o-phthalaldehyde. Attempts to reduce this blank fluorescence by recycling the cation-exchange paper through M NaOH and M HCl with water washing to neutrality after each treatment were unsuccessful since this process modified the paper characteristics so that the separation of histidine from homocarnosine was now impossible.

Paper chromatography. This technique was examined in the following experiments, using a number of solvent systems on Whatman No. 1 chromatography paper with marker quantities (20 µg) of histidine and homocarnosine. The chromatograms were developed for 18 hr. in an ascending direction.

No separation between histidine and homo-

carnosine was obtained with butan-1-ol: acetic acid: water (12:3:5 v/v) or propan-1-ol: acetic acid: water (12:3:5 v/v) solvent systems as the R_f (x 100) values of both histidine and homocarnosine were the same, being 11 in the former and 13 in the latter solvent system.

Although good separation between histidine and homocarnosine (R_f x 100 value being 32 for histidine and 20 for homocarnosine) was achieved with iso-propanol: Water: 12 N HCl (65:18.4: 16.6 v/v) solvent system (Nakajima et al., 1967), the method was unsuitable for fluorimetric purposes as the eluate of the paper, which was charred and brittle because of high concentration of hydrochloric acid in the solvent system, contained fine particles and was of dark coloration.

The following solvents were also tried but none proved satisfactory because of extensive streaking of the compounds: propan-1-ol: water (64:36 w/w), methanol water pyridine (15:5:1 by vol.), aqueous phenol solution (phenol: water, 4:1 w/v) alone or containing hydrochloric acid (0.2 ml conc HCl/100 ml) or acetic acid (1 ml acetic acid/100 ml).

In another experiment an attempt was made to separate histidine from homocarnosine by using

Whatman No. 3 MM chromatography paper with an iso-propanol: formic acid: water (8:1:1 v/v) solvent system as suggested by Nakajima et al., (1967). The paper was spotted with 20 ug of histidine and homocarnosine as before, and was developed for 30 hr in an ascending direction. Results showed incomplete separation between histidine and homocarnosine. The R_f (x 100) value for histidine was 15 and that for homocarnosine 10, with overlapping of the position of the forward front of the homocarnosine spot with that of the rear of the histidine spot.

Solvent systems containing ammonia were not tried in these trial experiments or in experiments using thin-layer chromatography discussed below because it had been found that traces of ammonia interfered with fluorimetric assay by giving rise to high blanks.

Thin-layer chromatography. Tests were carried out on 0.5 mm cellulose thin-layer plates with 20 ug amounts of the compounds using a number of solvent systems reported in the literature (Randerath, 1963; Smith, 1969), the chromatograms being developed in the ascending direction.

Development of the chromatogram in aqueous alcohol, either ethanol/water or propan-1-ol/water,

63:37 (w/w), gave no separation of histidine from homocarnosine because of trailing of both compounds.

Using propan-2-ol:formic acid:water (20:1:5 by vol.) the bulk of the applied substances separated from one another but because of some forward streaking of the homocarnosine with the leading edge merging with the histidine, the separation was unsatisfactory from the point of view of eventual quantification. The R_f (x 100) of the major amounts of the applied substances were 14 for histidine and 4 for homocarnosine. Attempts were made to improve the separation by substituting propan-1-ol for the propan-2-ol and acetic acid for the formic acid.

Using a system containing propan-1-ol acetic acid water (15:1:4, by vol.), the positions of histidine and homocarnosine applied as separate spots were well separated with R_f (x 100) values of 17 and 4 respectively. Unfortunately when a mixture containing the two substances was applied, separation was poor because of streaking of the components. Prolonging the solvent flow beyond the limit of the upper end of the thin-layer, by the application of a pad of filter paper did not lead to improvement in the separation (Fig 17, p 155).

Fig 17. Effect of the absence of sodium chloride in the solvent system on the separation of histidine and homocarnosine on a 0.5 mm cellulose-layer.
Histidine and homocarnosine, 20 ug of each, in 1 ul water was spotted on the line of origin on the layer. The chromatogram was developed for 13½ hr in ascending propan-1-ol: acetic acid: water (15:1:4, by vol.) solvent system containing no sodium chloride. A pad of Whatman No 3 MM paper was applied to the layer on the end furthest from the line of origin to allow overrunning of the solvent. Histidine was not separated from homocarnosine as backward tailing of the histidine spot intermingled with the forward streaking of the homocarnosine spot.

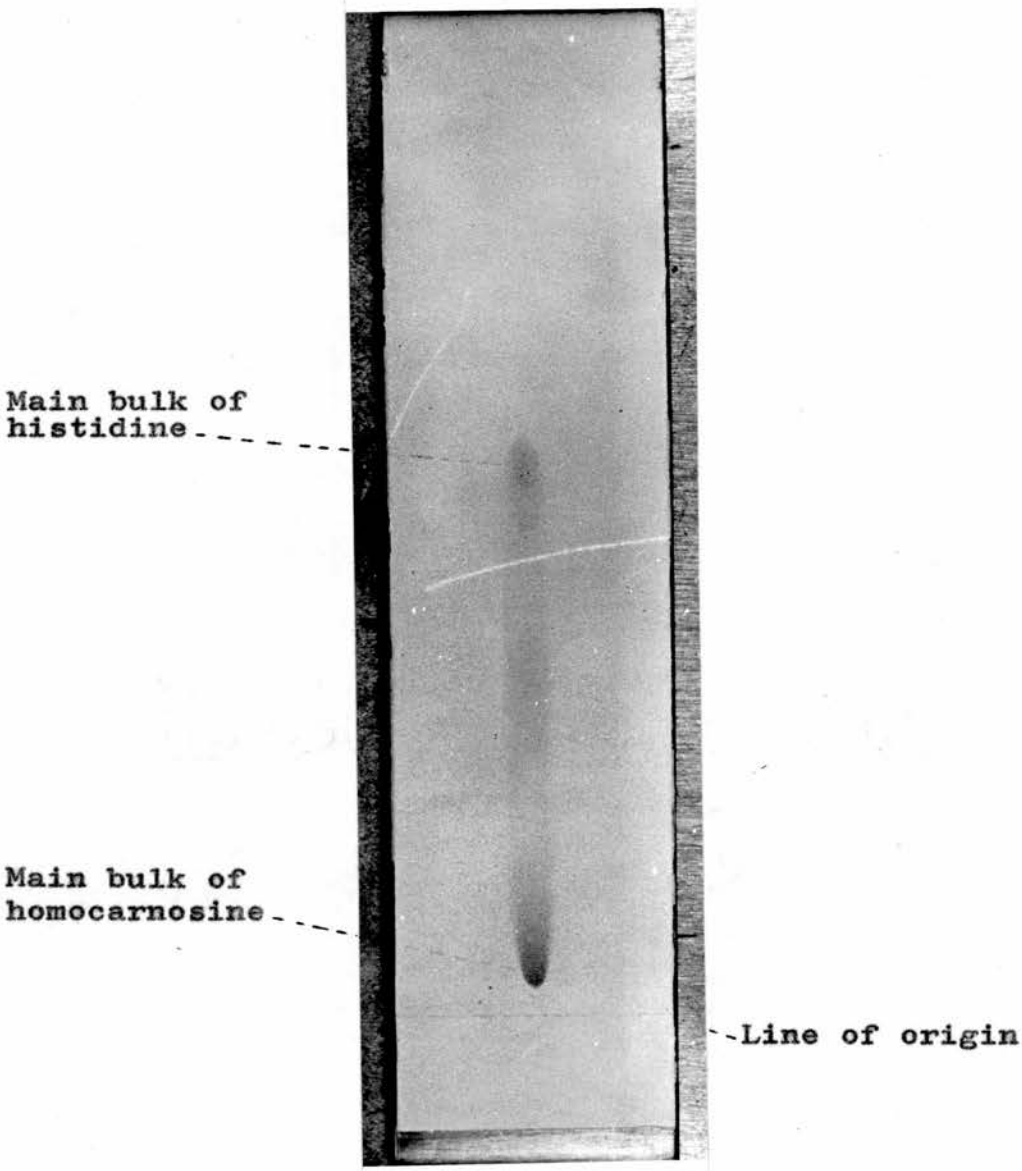


Fig 17. Effect of the absence of sodium chloride in the solvent system on the separation of histidine and homocarnosine on a 0.5 mm cellulose-layer.

It was found eventually that a satisfactory separation could be achieved by the addition of sodium chloride to the solvent mixture to give a concentration of 0.3 g/100 ml of solvent. The salt addition increased markedly the R_f value for homocarnosine while depressing slightly that of histidine (Table 13, p 157). Thus the difference between the R_f value of histidine and homocarnosine was much reduced by the salt addition but this was offset by the more advantageous effect of markedly diminishing the streaking and thus improving the quantitative separation. Adequate separation in spite of decreased R_f difference was obtained by over-running of the solvent beyond the upper edge of the adsorbent layer by application of a pad of filter paper as mentioned above (Table 13 , p 157 ; Fig 18 , p 159). Increasing the salt concentration to 0.6 g/100 ml solvent did not give any further advantage. It depressed the R_f values still further but the absolute separation of histidine from homocarnosine for the same distance of solvent flow remained unchanged (Table 13).

Carnosine is not adequately separated from histidine and homocarnosine on cellulose thin-layer chromatography in these solvent systems, but for reasons discussed later this does not constitute

Table 13

R_f (x 100) values of histidine, homocarnosine and carnosine (each 20 μg) on 0.5 mm cellulose thin-layer ascending chromatograms developed in propan-1-ol: acetic acid: water: (15:1:4 by vol) containing various concentrations of sodium chloride.

Compounds	Concentration of sodium chloride in solvent system (g/100 ml)		
	Nil	0.3	0.6
Histidine	26	17	14
Homocarnosine	4	21	18
Carnosine	24	18	16

Tailing of the histidine and the carnosine and forward streaking of the homocarnosine occurred in absence of sodium chloride.

Table 14

Location and extent of separation of histidine, homocarnosine and carnosine (20µg each) on a 0.5 mm cellulose thin-layer ascending chromatogram developed for 13.5 hr. in propan-1-ol: acetic acid: water (15:1:4 by vol) containing 0.3 g% (w/v) sodium chloride with over-running of the solvent.

Compounds	Location (cm) from origin	
	Lower limit	Upper limit
Histidine	5.0	7.5
Carnosine	6.5	8.5
Homocarnosine	8.5	11.0

Fig 18. Thin layer chromatogram of histidine, homocarnosine and carnosine on cellulose.

Extent of separation of histidine, homocarnosine and carnosine on the 0.5 mm cellulose layer (5 x 20 cm). Histidine is completely separated from homocarnosine while carnosine occupies a position intermediate between histidine and homocarnosine, partially overlapping the position of histidine. The chromatogram was developed for $13\frac{1}{2}$ hr in ascending propan-1-ol: acetic acid: water (15:1:4, by vol.) containing 0.3 g sodium chloride per 100 ml solvent. Mixture of histidine and homocarnosine, 20 ug of each, in 1 ul water was applied to the spot marked with (A) on the line of origin on the layer while carnosine, 20 ug in 1 ul, was applied to the spot marked with (B). A pad of Whatman chromatography paper No. 3MM was applied to the layer on the end furthest from the line of origin to allow overrunning of the solvent.

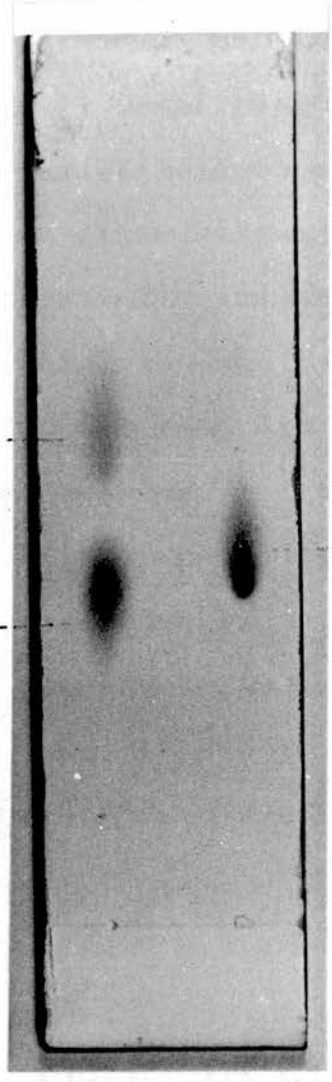


Fig 18. Thin layer chromatogram of histidine, homocarnosine and carnosine on cellulose. (see opposite page).

a problem in eventual separate determination of histidine and homocarnosine.

The separation of histidine and homocarnosine from other amino acids under the conditions of thin-layer chromatography. After histidine had been separated from homocarnosine on the cellulose thin-layer chromatography as described above, the technique was then tested for its effectiveness in separating these two compounds from other amino acids. A number of neutral amino acids which might be present in a crude tissue extract have iso-electric points slightly above pH 5.75 and consequently may be adsorbed to a greater or lesser degree on the "pH 5.75" ion-exchange resin column, and be eluted along with histine and homocarnosine and thus be present during the thin-layer chromatography for the separation of the latter compounds. The R_f values of some such neutral amino acids under the conditions of the thin-layer chromatography were determined (Table 15, p 161) and these proved to be considerably greater than those of histidine and homocarnosine. The histidine and homocarnosine eluates from a thin-layer chromatogram would therefore not contain any of these neutral amino acids so that their possible

Table 15

The R_f (x 100) values of histidine, homocarnosine and some of the neutral amino acids, whose iso-electric points are above pH 5.75 on 0.5 mm cellulose thin-layer chromatogram developed in propan-1-ol : acetic acid: water (15:1:4 by vol.) containing 0.3 g sodium chloride/100 ml solvent.

* Amino acids	R_f (x 100)
L-alanine	46
β-alanine	43
L-isoleucine	71
L-leucine	75
L-phenylalanine	72
L-proline	49
Sarcosine	48
L-valine	72
Histidine	17
Homocarnosine	21

* These amino acids, except histidine and homocarnosine, were obtained from Sigma Chemical Company and were of the purest grade.
Histidine was obtained from Koch-Light Laboratories Ltd and homocarnosine from Calbiochem (see Appendix, p 285).

interference with the fluorimetry need not be considered.

SEPARATION OF HISTIDINE FROM HOMOCARNOSINE BY THIN-LAYER CHROMATOGRAPHY ON CELLULOSE AND QUANTIFICATION OF THE RECOVERY OF THE SEPARATED COMPOUNDS

Details of the methodology of this thin-layer chromatography as eventually applied to extracts from biological material are given below.

Preparation of thin-layer plates.

Cleaning of glass carrier plates. Glass plates (200 mm x 50 mm, 4 mm thick) were freed from grease in the following way. They were left in chromic acid cleaning fluid overnight and then rinsed thoroughly with tap water and with distilled deionised water. They were dried in a vertical position in an oven at 45°C. Degreasing of the face of plate to which the layer was to be applied was completed by wiping it repeatedly in a direction along its length, with a piece of adsorbent gauge (Johnson and Johnson, B.P.C., sterile) soaked in methanol-chloroform mixture

(1:1, v/v), the plate meanwhile resting on the bench on a pad of the gauge. The plate was wiped dry with a piece of gauze and placed in the aligning tray preparatory to spreading the adsorbent.

Preparation of cellulose thin-layer. 40 g cellulose powder (Whatman, Thin-layer Chromedia, CC 41, microgranular cellulose powder) was suspended in 120 ml distilled deionised water and a smooth slurry obtained by blending in an Ato-mix (M.S.E. Ltd.) blender at full speed (12,000 r.p.m.) for 90 sec. The glass container was shaken gently to aid expulsion of air bubbles from the suspension.

The slurry was applied to the glass carrier plates, arranged in an aligning tray, with the aid of a spreader set to give a 0.5 mm layer of adsorbent. Twenty 200 x 50 mm plates or four 200 x 200 mm plates suitable for chromatographic use could be prepared simultaneously.

After 10 min. neighbouring plates were separated from one another and the cellulose layers allowed to dry overnight at room temperature in the horizontal position in the aligning tray. The plates were then transferred to a metal rack and stored in a dust free cupboard until used some 24 hr. later.

Thin-layer chromatograms.

A faint transverse line (line of origin) was drawn in pencil at right angles to the length of the plate (or, in the case of the 200 x 200 mm layers, to the eventual direction of solvent flow) and 25 mm above the lower margin.

Marker control chromatograms. A 1 ul portion of an aqueous solution of histidine, homocarnosine or carnosine (each 20 mg/ml) was applied to the line of origin to give a spot not greater than 5 mm diameter. Spots were located at least 15 mm from the side margins of the layer and 20 mm from a neighbouring spot. During application, the solvent was evaporated in a current of hot air.

Quantification of the recovery of histidine or homocarnosine from the chromatograms.

Histidine, 0.8 ug, or homocarnosine, 5 or 10 ug, in a volume of 50 ul of aqueous solution was transferred by repeated application with the aid of a Marburg microlitre pipette to the line of origin of a 200 x 50 mm plate. Care was taken to regulate the volume of the drops so that the spots were never greater than 5 mm in diameter, the spots being dried under a hot air (80-85^{o}C) stream as they were

applied. Application was restricted to the central 20 mm width of the plate to avoid errors due to uneven solvent flow at the margins of the layer.

A "blank" plate received an application of 50 µl distilled deionised water. Marker control solutions (preceding section) were applied to separate plates.

Development of chromatograms. A 360 mm long strip of 50 mm wide Whatman 3 MM chromatography paper was folded to give a pad 45 mm by 50 mm and 8 layers thick. A fold was made in the pad across the 50 mm width at a point 10-12 mm from one end. The pad was placed on the end of the 50 mm wide thin-layer plate distal to the origin so that the 10-12 mm section was in contact with the adsorbent layer and the remainder folded over the end of, and in contact with the back of, the carrier plate. The pad was held securely in position by a piece of cotton thread wound round a number of times and tied.

The prepared plates were transferred to a chromatography tank (Universal TLC chromatank, Shandon Scientific Co. Ltd.) containing 125 ml of a mixture of propan-1-ol: acetic acid: water (15:1:4 by vol.) containing 0.3 g sodium chloride/ 100 ml. The tank was quickly closed with a glass

lid, the sealing between the lid and the ground glass top edge of the tank being effected with a thin-layer of soft paraffin.

Neither equilibration of the plates nor lining the tank with solvent saturated filter paper to maintain saturation of the atmosphere in the tank with solvent vapors proved necessary with this solvent system (von Aux and Neher, 1963).

The chromatograms were run overnight (13.5 hr) at room temperature (about 21°C). The developed chromatograms were taken from the tank, the paper pads removed and the cellulose layer allowed to dry at room temperature during 24 hr. in a chromatography oven with a fan-induced current of air passing through.

Marker control chromatograms. The positions of the substances on the chromatograms were visualised by spraying the plates with 0.2% ninhydrin in acetone and the coloured products developed by heating for 30 min. at 100°C in an oven.

Elution of histidine and homocarnosine for quantification of the separated compounds.

Pencil lines were lightly drawn across the cellulose layer at right angles to the direction of the solvent flow at positions about 2 mm on

either side of the upper and lower limits of the histidine and of the homocarnosine locations as determined from the marker-control plates. The cellulose powder was scraped off from within the areas bounded by these lines on to a piece of aluminium foil. The cellulose powder carrying the histidine or the homocarnosine was then transferred to a 10 ml conical centrifuge tube. Distilled deionised water, 4.0 ml was added to the tube and the mouth of the tube closed with a piece of parafilm. The tube was shaken for 5-10 min. to produce a fine suspension of the cellulose in the water, and after about 90 min., centrifuged at 2000 r.p.m. for 30 min. The clear supernatant eluate was used for the fluorimetric estimations.

The cellulose from the corresponding areas of the "blank" chromatograms was treated similarly.

Fluorimetric estimation of histidine and homocarnosine in eluates from the chromatograms. A 1.0 ml portion of the appropriate eluate was transferred to a 10 ml glass-stoppered test-tube and the histidine or homocarnosine in the portion estimated fluorimetrically (histidine, p 85 ; homocarnosine, p 93).

