STUDIES ON THE BIOSYNTHESIS OF 5-HYDROXYTRYPTAMINE

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A thesis submitted by

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ABSTRACT

The studies reported here have aimed at understanding the first step in the biosynthesis of 5-hydroxytryptamine, that is the 5-hydroxylation of tryptophan with the production of 5-hydroxytryptophan. This reaction has been demonstrated in carcinoid tumour tissue, intestine and brain, and the enzyme catalysing the reaction, tryptophan 5-hydroxylase, has been partially purified from carcinoid tumour tissue and brain and has been shown to require a reduced pteridine cofactor for full activity. Tryptophan 5-hydroxylation by these tissues differs in several respects from that occurring in rat liver which is carried out by the non-specific activity of phenylalanine 4-hydroxylase.

It appears that the brain is capable of synthesising independently all the 5-hydroxytryptamine it needs and that tryptophan 5-hydroxylase is largely confined anatomically to the phylogenetically older parts of the brain and at the subcellular level to the presynaptic nerve endings, these localisations broadly following those of 5-hydroxytryptophan decarboxylase and 5-hydroxytryptamine.

Evidence is presented which suggests that the lowering of brain 5-hydroxytryptamine observed in experimental phenylketonuria results from the inhibition of the transport of tryptophan across the nerve cell membrane thereby depriving the cytoplasmic tryptophan 5-hydroxylase of its substrate.

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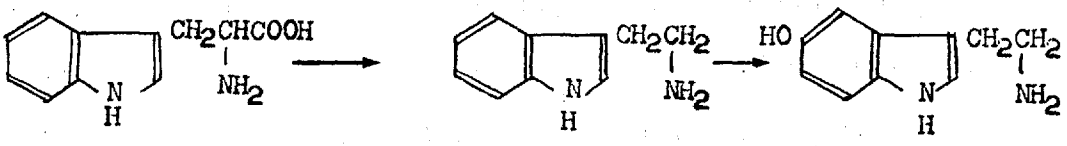
INTRODUCTION

In the gastrointestinal mucosa of many vertebrates are found cells which contain distinctive granules. These granules have well defined histochemical properties (chromaffinity, argentaffinity, ability to couple with diazonium salts and yellow fluorescence after fixation with formaldehyde). Erspamer and his colleagues (Erspamer, 1954) sought to identify the substance responsible for the peculiar properties of these granules in a series of investigations spread over nineteen years. They came to recognise that these cells, which they named "Enterochromaffin cells" were present not only in the gastrointestinal tract of vertebrates but also in the skin of certain species and gathered together in glandular formations such as the posterior salivary glands of the octopus. From tissues rich in these cells they were able to extract a substance having powerful pharmacological actions which they named "Enteramine". As this work was going on in Milan, marine animals were readily available. Erspamer and Asero (1953) chose two for their study on the isolation and characterisation of Enteramine. 30 kilos of posterior salivary gland tissue were removed from 30,000 octopuses (Octopus Vulgaris). From this tissue they extracted Enteramine as its picrate salt. The picrate salt of Enteramine was also prepared from the skin of 1020 Sicilian amphibians (Discoglossus Pictus). Sufficient material was recovered to allow the chemical and biochemical characterisation of Enteramine from these two sources as 5-hydroxytryptamine (5HT). The properties of the natural 5HT were compared with synthetic 5HT (Asero et al 1952) and found to be identical.

During the later stages of this work studies began independently (Rapport et al 1948) on the nature of the substance responsible for the vasoconstricting property of serum (O'Connor 1912). Rapport et al (1948) named this substance "Serotonin" and from beef serum a substance was isolated which on the basis of its colorimetric reactions, ultraviolet spectra, paper chromatographic and crystallisation properties was thought to be the creatinine sulphate salt of 5HT (Rapport 1949). When Hamlin and Fisher (1951) accomplished their synthesis of 5HT it was evident that Rapport had been correct in the structure he had assigned to Serotonin. It was not until 1952 however that Erspamer and colleagues and Rapport and his coworkers became mutually aware of each others work on enteramine and serotonin and that these two substances were identical.

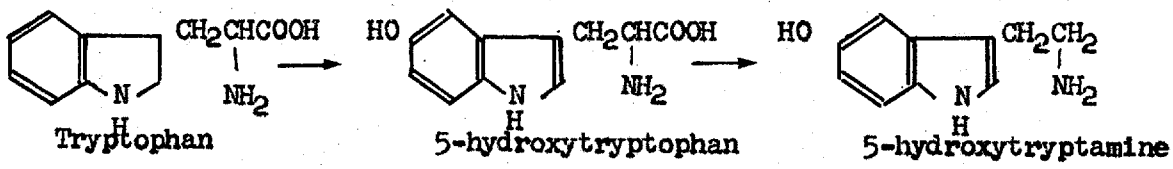
When the structure of 5HT became known ideas concerning its biosynthesis and metabolism began to form. Three possible routes for its biosynthesis appear to have been considered:

1. The 5-hydroxylation of tryptamine formed by the decarboxylation of tryptophan:

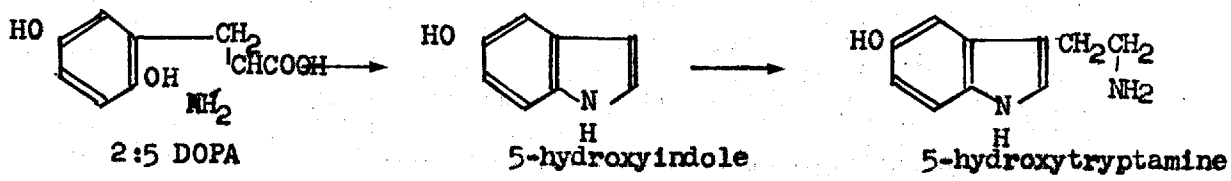


Tryptophan Tryptamine 5-hydroxytryptamine

2. The 5-hydroxylation of tryptophan with the formation of 5-hydroxytryptophan which is then decarboxylated to form 5-hydroxytryptamine:



3. The cyclisation of 2:5-dihydroxyphenylalanine (2:5-DOPA) to form 5-hydroxyindole with subsequent insertion of the β-ethylamine side chain.



Using 5-hydroxytryptophan synthesised by Ek and Witkop (1953) Udenfriend et al (1953) showed that homogenates of guinea pig kidney enzymatically decarboxylated 5-hydroxytryptophan (5HTP) to form 5HT. The same group of workers went on to partially purify this enzyme from guinea pig kidneys and demonstrate its requirement for pyridoxal phosphate (Clark et al 1954). At first the decarboxylation was thought to be specific for 5HTP but Lovenberg et al (1962) have shown that the enzyme prepared from guinea pig kidney, brain and intestine has a wide specificity for aromatic amino acids. This aromatic amino acid decarboxylase will catalyse the decarboxylation of the L isomers of dihydroxyphenylalanine (DOPA), 5HTP, tyrosine, phenylalanine, tryptophan, and surprisingly histidine, though the enzyme is not as active for histidine as the specific histidine decarboxylase (Weissbach et al 1961). Hagen (1960) has questioned whether all similar decarboxylases have such a wide specificity by demonstrating that decarboxylases prepared from phaeochromocytomas and carcinoid tumours, whilst decarboxylating DOPA and 5HTP will not decarboxylate the other aromatic amino acids.

Blaschko (1952) demonstrated that 5HT was oxidatively deaminated by guinea pig tissues and posterior salivary glands of Octopus Vulgaris and Titus and Udenfriend (1954) that the final product of this oxidative deamination was 5-hydroxyindole acetic acid (5HIAA). It remained for Weissbach et al (1957) to demonstrate that the direct product of the oxidative deamination of 5HT was 5-hydroxyindole acetaldehyde, this being converted to 5HIAA by alcohol dehydrogenase.

The position was reached therefore where it seemed that the probable route of biosynthesis of 5HT was via 5HTP and of metabolism to 5HIAA. As 5HTP is not an amino acid abundant in nature it was probable that it would have to be synthesised by the organism requiring it. With this information Udenfriend et al (1956) carried out a series of important experiments. They utilised the toad, Bufo Marinus, the venom glands of which contain large amounts of 5-hydroxy-N-methyl-tryptamine, N-N-dimethyl-5-hydroxytryptamine (Bufotenine), (β-[5-hydroxy-indolyl-(3)] -ethyl) trimethylammonium hydroxide (Bufotenidine), and 5-HT (Weiland et al 1934). DL-tryptophan-2-C^{14} was administered orally to the toad and later the toad was killed, the 5-hydroxyindoles extracted and subjected to paper chromatographic separation. Radioactive 5HTP was identified and recrystallised to constant specific activity as its p-iodophenyl-sulphonyl (pipsyl) derivative. This was the first definite demonstration that tryptophan could undergo 5-hydroxylation by living tissues. In addition to this they were also able to show that the administration of DL-tryptophan-2-C^{14} to rabbits led to labelling of the 5HT extracted from the platelets.

As a proportion of this thesis is concerned with the investigation of 5HT biosynthesis in carcinoid tumours, and as investigation of patients with the carcinoid syndrome helped greatly in elucidating the pathways of 5HT synthesis and metabolism a short consideration of these tumours and the clinical picture associated with them is necessary.

The term "carcinoid tumour" was coined by Orbendorfer (1907) to describe certain intestinal tumours which although carcinomatous ran a more benign course than the more common carcinomas. Gosset and Masson (1914) demonstrated that many of these carcinoid tumours contained granules which reacted with silver stains and Masson (1928) identified the cells containing these granules with the Kultschitzky cells of the crypts of Lieberkühn. He therefore called these tumours "Argentaffinomas". Since then it has been recognised that not all of these tumours have characteristic silver staining properties nor are they all located in the gastrointestinal tract. For this reason the term "Carcinoid tumour" is preferred to "Argentaffinoma". Cassidy (1934) described a case of abdominal neoplasm which presented with facial flushing and pulmonary valve disease but it was really Waldenstrom and Ljungberg (1953) who realised the association of carcinoid tumours with the clinical syndrome of flushing and valvular heart disease. Since then there have appeared many reports of carcinoid and other types of tumours associated with a clinical picture which,depending upon the particular case may commonly present with one or more of the following phenomena: flushing, which may become more or less permanent, diarrhoea, valvular disease of the heart, dependent oedema, pellagra-like

lesions of the skin and intermittent dyspnoea due to obstructive airway disease. This clinical picture is now known as the Carcinoid Syndrome and has been well described (see Waldenström 1958).

Lembeck (1953) was the first to demonstrate that a carcinoid tumour contained large amounts of 5HT and Page et al (1955) showed that patients with the carcinoid syndrome commonly excrete increased amounts of 5HIAA in the urine, the estimation of which is now the accepted diagnostic test for this syndrome. Initially many of the symptoms were attributed to an increased secretion of 5HT but the work of Robertson et al (1962) threw doubt upon whether 5HT was responsible for the flushing. Oates et al (1964) followed up this work and were able to demonstrate that the flushing was probably due to the release of a vasodilating kinin from the tumour, formed within the tumour by a kallikrein. Melmon et al (1965) have recently reported that this kinin is lysyl-bradykinin. The tumours associated with the carcinoid syndrome are therefore capable of producing at least two substances with marked pharmacological activities, and it is known that some also produce histamine. It is not unlikely that other substances are also produced, since the mechanisms by which the broncho-constriction and valvular lesions arise are still not well explained.

The pellagra-like lesions of the skin sometimes seen in severe cases are thought to be due to the diversion of tryptophan through the 5HT pathway which in these patients may account for 60% of the ingested tryptophan. This is thought to result in the diminished synthesis of nicotinic acid from tryptophan, though contributing

factors may be the malnutrition associated with a cachectic state and severe diarrhoea, which terminally many of these patients exhibit.

When it was realised that increased 5HT synthesis occurred in the carcinoid syndrome several investigations were done which helped in the initial formulation of the pathways of 5HT synthesis and metabolism.

Udenfriend et al (1956) administered tryptophan-2-C^{14} to a patient with the carcinoid syndrome and showed that the urinary 5HIAA became radioactive. That the tumour was dependent upon a supply of tryptophan for the synthesis of 5HT was shown in the investigations of Smith et al (1957) where the urinary level of 5HIAA excretion rose and fell para passu with the amount of tryptophan in the diet. In the normal dog however the amount of 5HIAA excreted in the urine could not be increased by the administration of tryptophan (Udenfriend et al 1956) which led these workers to suggest that 5HT biosynthesis is, under physiological conditions, occurring at a maximal rate. However since 5HIAA urinary excretion and tissue levels of 5HT can be easily increased by the administration of 5HTP one may assume that 5HTP decarboxylation is not the rat-limiting step. It is not unreasonable to speculate therefore that tryptophan 5-hydroxylation might be the rate limiting step in the biosynthesis of 5HT.

Scattered observations supported the hypothesis that 5HTP was the intermediate in the conversion of tryptophan to 5HT. Dalgleish (1956) and Sandler and Snow (1958) identified 5HTP in the urine of certain patients with the Carcinoid Syndrome. Donaldson et al (1959) administered labelled tryptophan to a patient with the Carcinoid Syndrome whose urine contained 5HTP and found that the 5HTP became labelled.

Initially Udenfriend et al (1953) dismissed tryptamine as an intermediate in the synthesis of 5HT as they could not demonstrate the formation of tryptamine from tryptophan. When however the enzymatic decarboxylation of tryptophan was revealed (Weissbach et al 1959) this possibility was reexamined but no evidence for this pathway could be found (Udenfriend et al 1959).

The hypothesis that 2:5-dihydroxyphenylalanine could be a precursor of 5HT would presume the hydroxylation of tyrosine, cyclisation of the side chain and the insertion of a β-ethylamine side chain. Chemical synthesis of 5-hydroxyindole by this route is possible (Cromartie and Harley Mason 1952). However the administration of labelled phenylalanine and tyrosine does not lead to the recovery of radioactive 5-hydroxytryptophan from the venom glands of Bufo Marinus (Udenfriend et al, 1956) nor have any of the various steps in this proposed sequence been shown to occur enzymatically.

All the evidence pointed to the sequence

Tryptophan ⟶ 5-hydroxytryptophan ⟶ 5-hydroxytryptamine

as being the physiological route of 5HT biosynthesis at least in animal tissues. However many plants contain 5HT as well as other amines such as noradrenaline, tyramine, dopamine and tryptamine (Waalkes et al 1958). It is not impossible that the biosynthesis of 5HT in plants could proceed by a route different from that in animal tissues. There are precedences for different biosynthetic pathways for important biological substances. For example depending upon the species of bacteria, algae, fungi or plant, lysine biosynthesis may occur from acetate and α-ketoglutarate via α-aminoadipic acid or from pyruvate and aspartate via α, -diaminopimelic

acid. No evidence has been adduced for both pathways existing in the same organism (Meister 1965).

However the possibilities that tryptamine or 2:5-DOPA are precursors of 5HT in certain species is fairly remote. There is the fact already stated, that noradrenaline, dopamine and 5HT tend to occur together in plants and there are certain similarities in the biosynthesis of these compounds which are worth considering. The starting point in the biosynthesis of these substances would appear to be the hydroxylation of an aromatic amino-acid, tyrosine for dopamine and noradrenaline and tryptophan for 5HT. DOPA and 5HTPare then decarboxylated to form dopamine and 5HT respectively. Up to this point then the biosynthetic routes are similar, but catecholamines and 5HT do show other metabolic reactions for instance oxidative deamination, O-methylation and N-methylation, though the importance of these varies from tissue to tissue and from amine to amine.

The studies which this thesis describes sprang from observations on a patient with the Carcinoid Syndrome (Appendix: Case 1). The urine of this patient contained large amounts of 5HTP and consideration of the significance of this highlighted the fact that very little was known about the enzymatic catalysis of tryptophan 5-hydroxylation. Udenfriend et al (1953) had briefly reported that tryptophan-2-C^{14} was converted to 5-hydroxytryptophan-2-C^{14} by liver homogenates. A detailed report of this work never appeared as the amount of conversion was thought to be insignificant. However in the light of later work demonstrating the 5-hydroxylation of tryptophan by rat liver (Freedland et al 1961[a]), which will be more fully discussed later, the earlier work may have been falsely dismissed.

Mitoma et al (1956) demonstrated the 5-hydroxylation of tryptophan in a bacterium, Chromobacterium Violaceum, an organism which makes a pigment Violacein, this pigment containing a 5-hydroxyindole structure (Beer et al 1954). They could not however obtain the tryptophan 5-hydroxylase activity when the bacterial cells were broken. They were able to show though that the bacterial cell would not hydroxylate phenylalanine and that the hydroxylating activity of these organisms differed from the microsomal aromatic hydroxylating system in liver. The latter has been the subject of many investigations and is responsible for the ring hydroxylation of many aromatic substances foreign to the body (Mitoma et al 1961). It is of interest to note that this latter hydroxylation involves the introduction of one atom of molecular oxygen, into the aromatic ring (Hayaishi et al 1956) and comes uder the heading of a "Mixed function oxidase", a class to which most enzymes catalysing aromatic hydroxylation probably belong (Mason 1957). In such reactions it is postulated that the hydroxylase (oxidase) is responsible for a coupled reaction involving a substrate, an electron donor and molecular oxygen. One atom of molecular oxygen is reduced, and the other introduced into the substrate. Precisely how such reactions occur is unknown but the overall reaction is represented by: Substrate + O_2 + RH_2 $\longrightarrow$ SO + R + H_2O (where RH_2 is the electron donor). Depending upon the hydroxylase the electron donor may be reduced NAD, reduced NADP, reduced pteridine compounds, or ascorbic acid (Hayaishi 1963). It is useful here to anticipate some of the results reported in this thesis to discuss in detail one of these "Mixed function oxidases", phenylalanine hydroxylase, as many of the properties of tryptophan 5-

hydroxylase which will be reported suggest a similarity between these two enzymes. Phenylalanine hydroxylation is reviewed in detail by Kaufman (1963), who has been largely responsible for working out the mechanism of the enzymatic conversion of phenylalanine to tyrosine. He views the reaction in the following way. The enzyme, phenylalanine hydroxylase, which in Kaufman's work is partially purified from rat liver, forms a complex with molecular oxygen. This brings out the first requirment fo the reaction when studied in vitro, that is the requirement for molecular oxygen (Eq. 1). By a reaction, the mechanism of which is unknown, the proposed enzyme-oxygen complex then introduces one atom of oxygen into the aromatic ring of phenylalanine to produce tyrosine and an enzyme complex containing only one atom of oxygen (Eq. 2). This atom of oxygen is then reduced to water by a reaction of the enzyme-oxygen complex with an electron donor which is oxidised during this process (Eq. 3).

$$E + O_2 = E - O_2 \qquad \text{(Eq. 1)}$$

$$E - O_2 + \text{phenylalanine} = \text{tyrosine} + E\text{-}O \qquad \text{(Eq. 2)}$$

$$E\text{-}O + XH_4 = E + XH_2 + H_2O \qquad \text{(Eq. 3)}$$

In a series of careful investigations Kaufman has shown that the electron donor is a tetrahydropteridine. It was found that tetrahydrofolate (THFA) as well as simpler, and often more stable tetrahydropteridines, could replace the naturally occurring cofactor which is now thought to be reduced biopterin (Kaufman 1963). Most of the resent studies on phenylalanine hydroxylation have utilised 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine. (In the studies to be reported on tryptophan 5-hydroxylase, tetrahydrofolic acid (THFA) and 6-7 dimethyl 5,6,7,8-tetrahydropteridine ($DMPH_4$) have been used in the investigation of electron donor requirements).