Standard solutions for calibration were prepared by dilution of 0.1, 0.2 and 0.3 ml stock solution of 1 µg/ml histidine or 10 µg/ml homocarnosine to 1.0 ml with eluate from a "blank" chromatogram.

After allowing for the non-specific "blank" fluorescence, the histidine or homocarnosine in the total eluates from the chromatogram was determined and the recovery of these substances calculated.

RESULTS

Typical positions of marker (20 µg) amount of histidine, homocarnosine and carnosine on the chromatograms developed under the described conditions are shown in Table 14 , p 158. As will be seen, a separation of 10 mm between the lower limit of the homocarnosine spot and the upper limit of the histidine area was obtained.

Recoveries of standard amounts of histidine and homocarnosine through the chromatography were satisfactory; (Table 16 , p169) an average of 91% being obtained from 0.8 µg histidine range 86-95%, 4 exps) and an average of 87% from 5 to 10 ug homocarnosine (range 84-100%, 5 exps.).

Eluates from the appropriate areas of the

Table 16

Recovery of histidine and homocarnosine from cellulose thin-layer chromatograms developed in propan-1-ol: acetic acid: water (15:1:4, by vol.) containing 0.3 g sodium chloride/100 ml solvent. The chromatograms were developed for 13.5 hr. in the ascending direction with over-running of the solvent.

Amount applied to chromatogram	Amount recovered in eluate		
ug	ug	Percentage	Mean Percentage
Histidine			
0.8	0.69	86	
0.8	0.74	92	91
0.8	0.76	95	
0.8	0.72	90	
Homocarnosine			
10.0	10.0	100	
10.0	9.8	98	
10.0	10.0	100	87
5.0	4.2	84	
5.0	4.5	90	

"blank" chromatogram gave readings equivalent to about 0.02 µg histidine and to about 0.2 µg homocarnosine in the sample for fluorimetry. Thus the fluorescence intensities of the 'reagent' blanks from the thin-layer chromatograms showed no significant difference from those obtained from simple water "blanks".

DISCUSSION

A number of solvent systems have been recommended (Randerath, 1963; Aures et al., 1968; Smith, 1969) for the separation of amino acids, peptides and imidazole compounds by thin-layer and paper chromatography, but homocarnosine was not included among the compounds studied. Attempts in the present work to separate histidine from homocarnosine on paper with many of these solvent systems were unsuccessful. Adsorbent layers other than cellulose powder have been used for amino acid separations and include such substances as sephadex (Determann, 1962), silica gel (Ehrhardt and Cramer, 1962), celite-starch (Shasha and Whistler, 1964) and diethylaminoethyl (DEAE)-cellulose (Llosa, Tertrin and Jutisz, 1964). However,

none of these reports included data regarding the separation of histidine from homocarnosine.

Separation of histidine from homocarnosine has been achieved in the present work using thin-layer chromatography with cellulose as adsorbent and propanol: acetic acid: water (15:1:4, by vol.) containing 0.3% (w/v) sodium chloride as developing solvent. The addition of salt to the solvent system had the effect of eliminating troublesome streaking, and consequent inadequate separation, of the compounds on the chromatogram which took place in its absence. This streaking was perhaps due to a 'salt' effect exerted by the compounds (Randerath, 1963; Smith, 1969) which in retrospect may have been applied to the plates in rather high (20 µg) amounts. This may also explain the streaking encountered in preliminary experiments using various solvent systems on paper chromatograms. The solution of this problem had the advantage that conditions of chromatographic development were found under which quantitative separation would still occur should relatively high amounts of the substances be present in extracts from biological materials.

With sodium chloride present in the solvent system, the separated compounds were confined on

the developed chromatogram to more compact areas with distinct boundaries. The salt addition also had an effect on the apparent R_f values of the compounds. In its absence the main bulk of the applied homocarnosine occupied a position close to the origin (R_f x 100 = 4, Table 13), although it was apparent that considerable forward streaking towards the solvent front had occurred. Under these conditions the main bulk of the histidine and carnosine occupied positions with R_f (x 100) values of 26 and 24 respectively although there was extensive tailing in each case. The marked difference in the R_f values of the bulk of the histidine and the homocarnosine suggested that the technique had possibilities for their quantitative separation if only the streaking phenomenon could be eliminated. The addition of sodium chloride had the desired effect but only at the expense of a considerable reduction in the difference between the R_f values. In the presence of the salt (0.3 g/100 ml solvent) the R_f (x 100) for homocarnosine increased from 4 to 21 while those for histidine and carnosine showed a decrease from 26 and 24 respectively to 17 and 18. It would appear that, in the case of homocarnosine, the sodium chloride exerted a 'salting out' effect increasing markedly

the relative solubility of the dipeptide in the mobile phase, possibly by reducing its solubility in the stationary phase and/or its adsorption to the cellulose. A similar explanation may account for the removal of the 'tailing' of the histidine and homocarnosine by the 'salting out' of the small amount of the compounds responsible for the phenomenon.

The decreasing R_f values with increasing salt concentration (Table 13) could be due to changes in the relative compositions of the mobile and stationary phases on the chromatogram in a direction favouring the distribution of the compounds towards the latter with increasing salt concentrations.

With the solvent system containing sodium chloride the R_f (x 100) values of the three substances histidine, carnosine and homocarnosine were 17, 18 and 21 respectively so that quantitative separation would not be obtained with a migration of the solvent restricted to the length (17.5 cm) of the cellulose layer. The absolute separation of histidine from homocarnosine was increased to a satisfactory extent by allowing over-running of the solvent by the application of a filter pad to the distal end of chromatogram. Carnosine was

still not separated from the other components and consequently if present in an extract, would be found in the final histidine or homocarnosine containing eluates, predominantly in the former. However, because of the relatively low fluorescence yield of its o-phthalaldehyde derivative (Table 5), carnosine would not interfere significantly with fluorimetry of histidine unless it was present in gross excess. More interference might be expected with the homocarnosine estimation because of the smaller difference in the fluorescence yields from the two dipeptides, but this would be offset by the greater degree of separation on the chromatogram so that in practice, interference would be minimal except in the presence of a relatively large quantity of carnosine in the chromatographed extract.

CHAPTER II

PART II - ESTIMATION OF HISTIDINE AND HOMOCARNOSINE IN MOUSE BRAIN: METHODOLOGY

ESTIMATION OF HISTIDINE AND HOMOCARNOSINE IN BRAIN

In previous sections, methods have been described for the separation and fluorimetric assay of histidine and homocarnosine. These model experiments were generally carried out with pure solutions of these substances in concentrations that might be expected to occur in brain. Higher concentrations of homocarnosine were employed in experiments with ion exchange resin columns so that it could be detected and conveniently measured in effluents and eluates.

The proposed method of analysis of brain tissue, summarised as a flow-sheet in Fig 19, p 176, is an extension of that previously described by Adam (1961) for the estimation of histamine in brain. Taken as a whole, the method should make it possible to estimate histamine, histidine and homocarnosine in a single extract of brain. Values for the concentration of histamine in mouse brain have been obtained by Stephen (1968) by the method of Adam (1961). In the present work, estimates were obtained for the concentration of histidine and homocarnosine in mouse brain. The effect of a previous dose of histidine

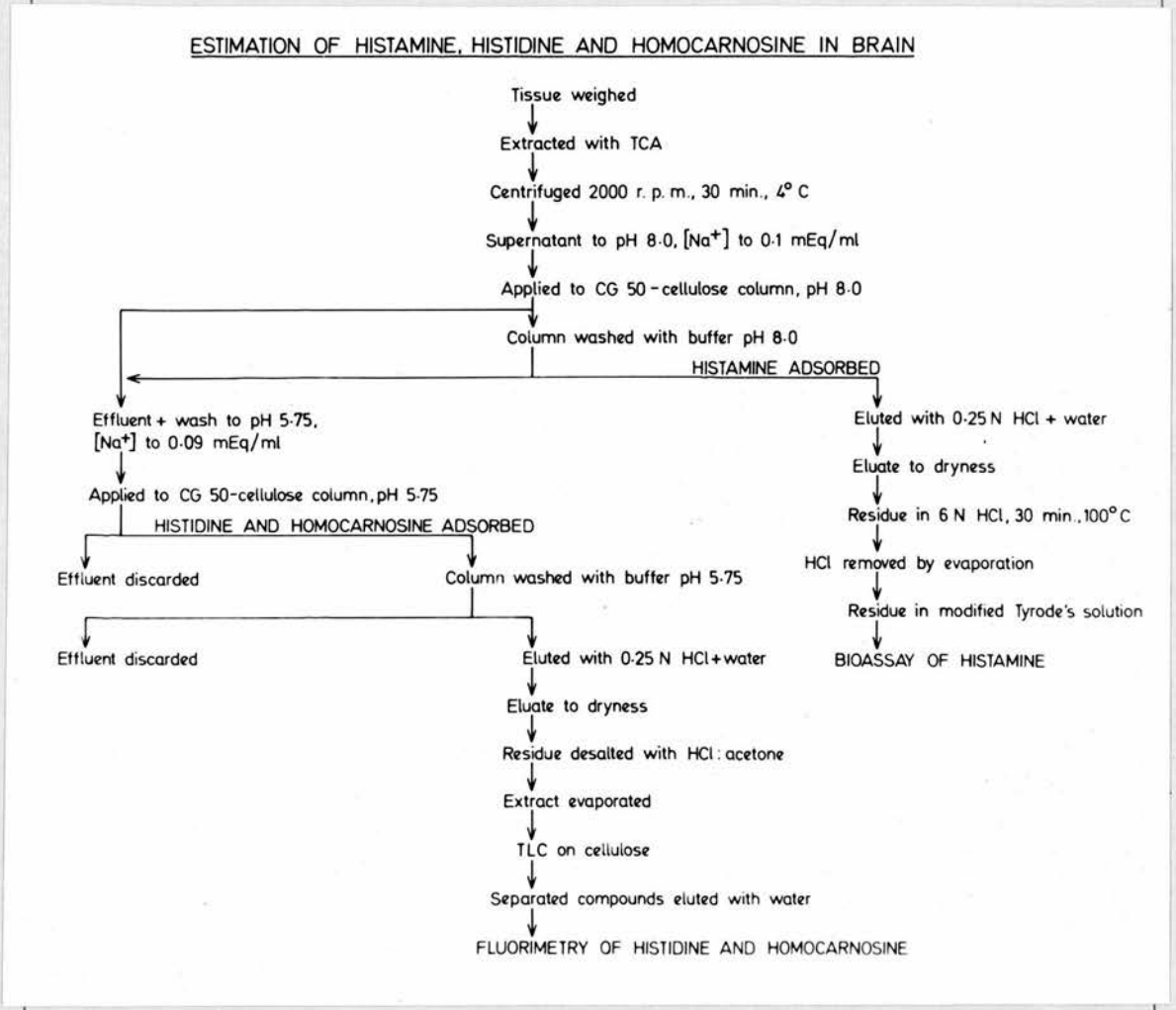


Fig 19. A flow-sheet of the procedure for extraction, purification and estimation of histamine, histidine and homocarnosine from a single brain sample.

(intraperitoneal injection) on these values was also studied. Unfortunately, owing to limitations of the working conditions of the laboratory it has not been possible to measure simultaneously the concentration of histamine in brain.

Animals. The animals used were male albino mice weighing between 25 and 35 g.

Histidine dosage. Histidine was given by intraperitoneal injection in a dose of 15 ug, 30 µg, 60 µg and 120 µg/g body weight to study the effect of such treatment on the histidine and homocarnosine concentrations of brain.

Histidine solution for injection. Histidine monohydrochloride monohydrate (Mol. wt. 209.6) was dissolved in water to give a concentration of 20.3 mg/ml. It was neutralised to pH 7.0 with sodium bicarbonate, 7.2 mg/ml. The osmolarity was calculated to be about 270 m osmoles/l, so that the solution was nearly isotonic (300 m osmoles/l).

This solution was used for administration of doses 120 µg/g (0.04 ml/5 g of the body weight) and suitable dilutions were made in saline for the lower doses so that the volume of solutions injected remained approximately constant in

spite of varying the dose of histidine. Control animals received the same volume of isotonic sodium chloride.

Removal of brain. Fifteen minutes after injection, the animal was killed by decapitation. The skull was exposed by a midline incision and a blade of a small pair of scissors was inserted into the foramen magnum taking care not to damage the brain. The cranium was cut horizontally on either side from the foramen magnum to the frontal bones. The upper part of the skull was thus removed as a single piece, exposing the entire brain. The spinal cord was cut at the level of first cervical vertebra, and the brain was removed by detaching it from the base of the skull with the aid of a blunt scoop.

The brain was weighed on a torsion balance of 1000 mg capacity.

Dissection of brain. The cerebellum with its peduncles was severed and discarded. The brain was then divided into two parts: a) the pons and medulla, and b) the remainder of the brain (Fig 20). After removing traces of blood with filter paper the samples were weighed on a torsion balance.

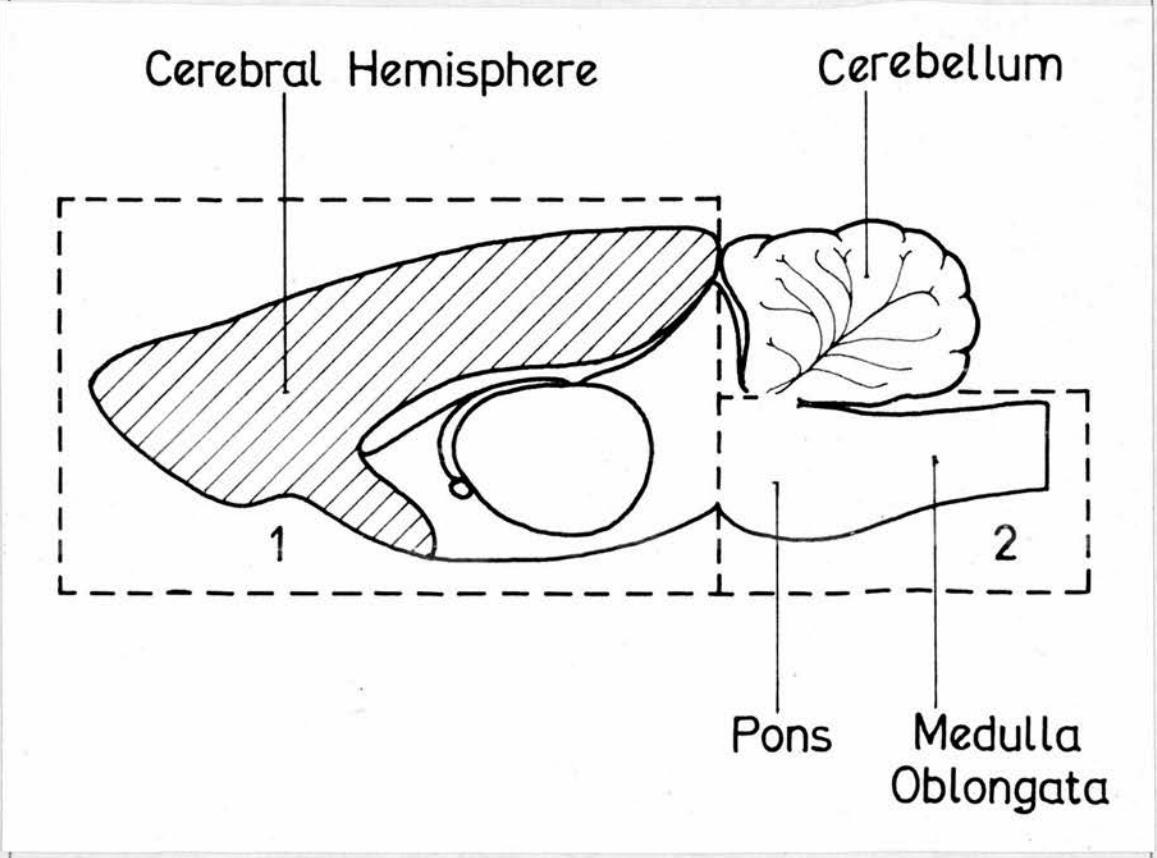


Fig 20. Diagram showing gross anatomical dissection of mouse brain.

(1) The portion containing mainly the cerebral hemispheres.

(2) The portion containing pons and medulla.

Pons and medulla. This was the brain stem extending from the superior cerebellar peduncles to the level of the first cervical vertebra. The weight of the individual samples varied from 83 to 98 mg.

Remainder of the brain. This was represented mainly by the cerebral hemispheres. The weight of the individual samples varied from 250 to 300 mg.

EXTRACTION AND PURIFICATION OF HISTIDINE AND HOMOCARNOSINE FROM BRAIN

All the samples were extracted within 5 to 7 min. after killing the animal.

Extraction of brain tissue with trichloroacetic acid.

The tissues were extracted and proteins precipitated by grinding with trichloroacetic acid.

For a sample weighing less than 100 mg, e.g. the 'pons and medulla', the extraction was carried out in a small tissue grinder. This consisted of a 15 ml M.S.E. heat resistant conical centrifuge tube, the taper of which had been ground with carborundum. The pestle was a glass rod moulded and ground to fit the taper

of the tube (Fig 21, p 182). The volume of the ground part of the tube was 1.25 ml. The tube was calibrated at the 5 ml capacity level.

For samples weighing more than 100 mg, that is, the portion of the brain containing the cerebral hemispheres, a 'Griffith' pattern grinder (Laboratory Glassblowers, Cat. No. 744) (Fig 21, p 182) was used.

Extraction of samples of pons and medulla. The sample was weighed and transferred from the torsion balance pan with the help of a fine glass rod and lowered into a measured volume of trichloroacetic acid (6% w/v) in the tissue grinder. The amount of trichloroacetic acid used was 5 µl/mg of tissue. The tissue was immediately ground and the process continued with the addition of a small volume (1 ml) of water. Water was then added to make the volume up to 5 ml. The suspension was thoroughly mixed and the tube was sealed with parafilm.

Extraction of samples of the 'remainder of the brain'

The sample was weighed and placed in a 'Griffith' type tissue grinder and ground in the required volume (5µl/mg of tissue) of trichloroacetic acid (6% w/v). The pestle and the inner side of the grinder were washed with a small volume (3 to 4 ml)

Fig 21. Homogenizers used for extraction of brain samples.

(A) For samples weighing less than 100 mg.

(B) For samples weighing more than 100 mg (see text, p 181).

Inner diameter of (A) at the open upper end 1.1 cm and at the lower ground-glass part 0.8 cm tapering down to 0.6 cm; and that of (B) at the open upper end 1.2 cm and at the lower ground-glass part 0.5 cm.