Kaufman found that during the conversion of phenylalanine to tyrosine by the rat liver enzyme the tetrahydropteridine was oxidised to a dihydropteridine, which is inactive, via an intermediate "Oxidised pteridine". This intermediate is capable of being reduced back to the active tetrahydropteridine by reduced NADP and an enzyme prepared from sheep liver, which may be regarded as a dihydropteridine reductase. Kaufman has presented evidence that the "Oxidised pteridine" capable of being reduced back to the active tetrahydropteridine is a 5,6-dihydropteridine and that the inactive final oxidised form is a 7,8-dihydropteridine. The overall reaction is therefore represented by eq. 4.

L-Phenylalanine + O_2 + tetrahydropteridine

↓ phenylalanine hydroxylase (rat liver)

↑ reduced NADP + Dihydropteridine reductase (sheep liver) (Eq. 4)

H_2O + L-Tyrosine + "Oxidised pteridine" ----→ 7,8-dihydropteridine

In terms of enzymological investigations carried out in vitro the necessity for the addition of reduced NADP and the dihydropteridine reductase should be overcome by adding an excess of the active tetrahydropteridine. However it is probable that quite a large excess of a tetrahydropteridine has to be added because these compounds are rather unstable and easily oxidised.

The results reported in this thesis will present evidence suggestive that tryptophan 5-hydroxylation proceeds by a similar reaction and the reports of Nagatsu et al (1964) on tyrosine hydroxylase and Levine and Sjoerdsma (1965) on the tryptophan 5-hydroxylase prepared from mouse mast cell tumours suggest that these enzymes also act through a similar mechanism.

In 1957 Parrat and West reported that certain mast cell tumours of rats contained large amounts of 5HT. Schindler (1958) incubated neoplastic ascitic mast cells of this type with DL-tryptophan-3-C^{14} and isolated from the incubation radioactive 5HT. He also produced solid mast cell tumours in mice and from these tumours prepared a cell-free supernatant extract which on incubation with radioactive tryptophan formed radioactive 5HT. No attempt was made to identify 5HTP as an intermediate.

Dalgleish and Dutton (1957) attempted to demonstrate the biosynthesis of 5HT from tryptophan in perfused liver and intestine but failed. Cooper and Melcer (1961) claimed to demonstrate the 5-hydroxylation of tryptophan and the subsequent decarboxylation of the 5HTP formed in cell free preparations of intestinal mucosa and kidneys of guinea pigs. They encountered trouble with non-enzymatic 5-hydroxylation of tryptophan which to some extent confuses the results they present. In addition the quantitative assay which they describe would probably not exclude the assay of tryptamine as 5HT, and this would account for the activity they found in kidney preparations with which tryptophan decarboxylation would be expected. They claimed that Cu^{++} and ascorbic acid were required for the full activity of intestinal tryptophan hydroxylase and as will be discussed later this is a potent combination in catalysing the non-enzymatic 5-hydroxylation of tryptophan. Even though there are many anomalies in this report which make it difficult to assess, nevertheless one is tempted to speculate that tryptophan 5-hydroxylation was being observed. Although in the studies to be reported later, tryptophan 5-hydroxylation has been demonstrated

in whole intestinal segments, Cooper and Melcer's work on cell-free preparations of intestinal mucosa could not be confirmed (see also Renson et al 1962). Another study which implicated the gastrointestinal tract as a site of 5HT biosynthesis was that of Bertaccini (1960). He found that the removal of the gastrointestinal tract from rats caused a marked fall in the urinary excretion of 5HIAA and in tissue concentration of 5HT (except in the brain and skin). He excluded tryptophan malabsorption as the cause of these changes and concluded that the gastrointestinal tract was the main site of 5HT biosynthesis in the body but that the brain and skin were also capable of synthesising 5HT (the latter probably by virtue of its mast cell population.)

These conclusions are relevant to the Carcinoid tumour. The majority of these tumours arise in the gastrointestinal tract and are made up of cells having the histochemical reactions of the argentaffin cells of the gastrointestinal mucosa which Ersparmer (1954) had implicated as the site of synthesis and storage of 5HT. The presumption has always been therefore that the tumour itself is directly responsible for the increased biosynthesis of 5HT seen in the carcinoid syndrome.

When the decision was made to investigate tryptophan 5-hydroxylation in isolated tissues, the choice of tissue for examination became important and some general considerations of this matter will be discussed. If an enzymatic reaction is to be studied in isolated tissue preparations then it is obvious that the more active the tissue is in respect of the reaction the easier the study is likely to be. At first sight a good criterion for choice of a tissue would seem to be the tissue concentration of one of the products of the reaction. However the tissue

concentration of a substance, particularly a substance with pharmacological activity, is a balance between synthesis, storage, destruction and release. For instance a tissue may contain a high concentration of a substance merely because that substance is stored there, as is the case with 5HT in platelets. If however a tissue does contain a high concentration of a substance and there is a reasonable indirect evidence that the substance is synthesised there such a tissue may be a good first choice for examination. Because clinical investigation on various aspects of the carcinoid syndrome was going on in the environment where the present studies were proposed, carcinoid tumours were removed at operation or metastatic carcinoid growth (which in the liver is usually well defined and abundant), was not too difficult to come by.

The greatest problem encountered in these studies has been the development of methods capable of demonstrating and assaying the enzymatic 5-hydroxylation of tryptophan. From the outset the enzymatic conversion of tryptophan to 5HTP was studied using radioactive tryptophan as a substrate. This was done for two reasons. Firstly it appeared from the literature that tryptophan hydroxylation was unlikely to be a quantitatively very active reaction. Secondly carcinoid tumours contain large amounts of 5-hydroxyindoles so that if the conversion of tryptophan to 5HTP was small it might prove difficult to demonstrate the de novo synthesis of 5-hydroxyindoles by non-isotopic methods.

The identification and assay of the radioactive 5-hydroxyindoles formed from tryptophan-3-C^{14} proved a very difficult technical problem. The methods designed to tackle this gradually developed as the study progressed and information about tryptophan 5-hydroxylation emerged. These

methods are therefore described in the context of the study as it evolved.

This investigation into the nature of tryptophan 5-hydroxylation as the first step in the biosynthesis of 5HT has passed through various phases. First carcinoid tumours were studied and the initial experiments failed to demonstrate tryptophan 5-hydroxylation. Freedland et al (1961a) then reported that tryptophan 5-hydroxylation occurred in rat liver preparations. The reaction was therefore studied in this tissue and the knowledge gained applied to the study of carcinoid tumour. Still no activity was found. Methods were then altered and improved and it became possible to demonstrate and study the conversion of tryptophan to 5HTP in carcinoid tumour tissue. These methods were then applied to the study of 5HT biosynthesis in intestinal segments and isolated brain tissue.

EXPERIMENTAL SECTION I

A. TRYPTOPHAN 5-HYDROXYLATION IN CARCINOID TUMOUR TISSUE

Introduction

The criteria used in the selection of carcinoid tumour tissues used in this study were:

(i) Cases from which tissue was removed should have presented with the carcinoid syndrome;

(ii) The urinary excretion of 5HIAA must have been raised and/or the tumour tissue must have contained an increased concentration of 5-hydroxyindoles. These criteria should ensure that, in vivo at least, the tumour tissue was actively synthesising 5HT.

The pattern of 5-hydroxyindoles excreted in the urine and present in the tumours of patients with the carcinoid syndrome is of interest. In most patients with the syndrome the main 5-hydroxyindole excreted in excess in the urine is 5HIAA and in tumours removed from such patients the predominant 5-hydroxyindole is 5HT, (often with a little 5HIAA) (own observations). Langemann (1958) has demonstrated that many carcinoid tumours contain both 5HTP decarboxylase and monoamine oxidase. However in a few patients the tumour and urinary pattern of 5-hydroxyindoles differ from this. These patients excrete in the urine large amounts of 5HTP, 5HT, and 5HIAA and tumours from such patients have been shown to contain large amounts of 5HTP as well as 5HT (Peart et al 1963). It has been shown (Oates and Sjoerdsma 1962) that the administration of 5HTP to man causes the excretion of 5HTP, 5HT and 5HIAA in the urine. It is therefore assumed that in these patients there is some defect in the

decarboxylation of 5HTP either because of a lack of 5HTP decarboxylase or because of some fault in intracellular organisation whereby 5HTP is released before it can be decarboxylated.

The first tumour studied (Appendix: Case 1) was of this type and on the assumption that it lacked the decarboxylase it was hoped that any 5HTP formed from tryptophan would remain as such in the tissue incubation mixture and not be further metabolised to 5HT. Initially therefore methods were developed to demonstrate the production of 5HTP which were applied to other tumours without fully appreciating that these latter tumours were rich in 5HTP decarboxylase.

Methods

L. Collection and Preparation of Carcinoid Tumour Tissue

(For details of tumours see Appendix)

Tumours which were collected at operation were immediately placed in ice in a vacuum flask, transported to the laboratory and stored in the deep freeze at -10°C to -15°C until used. Autopsy material (livers containing tumour metastases) was removed half to one and a half hours after death. The livers were stored at -10°C to -15°C until used.

Typical carcinoid tumour tissue is very fibrous and difficult to homogenise. The following procedure was there fore adopted. Pieces of the whole tumour or metastases macroscopically separated from the liver tissue around them were minced up with scissors. The mince was then pressed through several layers of wire gauze in a sodium press. The fine mince was weighed and homogenised in a tissue homogeniser, a glass tube with a teflon pestle (clearance 0.1 mm.) in 0.01 M potassium phosphate buffer pH 7, (1:1 w/v).

Whole homogenates were centrifuged at 100,000 g for 1 hour. The clear supernatant was decanted and kept and the particulate fraction was resuspended in 0.01 M potassium phosphate buffer, pH 7 to the original volume of whole homogenate taken. All these preparations were performed at 0° to 5°C.

2. Assay of Tryptophan 5-Hydroxylation

a. Deproteinisation At the end of the incubations to be described, in which 1 ml of either the whole homogenate, 100,000 g supernatant or 100,000 g particulate fraction were used, 2 ml of 0.8 N perchloric acid was added and the sample gently shaken. The denatured protein was removed by centrifugation at 1000 g for 10 mins and the supernatant kept. The precipitate was resuspended in 3 ml of 0.6 N perchloric acid, again centrifuged and the two supernatants pooled. This extract was adjusted to a pH of 7 with 1 N KOH and the crystalline potassium perchlorate removed by centrifugation at 500 g for 5 minutes.

b. Desalting This extract contains a high concentration of ions and its volume is about 10 ml. The 5-hydroxyindoles in this solution had to be extracted in a form suitable for high voltage paper electrophoresis, which had already been chosen as the method of identification. It was necessary therefore to desalt the extract and reduce the volume. The procedure adopted was essentially that described by Asatoor and Dalgleish (1956)

The deproteinised extract was adjusted to a pH of 4 with a few drops of glacial acetic acid. 0.25 G of charcoal previously deactivated with stearic acid (4 G stearic acid to 100 G charcoal) was added and the suspension allowed to stand with occasional mixing for 10-15 minutes. It

was then centrifuged at 2000 g for 10 minutes and the supernatant discarded. The precipitated charcoal was resuspended in 10 ml of distilled water and centrifuged again. This procedure was repeated twice more, discarding the supernatants each time and thereby washing the charcoal free of salts. The charcoal was then resuspended in 10 ml of an aqueous phenol solution (7G phenol/100 ml water) and allowed to stand for 10-15 minutes with occasional shaking. The suspension was centrifuged at 2000g for 10 minutes and the resultant supernatant filtered thfough No. 1 or No. 4 Whatman filter paper to remove the last traces of charcoal. Two further 10 ml volumes of the aqueous phenol solution were added and the procedures repeated. The phenolic eluates were pooled and evaporated to dryness at 70°C in a rotary evaporator under a vacuum of 10 mm Hg. The dry extract was dissolved in 10 ml of water and evaporated to dryness and this procedure repeated to remove last traces of phenol. The extract was washed from the flask with 1.5 ml x 3 volumes of water and the solution placed in small glass pots and dried overnight in a vacuum dessicator containing P_2O_5, NaOH and conc H_2SO_4 at 10 mm Hg. These dried extracts were then suitable for high voltage paper electrophoresis after they had been dissolved in a little water.

Initially interest was focused upon the recovery of 5HTP and it was found that, using this procedure, the recovery of 5HTP was about 70% when 2 µmoles of DL 5HTP was added (prior to the addition of perchloric acid) to aqueous solutions. The recovery of 5HT however was only 5-10% under the same conditions.

c. <u>The Separation of 5-hydroxyindoles by High Voltage Paper Electrophoresis</u> The dissociation constant of the 5-OH group of 5HT is 11.1 (Vane

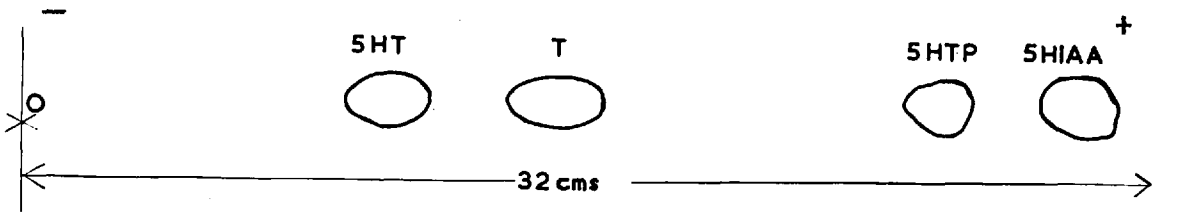


Fig. 1 High voltage paper electrophoresis at pH 12, showing separation of tryptophan (T), 5HTP, 5HT, and 5HIAA (For conditions see text). O = origin, - = cathode, ·· = anode. Stained with Erhlich's reagent

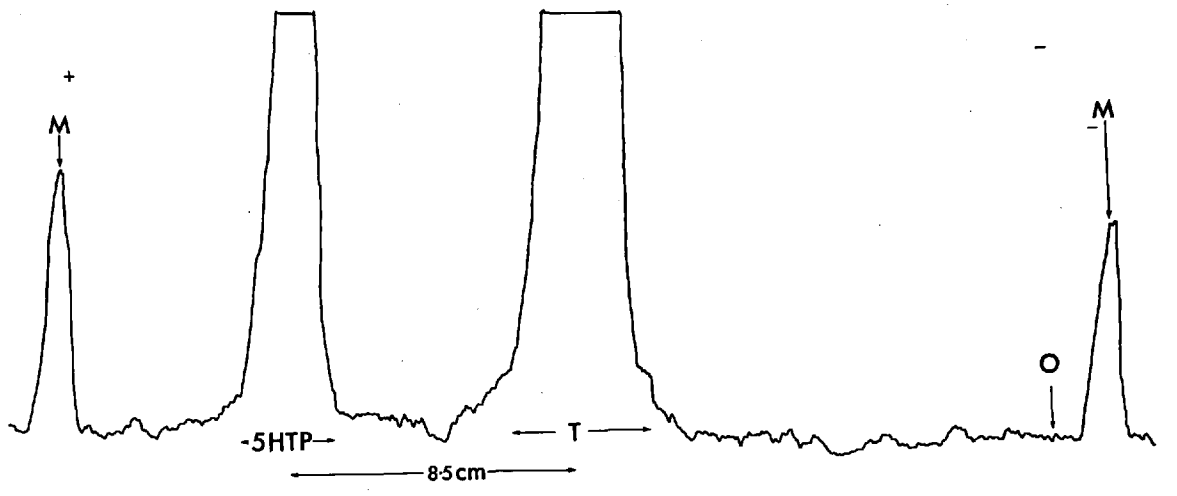


Fig. 2 High voltage paper electrophoresis, pH 12. Scan of electrophoretic strip showing peaks coinciding with 5HTP-3-C^{14} and tryptophan -3-C^{14}. (For conditions see text).

1959). It seemed likely that the dissociation constant of the 5-OH group of 5HTP would be in this region and therefore at pH values higher than this 5HTP would be expected to move more quickly toward the anode than tryptophan on high voltage paper electrophoresis. The apparatus used was one built in the laboratory but on the principles described by Efron (1960).

By trial and error the following solution was selected as the high voltage paper electrophoresis "buffer". Two stock solutions, A and B, are first prepared:

A. Sodium tetraborate ($Na_2B_4O_7$, $10H_2O$) 19.05 G/L

B. Sodium hydroxide (NaOH) 8 G/L

500 ml of A and 450 ml of B are mixed and the solution made up to nearly 2 L. The pH is then measured with a glass electrode and if necessary adjusted to 12 with 2 N NaOH.

Electrophoresis is carried out on 3 mm Whatman paper at 80-90 v/cm for 1 hour. Spots are applied one inch apart on strips of paper 5" in width or applied separately on strips of paper 1" in width, 4-5 strips being electrophoresed at one run. The origin is set well toward the cathode end of the paper if the separation of tryptophan, 5HT, 5HTP and 5HIAA is being studied as all these substances run toward the anode. Figure 1, shows the separation achieved. For interest is included a scan of part of an electrophoretic strip on which was spotted 2.5 μc/16μg of DL-3-C^{14} tryptophan and 0.5 μc/20μg of DL-3-C^{14} 5HTP (Figure 2).

Discrete separations can be achieved under ideal conditions, but the following precautions must be taken:

(i) The samples spotted must be virtually salt, protein and lipid free,

otherwise separations will be poor and trailing will occur.

(ii) The paper should not be overloaded. 40-60 µg of either tryptophan or the 5 hydroxyindoles is really the maximum amount for an initial spot 1 cm in diameter on Whatman 3 mm paper.

(iii) The electrophoretic solution must be changed with each run.

(iv) Although good separations are achieved the distance travelled from the origin is rather variable from run to run. However within any one run the separations are reliable. For this reason rather than use the distance travelled from the origin as the sole index of identification, standard strips upon which have been spotted pure substances have always been electrophoresed alongside the unknowns. Also in the experiments where the conversion of radioactive tryptophan to radioactive 5-hydroxyindoles has been studied, non-radioactive 5-hydroxyindoles have always been added as "Carriers" and of course these substances can be identified on the strip after scanning, by their staining reactions. One furbher point is of interest. Tryptophan, 5HT, 5HTP and 5HIAA when present in amounts of 20µg or more per spot fluoresce with a light greenish-blue tint on the electrophoretic strips run at pH 12 when these are dried and viewed under a Wood's lamp in U.V. light at 365 mµ. This property also aided location of these substances.

d. Staining Reactions Throughout the studies reported here 5-hydroxyindoles, tryptophan and related compounds have been located and identified on paper chromatograms and electrophoretograms by their staining reactions with Ehrlich's reagent (Jepson 1955).

e. Initial Analytical Procedure After incubation 2 µmoles of DL-5HTP were added and the sample deproteinised with perchloric acid. The extract

was passed through the charcoal method and the eventual dried extract dissolved in 0.2 ml of water. 0.02 ml aliquots were then spotted for high voltage paper electrophoresis at pH 12 as described. The strips were dried in a hot air oven at 50°C and then scanned for radioactivity in a chromatogram scanner (Isotope Developments Ltd. Scintillation counter 663). They were then stained with Ehrlich's reagent to locate the "carrier" 5HTP.

Expts 1 and 2. INITIAL ATTEMPTS TO DEMONSTRATE TRYPTOPHAN 5-HYDROXYLATION IN CARCINOID TUMOUR TISSUE

Tumours 1, 2, 3, 4 and 5 (See Appendix 1) were studied. Whole homogenates, 100,000g supernatant and particulate fractions alone and combined were examined for tryptophan 5-hydroxylation activity by the methods described. Various experimental conditions were tried and these are shown below.

(a) Using as a basis the findings of Cooper and Melcer (1961) who claimed to have demonstrated tryptophan hydroxylation in preparations of intestinal mucosa, tissue incubations were carried out as follows:

Tumour preparations examined	Volume added
Whole homogenates	1 ml.
100,000 G supernatant fraction	1 ml.
100,000 G particulate fraction	1 ml

Additions	Final Concentration
Potassium phosphate buffer pH 7.5	0.1 M
$CuCl_2$	2×10^{-3}M
Ascorbic Acid	1×10^{-3}M
DL tryptophan-3-C^{14} (specific activity 1μc/μmole)	1.2×10^{-3}M

The total volume of the incubation samples was 2 ml. Incubations were carried out for 1 hour at 37°C in a metabolic shaker either in air or in Thunberg tubes thrice evacuated and flushed with N_2. Boiled tissue was used as a control.

(b) Using as an analogy phenylalanine 4-hydroxylation (Udenfriend and Cooper, 1952 and Kaufman, 1958) the following incubations were carried out:

Tumour Preparations Examined	Added
Whole homogenates	1 ml
100,000 G Supernatant fraction	1 ml
100,000 G Particulate fraction	1 ml

Additions	Final Concentration
Potassium phosphate buffer pH 7	0.04 M
NADP	2×10^{-4} M
Glucose-6-phosphate (potassium salt)	0.04 M
Glucose-6-phosphate dehydrogenase	5 E.U.
$MgCl_2$	0.02 M
DL-tryptophan-3-C^{14} (specific activity 2.07 μc/μmole)	5.8×10^{-4} M

OR

Potassium phosphate buffer pH 7	0.04 M
Reduced NADP	2×10^{-4} M
THFA	2×10^{-4} M
NAD	2×10^{-4} M
$MgCl_2$	2×10^{-2} M
DL-tryptophan-3-C^{14} (specific activity 2.07 μc/μmole)	5.8×10^{-4} M

The total volume of the incubation samples was 2 ml. Incubations were carried out in tubes flushed with O_2 and corked, in a metabolic shaker at 37°C. Boiled tissues identically incubated acted as controls.

Results

1. By the methods of analysis used no radioactive 5-hydroxy-tryptophan could be demonstrated.

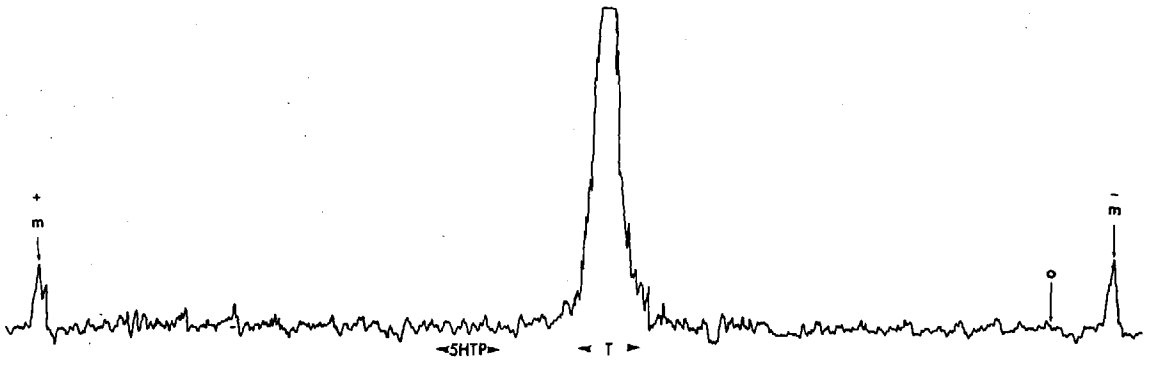


Fig. 3 Radioactive scan of high voltage paper electrophoresis (pH 12) of extract from an incubation of carcinoid tumour whole homogenate with DL-tryptophan-3-C^{14}. Stained with Erhlich's reagent. Note that no radioactivity is associated with the "Carrier", 5HTP.
(T = tryptophan)

2. It appeared from the size and density of the 5HTP spot on the paper electrophoretic strip after staining with the Ehrlich's reagent that about 50% of the carrier 5HTP was being recovered.

3. Although separations of tryptophan from 5-hydroxytryptophan were rather variable, separations were on many occasions excellent and had there been appreciable radioactivity associated with the 5HTP a peak should have resulted on the scan.

A representative strip showing good separation of carrier 5HTP from radioactive tryptophan is shown in figure 3. Note that no radioactivity is present in the area where 5HTP stained.

Discussion

The following appraisal of these negative results was made:

1. Had the tumours lost their activity? At the time this question was impossible to answer. In retrospect however the probable answer is no, since subsequently it was possible to demonstrate tryptophan hydroxylation in tumours stored at -10°C to -15°C for as long as nine months.

2. Had homogenisation destroyed some essential intracellular organisation necessary for full activity of tryptophan hydroxylase? By this is meant two things:- (a) That the intracellular localisation of the enzyme is in some way essential for its activity (Pardee, 1959); (b) That in the whole cell cofactors essential for enzymatic tryptophan hydroxylation are organised in space around the enzyme in optimum concentration. Breaking the cell then destroys this organisation and releases the cofactors whose effective concentration is then lowered

and which may be destroyed or inactivated by substances or enzymes with which in vivo they do not come into contact (Pardee 1959).

It seems very likely that the loss of cellular organisation did play some part in the negative results, since it was later demonstrated that although tryptophan hydroxylation could be demonstrated in tumour slices, frequently no activity could be demonstrated in cell free fractions prepared from these tumours. A similar situation has been reported by Mitoma et al (1956) with Chromobacterium Violaceum where it was shown that, whereas the whole bacterial cell would hydroxylate tryptophan, all cell free preparations were inactive.

3. Was tryptophan 5-hydroxylation inhibited by endogenous 5-hydroxyindoles? These tumours contained large amounts of 5-hydroxyindoles (see Appendix). As 5-hydroxyindoles are products of tryptophan hydroxylation it could be that in disorganised tumour tissues prepared by homogenisation tryptophan hydroxylation is inhibited by the high concentration of 5-hydroxyindoles present. Product inhibition of enzymatic reactions is a well recorded phenomenen (Walter and Freiden 1963) and very relevant is the work of Nagatsu et al (1964) on the inhibition of tyrosine hydroxylase by catecholamines.

4. Was allthe 5HTP converted to 5HT? In tumour 1 endogenous 5HTP was demonstrated and recovered after incubation, though no radbactivity could be found associated with it. This objection did not therefore apply to this tumour. However in the other tumours no endogenous 5HTP had been demonstrated on paper chromatography or electrophoresis and at a rough calculation this excludes the presence of more than about 1-2 µg of 5HTP/G of tumour compared with 200 µg or more /G of tumour for 5HT.

In these latter tumours therefore 5HTP might have been very quickly converted to 5HT by endogenous 5HTP decarboxylase. As the methods used in these inital experiments were developed primarily to detect the production of 5HTP it might have been that one was in fact missing tryptophan hydroxylation because all the 5HTP formed had been converted to 5HT.

5. Was the assay method sufficiently sensitive? Calculations made on the basis of later results show that the analytical procedure would not have detected less than a 1% conversion of the substrate L tryptophan to L 5HTP and it is very likely that under the conditions used here the conversion was less than this.

Optimistically it was still assumed that tryptophan hydroxylation did take place in these tumours and that the reasons for the negative results were:-

(i) Partial conversion of 5HTP to 5HT

(ii) Lowered tryptophan hydroxylation activity because of homogenisation.

(iii) Insensitivity of method.

B TRYPTOPHAN 5-HYDROXYLATION IN RAT LIVER PREPARATIONS

Introduction

At the time the initial negative tumour experiments were being done Freedland et al (1961a) reported that tryptophan 5-hydroxylation could be demonstrated in cell free preparations of rat liver. Dalgleish and Dutton (1957) had previously reported that liver was inactive in this respect but Freedland and his colleagues had taken

the unusual step of incubating their liver preparations with high concentrations of L-tryptophan (1.6×10^{-2}M). Having failed to demonstrate tryptophan hydroxylation in carcinoid tumours it was thought worthwhile to investigate the rat liver activity further and use it as a basis for the study of tumour hydroxylation.

Methods

(a) The Assay of Tryptophan 5-Hydroxylase in Rat Liver

To the incubation sample was added 4 volumes of 95% ethanol. The mixture was allowed to stand with occasional shaking for 5-10 minutes and then centrifuged at 1000 G for 10 minutes. The deproteinised supernatant fluid was taken and evaporated to dryness under vacuum at 50°C in a rotary evaporator. The dried extract was washed out of the flask with 1.5 x 3 ml of water and evaporated to dryness in small glass pots in a vacuum dessicator containing P_2O_5, NaOH, and conc. H_2SO_4. When dry, 3 ml of water was added, the extract dissolved and a 2 ml aliquot taken for assay by the colorimetric procedure described by Udenfriend et al (1955) and modified by Freedland et al (1961a). The optical density of the purple chromophore resulting from the nitrosonaphthol colorimetric reaction was measured at 540 mμ and compared with a standard solution containing a known amount of 5-hydroxytryptamine, a 2 cc aliquot of which had been carried through the colorimetric reaction. Two blanks were usually run, one consisting of 2 cc of water, the other a boiled tissue control or a control to which 4 vols of 95% ethanol had been added prior to incubation. The dried ethanolic extracts were also suitable for paper electrophoresis and chromatography.

(b) Preparation of Rat Liver

Rats were killed by a blow on the head, the livers immediately removed, weighed, minced with scissors and homogenised in the glass tube with a teflon pestle (as previously described for carcinoid tumours) in a solution of KCl 0.154 M containing 2.5 x 10^{-4}M NaOH (3 cc/G of liver). The whole homogenate was centrifuged at 20,000 g for 40 mins and the supernatant resulting used in the experiment to be described.

Before the cofactor requirements of this preparation were more precisely defined it was assumed that the rat liver tryptophan 5-hydroxylase activity would turn out to similar to the phenylalanine 4-hydroxylase activity, the properties of which had been studied by Kaufman (1959) and Udenfriend and Cooper (1952). In the initial experiments it will be seen that rather erratic and empirical additions were made, before a more systematic investigation of cofactor requirements was made.

Expt 3. THE DEMONSTRATION OF TRYPTOPHAN 5-HYDROXYLATION BY RAT LIVER

The following incubation mixture was set up:-

1 ml 20,000 g rat liver supernatant

L-tryptophan	final concentration	5 x 10^{-3}M
NAD	" "	5 x 10^{-4}M
Reduced NADP	" "	5 x 10^{-4}M
THFA	" "	2 x 10^{-3}M
Nicotinamide	" "	5 x 10^{-3}M
Potassium phosphate buffer, pH 7	"	0.1 M

Total volume of incubation, 2 ml. Boiled tissue identically incubated was used as a control. The samples were incubated for 1 hr at 37°C in an atmosphere of O_2 in a metabolic shaker. At the end of

incubation the samples were deproteinised, extracted and assayed as described.

Results

1 cc of a 20,000 g supernatant fraction prepared from a 3:1 (v/w) whole homogenate of rat liver produced 0.03 µmole of 5-hydroxyindoles in one hour (assayed as 5HT). This represents a 0.3% conversion to 5-hydroxyindoles of the 10 µmoles of L-tryptophan added.

Comments

(i) No purple chromophore appeared on assay of the control samples.

(ii) A visible purple chromophore was formed on assay of the experimental incubation.

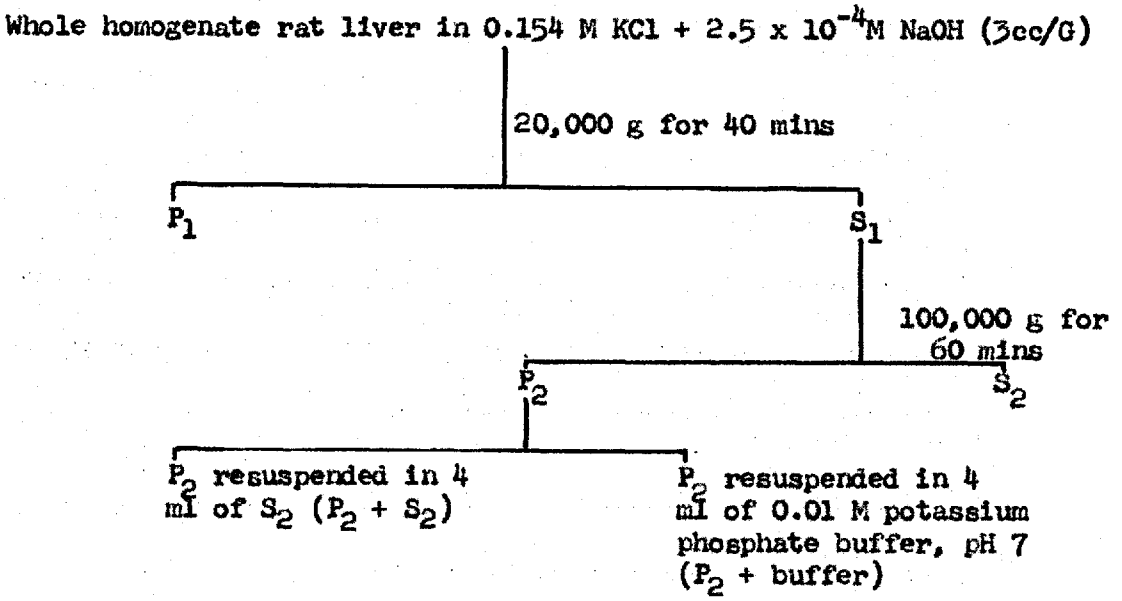
(iii) It was apparant therefore, assuming that the assay is specific for 5-hydroxyindoles (Udenfriend et al 1955) that rat liver possessed tryptophan 5-hydroxylating activity.

Expt 4. THE SUBCELLULAR FRACTIONATION OF TRYPTOPHAN 5-HYDROXYLASE FROM RAT LIVER

A 20,000 g supernatant fraction (S_1) of rat liver was prepared as already described. An aliquot of this was then centrifuged at 100,000 g for 60min in a Spinco ultracentrifuge (Model L). The resultant supernatant (S_2)was decanted and kept. Roughly half of the 100,000 g particulate fraction (P_2) was resuspended in 4 ml of S_2, the other half being resuspended in 4 ml of 0.01 M potassium phosphate buffer, pH 7. This subcellular fractionation is schematically illustrated in figure 4.

FIGURE 4

Subcellular Fractionation of Rat Liver Whole Homogenates



To 1 ml aliquots of fractions S_1, S_2, "P_2+S_2" and P_2+buffer" were added:

L-tryptophan	Final concentration		5×10^{-3}M
NAD	"	"	5×10^{-4}M
Nicotinamide	"	"	5×10^{-3}M
Potassium phosphate buffer pH 7		"	0.1 M

Total volume of incubation 2 ml. Tissue preparations to which were added 4 vols of 95% ethanol acted as controls. Incubations were carried out for 1 hr. at 37°C in O_2 in a metabolic shaker. The samples were deproteinised, extracted and assayed for 5 hydroxyindoles as described.

Results

TABLE I

Tryptophan 5-hydroxylation by subcellular fractions of rat liver

Fraction	Vol. (cc)	µmoles 5 hydroxyindoles produced/hr
S_1	1	0.12
S_2	1	0.185
$P_2 + S_2$	1	0.11
P_2 + buffer	1	0

Comments

This experiment showed that the tryptophan hydroxylase activity resided in the 100,000 g supernatant fraction, that no activity was present in the 100,000 g particulate fraction, and that perhaps a 100,000 g supernatant was slightly more active than a 20,000 g supernatant.

Expt. 5. STABILITY OF RAT LIVER TRYPTOPHAN 5-HYDROXYLATION

As these studies were being done to form a basis for the investigation of carcinoid tumour activity, the stability of the rat liver enzyme was investigated. If the carcinoid tumour hydroxylase was similar, one wanted to know how stable it was in the interval which might elapse between collection and "Immediate" incubation and also whether the activity deteriorated during storage in the deep freeze.

1. Stability at 37°C

1 ml. aliquots of a 20,000 g supernatant prepared as described were pre-incubated for various times at 37°C in air without shaking in the presence of NAD, nicotinamide and phosphate buffer, pH 7. (Concentrations as in the previous experiment). Total volume of incubation 2 ml.

At determined intervals L-tryptophan was added to a final concentration of 5×10^{-3}M, the tubes gassed with O_2 and shaken at 37°C for a further hour. At the end of incubation the samples were assayed for 5-hydroxyindoles.

Results

TABLE 2

The stability of tryptophan 5-hydroxylase in rat liver at 37°C

Preincubation Time	µmoles 5 hydroxyindoles produced/ml 20,000 g supernatant
0	0.12
15 mins	0.1
30 mins	0.117
60 mins	0.08
120 mins	0.05
180 mins	0.02

Comment

It will be seen that activity gradually declines over the 3 hours of the preincubation but that activity is still quite easily detectable then. Whether the decline in activity is due to a fall off in the effective concentration of essential cofactors or due to instability of the enzyme was not further investigated. The point was made that if activity could still be detected after 3 hours incubation at 37°C, then if the carcinoid tumour enzyme were similar it would be unlikely to become completely inactive during the period of collection and immediate preparation of tissue in the cold.

2. Stability at -10°C to -15°C

The last experiment did not exclude the possibility that the enzyme might become inactive during storage at -10°C to -15°C for longer periods. To test this a 100,000 g rat liver supernatant fraction was prepared as described and a 1 ml aliquot was assayed for tryptophan hydroxylase activity as in the last experiment. The rest was stored in the deep freeze for 24 hours, then thawed and a 1 ml aliquot again assayed for tryptophan hydroxylase activity

Results

TABLE 3

The stability of tryptophan 5-hydroxylase in rat liver at -10°C to -15°C

100,000 g supernatant	µmoles 5-hydroxyindoles produced/hr/ml
Fresh	0.14
Stored 24 hrs. at -10°C to -15°C	0.038

Comments

The fall in activity represents a decrease of 73% in tryptophan hydroxylase activity after storage for only 24 hours. This was a fairly depressing finding but confirmed the work of Kaufman (1957) on phenylalanine 4-hydroxylase. If the hydroxylating activity of carcinoid tumours was the same then the demonstration of this activity would have to be carried out on very fresh material. As it turned out however, the carcinoid tumour hydroxylase proved to be a great deal more stable than this.