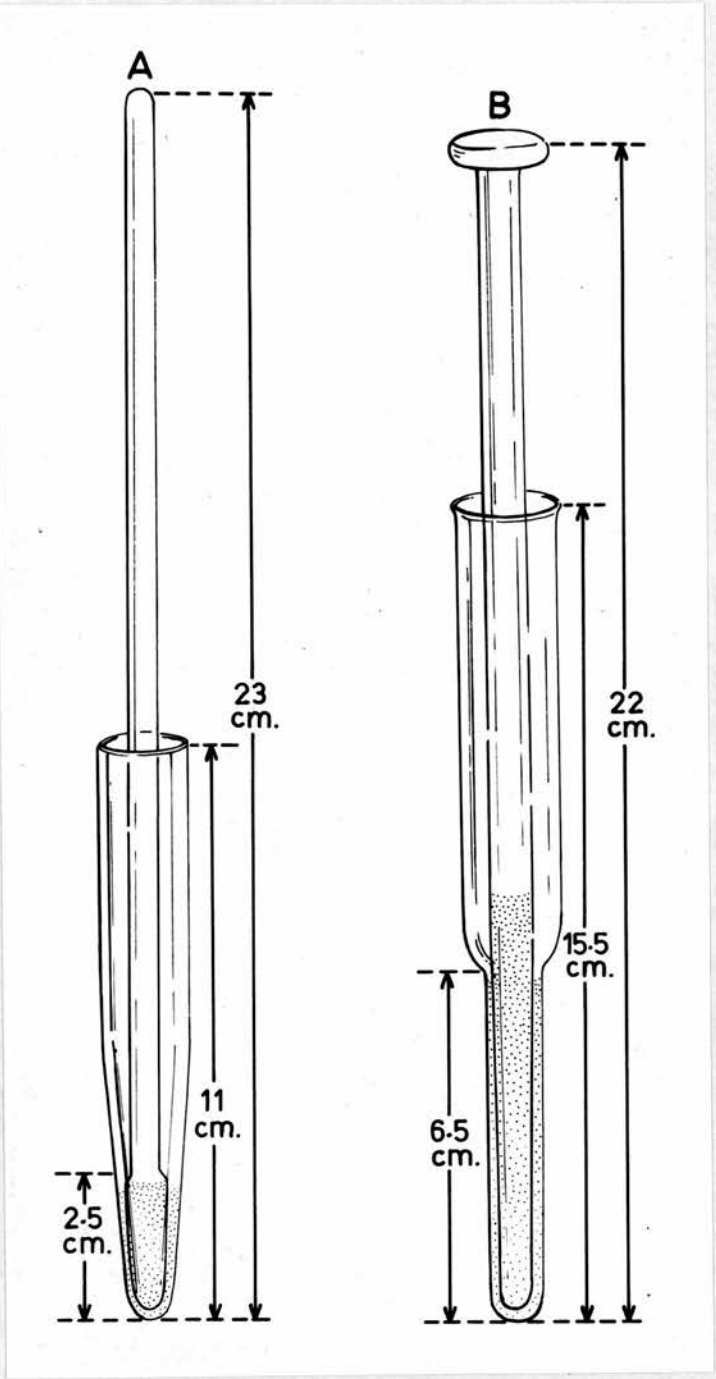


Fig 21. Homogenizers used for extraction of brain samples. (see opposite page).

of water and the suspension poured into a glass-stoppered graduated measuring cylinder (25 ml). The grinder was again washed with water which was also placed in the cylinder. The suspension in the cylinder was then made up with water to a final volume such that 1 ml contained 20 mg of tissue. After thorough mixing, an aliquot of 5 ml of the suspension was transferred with the aid of a Pasteur pipette to a graduated 10 ml conical centrifuge tube, which was then sealed with parafilm.

Removal of insoluble material from extracts.

The tubes containing the extracts were centrifuged at 2000 r.p.m. for 30 min at $4^{\circ}C$ in a refrigerated centrifuge. A Pasteur pipette was used to transfer 4.6 ml of each clear supernatant to a glass-stoppered 10 ml graduated test-tube.

Preparation of brain extracts for column chromatography. The extracts were prepared in two steps: (i) the aliquot was neutralised and (ii) its pH and Na^{+} concentration were adjusted.

Neutralisation of the extract. The 4.6 ml portion of the supernatant was neutralised with 0.1 N NaOH

delivered from a 1 ml microbufette. One drop of neutral red solution (0.01% w/v) was used as internal indicator. Approximately 0.02 ml of the alkali was required for each 5 µl of trichloroacetic acid used in the extraction of the brain sample. After neutralisation, one drop (0.02 ml) of 0.1 N HCl was added to make the solution just acidic, in order to stabilise the histamine present. This was done routinely although in point of fact histamine was not assayed in experiments reported here.

Adjustment of pH and Na^+ concentration of the solution. The solution was brought to pH 8.0 and to a total Na^+ concentration of approximately 100 mEq/litre by the addition (see below) of 0.2 M sodium phosphate buffer, pH 7.92, 390 mEq Na^+/litre. Distilled deionised water was then added to a final volume of 8.0 ml, and this buffered extract of the brain tissue applied, after mixing, to a CG-50 ion-exchange resin column adjusted to pH 8.0.

The volume of the buffer added depended on the amount of Na^+ added in the neutralisation of the extract; Na^+ derived from the tissue was ignored. The volume of the buffer required was based on the following calculation:

Final volume of solution = 8.0 ml with a total mEq Na^+ (0.1 mEq Na^+/ml) = 0.8

Vol. of 0.1 N-NaOH required for neutralisation of a portion of acid extract of tissue = x ml

$\therefore$ mEq Na^+ added as NaOH = 0.1x

$\therefore$ mEq Na^+ to be added in the buffer = 0.8 - 0.1x

mEq Na^+/ml in the buffer = 0.39

$\therefore$ Vol. of buffer solution required $= \frac{0.8 - 0.1x}{0.39}$ ml

For any given sample, the required volume of buffer was thus determined in relation to the volume of NaOH added in the neutralisation.

Ion exchange chromatography on Amberlite CG-50 at pH 8.0: separation of histamine from histidine and homocarnosine by adsorption of the amine.

A column was prepared by mixing 50 mg Amberlite CG-50 with 300 mg cellulose powder and was equilibrated with 0.05 M phosphate buffer, pH 8.0, Na^+ concentration 100 mEq/l as described on p123 .

The buffered supernatant extract from the brain tissue, 8.0 ml, was placed on the equilibrated column. The tube which had contained the extract was washed out with 2.0 ml of the 0.05 M phosphate buffer pH 8.0 and this too was applied to the column. A 'control' column received 10 ml of

the 0.05 M phosphate buffer pH 8.

Solutions were allowed to pass through the columns at a constant flow rate of 0.3 ml per min. followed by a wash with 5.0 ml of buffer solution. The effluent and the wash fluid were collected together in a 50 ml glass-stoppered tube. The total volume collected was 15 ml, and contained the histidine and homocarnosine, but none of the histamine, from the original sample.

Ion-exchange chromatography on Amberlite CG-50 at pH 5.75: adsorption of histidine and homocarnosine.

A column of a mixture of 300 mg Amberlite CG-50 and 200 mg cellulose powder was equilibrated to pH 5.75 with 0.1 M acetate buffer pH 5.75, 90 mEq Na^+/l (p 132).

The pH of the mixed effluent and wash fluid from the first ion-exchange column was adjusted to 5.75 ± 0.1 (glass electrode) by the drop-wise addition of M-acetic acid from a 1 ml micro-burette. The quantity required was between 0.6 and 0.7 ml.

The Na^+ concentration was then adjusted to 90 mEq/l by the addition of water. The effluent and wash fluid, 15 ml in all, contained 100 mEq Na^+/l or a total of 1.5 mEq Na^+. To give a solution with a concentration of 90 mEq/l, this

amount must be contained in 16.66 ml. The volume of water required to be added was therefore 1.66 ml minus the volume of M acetic acid needed to adjust the pH.

The combined effluent and wash fluid adjusted to pH 5.75 and Na^+ concentration of 90 mEq/l, was passed through the column at a rate of 0.3 ml/min. The test-tube containing the solution was washed out with 5 ml of the 0.1 M acetate buffer and this, too, was allowed to pass through the column.

Elution of histidine and homocarnosine. The adsorbed histidine and homocarnosine were eluted by the passage of 4.0 ml 0.25 N hydrochloric acid followed by 6.0 ml deionised distilled water at a rate of 0.2 ml/min. The eluate was collected in a test-tube (Fig 22, p 188) suitable for connection to a manifold attachment for evaporation.

Evaporation of eluates. Eluates were taken to dryness under reduced pressure (15-20 mm Hg) at 55°C using a Rotary Evapo-mix (Buchler Instruments, Inc.) (Fig 23). Ten such eluates could be evaporated simultaneously using this machine.

Desalting of dried eluates. Each dried eluate was taken up in 0.24 ml water and 0.26 ml conc.

Fig 22. **Glass-tube (c) used for collection of eluate from the cation exchange resin CG-50-cellulose column equilibrated at pH 5.75 and for subsequent evaporation of the eluate in Rotary Evapomix (Buchler Instrument Inc., Model No. 3 - 2100), together with parts necessary to connect the tube with the manifold of the Evapomix.**

(a) Splash-head

(b) Plastic reduction adapter into which tube (c) is inserted

(d) All the parts joined together. The open end of (d) is connected to the vacuum source.

The glass-tube (c) was also used for collection of the filtrate from the HCl: acetone extract of the dried eluate from the cation exchange resin CG-50-cellulose column adjusted to pH 5.75.

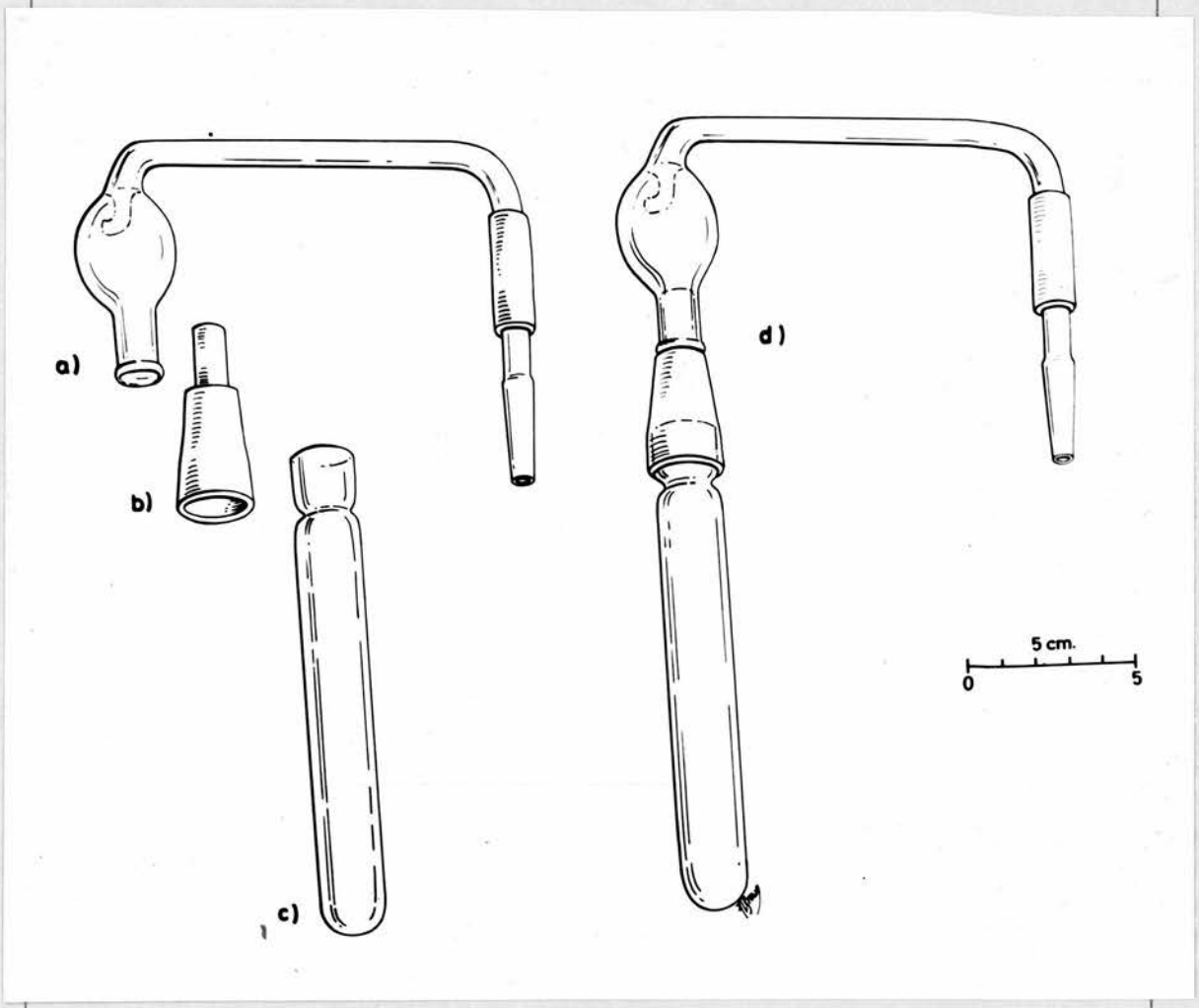


Fig 22. Glass-tube (c) used for collection of eluate from the cation exchange resin CG-50-cellulose column equilibrated at pH 5.75 and for subsequent evaporation of the eluate in Rotary Evapomix (Buchler Instrument Inc., Model No. 3 - 2100), together with parts necessary to connect the tube with the manifold of the Evapomix. (see opposite page).

Fig 23. Rotary Evapomix (Buchler Instrument Inc., Model No. 3 - 2100) used for the evaporation of eluates from cation exchange resin CG-50-cellulose columns and of the filtrates from the HCl-acetone extracts of the dried residue of eluates from the above column.

Fig 23. Rotary Evapomix (Buchler Instrument Inc., Model No. 3 - 2100) (see opposite page).

hydrochloric acid added followed by 10 ml acetone. The suspended salt was removed by filtration through a glass wool plug inserted in the taper of a glass tube as described on p 145. The test-tube which had contained the dried eluate was washed with a further 5 ml acetone and this was also passed through the filter. The combined filtrate and wash fluid was collected in a test-tube suitable for attachment to the Rotary Evapo-Mix machine.

Evaporation of filtrate. To remove the acetone, the filtrate (15.5 ml) was evaporated under reduced pressure (20 to 15 mm Hg) and at room temperature (21°C) to a volume of about 0.5 ml using the Rotary Evapo-Mix machine. The ambient temperature was then raised to 70°C (water bath) and the remaining part of the filtrate containing hydrochloric acid was evaporated to dryness; hot air at 75 - 80°C was blown on the sides of the tubes above the level of water to prevent condensation and reflux. It was found to take 10 min. to remove the acetone and a further 15 min. to complete the evaporation.

The histidine and homocarnosine in the dried extract was separated by thin-layer chromatography on cellulose.

Cellulose thin-layer chromatography: separation of histidine from homocarnosine.

The dried extract in each tube was taken up in 0.15 ml methanol and 0.15 ml water added. The solution was transferred to a 0.5 mm cellulose (Whatman, Chromomedia) layer on a 200 mm x 50 mm glass carrier plate (p 164). The transfer was made by repeated application using a glass capillary ('melting point') tube to the central 20 mm of the layer on a pencil line lightly drawn across the 50 mm width of the layer and 25 mm from one end. Care was taken to restrict the fluid band width to less than 5 mm; this was aided by rapid evaporation of the solvent in a stream of hot air from a hair dryer. Transfer was completed by washing the tube which had contained the extract, with 0.2 ml, 50% (v/v) methanol and applying this wash fluid to the cellulose layer.

Extracts from control ion-exchange columns through which only the reagents had been passed were treated similarly.

Each batch of cellulose layers carrying sample extracts contained one layer to which had been applied 1 µl of a 'histidine-homocarnosine' marker solution (Appendix p 286).

To each cellulose layer was attached, at the end distal to the point of application of the extract, a pad of 3 MM Whatman filter paper (p165) to allow over-running of the solvent during development of the chromatogram.

Development of thin-layer chromatogram. The chromatograms were developed for 13½ hr. in ascending propan-1-ol: acetic acid: water (15:1:4 by vol.) containing 0.3 g sodium chloride/100 ml and then dried for 24 hr. in a stream of air in a chromatography oven (p166).

Elution of separated histidine and homocarnosine.

The positions of histidine and homocarnosine on the marker plate were visualised by spraying the dried plate with 0.2% ninhydrin in acetone followed by heating for 30 min. at 100°C in an oven.

The cellulose from each corresponding area of each sample plate was scraped off, and shaken with 4.0 ml water to elute the histidine or homocarnosine; a clear eluate was obtained by removal of the suspended cellulose by centrifugation. The elution procedure is described on p 166 .

The supernatant fluid, approximately 3.5 ml, was transferred with the aid of a Pasteur pipette

to a 10 ml glass-stoppered tube. Of this solution, 2 ml was used for the fluorimetric assay and the remainder retained for a repeat estimation should this prove necessary.

Fluorimetric assay of histidine and homocarnosine.

A 1.0 ml portion of the eluate from the eluate of a thin-layer chromatogram was transferred to each of two 10 ml glass-stoppered tubes. To one was added 0.1 ml water; to the other was added 0.1 ml of a standard solution of histidine (2.0 µg/ml) or homocarnosine (10.0 µg/ml) whichever was appropriate. To each tube was added 0.2 ml N NaOH solution followed by 0.05 ml 0.5% (w/v) o-phthalaldehyde in methanol; the tube was stoppered and the solutions mixed.

The relative fluorescence intensities of the solutions were measured, for the histidine reaction product, 60 to 120 min. after adding the aldehyde and, for the homocarnosine reaction product, 10 to 120 min., after adding the aldehyde.

Estimates of the "blank" fluorescence deriving from reagents used in the separation technique were obtained by reacting the corresponding eluates from chromatogram of extracts from buffer solution processed through the ion-

exchange chromatography procedures in parallel with the samples.

Fluorimetry.

The relative fluorescence of the o-phthalaldehyde reaction product was measured in the Zeiss spectrophotofluorimeter using a slit width of 1.0 mm for the activation monochromator and 0.5 mm for the analysing monochromator.

For the histidine fluorophor, the fluorescence intensity was measured with the fluorescence analysing monochromator set at 437 nm (band pass 2.5 nm), from the peak height at 375 nm (band pass 14 nm) on the activation spectrum recorded from 300 nm to 437 nm.

For the homocarnosine fluorophor, the fluorescence intensity was measured with the fluorescence analysing monochromator set at 410 nm (band pass 2.5 nm), from peak height at 340 nm (band pass 10 nm) on the activation spectrum recorded from 300 to 410 nm.

Such activation spectra were recorded in duplicate for each sample (p 277).

The difference in the fluorescence intensities of a sample and a similar sample to which a known amount of histidine or homocarnosine had been added

was used as a measure of the relative fluorescence equivalent to that standard amount of histidine or homocarnosine. The use of such an internal standard for calibration would allow for any quenching or potentiation of the fluorescence (Appendix p 270) which might arise from the presence of other substances in the sample.

Calculation of the histidine or homocarnosine concentration of the tissue sample.

The concentration (μg/g) of histidine or homocarnosine in a brain sample can be calculated from the expression

$$C = \frac{y - b}{x - y} \times S \times 4 \times \frac{5}{4.6} \times \frac{1000}{w}$$

where C = concentration (μg/g) of histidine or homocarnosine in the brain sample.

y = fluorescence intensity of sample (aliquot 1 ml) at the characteristic wavelengths.

b = fluorescence intensity of the "blank" at the characteristic wavelengths.

x = fluorescence intensity of the sample (aliquot 1 ml) with added standard of histidine or homocarnosine at the characteristic wavelengths.

S = amount, in ug, of histidine or homocarnosine added as standard, to the sample aliquot.

w = weight of tissue sample in mg.

The factor 4 derives from the total volume (4.0 ml) of the final extract of which a 1.0 ml aliquot was used for fluorimetry.

The factor 5/4.6 corrects for the total volume of the initial tissue-trichloroacetic acid mixture (5 ml), of which 4.6 ml of supernatant fluid, obtained after centrifugation, was processed through the method. This correction factor makes the assumption that the histidine and homocarnosine were uniformly distributed in the supernatant fluid and precipitate.

An additional factor may be necessary in the above expression if a dilution of the eluate was required before assay in order to reduce the histidine or homocarnosine content of the solution for fluorimetry to within the limit of the range of linearity of the calibration curve i.e. to an amount below 0.6 µg for histidine or 3.0 µg for homocarnosine (p 79 and p 89).

CHAPTER III

ESTIMATION OF HISTIDINE AND HOMOCARNOSINE IN BRAIN TISSUE

THE ESTIMATION OF HISTIDINE AND HOMOCARNOSINE IN BRAIN TISSUE

The estimation of histidine and homocarnosine in brain tissue using the analytical procedures developed in the present work are considered in the following section under three headings. The first gives evidence of the specificity of the method, the second deals with the recovery of the two amino acids from buffer solutions and brain tissue extracts through the analytical procedure while the third presents the estimates of the histidine and homocarnosine concentrations in mouse brain and the effects thereon of histidine administration to the animals.