THE IDENTIFICATION OF THE PRODUCTS OF TRYPTOPHAN 5-HYDROXYLATION BY RAT LIVER

Although confirmation of tryptophan 5-hydroxylation by the assay method specific for 5-hydroxyindoles had been achieved, nevertheless more

precise identification of the products of the reaction was needed.

The assumption was made that any tryptophan hydroxylated would lead to the formation of 5-HTP, that this would be converted to 5HT by the aromatic amino acid decarboxylase present in the rat liver preparation, and that some of the 5HT might be oxidatively deaminated, if soluble monoamine oxidase was present (Weissbach et al 1957), producing 5HIAA. Methods were therefore needed for the extraction and identification of 5HT, 5HTP and 5HIAA.

Methods

(a) Deproteinisation with ethanol. This procedure has already been described on p. 30.

(b) Extraction of 5HT The dried ethanol extract was dissolved in 5 ml of water and the 5HT extracted from the solution into butanol and returned to 2 ml of 0.1 N HCl by the extraction procedure described by Bogdanski et al (1956). This final acid extract was evaporated to dryness in a vacuum dessicator. After dissolving the dried material in water, aliquots were used for the electrophoretic and chromatographic procedures to be described.

(c) Extraction of 5HIAA The dried ethanol extract was dissolved in 4 ml of water. 0.3 ml of 6 NHCl and enough NaCl to saturate the solution were added. This solution was then shaken with 20 ml of peroxide free ether for 10 mins. After separation of the two phases the ether phase was removed and evaporated to dryness at room temperature in a rotary evaporator at slightly reduced pressure (MacFarlane et al 1956). The dried extract was washed out of the flask with 1.5 ml x 3 volumes of

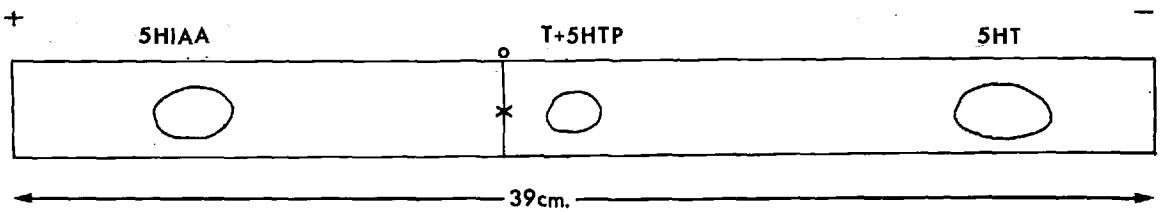


Fig. 5 High voltage paper electrophoresis at pH 6.1 showing the separation of tryptophan (T), 5HT, 5HTP and 5HIAA. For conditions see text. O = origin.

water and this solution dried again in a vacuum dessicator. The final dried extract in small glass pots was dissolved in 0.1 ml of water and aliquots subjected to paper electrophoresis and chromatography. This is only a slight modification of the method described by Udenfriend et al (1958a) and it avoids the return of the 5HIAA into phosphate buffer which leads to a salt laden solution unsuitable for electrophoresis and chromatography.

(d) High voltage paper electrophoresis

(i) At pH 12 in sodium hydroxide/sodium tetraborate solution

This method is described on p. 21.

(ii) At pH 6.1 The solution used is a mixture of pyridine, acetic acid and water prepared according to Efron (1960). High voltage paper electrophoresis is carried out at 80-90 V/cm for 30-45 mins. Spots are placed 1" apart at the midpoint (longitudinally) of a strip of No 1 Whatman paper 5-6" wide. The separations achieved on electrophoresis of standard solutions of tryptophan, 5HTP, 5HT and 5HIAA (20 µg of each spotted at the origin) are depicted in figure 5.

At pH 6.1 the separation of 5HT (towards the cathode) and 5HIAA (towards the anode) from the tryptophan are very good. Tryptamine runs slightly in front of 5HT and usually there is effective separation from it. This system does not effectively separate 5HTP from tryptophan. Standards were always electrophoresed alongside the experimental samples.

(e) Paper chromatography (Jepson 1960)

(i) Descending Paper Chromatography

Solvent: n-butanol:glacial acetic acid:water (120:30:50 v/v)

Paper - No 1 Whatman Development time - overnight

Staining - Ehrlich's reagent

"R_fs"	Tryptophan	50	5HT	52
	5HIAA	67	5HTP	32

(ii) Ascending paper chromatography

Solvent: Isopropanol: 880 ammonia: water (200:10:20 v/v)

Paper - No. 1 Whatman Development time - overnight

Staining - Ehrlich's reagent

"R_fs"	Tryptophan	25	5HT	56
	5HIAA	18	5HTP	15
	Tryptamine	83		

At this stage and with the apparatus available the R_fs were not highly predictable. For this reason standards were always run alongside the unknowns for definite identification.

Expt. 6 THE IDENTIFICATION OF THE PRODUCTS OF TRYPTOPHAN 5-HYDROXYLATION IN RAT LIVER

Five separate but identical incubations having the following composition were set up:

1 ml 100,000 g rat liver supernatant (prepared as already described)

L tryptophan 5×10^{-3}M ; NAD 5×10^{-4}M

Potassium phosphate buffer, pH 7 0.1 M; Nicotinamide 5×10^{-3}M

Total volume 2 ml.

The incubation tubes were flushed with O_2, corked and shaken gently for 1 hour at 37°C.

Sample 1

Procedure: Total 5-hydroxyindole production quantitatively assayed by colorimetric assay.

Sample 2

Procedure: Butanol extraction of 5HT

Electrophoresis at pH 6.1

Ascending paper chromatography in isopropanol: ammonia: water solvent

Sample 3

Procedure: Ether extraction of 5HIAA

Electrophoresis at pH 6.1

Sample 4

Procedure: Simple ethanol deproteinisation and extraction

Electrophoresis at pH 12

Chromatography in n-butanol:acetic acid: water

Chromatography in isopropanol:ammonia: water

Results

Quantitative

1 cc 100,000 g rat liver supernatant produced 0.14 μmoles of 5-hydroxy indoles in 1 hour.

Qualitative

Sample 2. Electrophoresis pH 6.1. Staining showed the presence of spots having the mobility and staining reactions of 5HT and tryptamine.

Chromatography Isopropanol:ammonia: water. Staining showed the presence of spots having the R_fs of 5HT and tryptamine.

Sample 3. Electrophoresis pH 6.1 - no 5HIAA could be detected

Sample 4. Electrophoresis pH 12 - staining showed the presence of a small amount of 5HTP, a larger amount of 5HT and a large trailing tryptophan "spot". Also present was a spot running just in front of

tryptophan having a bright light blue fluorescence under a Woods lamp and staining a bright yellow with Ehrlich's reagent. This was taken to be kynurenine, resulting from the action of tryptophan pyrollase on the substrate L-tryptophan (Knox and Mehler 1950).

Chromatography (n-butanol:acetic acid: water) - again 5HTP and 5HT were identified on staining.

Chromatography (Isopropanol: ammonia: water) - 5HT and tryptamine were definitely identified.

Comments

This experiment showed that the 5-hydroxyindoles produced were those expected. 5HTP had been identified as the intermediate in the production of 5HT. The absence of 5HIAA was not too surprising as monoamine oxidase is primarily a mitochondrial enzyme in liver (Hawkins 1952). The presence of tryptamine pointed to the activity of the aromatic amino acid decarboxylase (Lovenberg et al 1962).

Is tryptamine 5-hydroxylated by rat liver?

The incubation of rat liver supernatant with 5×10^{-3}M tryptamine, under conditions in which 5-hydroxyindoles were produced from tryptophan failed to demonstrate the production of any 5-hydroxyindoles. Jepson et al (1962) have demonstrated that tryptamine is 6-hydroxylated by rat liver microsomes.

Expt 7 CONFIRMATION OF THE 5-HYDROXYLATION OF TRYPTOPHAN IN RAT LIVER USING DL-TRYPTOPHAN-3-C^{14} AS A SUBSTRATE

Because isotopic methods were being used in the study of carcinoid tumours it was necessary to see whether tryptophan 5-hydroxylation in rat liver preparations could be demonstrated by these methods.

1 ml of a 100,000 g rat liver supernatant was incubated with the following additions:

Reduced NADP - 5×10^{-4}M		Reduced NAD - 5×10^{-4}M	
THFA	2×10^{-3}M	Nicotinamide	5×10^{-3}M

Potassium phosphate buffer pH 7, 0.1 M

To those samples in which total 5-hydroxyindole production was to be checked was added non-radioactive L-tryptophan to a final concentration of 5×10^{-3}M. Radioactive DL-tryptophan-3-C^{14} (specific activity 2.5 μc/μmole) was added at a final concentration of 1×10^{-3}M to those samples in which the aim was to demonstrate the production of radioactive 5-hydroxyindoles. Boiled tissue identically incubated acted as a control in both the non-isotopic and isotopic experiments. The total volume of all samples was 2 ml and incubations were carried out for 1 hr at 37°C in an atmosphere of O_2.

Analysis

1. In the samples incubated with non-isotopic tryptophan the total 5-hydroxyindole production was assayed colorimetrically.

2. Ethanolic extracts of the samples incubated with isotopic DL tryptophan-3-C^{14} were subjected to paper chromatography. The paper chromatographic strips were then scanned for radioactivity and stained with Ehrlich's reagent.

Results 1. Non-isotopic Method

Total 5-hydroxyindole production = 0.156 μmoles/hr/cc rat liver supernatant

2. Isotopic Method

(a) Paper chromatography in n-butanol: acetic acid: water. On the scan this showed a radioactive peak overlying a spot with the mobility and

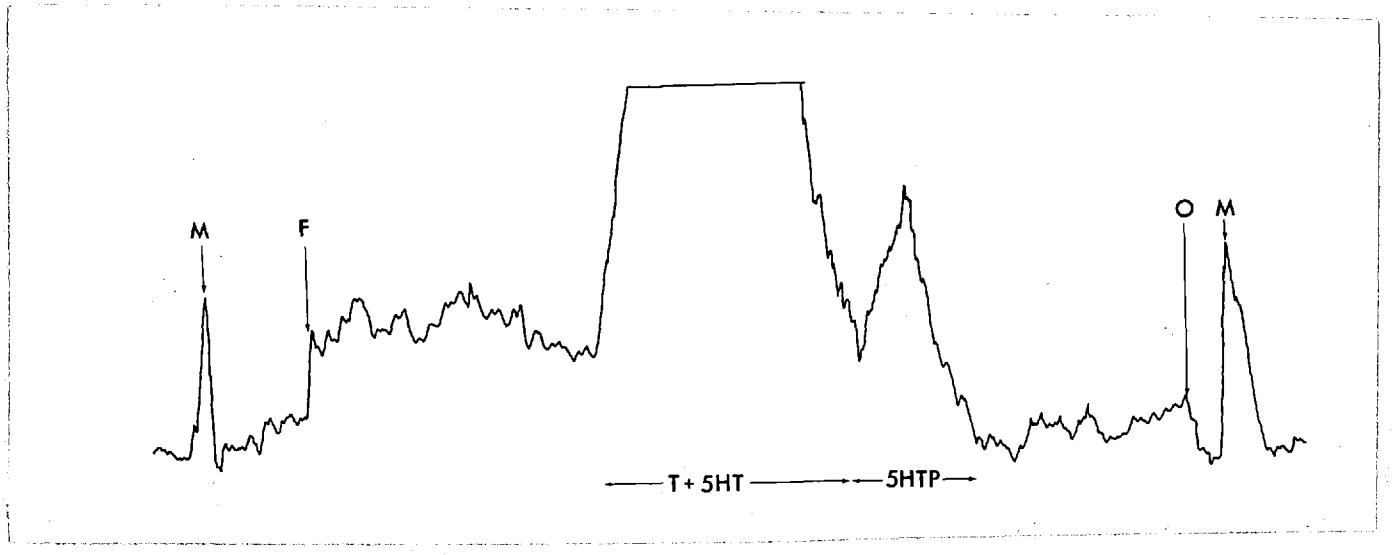


Fig. 6 Expt. 7. Scan of descending paper chromatogram of extract from the incubation of rat liver homogenate with DL-tryptophan-3-C^{14}. (For conditions see text). Note the peak overlying the 5HTP spot. O = origin, F = front, M = radioactive marker. Solvent = n-butanol: acetic acid: water, 120:30:50 v/v.

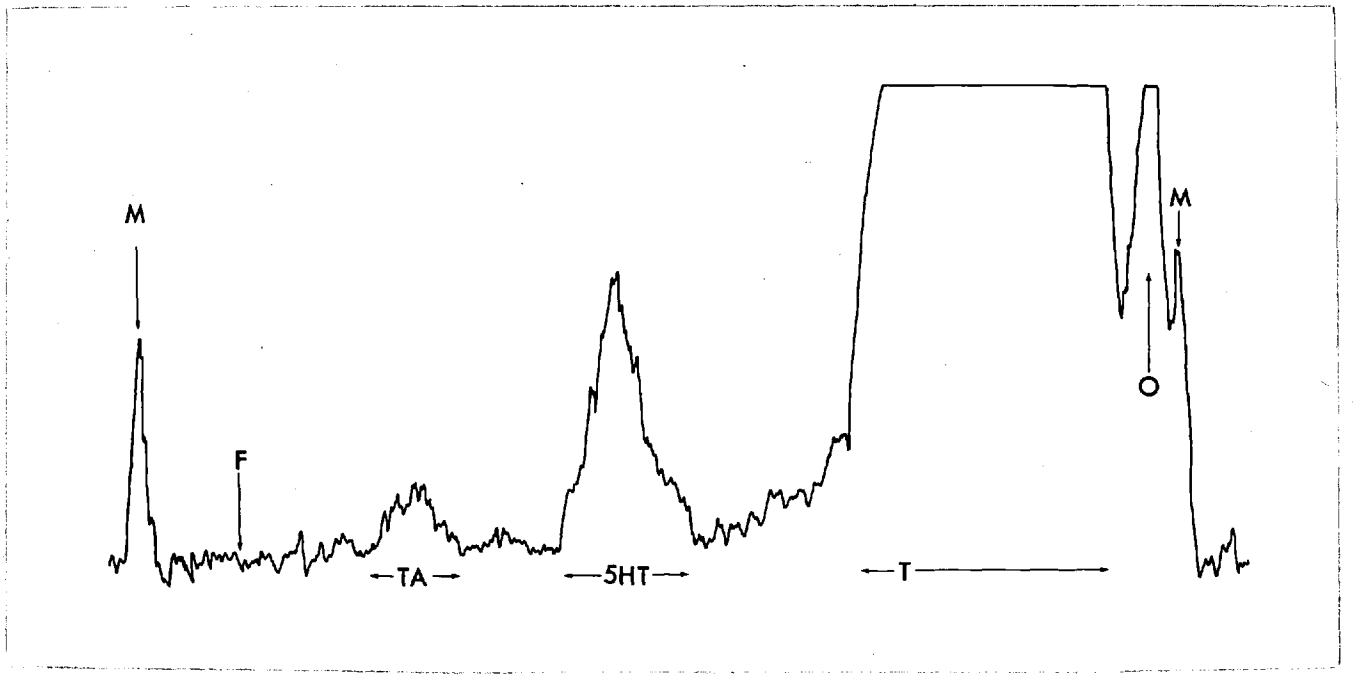


Fig. 7 Expt. 7. Scan of ascending paper chromatogram of extract from the incubation of rat liver homogenate with DL-tryptophan-3-C^{14}. (For conditions see text). Note peaks overlying 5HT, tryptamine (TA), and tryptophan (T). O = origin, M = marker, F = front.

staining characteristics of 5HTP (Figure 6).

(b) Paper chromatography in isopropanol: ammonia: water. On the scan this showed a radioactive peak overlying a spot with the mobility and staining characteristics of 5HT (Note also the small peak coinciding with tryptamine). (Figure 7).

3. No 5-hydroxyindole production was seen in the control samples by either the non-isotopic or isotopic methods.

Comments

These results confirmed, by isotopic means, the results obtained by non-isotopic and showed that the technique could be used at least qualitatively to demonstrate tryptophan 5-hydroxylation.

Expt. 8 THE EFFECT OF α-METHYL-DIHYDROXYPHENYLALANINE (α-METHYL DOPA) ON TRYPTOPHAN 5-HYDROXYLATION BY RAT LIVER

The comparative size and density of the 5HTP and 5HT spots produced in the previous experiments suggested that during the incubation at least 50% of the 5HTP was being decarboxylated to 5HT. It was thought that this decarboxylation could be blocked with α-methyl dopa (Sourkes 1954) but first an experiment was done to see whether this substance would effect tryptophan hydroxylation.

A 1 ml aliquot of a 100,000 g rat liver supernatant (stored overnight at -10°C) was incubated with the following additions:

L-tryptophan	5×10^{-3}M	Nicotinamide	5×10^{-3}M
NAD	5×10^{-4}M	Potassium phosphate pH 7, 0.1 M	
α-methyl dopa	1×10^{-3}M (when added)		

Incubation was carried out for 1 hr in tubes flushed with O_2 and corked at 37°C.

Results

TABLE 4

The effect of α-methyl dopa on tryptophan 5-hydroxylation by rat liver

Conditions	μmoles 5-hydroxyindole produced/hr/ml.supernatant
Without α-methyl dopa	0.038
With α-methyl dopa	0

Comments

Although the hydroxylation seen was quantitatively small this was probably due to the fact that the preparation had been stored. The significant point is however that α-methyl dopa appeared to inhibit the production of 5-hydroxyindoles by rat liver preparations. At the time the full significance of this was not appreciated. In the context of the work as it was progressing, these results meant that α-methyldopa could be used as a decarboxylase inhibitor if it was going to inhibit tryptophan hydroxylation. However Burkard et al (1964) have since confirmed that tryptophan 5-hydroxylation in rat liver is inhibited by α-methyldopa and Lovenberg et al (1965) have shown that α-methyldopa also inhibits a partially purified tryptophan 5-hydroxylase prepared from malignant mouse mast cells.

THE REMOVAL OF 5-HYDROXYINDOLES FROM HIGH-SPEED SUPERNATANT TISSUE PREPARATIONS BY GEL FILTRATION

In discussing the initial negative results using carcinoid tumours one of the points raised was the possible inhibitory effects of endogenous 5-hydroxyindoles upon tryptophan hydroxylation.

Attempts were made to demonstrate whether the addition of 5-hydroxyindoles to rat liver supernatants would inhibit tryptophan

hydroxylation and though these studies suggested that indeed inhibition did occur the technical obstacles to proving the point were so great that the problem was not further investigated.

Since it has now been shown that the tryptophan hydroxylase activity in rat liver preparations was in the high speed supernatant fraction a method was developed for application to carcinoid tumours whereby 5-hydroxyindoles could be quickly removed from supernatant preparations, leaving tryptophan 5-hydroxylase activity intact.