Specificity of the method for the estimation of histidine and homocarnosine in brain tissue

Several tests of the specificity of the method were carried out. These included (i) a study of the effectiveness of the separative procedure in the removal of other substances giving rise to fluorescent derivatives with o-phthalaldehyde and likely to be present in crude brain extracts; (ii) an investigation of

the possible artifactual formation of histidine from histidine derivatives in the course of the procedure, and (iii) tests of identity of the substances measured in the extract from brain tissue by chromatographic and fluorescence characteristics.

The effectiveness of the separative procedures.

Histamine (Shore et al., 1959 present work, p 98); arginine and agmatine (Cohn and Shore, 1961; the present work p 99); homocarnosine and carnosine (p 99) and histidine (Aures et al., 1968; present work, p 84) produce fluorophors by interaction with o-phthalaldehyde under similar experimental conditions. Of these substances, histamine (Adam et al., 1957, Adam, 1961; Adam and Hye, 1966), and arginine and agmatine (this work, p 125) are almost completely adsorbed on the column of cation exchange resin, Amberlite CG-50, at pH 8.0. The more weakly basic compounds, histidine, homocarnosine and carnosine pass into the effluent.

Histidine is separated from homocarnosine by chromatography on a thin-layer of cellulose. For reasons already discussed (p 173), the fact that carnosine is poorly separated from these compounds under the conditions of the chromato-

graphic development, and is located mainly in the 'histidine' position, is considered unlikely to lead to serious inaccuracy in the quantitative estimation of histidine or homocarnosine.

Test of possible artifactual formation of histidine. To remove the salts from the eluate of the cation exchange column, prior to the application of the solute to the cellulose thin-layer for the separation of histidine from homocarnosine, the eluate is evaporated to dryness, the residue taken up in hydrochloric acid and the salts precipitated by the addition of acetone. The desalted extract is evaporated to dryness under reduced pressure; the acetone is removed at room temperature but the residual, strongly acid solution, is evaporated at 70°C. It seemed possible that, under these conditions of extreme acidity and elevated temperature, histidine might be released from the histidine containing dipeptides or from N-acetylhistidine. Such histidine formation would, of course, lead to overestimates of the endogenous histidine in the tissue sample, and this possibility was examined in the following experiments, in which the acid conditions obtaining in the evaporation of desalted tissue extracts

were simulated.

Duplicate samples of a solution of 10 µg homocarnosine in 0.5 ml 6 N-HCl were evaporated under reduced pressure (15-20 mm Hg) at 70°C using a rotary Evapo-Mix. Each dried residue was taken up in 4.0 ml water and a 1.0 ml portion of each solution reacted with 0.2 ml N NaOH and 0.05 ml 0.5% o-phthalaldehyde for at least 1 hour before measuring the fluorescence at 340 nm (activation) and 410 nm (fluorescence). The fluorescence intensities of the solutions were compared with duplicate control samples containing 2.5 µg homocarnosine in 1 ml water and reacted with NaOH and o-phthalaldehyde concurrently with the evaporated samples.

The relative fluorescence intensities of the control solutions were 50 and 49 and of the test samples, 49 and 46. There was thus no real difference between the relative fluorescence intensities of the controls and the test solutions. If histidine had been formed from homocarnosine it would have been expected that the relative fluorescence intensities of the test samples would have been higher than those of the controls because of the much greater yield of fluorescence from histidine than from homocarnosine (p 96).

It is estimated that the production of about 0.12 µg histidine from the 10 µg homocarnosine (7.4 µg of potential histidine) would have been detected.

A similar experiment was carried out on duplicate samples of 0.5 ml 6 N-HCl to which was added a mixture of 1 µg carnosine and 0.5 µg each of N-acetylhistidine, 1-methylhistidine and 3-methylhistidine. The methylhistidines were included for the sake of completeness although demethylation to histidine was considered highly improbable. After evaporation each dry residue was dissolved in 1.0 ml water and reacted with o-phthalaldehyde in alkaline solutions as above. The fluorescence intensities of the samples were measured at 375 nm (activation) and 437 nm (fluorescence) and compared with the intensities simultaneously derived from a blank solution consisting of 1.0 ml water containing the alkali and o-phthalaldehyde and from a histidine solution containing 0.2 µg. The fluorescence intensities of the test samples (relative fluorescence 7 and 6.5) did not differ from that of the "blank" solution. Under the conditions of measurement the standard containing 0.2 µg histidine had a relative fluorescence of 36.

There was therefore, no evidence from these experiments that the presence of any of these histidine derivatives in an extract would lead to erroneous estimates of the endogenous histidine as a result of histidine formation during the evaporation of the strongly acidic solution at the desalting stage of the method of analysis.

Evidence of the identity of the 'histidine' and of the 'homocarnosine' measured fluorimetrically in extracts of brain tissue.

Acceptable criteria of identification depend on the parallel behaviours of the 'unknown' and the authentic substances in various physical and chemical tests. One such test seldom provides data for absolute identification, and corroborating evidence for several tests is necessary. In the assay of a substance in biological extracts, the amount of material available is usually severely limited and the number of tests of identification which can be applied routinely is, thereby, restricted. However the analytical method itself provides inbuilt tests which together afford evidence, within limits which should be recognised, for the identity of the substance finally estimated. In the present procedure for histidine and homo-

carnosine estimations, the material measured in the purified extracts of brain tissue must parallel the behaviour of the corresponding authentic substance in several respects: (1) its adsorption on a weak cation-exchange resin at pH 5.75 but not at pH 8; (2) its position on a cellulose thin-layer chromatogram and (3) the fluorescence characteristics of its fluorophor with o-phthalaldehyde with peak intensities at 375 nm (activation) and 435-440 nm (fluorescence) for the histidine fluorophor, and 340nm (activation) and 410 nm (fluorescence) for the homocarnosine fluorophor. Some supporting evidence of the validity of these tests as applied to extracts of brain tissue is given below.

A sample of mouse brain tissue (mainly cerebrum; aliquot processed equivalent to 100 mg wet weight) was extracted, purified and applied to a 5 cm wide cellulose thin-layer chromatogram as in the analytical method (p 180). After development of the chromatogram in propan-1-ol: acetic acid: water (15:1:4 by vol.) containing 0.3 g% sodium chloride (p 192), the areas of the dried layer corresponding to the positions of histidine and homocarnosine on a 'marker' chromatogram which was developed simultaneously,

were separated off and eluted in 4 ml water (p 192). The limits of these areas relative to the origin of the chromatogram were 5.0 to 7.7 cm (histidine) and 8.3 to 11.0 cm (homocarnosine).

Cellulose from other areas of the layer was also scraped off and eluted. The positions of these portions were 0.0 to 2.5 cm and 2.5 cm to 5 cm (these comprising the area between the origin and the lower limit of the 'histidine' position) and 11.0-13.5 cm (the area immediately beyond the upper limit of the 'homocarnosine' position) (Fig 24, p205); each portion of cellulose was eluted in 4 ml water. Finally the area between the upper limit of the 'histidine' and the lower limit of the 'homocarnosine' was eluted in 3 ml water. Portions of 1.0 ml of each eluate were treated with o-phthalaldehyde in the presence of alkali (p 193) and the fluorescence intensities measured.

Only the eluates from the areas of the sample chromatogram corresponding to the positions of histidine and homocarnosine on the 'marker' chromatogram contained fluorogenic material. Moreover the fluorophors from the appropriate eluates showed the fluorescence characteristics of those of histidine and homocarnosine. The intensities

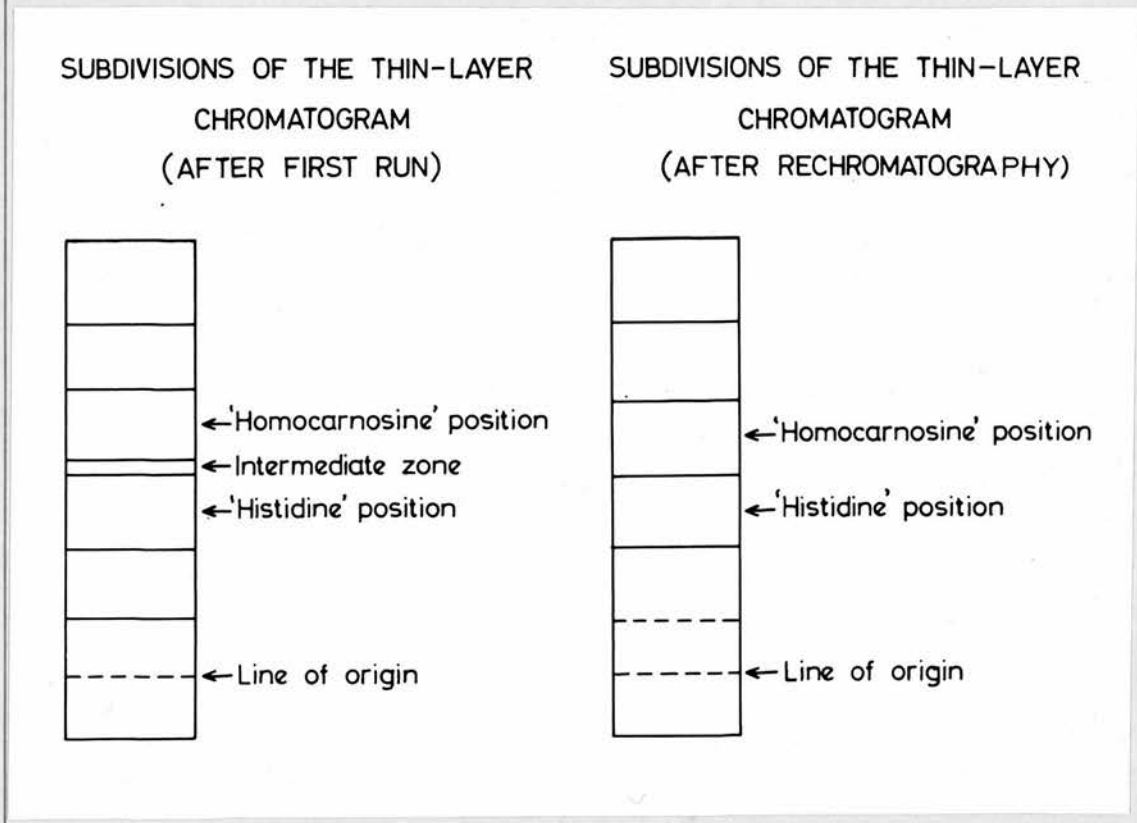


Fig 24. **Chromatographic characterisation of histidine and homocarnosine in brain extracts.** **(see text, p 203).**

Subdivisions of the cellulose thin-layers,

(a) after first run,

(b) after rechromatography.

Each area was eluted with 3 or 4 ml water, 1 ml of which was employed for measuring the fluorescence.

of fluorescence obtained from the eluates of the other areas were the same as those from eluates of corresponding areas of a 'blank' chromatogram.

These observations were confirmed in another experiment of a somewhat different design. Two samples of brain tissue were extracted, purified and submitted to thin-layer chromatography as in the analytical method (p180). The two eluates from the 'histidine' positions of the chromatograms were combined as were those from the 'homocarnosine' positions. The combined eluates were evaporated to dryness at 55°C under reduced pressure (15-20 mm Hg) using an Evapo-Mix machine. Each dry residue was taken up in 0.5 ml 50% methanol and rechromatographed on a 5 cm wide cellulose thin-layer using the same solvent as before. The dried developed chromatogram was divided at right angles to the solvent flow, into 5 areas each of 3 cm long starting 2 cm from the origin (Fig 24). The cellulose from each area was eluted in 4.0 ml water. A 1.0 ml portion of each eluate was treated with o-phthalaldehyde in alkali solution (p193) and the fluorescence of the samples measured.

From the results it was possible to map the distribution of fluorogenic material on the chromatograms. Fluorogenic material in the second

chromatogram of the supposed 'histidine'-containing eluates from the first chromatograms was confined to the position occupied by authentic histidine in a 'marker' chromatogram developed simultaneously; the fluorescence intensities of eluates from other areas of the chromatogram did not differ from those of eluates of a 'blank' chromatogram. Similarly the fluorogenic material in the second chromatogram of the supposed 'homocarnosine'-containing eluates from the first was confined to the region corresponding to homocarnosine in the 'marker' chromatogram. In each case the product of the o-phthalaldehyde reaction had the same fluorescence characteristics as those of the authentic compound. From the evidence obtained in these experiments it may be concluded that the substances measured in brain extracts had the chromatographic and fluorogenic characteristics of authentic histidine (Fig 25 , p 208) and homocarnosine (Fig 26 , p 209).

The results of these experiments also afford some evidence of the adequacy of the cellulose thin-layer chromatographic technique for the separation of histidine and homocarnosine in brain extracts. In the first experiment the total amount of histidine and of homocarnosine estimated

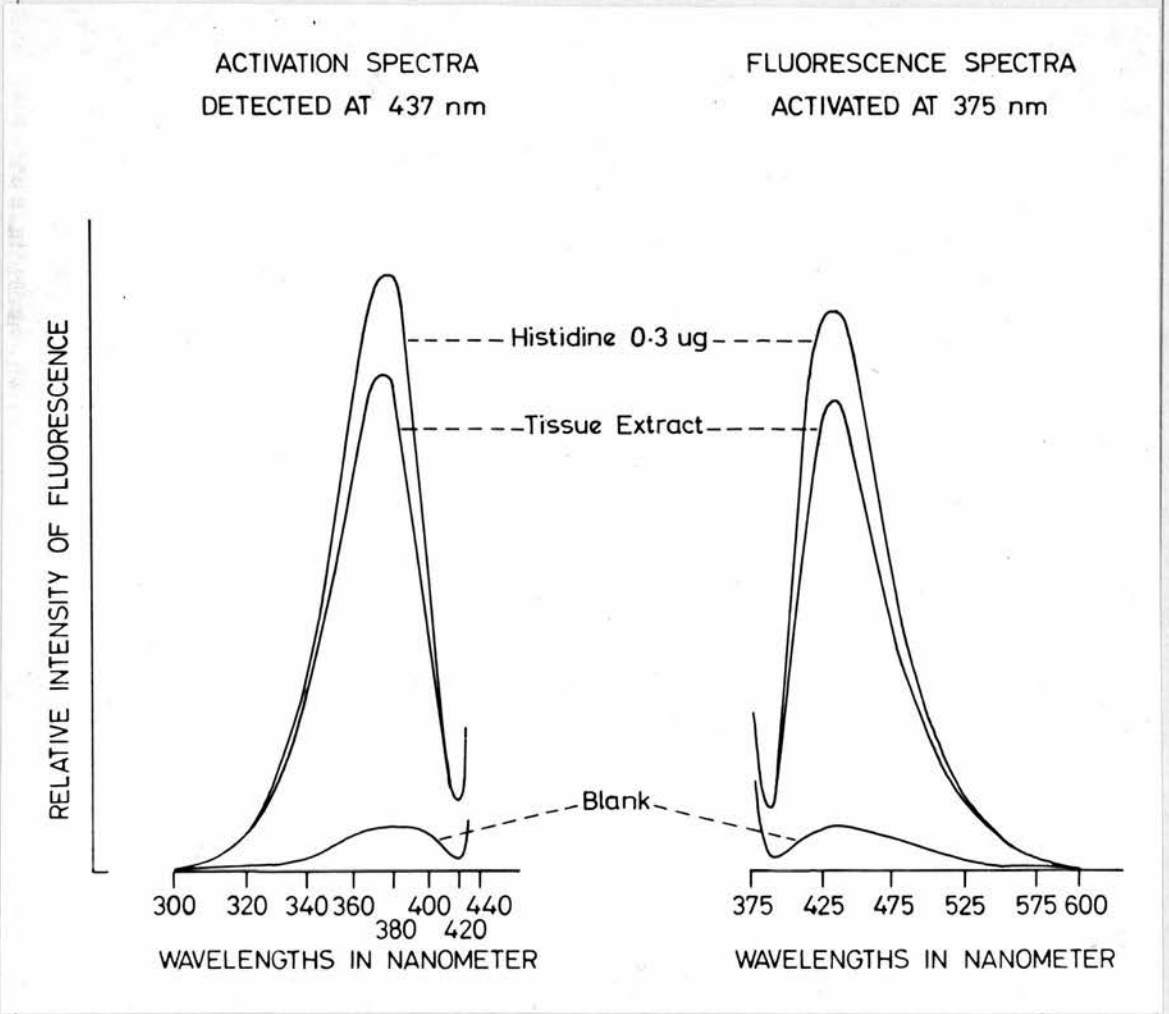


Fig 25. **Comparison of the activation and fluorescence spectra of the fluorophors derived from authentic histidine and from a 'histidine'-containing extract of brain tissue.**
Peak fluorescence was observed from both fluorophors at 375 nm (activation) and 437 nm (fluorescence).
Activation and fluorescence spectra of the control sample (blank) are also shown.

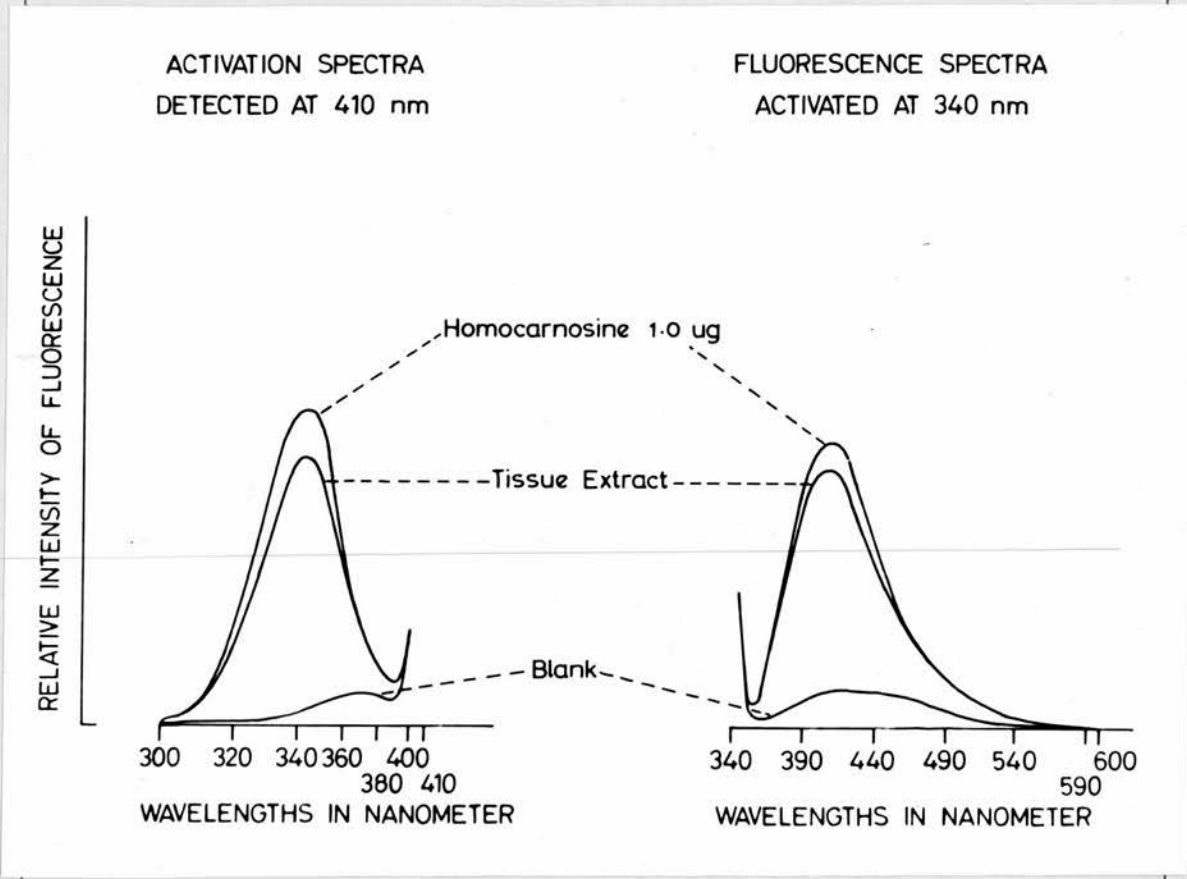


Fig 26. **Comparison of the activation and fluorescence spectra of the fluorophors derived from authentic homocarnosine and from a 'homocarnosine'-containing extract of brain tissue.**
Peak fluorescence was observed from both fluorophors at 340 nm (activation) and 410 nm (fluorescence).
Activation and fluorescence spectra of the control sample (blank) are also shown.

to be present in the respective eluates of the appropriate regions of the chromatogram was 0.8 µg for histidine and 2.0 µg for homocarnosine. The eluate of the cellulose layer intervening between the histidine and the homocarnosine regions contained no fluorogenic material attributable to either of these substances; amounts in excess of 0.06 µg histidine or 0.6 µg homocarnosine would have been detected.