Method

A whole homogenate of tumour 4 was prepared in 0.01 M potassium phosphate buffer pH 7 (3:1, v/w). A 100,000 g supernatant fraction from this was prepared in the same way as described for rat liver. This tumour contained about 150 µg/G of 5HT so that the supernatant fraction could be expected to contain about 40µg of 5HT/cc.

A column of Sephadex G 25 was prepared, 1 cm x 45 cms, and primed with 0.05 M potassium phosphate buffer pH 7. 5 cc of the tumour supernatant were placed on the column, allowed to enter the column and eluted with the same buffer. The fraction emerging (7 ccs) which was visibly opalescent and containing a small amount of haemoglobin was collected and assayed for its 5-hydroxyindole content by the nitroso-naphthol colorimetric procedure after deproteinisation with alcohol. No 5-hydroxyindoles were detected.

The next point investigated was whether the tryptophan 5-hydroxylase activity of rat liver would withstand this treatment.

Expt. 9 THE EFFECT OF GEL FILTRATION UPON THE TRYPTOPHAN 5-HYDROXYLASE ACTIVITY OF RAT LIVER SUPERNATANT

A 100,000 g rat liver supernatant from a 3:1 (v/w) whole homogenate was prepared as already described. 10 ccs of this was placed on a Sephadex G 25 column prepared as described above. On elution with 0.05 M potassium phosphate buffer pH 7 the fraction emerging which visibly contained haemoglobin was collected and its volume was 11 ccs ("Treated Supernatant"). This was compared for its tryptophan 5-hydroxylation activity with the supernatant not passed through the column ("Untreated Supernatant").

Either 1cc of "Untreated" 100,000 g supernatant or "Treated" supernatant was incubated with the following additions:

L tryptophan 5×10^{-3}M; Nicotinamide 5×10^{-3}M

Potassium phosphate buffer pH 7 0.1 M; NAD 5×10^{-4}M

The total volume of all samples was 2 ml. Boiled tissue acted as a control. Incubations were carried out for 1 hr at 37°C in tubes gassed with O_2 and corked. The samples were assayed for total 5-hydroxyindole production as previously described.

Results

TABLE 5

The effect of gel filtration on tryptophan 5-hydroxylation by rat liver

Rat liver supernatant	Activity in µmoles 5-hydroxyindoles produced /hr/ml of preparation	Volume of preparation	Total activity. µmoles 5-hydroxyindoles produced/ hour/total preparation
Untreated	0.144	10 ccs	1.44
Treated	0.075	11 ccs	0.825

It can be seen that the treated material had only 57.5% of the activity of the untreated material. It was thought likely that this was due to the removal of essential cofactors from the supernatant during its passage through the Sephadex and so investigations were done to more clearly define the cofactor requirements of the system.

Expts. 10 and 11 COFACTOR REQUIREMENTS OF RAT LIVER TRYPTOPHAN 5-HYDROXYLASE

The aim of these experiments was to find conditions which would restore the activity of supernatant fractions passed through sephadex. Again the basis for choice of the various additions made to the incubations was previous work carried out on phenylalanine 4-hydroxylase.

100,000 g rat liver supernatants were treated with sephadex G25 as described above. 1 ml aliquots of this material were incubated in a total volume of 2 ml with the addition of L-tryptophan 5×10^{-3}M and potassium phosphate buffer pH 7.0, 1 M. All samples were incubated for 1 hour at 37°C, shaking in oxygen. At the end of the incubation the samples were assayed for total 5-hydroxyindole production by the nitroso-naphthol colorimetric procedure. The various additions made, their final concentrations and the effect they had on the amount of tryptophan hydroxylated in two separate experiments are shown in tables 6 and 7.

Comments

From these studies it was apparent that of the cofactors used the best combination was reduced NAD, reduced NADP, THFA and nicotinamide. This combination appeared to fully restore the activity of 100,000 g rat liver supernatant passed through the sephadex column.

TABLE 6

Cofactor requirements of tryptophan 5-hydroxylase in rat liver

Additions (Final Conc.)	µmoles 5-hydroxyindole produced /hr/cc of treated supernatant	% activity
Reduced NAD 5×10^{-4}M + Reduced NADP 5×10^{-4}M) THFA 2×10^{-3}M + Nicotinamide 5×10^{-3}M)	0.19	100
Reduced NAD 5×10^{-4}M + THFA 2×10^{-3}M	0.169	89
Reduced NAD 1×10^{-3}M	0.122	64
Reduced NAD 5×10^{-4}M) Nicotinamide 5×10^{-3}M)	0.118	62
Reduced NAD 2.5×10^{-4}M	0.118	62
Reduced NAD 5×10^{-4}M	0.111	59
Reduced NADP 1×10^{-3}M	0.1	53
Reduced NADP 5×10^{-4}M	0.0995	52
Reduced NADP 2.5×10^{-4}M	0.0965	51
Reduced NADP 5×10^{-4}M) THFA 2×10^{-3}M)	0.095	50
Reduced NADP 5×10^{-4}M) Nicotinamide 5×10^{-3}M)	0.089	47

TABLE 7

NAD 5×10^{-4}M + ReducedNAD 5×10^{-4}M) NADP 5×10^{-4}M + Reduced NADP 5×10^{-4}M) THFA 2×10^{-3}M + Nicotinamide 5×10^{-3})	0.096	100
Reduced NADP 5×10^{-4}M + THFA 2×10^{-3}M	0.065	68
Reduced NAD 5×10^{-4}M	0.059	61
Reduced NADP 5×10^{-4}M	0.046	48
NAD 5×10^{-4}M	0.045	47
NADP 5×10^{-4}M	0.0296	31
THFA 2×10^{-3}M	0.0185	19
Nicotinamide 5×10^{-3}M	0.0185	19
No additions	0.0133	14

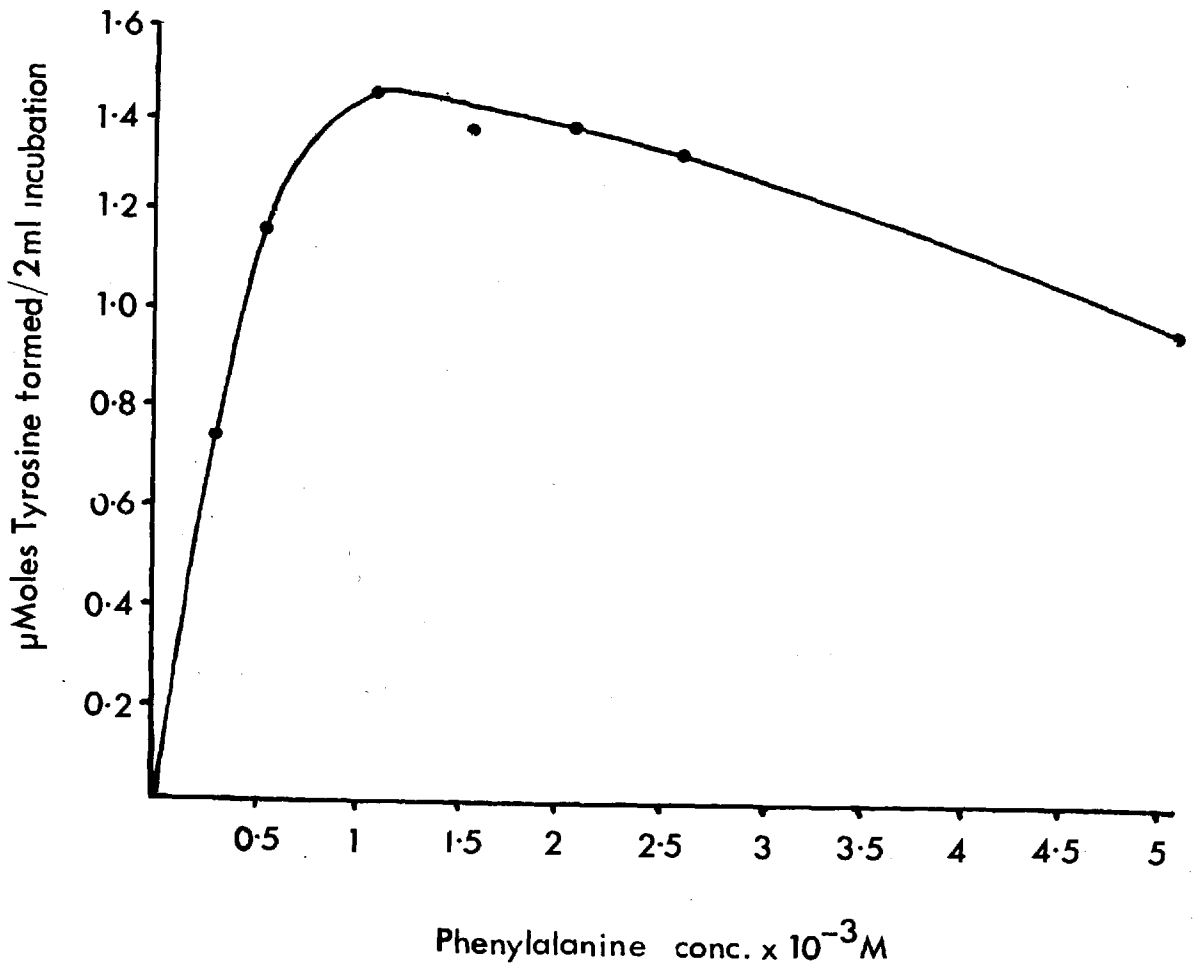


Fig. 8 The effect of increasing L-phenylalanine concentration upon tyrosine production by rat liver supernatant. For conditions see text.

STUDIES COMPARING PHENYLALANINE 4-HYDROXYLASE AND TRYPTOPHAN 5-HYDROXYLASE IN RAT LIVER

Freedland et al (1961b) followed up their work on the demonstration of tryptophan 5 hydroxylation in rat liver by showing that phenylalanine, tyrosine, phenyllactic acid and phenylpyruvic acid inhibit tryptophan hydroxylation by rat liver preparations and that their preparation of rat liver, now a 135,000 g supernatant fraction, also 4-hydroxylated phenylalanine. Some of these points were confirmed in the following experiments.

Expt. 12 THE DEMONSTRATION OF THE 4-HYDROXYLATION OF PHENYLALANINE BY RAT LIVER PREPARATIONS

Method

The assay of tyrosine was carried out according to the procedure described by Udenfriend and Cooper (1952). The enzyme preparation used was a 100,000 g rat liver supernatant fraction prepared and passed through the sephadex G 25 column as described. Incubations were carried out at 37°C for 1 hour in tubes gassed with O_2, corked and shaken.

All incubation samples contained reduced NAD 5×10^{-4}M, THFA 2×10^{-3}M, nicotinamide 5×10^{-3}M, and potassium phosphate buffer pH 7, 0.1 M, and 1 ml of sephadex treated rat liver supernatant. Total vol, 2 ml. Various amounts of L-phenylalanine were added and after incubation at 37°C for 1 hr in tubes gassed with O_2 the tyrosine content was assayed. The amounts of phenylalanine added and the corresponding amount of tyrosine produced can be seen in the graphical representation of the results in figure 8. Obviously the phenylalanine 4-hydroxylase in these preparations was very active, much more active than the tryptophan 5-hydroxylase activity.

Expt. 13 THE INHIBITION OF RAT LIVER TRYPTOPHAN 5-HYDROXYLASE BY PHENYLALANINE

Sephadex treated 100,000 g rat liver supernatant, 1 ml, was incubated for 1 hour at 37°C in O_2. Reduced NAD 5 x 10^{-4}M, THFA 2 x 10^{-3}M, nicotinamide 5 x 10^{-3}M, L tryptophan 5 x 10^{-3}M, and potassium phosphate buffer pH 7, 0.1 M, were added to give a total volume of 2 ml. L phenylalanine 1 µmole (5 x 10^{-4}M) was added to one tube. After incubation the total 5-hydroxyindole content of the samples was assayed colorimetrically.

Results

TABLE 8

The inhibition of tryptophan 5-hydroxylation in rat liver by phenylalanine

Sample	µmoles 5-hydroxyindoles produced/hour	Activity
- L-phenylalanine	0.089	100%
+ L-phenylalanine (5 x 10^{-4}M)	0.0332	38%

Comments

It is evident that phenylalanineis a potent inhibitor of tryptophan hydroxylation by rat liver preparations as suggested by Freedland et al (1961b). The relationship between phenylalanine 4-hydroxylation and tryptophan 5-hydroxylation in rat liver will be discussed later but at this stage it did seem rather strange that,if carcinoid tumours hydroxylated tryptophan by the same mechanism, increased tyrosine production had not been observed in the many biochemical studies on the carcinoid syndrome reported in the literature (Levine and Sjoerdsma 1963).

Expt. 14 CONFIRMATION OF PHENYLALANINE 4-HYDROXYLATION IN RAT LIVER BY ISOTOPIC METHODS

Because of the association between phenylalanine and tryptophan hydroxylation by rat liver it was obviously going to be necessary to investigate the capacity of carcinoid tumours to hydroxylate phenylalanine. As such activity was likely to be small, if present at all, methods were developed to demonstrate the formation of tyrosine-2-C^{14} from phenylalanine-2-C^{14}. If carcinoid tumours did hydroxylate tryptophan by the same mechanism as rat liver then there was a much greater chance of demonstrating phenylalanine 4-hydroxylation than tryptophan 5-hydroxylation. If however phenylalanine hydroxylation could not be demonstrated by sensitive isotopic methods then either an enzyme like that in rat liver had deteriorated to such an extent that it would certainly not be possible to demonstrate the much less active tryptophan 5-hydroxylation or tryptophan 5-hydroxylation in carcinoid tumours was catalysed by a different enzyme.

Method

At the end of the incubations, deproteinisation with ethanol was performed. The dried ethanol extract was dissolved in a little water and an aliquot subjected to descending paper chromatography in the n-butanol: acetic acid: water solvent already described on page After development overnight the chromatogram was dried and scanned for radioactivity and then stained with the nitrosonaphthol chromatographic stain described by Smith (1960).

1 cc of a 100,000 g rat liver supernatant treated with sephadex was incubated with the following additions:

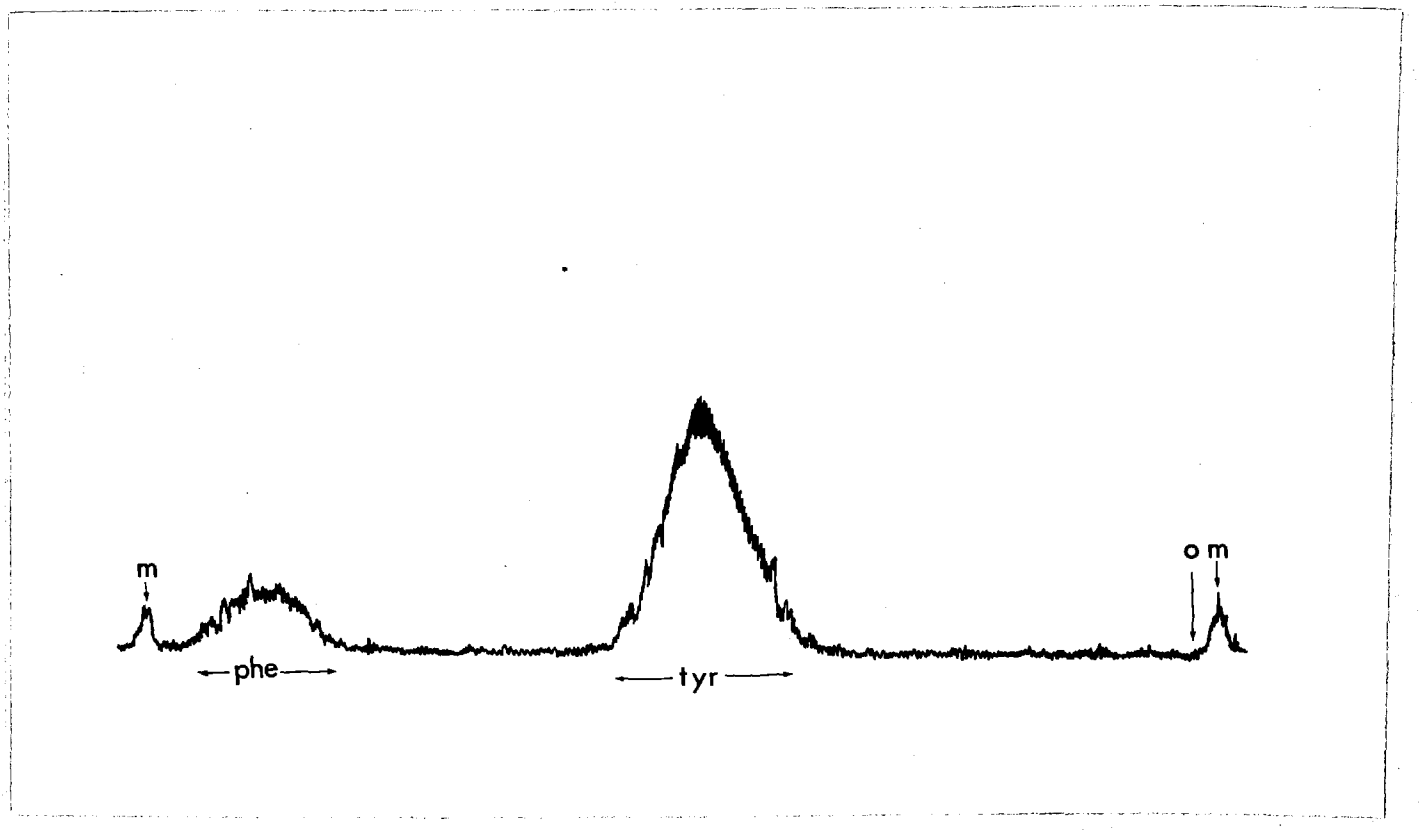


Fig. 9 Radioactive scan of descending paper chromatogram of extract from the incubation of rat liver supernatant with DL-phenylalanine-3-C^{14}. Note the radioactivity associated with tyrosine (tyr) and remaining phenylalanine (phe). O = origin, M = marker. Solvent - n-butanol: acetic acid: water, (120:30:50, v/v)

L-phenylalanine-2-C^{14} (specific activity 10 μc/μmole) 5×10^{-5}M

Reduced NAD (5×10^{-4}M) Reduced NADP (5×10^{-4}M)

THFA (2×10^{-3}M) Nicotinamide (5×10^{-3}M)

Potassium phosphate buffer, pH 7, 0.1 M.

Incubations were carried out for 1 hr at 37°C in an atmosphere of O_2. In the control sample boiled tissue was used.

Results

The result of scanning the chromatogram produced can be seen in figure 9. The large radioactive peak coincided with a spot having the characteristic staining properties of tyrosine. The small peak has the correct R_f value for phenylalanine.