In the second experiment the amount of histidine and homocarnosine involved in the chromatography were of the order of 1.1 µg histidine and 3.5 µg homocarnosine. No fluorogenic material attributable to the presence of the other compound was detected on the second chromatogram of the separated fractions obtained from the first chromatogram. Amounts of histidine and homocarnosine which would have been detected were 0.04 µg for histidine and 0.4 µg for homocarnosine.

Within the limitations imposed by the sensitivity of the method for the fluorimetric detection of histidine and homocarnosine, the evidence of the experimental results points to the quantitative separation of histidine from homocarnosine in brain extracts by the cellulose thin-layer chromatography technique. Stricter

monitoring of the separation would be possible by the addition of tracer amounts of radioactively labelled compounds to the brain extracts before chromatography.

Recovery of histidine and homocarnosine, from simple solution and brain extracts, through the analytical procedure.

Two types of experiments were carried out to test the recovery of histidine and homocarnosine through the analytical method. In the first a known quantity of histidine 0.5 to 2.0 µg and/or of homocarnosine 5 to 10.0 µg was added to 10 ml of 0.05 M phosphate buffer, pH 8.0 and 100 mEq Na^+/l. The solution was then processed through the analytical procedure starting at the stage involving the passage through an ion-exchange-cellulose column equilibrated to pH 8.0. Several such solutions were analysed in parallel and each batch contained one control sample consisting of 10 ml of buffer solution only. The latter provided final extracts for the estimations of the reagent blank of the method, which was used to correct

the sample estimates.

In the second type of recovery experiment, histidine and homocarnosine were added to a portion of a trichloroacetic acid extract of mouse brain. The extract was neutralised and its Na^+ concentration adjusted to 100 mEq/1 (p 184). To a 5.00 ml portion of the neutralised extract equivalent to 100 mg tissue was added known amounts of histidine and homocarnosine in a volume of 0.1 ml. The solution was then processed through the method in parallel with a like portion of the same extract to which no histidine or homocarnosine had been added. The latter provided estimates of the endogenous histidine and homocarnosine which were substracted from those of the sample containing the added substances to give the estimates for the exogenous histidine and homocarnosine. Appropriate reagent blank estimates for each batch of analyses were obtained by processing 10 ml of 0.05 M phosphate buffer pH 8.0 starting from the stage of the analytical procedure involving chromatography on an Amberlite CG-50-cellulose column equilibrated to pH 8.0.

For calibration of the fluorimetry, in most cases internal standards of either 0.1 or 0.2 µg histidine, or 1 or 2 µg homocarnosine as appropriate

were added to a portion of each final extract and the relative fluorescence of these standards was used to calculate the content of the substance in the sample solution (see results). In one histidine series the results were calculated from the relative fluorescence of standards prepared in the eluates of the control 'blank' thin-layer plates. Each sample and the corresponding standard, internal or external, were measured in the fluorometer within about 30 secs of each other and duplicate measurements of each taken.

Results

The results of these experiments are given in the accompanying Tables. Table 17 , p 214 and Table 18 , p 215 show the estimates for the recovery of histidine from buffer solutions and tissue extracts. Table 19 , p 216 and Table 20 , p 217 show estimates for the recovery of homocarnosine from buffer solution and tissue extracts. In each case the amount of histidine or homocarnosine, which had also been added to the sample, is quoted.

The histidine or homocarnosine content of the final extract from each sample from the separation procedure was calculated from the fluorescence derived from an internal standard

Table 17

Recovery of histidine from buffer solution (0.05 M Na-phosphate buffer, pH 8.0)

Mixtures of known amounts of histidine and homocarnosine were added to 10 ml buffer solution and processed through the analytical procedure.

Each recovery estimate was calculated from the individual sample internal standard with exception of results marked with ϕ, where the recovery estimates were calculated from the fluorescence of standard solutions prepared in blank extracts.

Sample No.	Amount of histidine added ug	Histidine recovered ug	Histidine recovered Percentage	Homocarnosine added ug
1	0.5	0.35	70	Nil
2	0.5	0.11	22	7.5
3	0.5	0.30	60	7.5
4	0.75	0.46	61	5.0
5	0.75	0.71	95	Nil
6	1.0	0.90	90	Nil
7	1.0	0.68	68	Nil
8	1.0	0.74	74	5.0
9	1.0	0.52	52	5.0
10	1.0	0.60 ϕ	60	Nil
11	1.0	0.45 ϕ	45	Nil
12	1.0	0.50 ϕ	50	Nil
		Mean recovery estimate from 1.0 ug is 63% ± 6 (S.E.M., 7 estimations).		
13	2.0	1.57	78	5.0
14	2.0	1.73	86	10.0
15	2.0	1.08	54	10.0
16	2.0	1.34	67	7.5
17	2.0	1.55 ϕ	78	Nil
18	2.0	1.68 ϕ	84	Nil
		Mean recovery estimate from 2.0 ug is 74% ± 5 (S.E.M., 6 estimations).		
		The Mean recovery from 0.5 to 2.0 ug histidine = 66% ± 4.3 (S.E.M., 18 estimations)		

Table 18

Recovery of histidine from trichloroacetic acid extracts of mouse brain.

Mixtures of known amounts of histidine and homocarnosine were added to trichloroacetic acid extracts of brain tissue and processed through the analytical procedure. 5.0 µg homocarnosine was added to each sample.

Histidine added µg	Exogenous + endogenous histidine estimate µg	Endogenous histidine estimate µg	Exogenous histidine i.e., recovery estimates µg	Per Cent
1.0	2.63	1.41	1.22	122
1.0	2.62	1.91	0.71	71
2.0	3.05	1.35	1.70	85

Table 19

Recovery of homocarnosine from buffer solution (0.05 M Na-phosphate buffer, pH 8.0.

Mixtures of known amounts of homocarnosine and histidine were added to 10 ml buffer solution and processed through the analytical procedure. Each recovery calculated from individual sample internal standards.

Sample No.	Amount of homocarnosine added µg	Homocarnosine recovered µg	Per Cent	Histidine added µg
1	5.0	2.6	52	Nil
2	5.0	2.12	42	0.75
3	5.0	2.75	55	1.0
4	5.0	1.85	37	1.0
5	5.0	2.2	44	2.0
		Mean recovery from 5.0 ug homocarnosine = 46% ± 3.3 (S.E.M., 5 estimations)		
6	7.5	5.1	68	0.5
7	7.5	4.5	60	0.5
8	7.5	4.1	55	Nil
9	10.0	7.3	73	2.0
10	10.0	6.0	60	2.0
		Mean recovery from 7.5-10.0 ug homocarnosine = 63% ± 3.2 (S.E.M., 5 estimations).		
		Mean recovery from 5 - 10 ug homocarnosine = 55% ± 3.6 (S.E.M., 10 estimations).		

Table 20

Recovery of homocarnosine added to trichloroacetic acid extracts of mouse brain.

Mixtures of known amounts of homocarnosine and histidine added to trichloroacetic acid extracts of brain tissue and processed through the analytical procedure.

Each estimate was calculated from individual sample internal standard.

Exp. No.	Amount of homo-carnosine added µg	Endogenous + exogenous homo-carnosine estimate µg	Endogenous homo-carnosine estimate µg	Exogenous homocarnosine i.e., recovery estimates µg	Percentage	Amount of histidine added µg
1	5.0	5.16	2.33	2.83	56	1.0
2	5.0	4.78	2.27	2.5	50	1.0
3	5.0	6.80	2.50	4.3	86	2.0

added to that sample (see methods p194).

Discussion

The mean recovery of histidine added in the range 0.5 µg to 2 µg to buffer solution was 66% (S.E.M. ± 4.3, range 22-95%, 18 estimations) (Table 17 , p 214). From the recoveries from each stage in the analytical process as determined in model experiments, a total recovery of some 70% might have been expected.

There appeared to be a tendency for a higher recovery from the larger amounts. From 2 µg, a mean recovery of 74% (S.E.M. ± 5, range 54-84%, 6 estimations) was obtained as compared with a mean recovery of 63% (S.E.M. ± 6, range 45-90%, 7 estimations) from 1 µg (Table 17 ,p 214). However, a Student's 't' - test showed no significant difference between the means ($t = 1.52$, $p > 0.1$). An average recovery of about 60% was also found in a limited number of experiments with 0.5 µg and 0.75 µg amounts. The recoveries from 1 µg and 2 µg histidine added to brain extracts ranged from 71% to 122% in three experiments. In all these experiments there was no evidence of an interference with the histidine

estimations which could be related to the presence of homocarnosine in the initial solution.

Homocarnosine in amounts of 5 µg to 10 µg added to buffer solution was recovered with a mean percentage of 55 (S.E.M. ± 3.6, range 37% to 73%, 10 estimations) (Table 19 , p 216). Again there was a tendency for larger amounts to give better recoveries; from 7.5 to 10 µg amounts a mean of 63% (S.E.M. ± 3.2, range 55 to 73%, 5 estimations) was obtained and be compared with a mean of 46% (S.E.M. ± 3.3, range 37 to 55%, 5 estimations) from 5 µg amounts (Table 19 , p 216). In three experiments in which 5 µg was added to brain extracts an average recovery of 64% was obtained.

There was no evidence of an interference in the homocarnosine estimations which could be related to the presence of histidine.

The percentage recovery of homocarnosine was lower than that of histidine and showed a dependence on the amount of homocarnosine present in the original sample.

The mean percentage recovery from 5 µg amounts of homocarnosine added to buffer was significantly different from that of 7.5 to 10 µg amounts ($t = 3.52$, $p < 0.01$). However the absolute loss of added substance did not differ significantly

between the two groups, ($t = 1.25$, $p > 0.2$), the mean loss from the 5 µg amounts being 2.7 µg and from the 7.5 to 10 µg amounts, 3.1 µg. This statistical analysis would appear to indicate mainly a constant rather than a proportional loss of homocarnosine. No suggestion can be offered at the present time as to where this might be occurring in the separation technique.

Similar analysis of the histidine recoveries from 1 µg and 2 µg amounts of histidine added to buffer solution showed no significant difference either percentage-wise ($t = 1.52$, $p > 0.1$) or in terms of absolute loss ($t = 1.17$, $p > 0.2$). It would appear that if there is the loss of a constant amount of histidine through the method it is not the main factor in the lowered recoveries. Having regard to the somewhat complicated separation technique and limits of sensitivity and discrimination of the fluorimetric methods, the recoveries of these relatively small amounts of histidine and homocarnosine would be considered acceptable.

As already mentioned in the method, each sample and the corresponding standard were measured in the fluorimeter within about 30 sec of one another and the readings made in duplicate. In

this way errors due to instrumental variations which were likely to occur on occasion were avoided as much as possible. The validity of the results obtained in this manner was examined in a single experiment concerned with the recoveries from solutions in which, in addition to internal standards, external standards were monitored at the same time. The former showed throughout the series of analyses a variation in the measurement of relative fluorescence but this variation was paralleled by a similar change in an external standard measured concurrently. The observed differences in the relative fluorescence of the internal standards from different samples were thus attributable to instrumental fluctuations rather than to materials in the samples quenching or potentiating fluorescence to varying degrees.

The percentage recoveries of histidine and homocarnosine added to tissue extracts were similar to those from buffer solutions. They might be expected to show a greater variation because each recovery estimate is dependent on two measurements, that for the endogenous substance and that for the endogenous plus the standard amount added; both these measurements would be subjected to variable losses through the method and consequently

variation in the recovery estimate is likely to be greater than that from buffer solution in which only one measurement is involved.

ESTIMATES OF THE HISTIDINE AND HOMOCARNOSINE CONCENTRATIONS IN MOUSE BRAIN

After the removal of the brain from the skull, the cerebellum with its peduncles was severed and discarded. The brain was then divided into two parts - the region of pons and medulla extending from the levels of superior cerebellar peduncles to the level of first cervical vertebra, and the remainder of the brain containing mainly the 'cerebral hemispheres' (p 178). The estimates of the concentration of histidine in µg/g wet weight of tissue in these two parts of brain from individual animals are shown in Table 21, p 223. The mean value in the 'pons and medulla' was 9.7 µg/g ± 1.1 (S.E.M., 7 estimations) the range being 6.1 to 13.7 µg/g. The concentration in the part which mainly contains cerebral hemispheres was 10.5 µg ± 0.91 (S.E.M., 12 estimations), the range being 6.5 to 14.8 µg/g. The difference

Table 21

Histidine and homocarnosine concentrations of mouse brain.

Values are uncorrected for recovery through the method.

Animal No.	Histidine concentration ug/g		Homocarnosine concentration ug/g	
	Pons and medulla	Cerebral hemispheres	Pons and medulla	Cerebral hemispheres
1	8.2	8.7	-	-
2	10.1	7.0	35.5	-
3	13.7	13.5	30.2	
4	9.7	-	30.2	-
5	6.1	12.7	-	-
6	13.0	6.5		
7	7.1	-	-	-
8	-	14.8	-	26.2
9	-	14.8	-	25.7
10	-	13.1	-	20.5
11	-	7.1	-	23.1
12	-	7.8	-	13.1
13	-	9.8	-	11.3
14	-	9.9	-	13.8
	Mean = 9.7 ± 1.1 (S.E.M.)	Mean = 10.5 ± 0.91 (S.E.M.)	Mean = 32.00 ± 1.76 (S.E.M.)	Mean = 19.0 ± 2.41 (S.E.M.)
	Mean = 10.2 ug/g ± 0.68 (S.E., 19 estimations)			

between the histidine concentrations of these two parts of the brain was not significant ($p > 0.5$).

Estimates of the concentration of homocarnosine in these two parts of mouse brain are also shown in Table 21 , p 223. The mean value of the three estimates in pons and medulla was 32.0 µg/g ± 1.76 (S.E.M.), the range being 30.2 to 35.5 µg/g. The individual estimates of the part which contains cerebral hemispheres vary from 11.3 to 26.2 µg/g of fresh tissue, the mean of the values being 19.0 µg/g (± S.E.M. 2.41, 7 estimations). Although the homocarnosine concentration appears to be significantly higher in the pons and medulla ($p < 0.005$), it is considered that further estimates would be required before this can be regarded as valid. In particular, comparison of estimates of both parts of the same brain would be desirable. This has not, as yet, been done.

The above estimates have not been corrected for the recovery through the method.

The effect of an intraperitoneal injection of histidine on the concentrations of histidine and homocarnosine in mouse brain has been studied. The dose injected varied from 15 µg to 120 µg/g body weight and the animals were killed 15 min later. The analyses were carried out on the 'cerebral

Table 22

Effect of an intraperitoneal injection of histidine on the histidine concentration of the 'cerebral hemispheres' of mouse brain.

The dose injected varied from 15 to 120 µg/g body weight. The control animals received isotonic saline 0.04 ml for each 5 g body weight, a volume the same as the histidine solution injected. The animals were killed 15 min after injection.

Controls: saline only	15 µg/g	Histidine dose 30 µg/g	60 µg/g	120 µg/g
HISTIDINE concentration (µg/g wet weight) in cerebral hemispheres				
9.8	12.2	21.8	11.3	15.7
10.0	10.7	21.8	17.0	16.6
7.0	14.8	11.3	23.2	
7.8				
Mean = 8.65 ± 0.74 (S.E.M.)	Mean = 12.57 ± 1.2 (S.E.M.)	Mean = 18.3 ± 3.5 (S.E.M.)	Mean = 17.2 ± 3.44 (S.E.M.)	Mean = 16.15 ± 0.45 (S.E.M.)

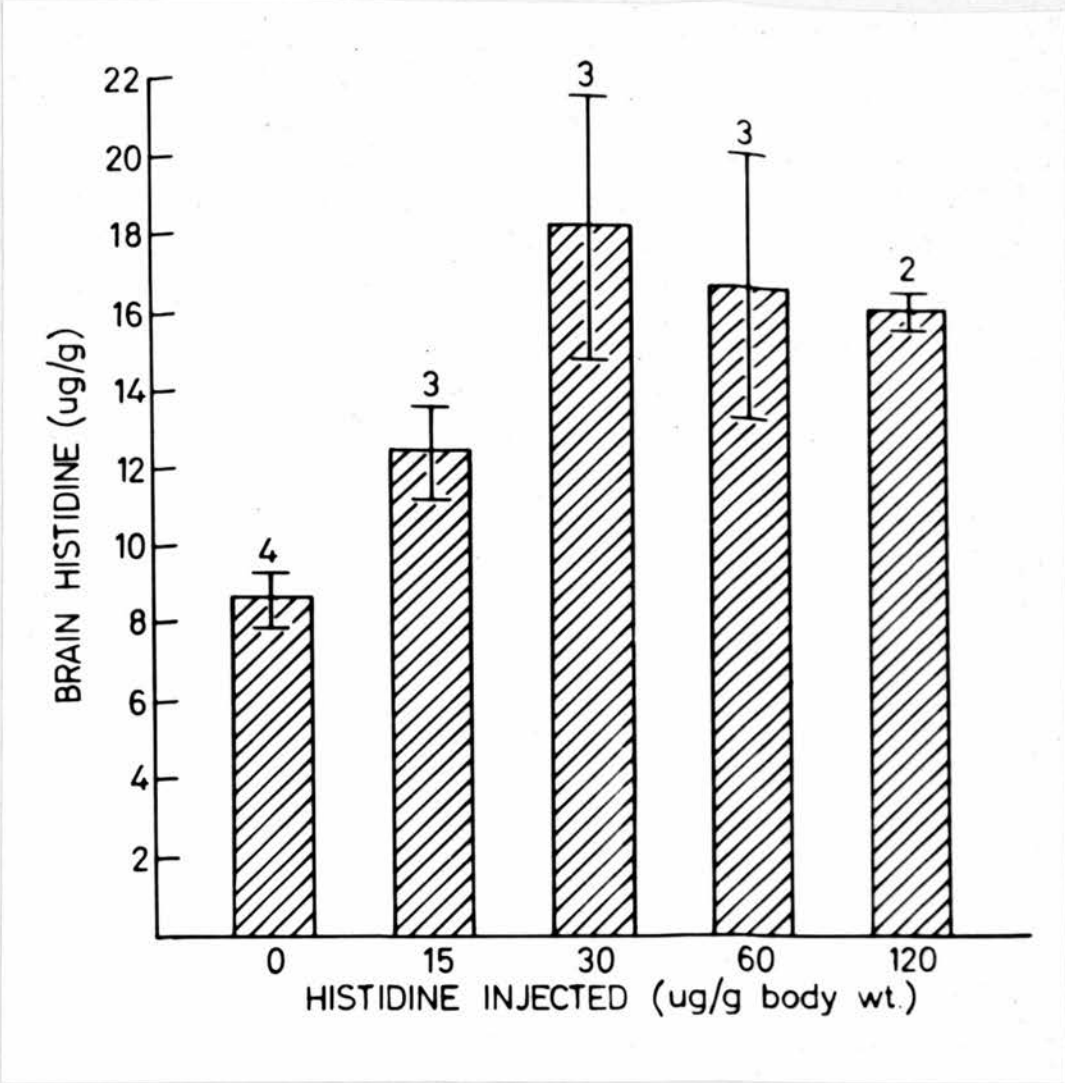


Fig 27. Histogram showing the relation of the concentration of histidine in mouse brain ('cerebral hemispheres') to the dose of histidine administered intraperitoneally 15 min before killing. I = ± S.E. with number of observations indicated on top.

Table 23

Effect of an intraperitoneal injection of histidine on the homocarnosine concentration of the 'cerebral hemispheres' of mouse brain. The control animals received 0.04 ml of isotonic saline for each 5 g body weight. The animals were killed 15 min after injection.

Control animals	Histidine dose			
	15 µg/g	30 µg/g	60 µg/g	120 µg/g
	HOMOCARNOSINE concentration (µg/g wet weight) in 'cerebral hemispheres'			
11.3	9.6	13.1	20.1	15.3
13.1	15.3	15.7	14.4	30.5
23.1	12.6	9.6	13.1	
13.1				
Mean = 15.15 ± 2.68 (S.E.M.)	Mean = 12.5 ± 1.65 (S.E.M.)	Mean = 12.8 ± 1.77 (S.E.M.)	Mean = 15.87 ± 2.15 (S.E.M.)	Mean = 22.9 ± 7.6 (S.E.M.)

hemispheres' regions of the brains as defined above. The histidine concentration in the brain increased with all doses (Table 22 , p 225) but showed a maximum with doses at and in excess of 30 ug/g (Fig 27 , p 226). With the lowest dose of histidine administered, 15 ug/g a significant rise ($p < 0.05$) occurred. Compared with the group of animals which had received 30-120 ug/g those which had received 15 ug/g showed a significantly ($p < 0.05$) smaller rise in histidine concentration.

No alteration in the homocarnosine concentration was detectable at any dose level (Table 23 , p 227).

CHAPTER IV

GENERAL DISCUSSION

GENERAL DISCUSSION

The work described in this thesis is considered in two parts: the first deals with the development of methods for the fluorimetric estimation of histidine and homocarnosine in brain tissue; the second with the application of the methods to a study of the histidine and homocarnosine content of mouse brain and the effects of histidine loading on the brain concentrations of these substances.

PART I

Development of methods for the fluorimetric estimation of histidine and homocarnosine in small quantities of brain tissue

Various methods for the estimation of histidine in pure solutions and in biological materials have been described in the literature and some of these methods have applied to the estimation of histidine in brain. These have already been discussed in some detail together with the reasons for their unsuitability for present purposes (p 63).

In the course of the development of a fluorimetric method of histidine estimation in the present study it was observed that homocarnosine could be estimated similarly. Measurements of homocarnosine concentrations in brain tissue from various species (Abraham et al., 1962; Kanazawa and Sano, 1967; Yockey and Marshall, 1969) have hitherto depended on a colorimetric method originally described by Abraham et al., (1962) and based on the Pauly reaction.

In the present work a method for the fluorimetric assay of small amounts of histidine and of homocarnosine in pure solution or in extracts of brain tissue, has been detailed. The method was developed in two stages, first, the experimental conditions for the fluorimetric estimation of histidine and of homocarnosine in pure solutions were defined, and second, a method for the separation of histidine and homocarnosine, from each other, and from other interfering substances to an extent suitable for their fluorimetric assays was worked out in model experiments with the pure substances.

During the development of methods for the independent fluorimetric assay of histidine and

the histidine containing dipeptide, homocarnosine, based on fluorophor formation by reaction with o-phthalaldehyde in alkaline solution, it was found that histamine, agmatine, arginine and carnosine also produced fluorescent products under the same reaction conditions.

Methodology was developed for the separation of histidine and homocarnosine from these interfering substances and other amino acids in an attempt to obtain, for assay, extracts which would simulate pure solutions to a maximum possible extent with reasonably practical manipulations. These separative procedures include column chromatography and thin-layer chromatography.

Of the compounds known to react with o-phthalaldehyde under the same experimental conditions as those of histidine and homocarnosine to produce fluorophors,histamine, agmatine and arginine are adsorbed on the cation exchange resin column composed of Amberlite CG-50 and cellulose powder, and equilibrated at pH 8.0 (see p 121), and are, thus separated from histidine and homocarnosine which pass into the effluent along with the acidic and neutral amino acids. The other basic amino acids, lysine (iso-electric point, 9.47 p111), hydroxylysine

(iso-electric point, pH 9.15 p_{111}) and ornithine (iso-electric point, pH 9.74 p_{111}) would also be adsorbed on these columns, since their iso-electric points are at pH values more than 1.0 unit higher than that obtaining under the conditions of the chromatography. Carnosine, with an iso-electric point at pH 8.1 (p_{111}) would likely be present in the effluent to some extent.

By the use of a second cation exchange resin column composed of Amberlite CG-50 and cellulose powder equilibrated at pH 5.75, histidine and homocarnosine were separated from the acidic and those of the neutral amino acids whose iso-electric points are at pH values lower than pH 5.75. The latter compounds pass into the effluent while histidine and homocarnosine are adsorbed on the columns. Although the acidic and neutral amino acids have not been reported to react with o-phthalaldehyde under the conditions of the experiment, removal of these acids would preclude possible overloading of the thin-layer chromatogram used to separate histidine from homocarnosine in the next stage of the method.

Since the iso-electric point of carnosine is 8.1, it would be adsorbed on the columns and therefore would be present along with histidine

and homocarnosine in the hydrochloric acid eluates of the columns.

The final separation of histidine from homocarnosine was achieved by thin-layer chromatography. As a preparatory step for the chromatography the sodium chloride present in the dried residue of the eluate from the second ion-exchange column was removed. This was carried out by extracting the components to be chromatographed with HCl and acetone and then filtering the extract through packed glass-wool to remove the insoluble salt. Less than 2% of the total sodium chloride expected to be present in an eluate from the ion-exchange column was present in the filtrate. Hydrochloric acid and acetone were then removed by evaporation.

Separation of histidine from homocarnosine in the desalted eluate from the ion-exchange column was obtained by chromatography on a 0.5 mm layer of cellulose, the chromatogram being developed in propan-1-ol: acetic acid: water (15:1:4 by vol) solvent system containing 0.3 g sodium chloride per 100 ml. Quantitative separation was achieved only with the presence of the sodium chloride in the solvent mixture and by 'over-running' of the solvent. This chromatography also separated the histidine and the homocarnosine from a number

of neutral amino acids whose iso-electric points are at higher pH values than pH 5.75 (Table 6, p 111), and which could be adsorbed on the column at pH 5.75 and then eluted along with histidine and homocarnosine. Although carnosine partially occupies the position of histidine on the thin-layer plate, the intensity of its fluorescence is too low to interfere with the fluorimetric assay of histidine (p 96).

The separated histidine and homocarnosine were eluted from the thin-layer chromatogram and estimated fluorimetrically following reaction with o-phthalaldehyde.

Various methods for the quantitative separation of histidine from homocarnosine in tissue extracts have been described previously in the literature. These include fractional elution after adsorption on a strong cation exchange resin (Pisano et al., 1961; Abraham et al., 1962), and paper electrophoresis (Kanazawa and Sano, 1967). These methods were, however, used for relatively large amounts and did not appear to be readily applicable to the separation of the small amounts of histidine and homocarnosine which it was hoped to estimate.

The separation method discussed above was

developed in model experiments using the authentic substances. It was an extension of that of Adam (1961) for the separation of histamine in brain tissue prior to assay of the amine, and thus opened up the possibility of estimating histamine, histidine and homocarnosine in a single sample of brain. Adam (1961) applied buffered trichloroacetic acid extracts of the brain tissue to cation exchange CG-50-cellulose columns equilibrated at pH 8.0 and eluted the adsorbed histamine which was measured biologically. In the present work it has been shown that after the application of a similarly prepared brain tissue extract to such a column, the effluent contained the histidine and homocarnosine which could then be measured fluorimetrically after further purification and separation. A flow sheet of the method which would enable the measurement of histamine, histidine and homocarnosine has been presented on page 176.

In the literature consulted no method for the measurement of the histamine, histidine and homocarnosine in the same tissue sample appears to have been developed till now.

Fluorimetry of histidine and homocarnosine. It has been found that histidine and homocarnosine react with o-phthalaldehyde to produce a fluorophor in a manner similar to histamine (Shore *et al.*, 1959).

In the method developed by these workers for histamine estimation, the amine and the aldehyde were allowed to react in alkaline medium for 4 min before acidifying to stabilise the fluorophor. Histidine was also found to react under these conditions and they were used by Pisano *et al.*, (1961) in their method of histidine estimation. However, the sensitivity of the method is relatively poor, a minimum of 1.5 µg of the amino acid being required in the sample.

In the present work it has been found that the conditions are not optimal in two respects for the development and measurement of the fluorophor from histidine. The time of reaction with o-phthalaldehyde was found to be too short, a 60 min reaction time being required to allow maximum fluorophor production. Subsequent

acidification of the reaction medium resulted in a considerable decrease in fluorescence intensity. The intensity of fluorescence of the histidine-o-phthalaldehyde fluorophor remained unchanged in the alkaline conditions of the reaction medium for at least an hour after the attainment of maximal fluorescence.

Under the experimental conditions devised in the present work it proved possible to measure 0.02 µg histiding in 1.0 ml pure solution, and provided the basis of a method for the estimation of the amino acid in tissues, possessing a sensitivity considerably greater than any other known method. For example, the concentration of endogenous histidine in brain is such that less than 100 mg tissue would be required for the estimation.

Probably for reasons similar to those discussed above, the method of Ambrose et al., (1969) for the fluorimetric measurement of histidine is also less sensitive than the present method. These workers allowed histidine to react with o-phthalaldehyde in alkaline medium for 15 min at 30°C and then for another 15 min in acid conditions before measuring the fluorescence.

The minimum measureable amount of histidine in the sample was reported to be 0.5 µg, so the method is more sensitive than that of Pisano et al., (1961) probably because the reaction between histidine and o-phthalaldehyde is allowed to continue for 15 min, instead of 4 min (Pisano et al., 1961) in alkaline medium before acidifying the sample.

It has also been found in the present work that the fluorophor production with o-phthalaldehyde from homocarnosine is favoured by an alkaline pH, and that maximum fluorescence develops within 10 min, as compared with 60 min for histidine. As with the histidine fluorophor, acidification of the medium decreased the fluorescence significantly. The sensitivity of the method for homocarnosine estimation under the experimental conditions set out in this work is about 0.2 µg in 1.0 ml pure solution, i.e. about one-tenth of the sensitivity of the histidine estimation. This is to be compared with the sensitivity of the method of Abraham et al., (1962), a colorimetric method based on the Pauly reaction, in which the lowest amount that could be measured was 4.8 µg.

If a method of homocarnosine estimation of greater sensitivity were required this could

presumably be achieved by the prior hydrolysis of the peptide with subsequent estimation of the liberated histidine. Such a method would, however, require the preliminary separation of the homocarnosine from other histidine peptides such as carnosine which could itself then be estimated in a similar manner. Carnosine does react with o-phthalaldehyde in an alkaline medium to give a fluorophore but the molecular yield of fluorescence is very low, about 10 µg of the dipeptide being required in simple solution to give a fluorescence double that of the blank.

PART II

Application of the analytical method to the estimation of histidine and homocarnosine concentrations in mouse brain and to a study of the effects of histidine loading on the brain concentrations of those substances.

The application of the method to the estimation of histidine and homocarnosine in small (100 mg) samples of brain tissue has been described, (p 175) and its limitations have been discussed (p 218). The estimated concentrations of histidine in mouse brain 10.2 µg/g ± 0.68 (S.E.

19 estimations, Table 21 , p 223) agree substantially with those reported by other workers (Table 3 , p 68 and Table 24 , p 241). Further, the results showed no significant difference between the concentration of histidine in the pons and medulla region and in the cerebral hemispheres 9.7 µg/g ± 1.1 (S.E.M.) and 10.5 µg/g ± 0.91 (S.E.M.) respectively ($P > 0.5$). Other workers using other species have reported regional differences in the concentration of the amino acid (Table 24 , p 241). Shaw and Heine (1965) found that in the rat brain the concentration of histidine was highest in the cerebellum, lowest in the cerebral hemispheres and intermediate in the mid-brain and pons-medulla. However, in the same species, Kandera et al., (1968) reports higher concentration in the cerebral hemispheres and cerebellum than in the mid-brain and pons-medulla. In the cat brain, higher concentrations of histidine were observed in cerebellum and mesencephalon (tectum) than in thalamus, cortex (temporal) and corpus callosum (Battistin et al., 1969). In all these cases, histidine was estimated using an automatic amino acid analyser.

The homocarnosine concentration was found to be higher in the pons-medulla region than in

References cited in Table 24

Reference	Method of estimation
(a) This thesis	Fluorimetry
(b) Okumura *et al.*,(1959)	Ion-exchange separations (Ninhydrin Reaction)
(c) Shaw and Heine (1965)	Autoanalyzer (Ninhydrin Reaction)
(d) Levi *et al.*, (1967)	" "
(e) Kandera *et al.*,(1968)	" "
(f) Battistin *et al.*,(1969)	" "

Table 24

Regional distribution of HISTIDINE in brain of various species

Mean values (ug/g). S.E.M. in parenthesis. Number of estimates //.

REGION	Species			
	CAT	DOG	MOUSE	RAT
Cerebrum				
Whole	-	-	10.5 (0.9) /12/ a	10.3 /4/ e
	-	-	12.6 (0.4) /3/ d	17.5 (1.0) /10/ c
Cortex	-	12.0 /1/ b	-	-
White matter	-	22.0 /1/ b	-	-
Mesencaphalon				
Mid-brain	-	-	-	9.2 /4/ e
				19.1 (1.45) /10/ c
Tectum	26.0 /4/ f	-	-	-
Diencephalon				
Thalamus	22.8 /4/ f	-	-	-
Telencephalon				
Temporal cortex	21.8 /4/ f	-	-	-
Corpus callosum	20.5 /4/ f	-	-	-
Rhombencephalon				
Pons-medulla	-	-	9.7 (1.10) /7/ a	6.8 /4/ e
				22.4 (1.45) /10/ c
Medulla	-	10.0 /1/ b	-	-
Cerebellum	-	-	-	9.6 /4/ e
				26.0 (1.0) /10/ e
vermix	-	27.0 /1/ b	-	-
cortex	26.6 /4/ f	-	-	-

Reference on the opposite page.

the cerebral hemispheres (32.0 µg/g and 19.0 µg/g respectively, Table 21 , p 223). Apart from this observation the possibility of regional differences in the homocarnosine concentration of mouse brain does not appear to have been investigated; however, such differences have reported in the brain of other species (Table 25 , p 243). Abraham et al., (1962) observed that in human brain homocarnosine concentration was highest in the cerebrum and lowest in the cerebellum and intermediate in the brain stem. In bovine brain these workers noted that the values were highest in brain stem, lowest in cerebellum and intermediate in the cerebrum while in monkey brain the values were, as in bovine brain, highest in brain stem, but lowest in cerebrum and intermediate in the cerebellum. In more detailed studies in human brain Kanazawa and Sano (1967) observed that the homocarnosine concentrations were higher in the thalamus, hyopthalamus and cerebellum and lowest in the medulla. The values obtained for homocarnosine concentration in 'the pons and medulla' in the present work are lower than the mean of the values obtained for the pons and the medulla separately by Kanazawa and Sano (1967) (Table 21 , p 223 and Table 25 , p 243).

Table 25

Regional distribution of HOMOCARNOSINE in brain of different species

Mean value (µg/g). S.E.M. in parenthesis. Number of Estimates //.

REGION	Species		
	MAN	MONKEY	OX
Cerebrum			
Whole	80 /1/ a	20 /2/ a	35 /1/ a
Frontal cortex	20 (12) /5/ a 63 (10) /4/ b		
white	63 (19) /6/ a 78 (12) /4/ b		
Parietal cortex	91 (7) /4/ b		
white	86 (14) /5/ b		
Temporal cortex	58 (10) /5/ b		
white	64 (12) /4/ b		
Brain stem	50 /1/ a	37 /2/ a	56 /1/ a
Hypothalamus	145 (9) /2/ b		
Thalamus	135 (19) /4/ b		
Rhombencephalon			
Pons	72 (10) /5/ b		
Medulla	55 /1/ b		
Cerebellum			
whole	38 /1/ a	24 /1/ a	25 /1/ a
cortex	124 (5) /5/ b		
white	126 (16) /5/ b		

Reference	Method of estimation
(a) Abraham *et al.*, 1962	Pauly Reaction
(b) Kanazawa and Sano, 1967	" "

The values of homocarnosine concentration of the 'cerebral hemispheres' reported in the present work are in fair agreement with the estimates by Abraham et al., (1962) for the cerebral hemispheres of monkey brain, but much less than those they obtained for human and bovine brain (Table 25 , p 243).

Higher concentrations of homocarnosine in the grey matter of human cortex reported by Abraham et al., (1962) were not confirmed by Kanazawa and Sano (1967) who found that the concentration was almost the same as that in the white matter.

In all the previous work referred to above the homocarnosine was estimated spectrophotometrically using the diazo coupling reaction based on the Pauly reaction.

Validity of the estimates of histidine and homocarnosine in mouse brain tissue. The substances estimated as histidine and homocarnosine conform to the behaviour of authentic histidine and homocarnosine in several respects which have been discussed in detail previously (p 197). In brief, they include similar behaviour on ion-exchange chromatography, thin-layer chromatography and reaction with

o-phthalaldehyde to yield fluorophors with the appropriate fluorescence characteristics. These attributes together with the elimination of substances interfering in the fluorimetry, as demonstrated by the absence of potentiation or quenching effects, suggest that the values obtained for the concentration of histidine and homocarnosine are not erroneously high or low, despite the likely presence of other endogenous substances. A possible interference by homoanserine (γ-aminobutyryl-1-methylhistidine) which is also present in brain (Nakajima et al., 1967) is not excluded. Since 1-methylhistidine does not react with o-phthalaldehyde to form a fluorophor, errors due to the presence of homoanserine during the fluorimetry of histidine or homocarnosine are likely to be minimal. Unfortunately no authentic sample of homoanserine was available for study.

Further tests of identification of the assumed histidine and homocarnosine could be applied, for example, in parallel assays. Thus the compound estimated as histidine could be converted to histamine by a specific histidine decarboxylase, the amine being then estimated biologically. The assumed homocarnosine could be hydrolysed by the enzyme carnosinase (Pisano et al., 1961) and the liberated histidine and

γ-aminobutyric acid identified and estimated.

Histidine loading. The passage of a number of amino acids into brain after intravenous injection has been observed by many workers (Greenberg and Winnick, 1948; Schwerin, Bessman and Waelsch, 1950; Lajtha, Berl and Waelsch, 1959 and Richter, 1959), but histidine was not included in these studies.

A rise in the concentration of brain histidine was reported by Kamin and Handler (1951) after intravenous infusion of the amino acid in dogs. Nakamura (1963) injected histidine by the intraperitoneal route in rats and observed a rise in the histidine concentration of brain which was maximum 30 min after the injection and then gradually decreased.

It was found in the present work that after intraperitoneal injection of histidine into mice the concentration of the amino acid increased in the brain (Table 22, p 225, and Fig 27, p 226). The histidine concentration, however, did not rise progressively with dose and was maximal at a dose level of 30 ug per gram body weight or above. The appearance of a plateau level is evidence against the observed rise being due to elevation

of the histidine in the blood trapped in the brain capillaries because this would be expected to rise in relation to the dose.

It is unlikely that steady state conditions had been achieved in the 15 minutes which passed between the time of injection and the time of killing of the animals, and it is probable that the plateau level reached irrespective of the dose above 30 µg/g reflects the saturation of some carrier mediated process which has been demonstrated both *in vivo* (Nakamura, 1963) and *in vitro* (Neame, 1961, 1964; Nakamura, 1963; Levi *et al.*, 1967; Battistin *et al.*, 1969). It seems improbable that the peritoneal cavity would be the site of such a process, and its more likely situation, as evidenced from above work, would be between the blood and the brain.