Summary of studies on tryptophan 5-hydroxylation in rat liver

1. The enzymatic 5-hydroxylation of tryptophan by rat liver preparations in vitro had been confirmed.
2. 5-hydroxytryptophan had been shown to be the intermediate in the biosynthesis of 5HT.
3. The tryptophan 5-hydroxylase activity lay in the 100,000 g supernatant fraction of rat liver.
4. This fraction could be freed of 5-hydroxyindoles and its tryptophan 5-hydroxylase activity fully restored by the addition of certain cofactors.
5. The same rat liver preparation had marked phenylalanine 4-hydroxylase activity and phenylalanine inhibited tryptophan 5-hydroxylation by rat liver
6. The tryptophan 5-hydroxylase in rat liver was stable enough to allow fairly extensive manipulations in the cold, but storage at -10°C to -15°C led to marked loss of activity.

C. Expt 15 THE EXAMINATION OF CARCINOID TUMOUR TISSUE FOR TRYPTOPHAN 5-HYDROXYLASE ACTIVITY SIMILAR TO THAT FOUND IN RAT LIVER

Description of Experiments and Results

Using the information and technical knowledge gained from the study of tryptophan 5-hydroxylation in rat liver, attempts were made to demonstrate similar activity in carcinoid tumour tissue. Tumour 6, metastases from a liver removed at an autopsy carried out within one hour of death was prepared and studied within 4 hrs of its collection, all preparations being carried out at 0°-4°C.

Whole homogenates, 20,000 g supernatant and particulate fractions, and a 100,000 g supernatant fraction were prepared as described for rat liver. In addition an aliquot of the 100,000 g supernatant was rendered free of 5-hydroxyindoles by gel filtration using sephadex G 25. These preparations were incubated with DL-tryptophan-3-C^{14} under those conditions shown to be optimal for tryptophan 5-hydroxylation in rat liver. At the end of the incubations, "Carrier" 5HTP, 5HT and 5HIAA were added and the deproteinised extracts extensively analysed by high voltage paper electrophoresis and paper chromatography scanning the paper strips for radioactivity.

No radioactivity could be found in association with any of the "Carrier" 5-hydroxyindoles despite good recovery of the latter. In addition the supernatant rendered free of 5-hydroxyindoles possessed no tryptophan or phenylalanine hydroxylase activity; using non-radioactive L-tryptophan and L-phenylalanine as substrates and colorimetric assays for 5-hydroxyindoles and tyrosine. The same negative results were obtained in studies on tumours 4 and 5.

Conclusions

One was forced to conclude that if the 5-hydroxylation of tryptophan did take place in carcinoid tumours (and I must say that I began to have doubts about this) then the reaction was different probably qualitatively and quantitatively from that occurring in rat liver.

D. TRYPTOPHAN 5-HYDROXYLATION IN CARCINOID TUMOUR SLICES

Reappraisal of the problem

1. I decided to study the hydroxylation of tryptophan in slices of carcinoid tumour where, it was hoped, a minimum of disruption of intracellular organisation would be caused and perhaps no dissipation of, as yet unknown, cofactors would result.

2. A method needed to be developed in which any radioactive 5HTP formed from DL-tryptophan-3-C^{14} during incubation would remain in the incubation as 5HTP and not be decarboxylated to 5HT thereby dissipating the radioactivity throughout the 5-hydroxyindole pathway. In this way there would be the maximum opportunity of picking up the radioactivity associated with one 5-hydroxyindole. Some way of blocking decarboxylation without blocking hydroxylation was needed.

3. Lastly the analytical procedures needed to be made qualitatively more precise and quantitatively more sensitive. Here follows an account of how the methodological difficulties were overcome.

Methods

(a) Inhibition of 5HTP decarboxylation

In 1962 Drain et al reported that 3-hydroxybenzyloxyamine (NSD 1024) was a potent inhibitor of 5-HTP decarboxylation both in vivo and

in vitro. The effectiveness of this compound in inhibiting the decarboxylation of 5HTP in carcinoid tumours was investigated comparing it with α-methyl dopa.

A 100,000 g supernatant fraction of tumour 5 was treated with sephadex G 25 to remove 5-hydroxyindoles as previously described. 1 ml of this "treated" tumour supernatant was incubated for 1 hr in a total volume of 2 ml at 37°C in air, gently shaking. Boiled tissue acted as a control. To all tubes the following additions were made (shown as final concentration):-

DL-5HTP 2.5×10^{-3}M Pyridoxal phosphate 4×10^{-5}M

Potassium phosphate buffer pH 7.5, 0.1 M

The decarboxylation of 5HTP was separately studied (i) Without the addition of a decarboxylase inhibitor; (ii) In the presence of α-methyldopa 1×10^{-3}M and (iii) with the addition of NSD 1024 2.5×10^{-3}M.

At the end of the incubation, ethanol deproteinisation and extraction of the 5-hydroxyindoles was performed and from the ethanol extract the 5HT was extracted with butanol as already described on p. 57. However the butanol was twice washed with borate buffer pH 10 to ensure the removal of 5HTP (Udenfriend et al 1958b). After the 5HT had been returned to 0.1 N HCl it was assayed by the nitrosonaphthol colorimetric procedure

Results

TABLE 9

The Effect of α methyldopa and NSD 1024 on 5HTP decarboxylation by carcinoid tumour tissue

Conditions	% inhibition of 5HTP decarboxylation
No decarboxylase inhibitor	0
α-methyldopa 1×10^{-3}M	86
NSD 1024 2.5×10^{-3}M	100

As α methyl dopa was not as efficient as NSD 1024 as a decarboxylase inhibitor and as it had also been shown to inhibit tryptophan hydroxylation by rat liver, NSD 1024 was chosen as a decarboxylase inhibitor.

(b) Assay of tryptophan 5-hydroxylation in carcinoid tumours

Assay method I and II

1. All incubations were carried out at 37°C in flasks (for slices) or tubes (for whole homogenates and subcellular fractions) flushed with O_2 and corked, unless otherwise indicated. DL-tryptophan-3-C^{14} was added as the substrate and NSD 1024 to inhibit the decarboxylation of 5HTP.

2. At the end of incubation 2 or 4 µmoles of DL 5HTP were added and the slices homogenised in the incubation medium.

3. Samples were deproteinised by the addition of perchloric acid (see p. 19).

4. The 5HTP in the protein free extract was absorbed onto and eluted from charcoal deactivated with 4% stearic acid (see p. 19).

5. The final dried and phenol-free eluate from the charcoal was dissolved in 0.1 ml of water all of which was subjected to two-way ascending paper chromatography.

(i) Two-way ascending paper chromatography 10" x 10" squares of Whatman 3 mm chromatographic paper were held in a Universal Frame (Shandon) and two-way paper chromatography performed (Jepson 1960) with the following solvents: Solvent 1 - isopropanol: 880 ammonia: water (200: 10; 20 v/v). This was the first solvent used and the chromatogram was developed overnight. After drying the frame was turned over and

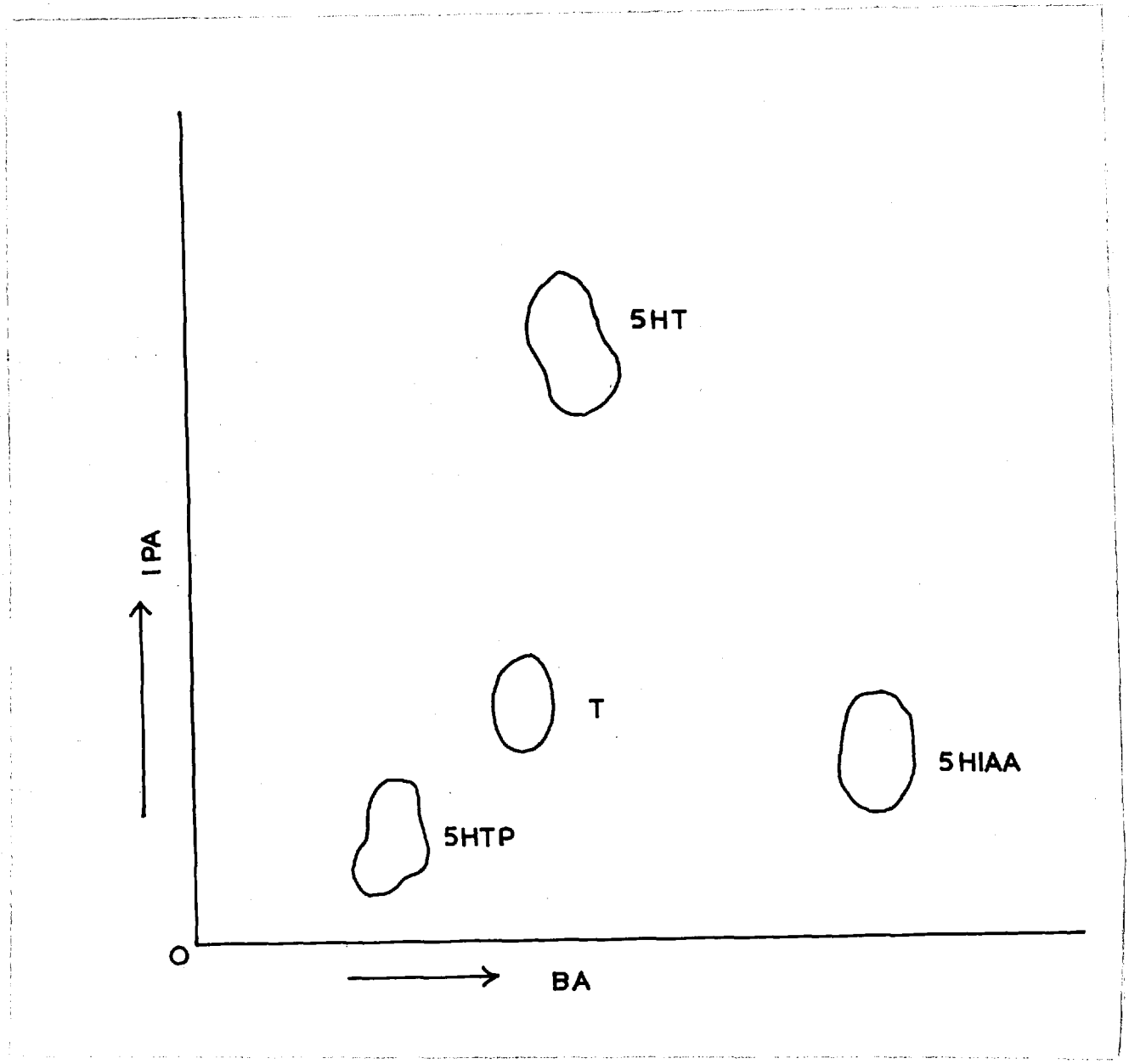


Fig. 10 Tracing of two way ascending paper chromatogram showing the separation and position of tryptophan (T), 5HT, 5HTP and 5HIAA.
IPA = Isopropanol: ammonia: water (200:10:20, v/v)
BA = n-butanol: acetic acid: water (120:30:50, v/v)

chromatographed in solvent 2 (n butanol: glacial acetic acid: water [120: 30: 50 v/v]). This development either took place the next day for 7-8 hours or over the next night for 14 hours. The chromatograms were all dried in a hot air oven the temperature of which was not allowed to exceed 50°C.

(ii) Position of relevant substances on two-way paper chromatography Because solvents were allowed to "run over the top", precise R_f values have little meaning. However the relative positions of tryptophan, 5HTP, 5HT and 5HIAA on these chromatograms is extremely reliable. As the carrier 5HTP and the 5HT produced from it by decarboxylation fluoresce a lightish blue under a Wood's lamp this also aided location. A standard chromatogram of tryptophan, 5HTP, 5HT, 5HIAA and sometimes tryptamine was always run in the same frame as the experimental samples and after drying stained with Ehrlich's reagent. The "unknown" chromatograms were compared with these standards. An example of a typical standard chromatogram is shown in figure 10.

6. Radioautography of paper chromatograms This was carried out by placing the chromatogram up against X-ray film (Ilflex or Kodirex), in a special folder and leaving in the dark for 4-7 days. The X-ray film was then developed and fixed.

7. Elution of 5HTP from the first paper chromatogram After the position of the 5HTP was located and the initial radioautogram had been compared with the chromatogram, the 5HTP area was cut out, chopped up with scissors and the paper mixed with 1.5 ml x 3 volumes of water. The resulting pulp was separated by centrifugation and the supernatant fluid evaporated to dryness in a vacuum dessicator.

When H.V. paper electrophoresis was performed the dried 5HTP eluate was dissolved in 0.1 ml of water and the whole amount spotted.

If enzymatic decarboxylation was to follow, then it was dissolved in 0.4 ml of water.

8. High voltage paper electrophoresis This was performed in initial experiments to ensure as far as possible the identification of 5HTP. It was carried out on the 5HTP eluate from the initial chromatogram at pH 12 in the way described on p. 21. After the strip had been dried it was scanned for radioactivity and the 5HTP located by its position by comparison with a standard 5HTP strip run alongside and by the fluorescence of the 5HTP. The 5HTP was then eluted from this strip in the same way as it was eluted from the first chromatogram and evaporated to dryness in the same way. It was then dissolved in 0.4 ml of water ready for decarboxylation.

9. Enzymatic decarboxylation of 5HTP It might be thought that two way paper chromatography followed by high-voltage paper electrophoresis was a sufficient identification of 5HTP. Nevertheless, if one could demonstrate that, on incubation in a 5HTP decarboxylase system, the radioactivity which first appeared to be associated with 5HTP now appeared to be associated with 5HT, the case for identification would be virtually proved.

In addition to this point however is a technical one. High voltage electrophoresis at pH 12 is not very reliable and if one run did not work then the results of the previous week's work was wasted. However the enzymatic decarboxylation with subsequent chromatography

Fig. 11 Radioautogram of a two way ascending paper chromatogram of "Pure" DL-tryptophan-3-C^{14}. Note that there is some ill-defined radioactivity in the area of 5HTP.

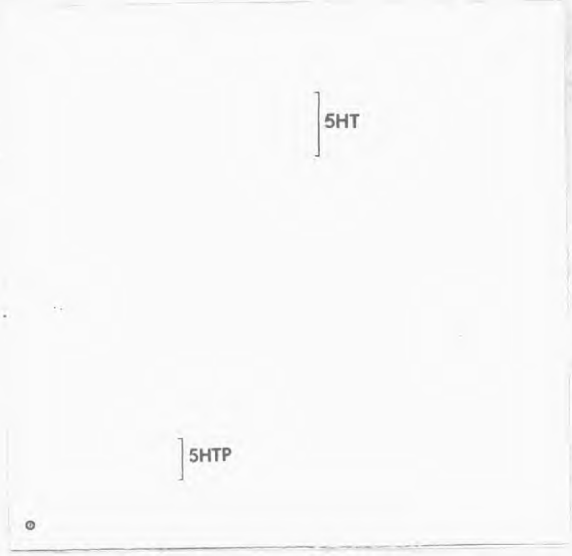


Fig. 12 Radioautogram of a two way ascending paper chromatogram of material eluted from the 5HTP area on the chromatogram depicted in Fig. 11, which has been subjected to enzymatic decarboxylation. Note there is no radioactivity in the area of 5HT.

proved to be very reliable and also very necessary if electrophoresis was not carried out. The reason for this is as follows.

The DL-tryptophan-3-C^{14} used in these experiments proved to be rather unstable in this two-solvent chromatographic system. Radioautograms of "pure" DL tryptophan-3-C^{14} showed many radioactive smudges one or more of which might be contaminating in the 5HTP area (see figure 11). However subjecting these areas to decarboxylation and subsequent chromatograph for 5HT no radioactivity was seen anywhere near the 5HT area (see figure 12). Introduction of decarboxylation therefore rid the system of these contaminating artefacts and allowed precise assay of the hydroxylation, by assay of the 5HT.

Fortunately it was found that the R_f of the NSD 1024 in the butanol solvent was 80 compared with 27 for the 5HTP. In this way the decarboxylase inhibitor was removed from the 5HTP prior to decarboxylation.

10. Standard method of decarboxylation of 5HTP The 5HTP eluate from the initial chromatogram or from the high voltage electrophoresis was enzymatically decarboxylated by a stereospecific L-aromatic amino acid decarboxylase prepared from guinea pig kidneys and partially purified by ammonium sulphate precipitation to stage 3 in the preparation described by Clark et al (1954). All preparations were tested for their activity at first by the methods of Clark et al (1954) and later by the method of Lovenberg et al (1962). All preparations when tested were capable of decarboxylating 0.75 µmoles of L 5HTP/hour/0.2 ml enzyme solution. This was usually very adequate to deal with the amount of 5HTP recovered from chromatograms or electrophoretograms. The decarboxylase was stable at

-10°C for at least two months. The incubation conditions employed were as follows:

5HTP eluate 0.4 ml (from chromatogram or electrophoretogram)

Decarboxylase 0.2 ml Pyridoxal phosphate 4×10^{-5}M

Potassium phosphate buffer pH 7.5, 0.05 M

Iproniazid 5.5×10^{-3}M Total volume 0.8 ml.

The system was preincubated at 37°C for 5 minutes, prior to the addition of the 5HTP eluate to inhibit any monoamine oxidase present. Then the sample was incubated in air for 45 minutes at 37°C being gently shaken. The sample was deproteinised by the addition of 4 vols of 95% ethanol and the clear supernatant, after centrifugation, decanted and evaporated to dryness in a vacuum dessicator. The dried extract was dissolved in 0.1 ml of water and all of it subjected to two-way paper ascending chromatography in the solvents already described. Radioautograms of the chromatograms were prepared.

11. Location and assay of 5HT The 5HT in initial qualitative experiments was identified by staining the dry chromatogram with Ehrlich's reagent. In quantitative experiments however the 5HT "spot" was located by its position as compared with a standard chromatogram and by its fluorescence. The spot was cut out and the 5HT eluted with water as described previously for 5HTP. This eluate was dried in a vacuum dessicator.

Initially this dried 5HT eluate was assayed for the amount of 5HT present by dissolving it in 1 ml of water and taking aliquots for fluorescent assay, after suitable dilution, in 3 N HCl (Udenfriend et al 1958b). In later experiments however it was found more convenient

to dissolve the 5HT extract in 0.5 ml of water and assay a 0.25 ml aliquot by the nitrosonaphthol colorimetric procedure (Udenfriend et al 1955), modifying the volume relationships of the reagents used proportionately.

The radioactivity of the 5HT eluate was assayed by counting known aliquots dried on aluminium planchettes to "infinite thinness" in a gas flow counter (Nuclear Chicago, Model D 47), the efficiency of which was 30%. Knowing the amount of DL 5HTP added as carrier and the amount and specific activity of the 5HT finally recovered it is possible to calculate the amount of 5HTP formed from the substrate tryptophan.

Figure 13 shows the scheme of Assay, Method I. When high voltage paper electrophoresis was omitted the procedure will be referred to as Assay Method II.

Expt. 16 QUALITATIVE DEMONSTRATION OF TRYPTOPHAN 5-HYDROXYLASE IN SLICES OF CARCINOID TUMOUR

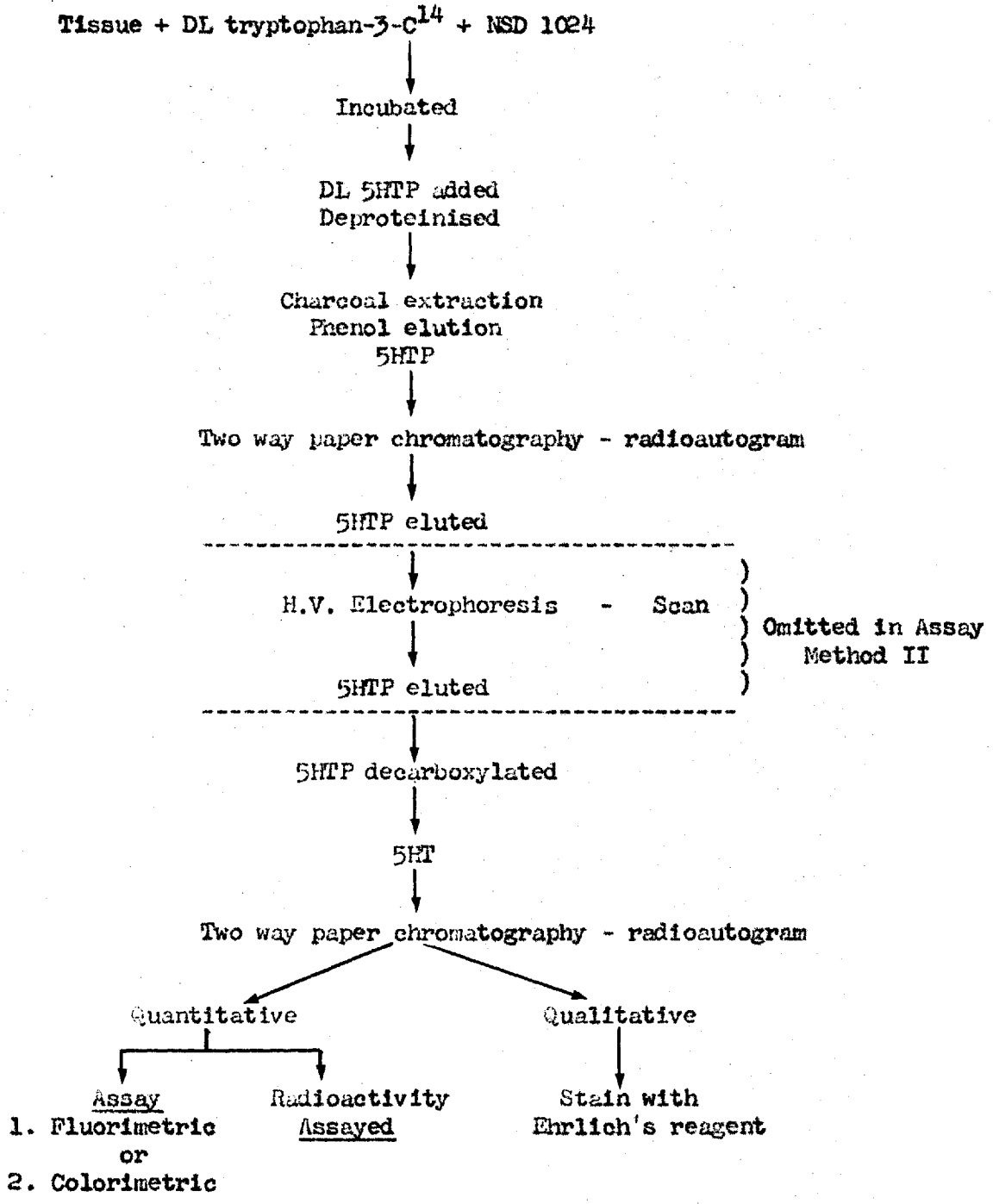
Slices were prepared (Elliott 1955) from hepatic metastases of tumour 6 (see appendix) macroscopically distinct from liver tissue and these were incubated under the following conditions:

Slices of tumour, 2.4 G wet wt. Ringer phosphate solution pH 7.4, 5ml
DL-tryptophan-3-C^{14} (specific activity 32 μc/μmole) 308 mμmoles added
NSD 1024 2.5×10^{-3}M Total volume of incubation 5.8 ml.

2.4 G of boiled tumour slices were identically incubated as a control. Incubation was carried out for 1 hour with shaking at 37°C in 50 ml flasks flushed with O_2 and corked. At the end of the incubation 4 μmoles of DL-5HTP were added as carrier. Assay method I was then carried out except that the final 5HT chromatogram was stained with Ehrlich's reagent instead of quantitatively assaying the 5HT.

Figure 13

Scheme: ASSAY METHODS I & II



Results

(Figures 14 to 19)

1. The radioautograms of the initial "5HTP" chromatogram show virtually no 5HTP in the control but marked radioactivity in the 5HTP area in the experimental sample (figs 14 and 15).

2. The scan of the electrophoretic strip showed radioactivity associated with the 5HTP spot in the experimental sample but not in the control (figs. 16 and 17).

3. The final radioautograph of the chromatogram of the decarboxylated 5HT extract shows radioactivity in the area of 5HT in the experimental but not in the control sample whereas in both "Carrier" 5HT stained with equal intensity indicating roughly equal recovery (figs. 18 and 19).

4. Also it can be seen that little radioactivity is present in the 5HTP area on the final chromatogram whereas in this area 5HTP stained quite densely with Ehrlich's reagent (fig. 19).

The carrier added at the end of incubation was DL-5HTP. On decarboxylation one may assume that all the L-5HTP was decarboxylated by the L-specific decarboxylase to 5HT which was radioactive, whereas the D-5HTP remaining was non-radioactive. This was strongly suggestive that the hydroxylation had been specific for the L-isomer of tryptophan.

However it is known that non-specific non-enzymatic hydroxylation of a number of aromatic compounds occurs in the presence of O_2, ascorbic acid, Fe^{2+} and EDTA (Udenfriend 1954) and tryptophan is hydroxylated to 5HTP by this system (Dalgleish 1955).

Though such a system seems unphysiological it nevertheless seemed important to exclude its participation here. If, as seemed

Fig. 14 Expt. 16 (Control) Radioautogram of two-way ascending paper chromatogram of extract from the incubation of BOILED tumour slices with DL-tryptophan-3-C^{14}. Note there is little radioactivity in the area of 5HTP.

Fig. 15 Expt. 16 (Experimental) Radioautogram of two way ascending paper chromatogram of extract from the incubation of tumour slices with DL-tryptophan-3-C^{14}. Note the radioactivity in the area of 5HTP.

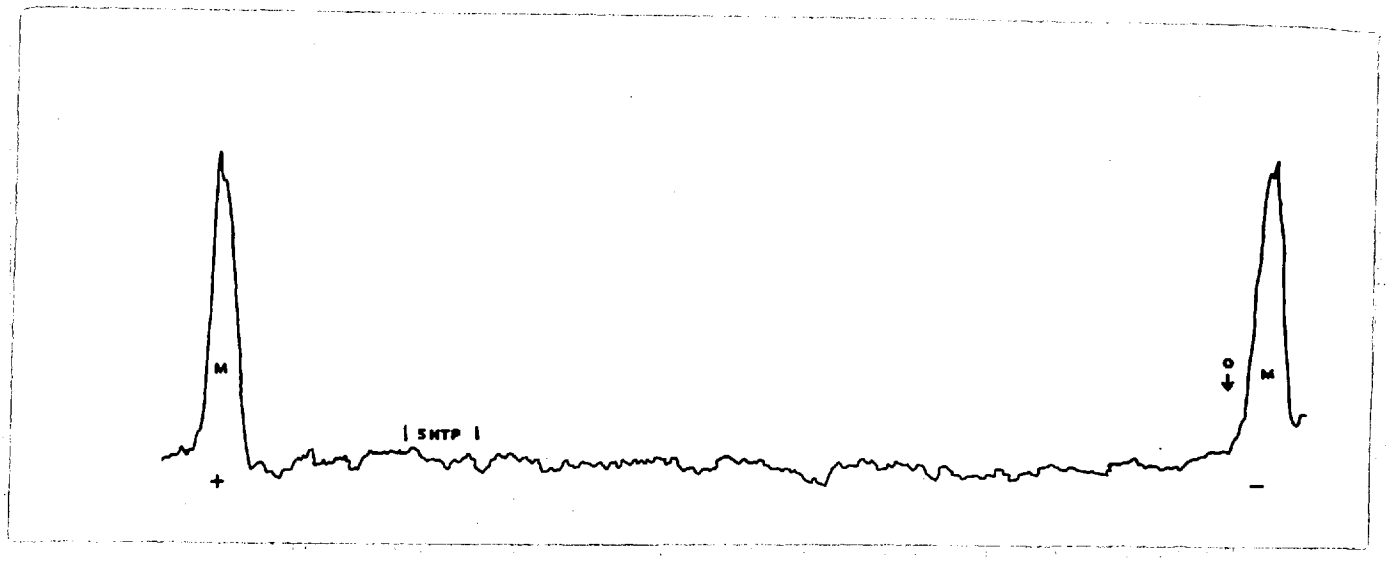


Fig. 16 Expt. 16 (Control) Radioactive scan of high voltage paper electrophoretic strip (pH 12) of eluate from 5HTP area on the chromatogram from which the radioautogram scan in Fig. 14 was prepared (i.e. Control incubation). Note there is no radioactivity associated with the 5HTP.

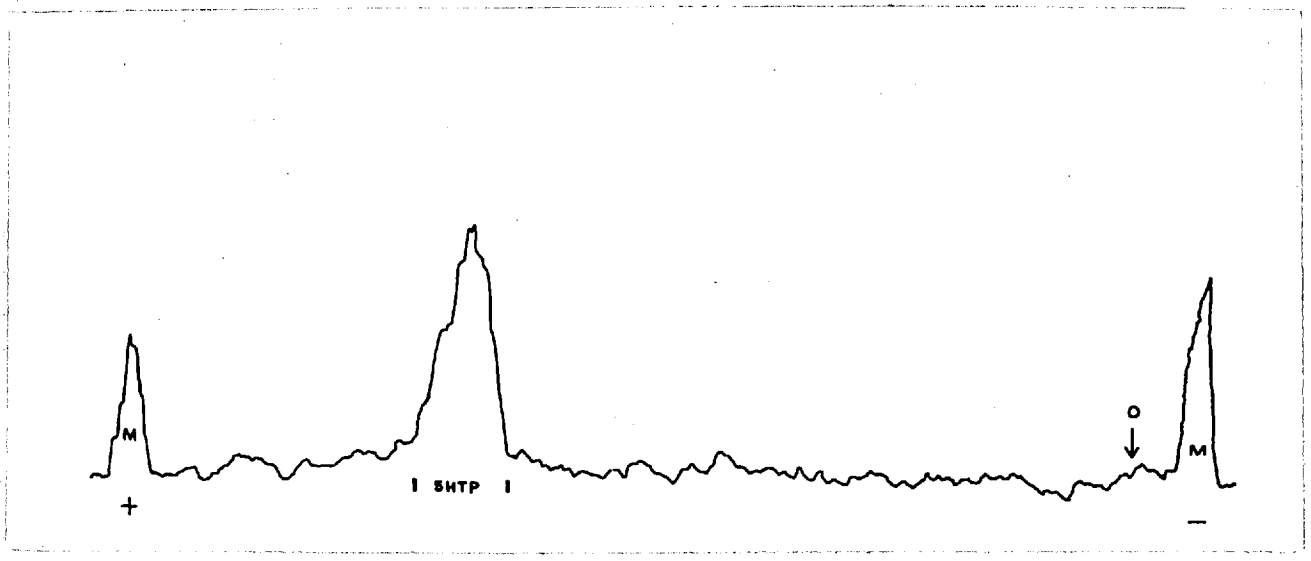


Fig. 17 Expt. 16 (Experimental) Radioactive scan of high voltage paper electrophoretic strip (pH 12) of eluate from 5HTP area on the chromatogram from which the radioautogram scan in Fig. 15 was prepared (i.e. Expt. incubation). Note the radioactivity associated with 5HTP.

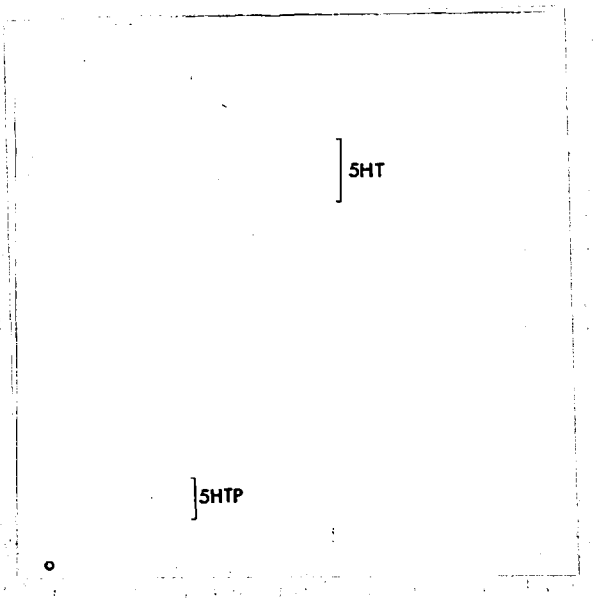


Fig. 18 Expt. 16 (Control) Radioautogram of a two way ascending paper chromatogram enzymatically decarboxylated 5HTP eluted from the strip depicted in fig. 16. Note there is no radioactivity in the area of 5HT.

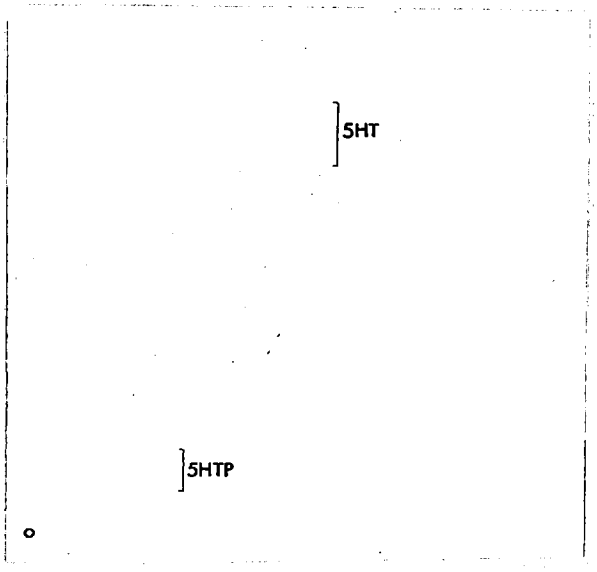


Fig. 19 Expt. 16 (Experimental) Radioautogram of a two way ascending paper chromatogram of enzymatically decarboxylated 5HTP eluted from the strip depicted in Fig. 17. Note the radioactivity associated with 5HT, but that very little radioactivity is associated with the remaining D 5HTP.

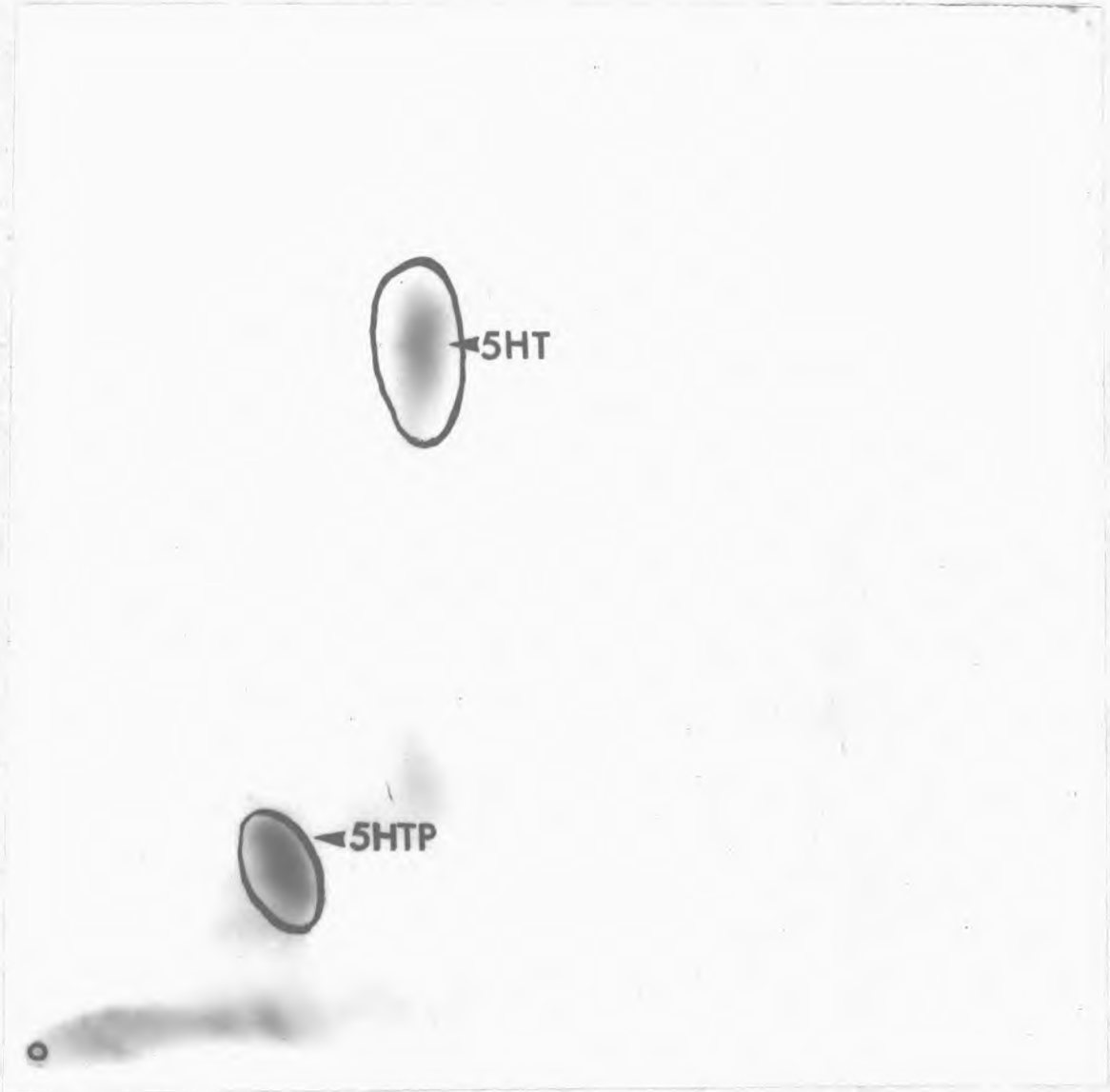


Fig. 20 Expt. 17 Radioautogram of a two way ascending paper chromatogram of enzymatically decarboxylated 5HTP prepared from DL-tryptophan-3-C^{14} by non-enzymatic hydroxylation. Note the roughly equal density of the 5HT and D 5HTP areas.

likely, such non-enzymatic hydroxylation could be shown not to be stereospecific then it could be distinguished from the hydroxylation seen with carcinoid tumours.