No change in the homocarnosine content of mouse brain was found 15 min after the intraperitoneal injection of histidine in spite of a rise in the brain histidine level (Table 23, p 227). Although there is little conclusive evidence in the literature concerning the source of the homocarnosine found in brain such evidence as there is points to its formation in that organ. Distribution studies have, with the exception of

one report of its presence in rabbit liver (Kanazawa and Sano, 1967), indicated its absence from tissues other than the central nervous system. It has been suggested that this unique distribution is related not to a specific localisation of synthesising enzyme but rather to the fact that one of its precursors, Y-aminobutyric acid is restricted to brain tissue (Pisano, 1969). Further evidence of its cerebral formation is given by the finding that homocarnosine was not taken up by brain slices when these were incubated in a medium containing the dipeptide (Abraham, Pisano and Udenfriend, 1964).

Although the presence of a homocarnosine synthesising enzyme in mammalian brain does not appear to have been demonstrated, some evidence of its presence in the frog brain is afforded by the *in vivo* and *in vitro* studies of Yockey and Marshall (1969). They administered ^{14}C- histidine to frogs intracerebrally and observed that, over a 2 hr period, the specific activity of the brain histidine decreased while that of the homocarnosine and carnosine increased to a maximum within this period. Intraperitoneal or intracardiac injection did not lead to labelling of the histidine containing peptides in the brain from which observation

it may be inferred that ^{14}C- homocarnosine detected in brain after intracerebral injection of ^{14}C-histidine did not arise by peripheral formation from ^{14}C-histidine escaping to the periphery and subsequent uptake of the dipeptide into the brain. After intraperitoneal or intracardiac injection there was a slight increase in the radioactivity of brain histidine which was related to time and dose. Presumably the peripherally administered labelled histidine was diluted by the endogenous histidine to such an extent that adequate labelling of the brain histidine did not take place to allow demonstration of homocarnosine formation.

These workers also observed that after incubation in vitro, both intact and homogenized frog brain formed ^{14}C-homocarnosine from ^{14}C-histidine.

From the results of the present work it appears that the 15 min time interval between the administration of histidine and killing of the animals may not have been long enough for a sufficiently higher-than-normal concentration of histidine to be built up in the brain for a synthesising enzyme system to incorporate histidine into homocarnosine to produce a detectable increase

in the homocarnosine concentration. It may, also, be that the synthesising enzyme is rate-limiting and saturated at the normal concentration of histidine or of γ-aminobutyric acid in the brain. A rise in histidine concentration would not therefore affect the steady state equilibrium although incorporation of exogenous histidine would be detectable by use of radioactively labelled histidine. Further investigations are obviously necessary to study the various aspects of cerebral homocarnosine formation.

Abou (1968) observed that the histamine concentration increased in certain areas of rabbit brain after intravenous infusion of histidine. Since histamine does not pass from blood into brain (Halpern et al., 1959; Adam et al., 1964) the rise in histamine concentration in brain was probably due to passage of histidine from the blood circulation into brain where it was converted to histamine. Extension of these studies to investigate a possible relationship between the elevated histamine levels and raised histidine concentration in various regions of the brain is necessary. Such a study is now possible with the methodology described in this thesis.

High concentration of histidine in blood and urine associated with mild mental retardation and speech defects in a clinical condition known as histidinaemia was first reported by Ghadimi, Partington and Hunter (1961). The disease, one of the inborn metabolic disorders in children is believed to be caused by absence or deficiency of the enzyme histidase responsible for the conversion of histidine into urocanic acid (Ghadimi, Partington and Hunter, 1962; La Du, Howell, Jacoby, Seegmiller, Sober, Zannoni, Canby and Zeigler, 1963; Zannoni and La Du, 1963).

The histidine concentration in plasma of a patient may vary from 20 to 120 µg/ml (Ghadimi et al., 1962), the most commonly encountered value being about 90 µg/ml (Ghadimi and Partington, 1967); in healthy subjects the concentration of histidine in plasma varies from 10 to 20 µg/ml Shepherd and Mackay, 1967). The histidine concentration in cerebro-spinal fluid also rises in this disease (Ghadimi and Partington, 1967).

No ready explanation of the relationship between the increased level of plasma histidine and the degree of mental retardation and speech defects has been found in most cases. La Du (1967) observed that some of his mentally retarded

patients had blood histidine levels lower than those of two mentally normal subjects whose blood histidine levels were consistently between 130 and 200 µg/ml. According to Waisman (1967) the mental retardation observed in some of the children may be familial rather than due to histidinaemia. Association of the speech defect with histidinaemia may represent a selective type of damage to the central nervous system; this has not yet been clearly substantiated. The abnormal metabolites of histidine, such as imidazole pyruvic acid, which are present in this disorder (La Du et al., 1963) might produce subtle but wide-spread brain damage which is manifested only in the more complex central nervous functions, such as speech. La Du et al., (1963) found poor auditory memory span in their two cases in the presence of normal hearing.

In another type of clinical condition characterised by myoclonic and grand mal seizures during the first 3 months of life and later by psychomotor retardation, Perry, Hansen, Tischler, Bunting and Berry (1967) observed about 2.5 µg homocarnosine per ml of cerebro-spinal fluid. The values reported were 10 times more than normal values which vary from a trace to 0.25 µg/ml

(Abraham *et al.*, 1962).

The method developed in the present work for the measurement of histidine and homocarnosine in small brain samples may find an application in the studies of such neurologic disorders.

APPENDIX

APPENDIX 1

FLUORIMETRY

Fluorescence and its measurement.

Principle. A compound is said to be fluorescent when its molecules, after absorbing light of a particular wavelength, emit light of different wavelength; in solution at normal temperature the emitted light is of higher wavelength than that of the absorbed light. Fluorescent compounds show peak(s) of fluorescent intensity when they absorb i.e. when the molecules are activated or excited by light of particular wavelength(s). The emitted light i.e. the fluorescence, also shows maximum intensity at a particular wavelength(s). Thus the fluorescence of a compound is characterised by peak intensities at particular wavelengths for the activation light and for the fluorescence. Determination of these maxima, by recording the activation and the fluorescence spectra, assists in the identification of the substance and increases the specificity of the estimation when fluorescence is used in quantification. Such characteristics, however, are seldom absolute and closely related compounds

show the same or very similar parameters.

With suitable instrumentation to measure the intensity of the emitted light, the fluorescence of a compound may be used to determine its concentration. Methods for the estimation of many compounds of biological interest have been developed in recent years; in some cases, to increase sensitivity and/or specificity advantage is taken of the fluorescence of derivatives of a biological compound which itself fluoresces only weakly or not at all or shows fluorescence characteristics which are insufficiently specific to the compound (Udenfriend 1962).

Instrumentation

Instruments used for recording activation and fluorescence spectra are known as spectrophoto-fluorimeters. These instruments have three basic components, (i) a source of light to activate the potentially fluorescent molecules, (ii) two light filtering systems, one to select from the whole spectrum of light emitted by the light source the wavelengths most suitable for activation and the other to select the wavelengths

of the fluorescent light reaching the detector system and (iii) a light detection apparatus to pick up the emitted fluorescent light.

The solution of the fluorescing substance is contained in a cuvette the walls of which are made of glass, or of silica if the solution is to be irradiated with ultra-violet light. The cuvette is located in a compartment of the instrument in such a way that the solution may be exposed to the activating light and the fluorescence of the solution detected. Since the intensity of the emitted fluorescent light is generally of many orders of magnitude less than that of the activating light the emitted light is usually detected along a path at right angles to that of the activating light. The contribution of the latter to the emergent light is thus considerably reduced and is limited to the activating light reaching the emergent beam by reflection from the walls of the cuvette and from particles in the solution. Steps are taken to reduce such reflections to a minimum.

A spectrophotofluorimeter manufactured by Carl Zeiss, Oberkechen/Wurtt (West Germany) was used in the present work and details mentioned below refer specifically to this instrument.

(i) The source of light is a xenon arc lamp (450 watt) which is capable of delivering high intensity light throughout the U-V and visible spectra.

(ii) The filtering system or monochromator, is a device for selecting a narrow spectral band from the continuous spectrum of the light entering it. The incident light is dispersed by means of a prism or diffraction grating and the desired spectral band of light emerging from the monochromator is selected by suitable optics.

Two such monochromators are incorporated in the instrument, one being interposed between the activating light source (the xenon arc lamp) and the sample being irradiated, and the other between the sample and the light detector system to permit spectral analyses of the fluorescent light (Fig 28, p 258). In recording spectrophotofluorimeters such as the Zeiss instrument, motorised drive systems are incorporated in the monochromator assemblies. This allows the emergent spectral bands to be changed automatically in a continuously progressive manner, the wavelengths being monitored on a moving scale at the front of the instrument. With a suitable recording system a continuous spectrum of the fluorescence

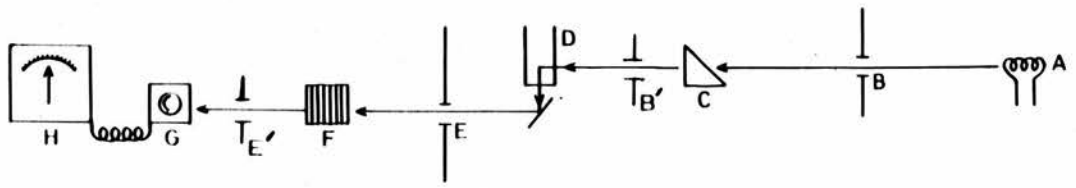


Fig 28. A schematic diagram of the optical path through Zeiss spectro-photofluorimeter.

(A) Source of light, xenon arc lamp.

(B) and (B') adjustable slits for prism monochromator.

(C) Prism monochromator

(D) Sample cuvette

(E) and (E') adjustable slits for grating monochromator

(F) Grating monochromator

(G) Photomultiplier tube

(H) Recorder.

intensities of the sample in response to alterations in the wavelength of activating light may be obtained; alternatively, a spectral analysis of the intensities of the fluorescence when the sample is activated with a light of constant wavelength, can be recorded. Such recorded spectra are not true spectra because the light source (the xenon arc lamp) does not have the same intensity of output over the whole spectral range nor does the light detector system (photo-multiplier tube, see below) give the same response to the same intensity of light at different wavelengths.

The spectral band widths of the light leaving the monochromator can be varied by varying the width of the slits situated at the entrance to and the exit of the light from the monochromator. In the Zeiss instrument both slits are adjusted jointly and may be set to widths varying up to 2 mm by a graduated control. Better resolution can be obtained by reducing the band-widths of the light activating the sample and/or the light reaching the detector; this, however, diminishes the fluorescence and/or the response of the detector due to the decreased intensity of the light.

In measuring very low concentration of a

fluorophor, as often occurs in the assay of substances from biological sources, the adjustment of the slit widths is important because it determines both the specificity and sensitivity of the measurement. When the slits are narrowed, specificity is increased but sensitivity is reduced; the band widths of the activating and fluorescent light are restricted and the wavelengths approximate closely those of the characteristics of the fluorophor. The reverse obtains when the slits are widened. Consequently, the final choice of slit widths is a compromise between the desirability of using very narrow slits for greater specificity and the necessity of widening the slits in order to obtain a measurable response.

In the fluorimetry reported in this thesis (p 71) the slits widths were set routinely at 1.0 mm for the 'activation' monochromator and at 0.5 mm for 'fluorescence' monochromator.

For reasons unconnected with this study, the instrument was fitted with a 'prism' type monochromator on the activating side and a 'grating' monochromator for spectral analysis of the fluorescence. Because of the differences in the dispersal characteristics of the two

types, the band pass for a given slit width increases with wavelength in 'prism' monochromator while remaining constant with the 'grating' monochromator. Also with a constant speed of the motorised drive to the monochromator the rate of change of the light wavelength emerging from the 'prism' monochromator increases as it progresses from the shorter to the longer wavelengths. In contrast, the rate of change of wavelengths of the light emerging from the 'grating' monochromator remains constant. The differences are reflected in the unequal intervals on the wavelength axes of activation spectra recorded in this thesis((pp 208 & 209).

(iii) Light detector. The emitted fluorescence is detected by a photomultiplier tube which in the instrument used was a I P 28 type (Radio Corporation of America, Harrison, New Jersey, N.J., USA) which is most sensitive over the spectral range of 400 to 550 nm. The amplified light-current is fed to a microammeter and to a 'Servoscribe' (Smith Industries Ltd.) potentiometric recorder. Relative fluorescence intensities of solutions were measured routinely from the recorder tracings.

Cuvettes. The solution for the fluorimetric

measurement was contained in a micro-cuvette (made of a synthetic silica, 'Quartz-Suprasil' which required a minimum volume of 0.5 ml.

Recorded fluorescence characteristics of fluorophors

Differences in the characteristics of individual xenon arc lamps and/or photomultiplier tubes nominally of the same type are among the reasons for minor differences in the wavelengths for the maximal activation and fluorescence of a particular fluorophor determined by different instruments. Fluorescent characteristics reported in this thesis are uncorrected for such instrumental variations.

Determination of the fluorescence characteristic of a compound. Manual manipulation of the monochromator settings while monitoring the instrument response can be used to determine the wavelength producing maximum activation and that at which the fluorescence is maximal. In practice these characteristics may be more precisely determined by recording the activation and emission spectra. The analysing monochromator

is set to pass light of wavelength close to that of the maximum fluorescence as determined manually and the activation spectrum recorded. From the latter the wavelength producing maximum activation is determined and the activation monochromator set at this value while the fluorescence spectrum is recorded. From this spectrum the wavelength of maximal fluorescence intensity may be obtained. Relatively high concentrations of the fluorophor and the use of very narrow slit widths on the monochromators will increase the precision of the determination of these fluorescence characteristics.

Examples of such recorded spectra for the o-phthalaldehyde reaction products with histidine and homocarnosine are shown in Figs 4 & 9, pp 75 & 87. These spectra also show part of the so-called 'light scatter peak' which is distinguishable from the specific fluorophor peak by showing a maximum when the emission and activation wavelength coincide. This peak is usually much greater than that due to the specific fluorescence when the fluorophor is present in very low concentration and presents problems in measurement of the fluorophor if the activation and fluorescence maxima are close to one another,

because parts of the two peaks merge. The size of the light scatter peak tends to be variable since it is readily influenced by fine particles present in the solutions to variable degrees. Such light scatter peaks may be minimised by the use for fluorimetry, only of solutions which are visibly free from suspended particulate matter.

In point of fact, in the studies reported in this thesis light scattering presented no problems in the fluorimetry because with each fluorophor, the wavelength of maximum activation and that of maximum fluorescence were sufficiently different so that there was no overlap of activation and fluorescence wavelengths using the band widths (as determined by the slit widths of the monochromators) employed in the fluorimetry.

Practical considerations for the fluorimetric assay of a substance

In order to obtain maximum specificity and sensitivity in a fluorimetric assay it is essential that any contribution to the specific fluorescence by fluorescence of an unspecific nature should be reduced to the minimum and that this irreducible minimum should be capable of being measured so that its contribution to the specific fluorescence

may be allowed for. Such non-specific fluorescence can stem from the reagents used in the analytical procedure and, in preparations of biological materials, from endogenous substances other than that to be assayed, which are present in the final extract for measurement. In the former case reduction to a minimum may be achieved by use of reagents of the highest grade of purity and this may require the further purification of the commercially available reagents by procedures such as distillation, or recrystallisation (see for example, p 282, purification of o-phthalaldehyde used in the present study).

Interference from non-specific fluorescence.

The following are some of the sources of non-specific fluorescence.

Water supply. The water supply is often a source of non-specific or other types of interference with specific fluorimetry (Laverty and Sharman , 1965). In this laboratory it has been the practice to use glass-distilled water subsequently passed through a mixed-bed ion exchanger ('Elgastat'), and such water has been found satisfactory for the preparations of aqueous reagents. For all purposes in the

present work such distilled deionised water was used except when otherwise mentioned.

Cleansing of glassware. Thorough cleansing of glassware is an essential requirement for the fluorimetry of very low concentrations of fluorophors. Contamination with acid or alkali or with some unknown substance may completely defeat the purpose of the experiment. In the present work, glassware was steeped overnight in chromic sulphuric cleaning solution (Appendix 2 , p 280) and thoroughly rinsed (20 times) with tap water to remove the cleaning fluid, followed by thorough rinsing (20 times) in distilled deionised water. Non-graduated glassware was dried in a closed oven at 45°C; graduated glassware was drained dry in a dust free situation at room temperature. Clean glassware was stored under conditions preventing recontamination before use.

Non-specific fluorescence in biological extracts.

Problems of non-specific fluorescence arising from other substances in biological materials may be diminished by elimination of at least some of these substances by separative techniques such as solvent extraction, or chromatography in one or more of its various forms. The application

of some of these techniques for this purpose is illustrated in pp 109-174 of this thesis.

'Blank' fluorescence.

Although non-specific fluorescence, usually referred to as the 'blank' fluorescence, can often be reduced to a considerable degree by attention to the points outlined above, it can seldom be eliminated and therefore must be determined in order to allow correct measurement of the intensity of the specific fluorescence. The assessment of the 'blank' in the measurement of the fluorescence derived from a simple aqueous solution of the fluorophor such as one uses in calibrating the fluorimeter, presents no problem since the 'blank' is readily obtained by measuring the fluorescence of an equal volume of water which has been treated with any necessary reagents in the same manner as the sample.

The assessment of the true blank fluorescence in the estimation of a specific substance in, say, an extract derived from a biological material presents a much more difficult, and in some cases an insuperable, problem. In this case the blank fluorescence derives not only from reagents used in the analytical procedure but also from the other substances in the tissue which are

present in the final extract. These other substances may either fluoresce per se at wavelengths close to those characteristic of the fluorophor being measured or interact with any reagents necessary for the production of the fluorophor, to produce fluorescence similar to that of the specific substance.

If, in the former situation, the specific fluorophor is derived from the compound to be measured by reaction with other substances the alteration of the order of addition of the reagents may prevent the formation or allow the rapid destruction of the specific fluorophor. If the assumption is made that substances contributing to the 'blank' fluorescence do not behave similarly, an estimate of the 'blank' can be so obtained. These are many examples of such manoeuvres being employed in the fluorimetry of compounds of biological interest. For example, in the assay of histamine by the o-phthalaldehyde method, Shore et al., (1959) estimated the 'blank' by determining the fluorescence of an aliquot of the sample solution treated with excess hydrochloric acid before the addition of the aldehyde and sodium hydroxide. The acid conditions maintained by the order of addition of the

reagents, prevent the formation of the histamine fluorophor which only takes place in alkaline medium, the fluorophor being stabilised by the subsequent addition of the excess hydrochloride acid. Seldom can the validity of such a 'blank' estimation be determined and its use may lead to erroneous results.

The estimations of histamine in brain by the fluorimetric method of Shore et al (1959) are a case in point. These were later shown by Kremzner and Pfeiffer (1966) and Shaw (1968) to be overestimates because of the presence, in the extracts, of spermidine which reacted similarly to histamine both under the conditions for developing the fluorophor and for determining the 'blank' value.

With some fluorimetric methods of estimation even the procedure outlined above is not possible and the best estimate of the 'blank' is that of a 'reagent blank' obtained by processing water instead of the crude tissue extract through the analytical procedure. This method of assessing the 'blank' for fluorimetric estimations was employed in the work reported in this thesis.

It is evident that adequate separation techniques should be applied to obtain the tissue extract for fluorimetry as 'chemically pure' as

possible. Unavoidable losses of the compound of interest, its usually low initial concentration in the tissue, the sensitivity of the assay procedure and the labour involved impose limits to the extent to which such purification procedures may be employed prior to the assay. Thus the applied analytical procedure is inevitably a compromise between what is desirable and what is feasible in practice.

Potentiation or quenching of fluorescence - the use of 'internal standards'.

In addition to contributing to the 'blank' fluorescence, other substances in a tissue extract may interfere with the fluorimetry of the specific fluorophor in such a way as to potentiate or reduce (quench) its intensity. The extent of such effects may be determined in one or other of two ways using so-called 'internal standards'.

In the first a known quantity of the fluorophor or its precursor is added to a portion of the final extract of the tissue sample and any necessary reaction carried out in parallel with similar treatment to a second, like portion of the sample with no addition of standard. Comparison

of the increment of fluorescence intensity with the reading obtained from the same amount of the fluorophor in simple solution will show the presence or the absence of such quenching or potentiation of the fluorescence and the correction to be applied to the sample reading.

In the second a known quantity of the substance to be measured in the tissue is added to a portion of the crude tissue preparation and this is processed through the whole of the analytical procedure along with a second portion of the crude tissue extract to which no such addition is made. The difference in the intensities of the fluorescence of the final extracts is a measure of the fluorescence derived from the known amount of the added substance. This measurement can be used in calculating directly the concentration of the compound in the original crude tissue extract since the 'internal standard' has been subjected to the same manipulations as the endogenous material. Such a measurement takes into account not only the recovery of the substance through the separative procedures necessary to produce an extract suitable for fluorimetry but also any potentiation or quenching of the specific fluorophor, provided

the loss of the compound both from the 'test' and 'standard' sample to be measured are proportional.

In the use of such 'internal standards' it is essential that the amount of the added substance should be of the same order of magnitude as the endogenous substance in the sample. Estimates of the recovery through the analytical procedure and/or of the extent of interference in the fluorimetry may well give results inapplicable to the small amounts of endogenous substance if they are derived from observations obtained from a relatively large amount of added standard.

In practice in the work reported in this thesis, the occurrence of potentiation or quenching of the fluorescence intensity of the specific fluorophor was allowed for in the following way. To a portion of the final extract for fluorimetry was added a known amount of the substance to be measured contained in a small volume of distilled deionised water (the 'standard'). The same volume of water was added to another portion of the extract (the 'test'). Fluorophor production was carried out in both samples in parallel and the respective intensities of fluorescence measured. The observed difference in the fluorescence of the samples was a measure of that due to the known

amount of standard and from this the content of the substance in the test sample was calculated after allowance had been made for non-specific 'blank' fluorescence. Potentiation or quenching of the fluorescence, if any, would be expected to occur proportionately on the unknown amount of substance present in the 'test' sample.

Other factors influencing fluorescence intensity

The pH of the solution for fluorimetry may be of importance to stabilise the fluorophor, to provide conditions of maximal fluorescence or to increase the specificity of the measurement. In the o-phthalaldehyde method of estimating histamine the solution is acidified to stabilise the fluorophor formed under alkaline conditions (Shore et al., 1959). In the assay of 5-hydroxyindoles the fluorescence may be measured in strongly acidic solution in which contribution to the fluorescence by indoles not carrying a 5-OR substituent is eliminated (Udenfriend, 1962). Many potential fluorophors are weak acids, bases or amphoteric substances and show

fluorescence only in the ionised or unionised state. The fluorescence intensity derived from such substances will obviously be influenced by the pH of the solution (Udenfriend, 1962).

In the present work fluorophors of histidine and homocarnosine were prepared in alkaline conditions. It was found that acidification of the medium prevented the formation of histidine-o-phthalaldehyde fluorophor and that the intensity of fluorescence was significantly reduced when the medium was acidified after the reaction had been completed (p 79). Similar results were also obtained with homocarnosine-o-phthalaldehyde fluorophor (p 91).

Temperature of the solution. The temperature of the solution may also affect the fluorescence emitted by a fluorophor, the intensity decreasing with rise of temperature as observed with indole acetic acid (Udenfriend, 1962). The measurement of a temperature sensitive fluorescence requires not only control of the initial temperature of the solution in the cuvette but a device for maintaining the cuvette compartment at constant temperature, for example by circulating water at the suitable temperature through an outer jacket

enclosing the cuvette compartment. Without such a means of stabilisation the temperature in the cuvette compartment may rise considerably during a series of measurements as a result of heat transfer from the lamp providing the excitation light.

In experiments, described in the present work (pp74&86) designed to follow the time-course of fluorophor production from the o-phthalaldehyde reaction with histidine and with homocarnosine, the reaction was allowed to proceed in a cuvette located in the cuvette compartment of the fluorimeter. No significant variation in the intensity of fluorescence from either fluorophor was evident over a period of at least one hour following completion of the reaction. In the case of the homocarnosine fluorophor the reaction was evidently complete at room temperature within the ten min time lapse which occurred between mixing the reagents and the insertion of the solution into the fluorimeter. It appeared therefore that the fluorescence intensities of these fluorophors were not critically temperature dependent as no steps were taken to maintain the cuvette compartment, and hence the solution in the cuvette, at constant temperature during the

protracted series of measurements.

The linear relation of relative fluorescence to concentration and the sensitivity of a fluorimetric assay.

It is desirable in the fluorimetric assay of an unknown sample that the fluorescence intensity should be within the range showing a linear relation between the relative fluorescence and the concentration of the substance being measured. At higher concentrations, phenomena such as 'self-quenching' (Udenfriend 1962) operate to cause departure from linearity. The range of concentration over which a linear relation holds is determined by measurement of the relative fluorescence of standard solutions. In the present work linearity has been shown to extend over the range 0.02 to 0.6 µg/ml for histidine and 0.2 to 3 µg/ml for homocarnosine.

The lower limit of the range is set by the limit of sensitivity of the method, i.e. the smallest amount of substance that can be measured accurately.

This depends on (1) the fluorescence yield of the substance and (2) the size of the 'blank'. In samples derived from say, tissue extract, a

third factor of potentiation or quenching may operate.

In the work described in this thesis, an estimation was not considered valid unless the sample produced a relative fluorescence at least twice that of the appropriate 'blank' at the wavelengths characteristic of the fluorophor.

Instrumental fluctuations.

Instrumental fluctuations of a short or long term nature can arise in fluorimetry because of variations in the intensity of emission of the light source or variation in the response of the photomultiplier.

It is essential therefore that standards should be included with each batch of samples and that more than one measurement of the relative fluorescence of a sample should be recorded.

In order to minimise such possible instrumental errors in carrying out the estimations recorded in this thesis the fluorescence of an 'unknown' sample and that of an appropriate external or internal standard (p 213) were measured as closely together as possible in time and at least duplicate readings of the fluorescence intensity of each solution were taken.

APPENDIX 2

CHEMICAL REAGENTS AND PREPARATION OF STANDARD AND OF BUFFER SOLUTIONS USED IN THE ANALYTICAL PROCEDURES

INORGANIC REAGENTS

Water. In the preparation of aqueous solutions, in the final rinsing of cleaned glassware and in the experimental procedures where the use of water was required, water which had been distilled from glass and subsequently passed through a column of mixed-bed column of ion-exchange resins ('Elgastat') was used. Reference to 'water' in this thesis thus generally signifies the use of such distilled deionised water.

Inorganic salts were of 'analytical reagent grade' standard of purity. Generally they were obtained from BDH Chemicals Ltd. and were of their 'ANALAR' range. They included

Silver nitrate

Sodium chloride

Sodium dihydrogen orthophosphate, $NaH_2PO_4 \cdot 2H_2O$

Disodium hydrogen orthophosphate, $Na_2H\ PO_4$

Sodium hydroxide, pellet form

Potassium hydroxide, pellet form

Hydrochloric acid. Concentrated, Sp.Gr. 1.18, Micro-analytical reagent (BDH Chemicals Ltd.), approximately 11.7 M.

6N HCl. Prepared by appropriate dilution of concentrated hydrochloric acid.

3N HCl. Prepared from concentrated hydrochloric acid and standardised against N NaOH using neutral red as internal indicator.

N HCl. Prepared from N HCl 'concentrated volumetric solution' supplied by BDH Chemicals Ltd.

0.25 N HCl. Prepared by dilution of the N HCl above.

0.1 N HCl. Prepared from 0.1 N HCl 'concentrated volumetric solution' supplied by BDH Chemicals Ltd.

Sodium hydroxide N NaOH. Prepared from sodium hydroxide (ANALAR) pellets. Standardised against N HCl using phenolphthalein as internal indicator.

0.1 N NaOH. Prepared by dilution of N NaOH and restandardised against 0.1 N HCl.

N NaOH, (used in fluorimetry). Volumetric solution as supplied by BDH Chemicals Ltd.

Sodium chloride solution, 0.9 mEq Na^+/ml. 5.26 g sodium chloride dissolved and made up to 100 ml with water.

Solutions of sodium chloride, for calibration of

flame photometer, containing 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mEq Na^+/ml were stored in polythene bottles.

ORGANIC REAGENTS AND SOLUTIONS

Acetic acid, glacial, 'ANALAR' BDH Chemicals Ltd. For preparation of solvents for chromatography.

1.0 N acetic acid. Prepared from concentrated volumetric solution supplied by BDH Chemicals Ltd.

0.2 and 0.1 N acetic acid solution for preparation of buffer solutions (see below), prepared by dilution of 1.0 N acetic acid volumetric solution.

Sodium acetate CH_3COONa, 'ANALAR', BDH Chemicals Ltd.

Cellulose powder, for mixing with 'Amberlite CG-50' resin for column chromatography, Whatman standard grade for chromatography.

Cellulose powder for thin-layer chromatography, Whatman thin-layer Chromedia, CC41 microgranular cellulose powder.

Chromic acid cleaning fluid. 175 g sodium dichromate (BDH Chemicals Ltd.) dissolved in the minimum amount of tap water at room

temperature and diluted with 2.5 l conc sulphuric acid (commercial grade).

Ion exchange resin. 'Amberlite' resin CG-50, chromatographic grade, Type 1, 100-200 mesh (Rohm and Haas Company, Philadelphia, U.S.A.) obtained from BDH Chemicals Ltd. Before use, fines were removed from the resin by sedimentation in water. A 30 g portion of the resin was stirred vigorously with a glass rod in 1 litre of water in a beaker. The suspension was allowed to sediment for 10 min and the supernatant fluid containing the fine particles was decanted off. The process of suspension and sedimentation was repeated six times in all. The final deposit was dried in an evaporating basin in an oven at 45°C for 48 hr and the dried cake was ground gently until a free-running preparation was obtained. Removal of the fines avoided clogging of the ion-exchange columns and ensured a satisfactory flow rate.

Neutral red, pH indicator, 0.01% in water prepared from neutral red (redox indicator) BDH Chemicals Ltd.

Ninhydrin, 'ANALAR' BDH Chemicals Ltd., 0.2% in

acetone ('analytical reagent', May and Baker Ltd.).

o-phthalaldehyde. BDH Chemicals Ltd. This was purified before use by recrystallisation from ligroin (see 'organic solvent' section below) in the following way:

About 1 g of o-phthalaldehyde was dissolved in 100 ml ligroin at about 80°C on a water bath. The hot solution was decanted from undissolved material into a beaker which was then covered with aluminium foil and kept at 4°C overnight. The long needles of o-phthalaldehyde which had deposited at the bottom of the beaker were dislodged with a clean glass rod and filtered off on a Buchner funnel. Recrystallised o-phthalaldehyde retained in the Buchner funnel was further washed with ligroin. The funnel was then covered with aluminium foil and the crystals allowed to dry at room temperature (21°C). The dried o-phthalaldehyde was stored at 4°C in a brown wide-mouth glass-stoppered bottle and further protected from light by covering the bottle in black paper.

Trichloroacetic acid 'for protein precipitation', BDH Chemicals Ltd. 6% (w/v) in water.

ORGANIC SOLVENTS

Unless otherwise stated these were obtained from BDH Chemicals Ltd.

Acetone. Analytical reagent, May and Baker Ltd. Distilled twice.

Chloroform. 'ANALAR' grade.

Ligroin. Reagent grade.

Methanol. 'ANALAR' grade. This was purified further by distillation first from potassium hydroxide (pellets form, BDH Chemicals Ltd.) (30 g/l) to which was added a small quantity of silver nitrate (about 2 g/l) and then from potassium hydrogen sulphate (30 g/l). Distillation was stopped when about 50 ml alcohol remained in the flask.

Propan-1-ol. Laboratory reagent grade. This was purified before use in the same way as the methanol.

Propan-2-ol. 'ANALAR' grade.

AMINO ACIDS, PEPTIDES AND AMINES

The solutions were stored at $4^{\circ}C$.

Agmatine obtained as sulphate, Mol. wt. 228.28, from L. Light and Co. Ltd. Stock solution of 100 ug agmatine/ml was prepared by dissolving 17.5 mg in water and diluting to 100 ml. A working solution of 1 µg/ml was prepared by diluting the stock solution.

Arginine obtained as L-arginine hydrochloride, Mol. wt. 211, grade A, Calbiochem. Stock solution of 100 µg arginine/ml was prepared by dissolving 12 mg in water and diluting to 100 ml. Working standard solutions containing 1 and 10 ug arginine/ml were prepared by appropriate dilution of the stock solution.

Carnosine, β-alanyl histidine, Mol. wt. 226, obtained from Calbiochem (grade A). A stock solution of 100 µg carnosine/ml was prepared by dissolving 10 mg of the peptide in 100 ml water. A working standard solution of 10 µg carnosine/ml was prepared by making appropriate dilution of the stock solution.

Histamine obtained as histamine acid phosphate, Mol. wt. 307.1, 'ANALAR' reagent, BDH Chemicals Ltd. A stock solution of 100 µg base per ml was prepared by dissolving 27.7 mg of the compound in water and diluting to 100 ml.

Standard solutions containing 10 ug, and 1.0 µg base per ml was prepared by appropriate dilution of portions of the stock solution. A solution of 0.1 µg base/ml was prepared by further dilution of a portion of the standard solution containing 1.0 µg base per ml.

Histidine obtained as L-histidine monohydrochloride monohydrate, Mol. wt. 209.63, grade puriss, Koch-Light Laboratories Ltd. A stock solution of 100 µg histidine per ml was prepared by dissolving 13.5 mg of the compound in water and diluting to 100 ml. Standard solutions containing 10.0 ug and 1.0 µg per ml were made by appropriate dilution of portions of the stock solution. A solution of 0.1 µg per ml was prepared by dilution of a portion of the standard solution containing 1.0 µg per ml.

Homocarnosine, -aminobutyryl histidine, obtained as sulphate, Mol. wt. 338.35, from Calbiochem (grade A). A stock solution of 100 µg homocarnosine/ml was prepared by dissolving 14.1 mg of the compound in water, and diluting to 100 ml. Working standard solutions of 10 µg/ml and 1 µg/ml were prepared by appropriate dilution

of portions of the stock solution.

Marker solutions for paper and thin layer chromatography. Solutions of carnosine, histidine hydrochloride and homocarnosine sulphate and of a mixture of histidine and homocarnosine, each substance in concentration of 20 µg/ml in water were used as 'markers' in control paper and cellulose thin layer chromatograms.

1-Methylhistidine obtained as L-1-methylhistidine monohydrate, Mol. wt. 187.2, from Calbiochem (grade A). A stock solution of 100 ug 1-methyl-histidine/ml was prepared by dissolving 11.0 mg in water and diluting to 100 ml. Working standard solutions of 10 µg/ml and 1 µg/ml were made by appropriate dilutions of portions of the stock solution with water.

3-Methylhistidine obtained as L-3-methylhistidine, Mol. wt. 169.7, from Calbiochem (grade A). A stock solution of 100 µg/ml was prepared by dissolving 10 mg of the amino acid in water and diluting to 100 ml with water. Working standard solutions of 10 µg/ml and 1 µg/ml were made from the stock solution by appropriate dilution in water.

N acetylhistidine obtained as N-acetyl-L-histidine monohydrate, Mol. wt. 215.2, from calbiochem (grade A). A stock solution of 100 µg/ml was prepared by dissolving 11 mg in water and diluting to 100 ml. Working standard solutions containing 10 µg/ml and 1 µg/ml were made by appropriate dilution of the portions of the stock solution.

Spermidine obtained as spermidine trihydrochloride, Mol. wt. 254.6, from Calbiochem (grade A). A stock solution containing 100 µg spermidine/ml was prepared by dissolving 17.5 mg of the compound in water and diluting to 100 ml. Working standard solutions of 10 µg/ml and 1 µg/ml were made by appropriate dilution of the portions of the stock solution.

Adrenaline obtained as Adrenaline hydrogen tartrate from BDH Chemicals Ltd., Mol. wt. 333.30. A stock solution of 1 mg base/ml was prepared by dissolving 12.49 mg of the compound in 6.87 ml 0.01 N HCl. Appropriate dilutions of this and the following amines were prepared in water.

Noradrenaline obtained as Noradrenaline hydrogen tartrate, from Bayer Products Co., Mol. wt.

317.72. 9.72 mg of the compound was dissolved in 5.15 ml 0.01 N HCl to prepare a stock solution of 1 mg base/ml.

Dopamine obtained as 3-Hydroxytyramine HCl from Calbiochem, grade A, Mol. wt. 189.7 . A stock solution of 1 mg base/ml was prepared by dissolving 4.53 mg in 3.67 ml 0.01 N HCl.

5-Hydroxytryptamine obtained as 5-Hydroxytryptamine creatinine sulphate from BDH Chemicals Ltd., Mol. wt. 405.44, A stock solution of 1 mg base/ml was prepared by dissolving 10.3 mg of the amine in 4.48 ml 0.01 N HCl.

Other chemicals used infrequently are specified at the appropriate points in the text. They were of the purest grade commercially obtainable and were used without further treatment.

PREPARATION OF BUFFER SOLUTIONS FOR ION-EXCHANGE CHROMATOGRAPHY

The pH of buffer solutions prepared as described below were checked before use with a pH meter (Beckman 'Zeromatic').

0.1 M acetate buffer, pH 3.75, 100 mEq Na^+/1.

An aqueous solution of 0.82 g sodium acetate (anhydrous) in 100 ml was diluted to 1000 ml with 0.1 N acetic acid.

The Na^+ concentration of the buffer was adjusted to 100 mEq/1 by addition of 5.22 g sodium chloride.

0.2 M acetate buffer, pH 4.75, 100 mEq Na^+/1.

To an aqueous solution of 8.2 g sodium acetate (anhydrous) in 500 ml was added 500 ml 0.2 N acetic acid.

0.1 M acetate buffer, pH 5.75, 100 mEq Na^+/1.

To an aqueous solution of 8.2 g sodium acetate (anhydrous) in 1000 ml was added 100 ml 0.1 N acetic acid.

The Na^+ concentration, 90 mEq/1 was raised to 100 mEq/1 by the addition of 0.58 g sodium chloride.

0.066 M phosphate buffer, pH 6.8, 100 mEq Na^+/1.

To 500 ml 0.1 M sodium dihydrogen orthophosphate (7.8 g NaH_2PO_4, $2H_2O$/500 ml) was added 500 ml 0.1 M disodium hydrogen orthophosphate (7.1 g Na_2HPO_4/500 ml).

The Na^+ concentration of this solution, 150 mEq/1, was reduced to 100 mEq/1 by dilution to 1500 ml, thus changing the molarity of the buffer from 0.1 to 0.066 M.

<u>0.055 M phosphate buffer, pH 7.4, 100 mEq/1</u>.

To 200 ml 0.1 M sodium dihydrogen orthophosphate (15.6, NaH_2PO_4, $2H_2O$/1) was added 800 ml 0.1 M disodium hydrogen orthophosphate (14.2 g Na_2HPO_4/1).

The Na^+ concentration of the buffer was adjusted to the required value by the addition of 800 ml water which reduced the molarity of the solution to 0.055 M.

<u>0.05 M phosphate buffer, pH 8.0, 100 mEq Na^+/1</u>.

26.5 ml 0.2 M sodium dihydrogen orthophosphate (31.2 g, NaH_2PO_4, $2H_2O$/1) was diluted to 500 ml with 0.2 M disodium hydrogen orthophosphate (28.4 g Na_2HPO_4/1).

The Na^+ concentration of this solution (390 mEq/1) was reduced to 100 mEq/1 by dilution of 256 ml of the buffer solution to 1000 ml. The molarity of the buffer solution was thus decreased from 0.2 to 0.05 M.

It will be noted that all these buffer

solutions have been adjusted to contain approximately 100 mEq Na^+/l. This adjustment was made because it had been shown by Adam *et al.*, (1957) that the adsorption of histamine on such ion-exchange resin columns was dependent on the Na^+ concentration of the applied solution. It was considered advisable in this attempted extension of the method to include the estimation of histidine to adhere to this condition. The influence of the Na^+ concentration on the adsorption of histidine and other substances discussed in this thesis has not been specifically investigated.

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