Expt. 17 THE NON-ENZYMATIC, NON-STEREOSPECIFIC 5-HYDROXYLATION OF TRYPTOPHAN

The incubation conditions were as follows:

DL-tryptophan-3-C^{14} (specific activity 32 μc/μmole) 308 μmoles added

Potassium phosphate buffer (pH 7.4) 0.04 M

$FeSO_4$ 6×10^{-3}M EDTA 4×10^{-2}M

Ascorbic acid Total volume, 2.5 ml.

Incubation was carried out in tubes flushed with O_2, corked and shaken for 1 hour at 37°C. At the end of incubation 4 μmoles of DL-5HTP were added as carrier and then the sample carried through assay method I except that again the 5HT on the final chromatogram was stained with Ehrlich's reagent instead of being quantitatively assayed.

Results

Figure 20 shows the radioautogram of this final chromatogram. Several spots are present but attention should be focussed upon the 5HTP and 5HT spots. One can see that there is roughly an equal amount of radioactivity in the 5HTP area and 5HT area and on staining the amount of 5HTP and 5HT in these two areas was approximately equal. This suggests that both the D and L isomers of tryptophan had been non-enzymatically hydroxylated and that only half of the recovered 5HTP, the L isomer, had been decarboxylated to 5HT, the rest of the radioactivity remaining with the D-5HTP. This contrasts with apparent stereospecificity of the tumour hydroxylation for the L isomer of tryptophan and very strongly suggests its enzymatic nature.

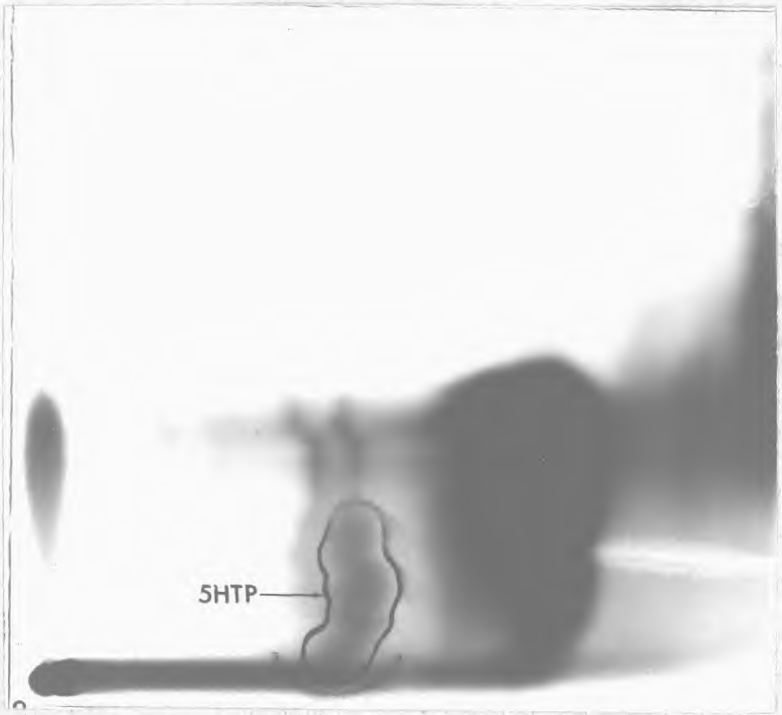


Fig. 21 Expt. 18. Radioautogram of two way paper chromatogram of extract from the incubation of DL-tryptophan-3-C^{14} with carcinoid tumour slices. (For conditions see text). Note the radioactivity associated with 5HTP.

Fig. 22. Expt. 18 Scan of one way ascending chromatogram of the extract from the enzymatic decarboxylation of 5HTP eluted from the chromatogram represented in Fig. 21. Note the radioactivity associated with 5HT and absence of radioactivity in the area of remaining D 5HTP (5HTP). O = origin, f = front, m = markers.
Solvent - isopropanol: ammonia: water.

Expt. 18 QUANTITATIVE ESTIMATION OF TRYPTOPHAN 5-HYDROXYLATION BY CARCINOID TUMOUR SLICES (7)

The preceding results were so clear cut and the amount of radioactivity residing in the final 5HT spot looked large enough to be easily assayed. The following experiment was therefore performed:

Tumour (7) slices, 3 G wet wt.

Ringer phosphate solution pH 7.4, 5 ccs.

DL-tryptophan-3-C^{14}, specific activity 32μc/μmole, Final conc. 6.2×10^{-5}M

NSD 1024, 2.5×10^{-4}M

The total volume of incubation was 5.8 ml. Boiled slices identically incubated acted as a control. Incubations were carried out in 50 ml flasks flushed with O_2 and corked, for 1 hour at 37°C. At the end of incubation 4 μmoles of DL-5HTP were added as carrier. (Paper chromatography of extracts of this tumour had shown that 5HTP content of this tumour was less than 1 μg/G tumour tissue, a level unlikely to interfere with quantitative assay performed here). Assay method II was then carried out and followed with radioautograms of the chromatograms.

Results

Figure 21 shows the radioautogram of the initial chromatogram of the experimental sample and the large amount of radioactivity in the 5HTP area. Figure 22 shows a scan for radioactivity of a one-way ascending paper chromatogram prepared in the "isopropanol: ammonia: water" solvent of the extract from the decarboxylated 5HTP. The 5HT was then eluted and the amount of 5HT fluorimetrically assayed and its radioactivity determined as described.

TABLE 10

Tryptophan 5-hydroxylation by carcinoid tumour (7) slices

	Carrier DL-5HTP added µmoles	5HT recovered µmoles	Specific activity of 5HT c/min/µmole	µmoles 5HTP produced/G tissue/hr.	% tryptophan 5-hydroxylated *
Experimental	4	0.05	5.6×10^4	700	1.36
Control	4	0.036	0	0	0

* Calculated on the basis that only the L isomer is hydroxylated.

Recrystallisation of 5HT to constant specific activity

The specific activity of the recovered 5HT was high enough to consider its recrystallisation to constant specific activity, as a proof of identity.

Methods

5HT base was prepared from 5HT creatinine sulphate by the method described by Chadwick and Wilknison (1960). The final solution in 0.1 N HCl contained 66 µmoles of 5HT base/cc, when assayed by the nitroso-naphthol colorimetric procedure. The procedure then followed was that reported by Rapport (1949).

To the remaining 5HT eluate from the final chromatogram of the last experiment dissolved in 1 cc of water was added 66 µmoles of 5HT base (1 cc). To this mixture was added 2 cc of a saturated solution of picric acid. This mixture was cooled to 0°C and allowed to stand. In a few hours crystals of 5HT picrate formed. These were separated from the mother liquor by centrifugation and dissolved in 2 ml of 0.1 N HCl.

Aliquots of this solution were taken for the colorimetric assay of 5HT and for estimation of radioactivity at infinite thinness in a gas flow counter. This procedure was repeated twice.

Results

The 5HT recrystallised as its picrate complex to a specific activity of 18.3, 19.4 and 18.5 c/min/μmole on three successive recrystallisations. The melting point of these crystals was 186°C-189°C with decomposition. The reported m.p. of the picrate is 185-194°C (Asero et al, 1952, Erspamer and Asero 1953, Hamlin and Fischer 1951). The melting point of the mixture was the same as that of the picrate prepared from the 5HT base alone.

Confirmatory experiments on tryptophan 5-hydroxylation in carcinoid tumours

Expt 19 QUALITATIVE

Slices of tumours 7 and 8 were incubated in exactly the same way as in the previous experiment, and treated by assay method II.

Results

1. Radioactivity was seen on the radioautograms of the first chromatogram in the area of 5HTP.

2. Upon decarboxylation of this 5HTP, the radioactivity appeared in the 5HT area on the radioautogram of the final chromatogram.

Expt. 20 QUANTITATIVE ESTIMATION OF TRYPTOPHAN 5-HYDROXYLATION IN SLICES OF TUMOUR 9

Slices of tumour 9 were incubated under the following conditions:

Slices of tumour 9 (4 G wet wt).

DL-tryptophan-3-C^{14} (630 mμmoles/20μc) added.

NSD 1024. 2.5×10^{-4}M

Ringer phosphate solution, pH 7.4, 5 ml.

Incubation was carried out for 1 hr at 37°C in a flask flushed with O_2, corked and gently shaken. At the end of incubation 4 µmoles of DL-5HTP were added and the sample treated by assay method II.

Results

Qualitative

1. Radioactivity was seen on radioautograms of first chromatogram in area of 5HTP.

2. After decarboxylation radioactivity now appeared in the area of 5HT on the radioautogram of the final chromatogram.

Quantitative

The 5HT was eluted from the final chromatogram. Its amount was assayed colorimetrically and its radioactivity assayed at infinite thinness in a gas flow counter.

Table 11

Tryptophan 5-hydroxylation by carcinoid tumour (9) slices

DL-5HTP added µmoles	5HT recovered µmoles	Specific activity of 5HT c/min/µmole	5HTP (mµmoles) produced/G tissue/hour	% L-tryptophan hydroxylated by whole sample
4	0.11	1.13×10^5	1.02	3.2

Expt. 21 THE CONVERSION OF TRYPTOPHAN TO 5HT BY CARCINOID TUMOUR (7) SLICES

Slices of tumour 7 were incubated under the conditions described on p. 76 except that iproniazid 3.7×10^{-3}M was substituted for NSD 1024. The incubation sample was deproteinised with ethanol and the dried ethanolic extract extracted with butanol to remove 5HT and this was returned to 0.1 N HCl and evaporated to dryness in a vacuum dessicator.

Fig. 23 Expt. 21 (Control) Radioautogram of two way ascending paper chromatogram of a butanol extract of the incubation of BOILED carcinoid tumour slices with DL-tryptophan-3-C^{14}. Note the absence of radioactivity in the area of 5HT.

Fig. 24 Expt. 21 (Experimental) Radioautograms of two way ascending paper chromatogram of a butanol extract of the incubation of carcinoid tumour slices with DL-tryptophan-3-C^{14}. Note the radioactivity associated with 5HT.

It was then dissolved in 0.1 ml of water and subjected to two-way ascending paper chromatography in the solvents already described.

Results

Although only qualitative it will be seen that there is no radioactivity in the area of the 5HT in the control sample (where the 5HT endogenously present in the tumour stained, fig. 23) whereas radioactivity is present in the 5HT area in the experimental sample (fig. 24).

Discussion

There seems no doubt that, from these experiments, tryptophan is 5-hydroxylated by carcinoid tumours and that the direct product of this reaction is 5HTP, and that if the biosynthetic pathway for 5HT is not blocked at the decarboxylation stage, the tumours will complete the biosynthesis of 5HT.

Two points seemed important to investigate at this stage.

1. To ensure that one was really observing tumour hydroxylation and not the hydroxylation of tryptophan in the human liver from which the metastases had been removed. (This point had to some extent been proved by the observations on tumour 8 which was a primary module from the ileum and which could not therefore have been contaminated by liver).
2. Would these tumours incubated under the same conditions 4-hydroxylate phenylalanine to form tyrosine? If one could establish that under the conditions in which tryptophan 5-hydroxylation took place phenylalanine 4-hydroxylation did not occur then there would be good evidence that the tumour hydroxylating enzyme was different from that in rat liver.

Expt. 22 THE ABSENCE OF TRYPTOPHAN 5-HYDROXYLATION IN LIVER CONTAINING METASTATIC CARCINOID TUMOUR (TUMOUR 6)

Slices of liver macroscopically free of tumour and slices of carcinoid metastases from the same liver were separately incubated with boiled slices of the respective tissues acting as controls under the conditions described in experiment 18 on page 73 . After incubation DL 5HTP (4 μmoles) was added and the initial steps of assay method I carried out.

Results

Whereas the production of radioactive 5HTP by the experimental tumour samples was easily demonstrable, no radioactive 5HTP could be detected in the extract from the liver slice incubation.

Comments

This experiment showed that under conditions in which tryptophan 5-hydroxylation was easily demonstrable in tumour slices, it was not demonstrable in liver slices, which had been taken from the same specimen stored under the same conditions and prepared in the same way. Could these results be explained though by the assumption that much greater amounts of the same enzyme were present in the tumour than in the liver and that whilst the activity of the liver had deteriorated to undetectable levels the activity of the tumour was still detectable because of its initial higher activity? If this were so, and indeed phenylalanine 4-hydroxylase was present in the tumour and carrying out tryptophan 5-hydroxylation there, then phenylalanine 4-hydroxylation should be easily demonstrable, for in liver the activity of phenylalanine 4-hydroxylase is 30 times greater for phenylalanine than

tryptophan (Renson et al 1962).

Expt. 23 THE ABSENCE OF PHENYLALANINE 4-HYDROXYLASE FROM CARCINOID TUMOUR AND "CARCINOID LIVER"

Slices of hepatic metastases from tumour 6 and slices of normal liver in which these metastases lay were incubated with DL-phenylalanine-3-C^{14} under the following conditions:

Liver slices or metastatic tumour slices, 2.5 G wet wt;

DL phenylalanine-3-C^{14} (2 μmoles added) (specific activity 5μc/μmole)

Ringer phosphate solution, pH 7.4, 5 ml.

The samples were incubated, shaking for 1 hr at 37°C in 50 ml flasks flushed with O_2 and corked.

At the end of incubation 4 μmoles of L-tyrosine were added and the sample deproteinised with 0.8 N perchloric acid. The extract, neutralised with 1 N KOH was passed through a small column of Zeocarb 225 (H^+). This column was washed with water and the tyrosine eluted with 10 ml of 2N ammonia. The eluate was evaporated to dryness in a rotary evaporator at 70°C under a vacuum. The dried extract was dissolved in 0.1 ml of water, 0.05 ml of which was spotted for paper chromatography in n-butanol: acetic:acid: water (p. 38).

Results

Radioautograms of these chromatograms showed no radioactivity in the tyrosine area in any of the samples although the "Carrier" tyrosine was recovered as shown by its staining reaction on the chromatogram with the nitrosonaphthol reagent (Smith 1960).

These experiments established therefore that the tryptophan 5-hydroxylation activity in carcinoid tumours differed markedly in its specificity from the phenylalanine 4-hydroxylation activity seen in rat liver

Expt. 24 TRYPTOPHAN 5-HYDROXYLATION IN WHOLE HOMOGENATES OF CARCINOID TUMOURS

Although tryptophan 5-hydroxylation had been demonstrated using slices of carcinoid tumour it proved very difficult to demonstrate this activity in homogenised tissue. Whole homogenates were prepared from tumours 4, 5, 7 and 8 and attempts were made to demonstrate tryptophan 5-hydroxylation using DL tryptophan-3-C^{14} as a substrate and NSD 1024 as a decarboxylase inhibitor and analysing for 5HTP by assay method II. In no case was any radioactive 5HTP or 5HT recovered. This was surprising as slices of the last two tumours (7 and 8) were shown to be active in incubations carried out at the same time. Obviously disorganisation of the tissue rendered tryptophan 5-hydroxylase inactive in some way. However whole homogenates of tumour 9 were shown to retain their tryptophan 5-hydroxylase activity and in the following experiment the activity of the homogenate was compared with that of slices.

Preparation of Tissue and Incubation Conditions

Slices were cut from hepatic metastases of tumour 9. Metastases were homogenised in Ringer phosphate solution pH 7.4 (1cc/1G tumour tissue) as described on p.18 . This tumour proved to be much less fibrous than previous tumours and therefore easier to homogenise.

Slice Incubation

The slice incubation was in fact that reported on p.17 , the results of which are repeated below.

Whole Homogenate Incubation

Whole homogenate tumour 9 (1.1 w/v) 2 ml; NSD 1024 2.5×10^{-3}M

DL tryptophan-3-C^{14} 10 µc/315 mµmoles (final concentration 1.26×10^{-4}M)

The total volume of the sample was 2.5 ml.

The incubation mixture was placed in a 50 ml flask, flushed with O_2 and corked, and incubated at 37°C with shaking for 2 hours. At the end of the incubation 4 µmoles of DL-5HTP were added and the sample treated by assay method II.

Results

Qualitative (whole homogenate and slices)

Radioactivity was seen in the area of 5HTP on the radioautograms of the first chromatograms, and after decarboxylation this radioactivity appeared in the area of 5HT on the radioautograms of the final chromatograms.

Quantitative TABLE 12

Tryptophan 5-hydroxylation in carcinoid tumour (slices and whole homogenate)

Preparation	DL-5HTP added µmoles	5HT recovered µmoles	specific activity of 5HT c/min/µmole	mµmoles 5HTP formed /G of tissue/hr.
Slices	4	0.11	1.13×10^5	1.02
Whole homogenate	4	0.17	5.4×10^3	1.22

Discussion

Allowing for the fact that the concentration of DL tryptophan-3-C^{14} were different one can see that the activity of slices and whole homogenate of this tumour in respect of tryptophan 5-hydroxylation were similar. Why should this tumour be active as a whole homogenate and not the others? Three possibilities are considered:

1. This tumour was of bronchial origin, the others were of intestinal origin. This different derivation may have been responsible in some way.

2. This tumour was much more easily homogenised than the others and less vigorous methods had to be used. I doubt whether this really accounts for its activity, because it has been possible to partially purify a tumour tryptophan 5-hydroxylase in a soluble form from metastases of a tumour treated by much more vigorous methods, in the whole homogenate of which no activity was present.

3. The most obvious difference between this tumour and the others tested was in the endogenous content of 5HT. This tumour contained between 5 - 10 μgms of 5HT/G of tumour tissue. Could it have been that the much higher 5HT content of the other tumours on homogenisation caused inhibition of tryptophan hydroxylation? This point is tested in the next experiment to be described and the answer is probably no! The reason why the whole homogenate of this tumour was active and the others inactive remains unknown.

Expt. 25 THE EFFECT OF 5HT, PHENYLALANINE AND AMETHOPTERIN UPON TRYPTOPHAN HYDROXYLATION BY CARCINOID TUMOURS

Introduction

5HT Because of the reasoning above, the effect of 5HT on tryptophan 5-hydroxylation by whole homogenates of carcinoid tumour (9) was studied.

Phenylalanine We have seen that phenylalanine inhibits tryptophan 5-hydroxylation by the rat liver and it was of some interest to see whether phenylalanine would inhibit tumour tryptophan hydroxylation even though these tumours do not appear to hydroxylate phenylalanine.

Amethopterin Amethopterin is a potent inhibitor of phenylalanine 4-hydroxylase in rat liver (Kaufman and Levenburg 1959). The mechanism of its inhibition is thought to be via its inhibiting effect upon dihydrofolate reductase (Osborn et al 1958) which is necessary for the reduction

of the dihydropteridine to the active tetrahydropteridine.

Experiment 25

A whole homogenate 1:1 w/v) was prepared in Ringer phosphate solution, pH 7.4, and was incubated under the following conditions:

Whole tumour homogenate 1:1 w/v) in Ringer phosphate solution, 2 ml;

DL tryptophan-3-C^{14} (10 μc/315 mμmoles) 1.26×10^{-4}M;

NSD 1024, 2.5×10^{-3}M.

The total volume of the incubation was 2.5 ml. The following additions were made to separate incubations:

(1) No further additions

(2) 5HT creatinine sulphate (2 μmoles of 5HT base) 8×10^{-4}M.

(3) L phenylalanine (2 μmoles) 8×10^{-4}M.

(4) Amethopterin 5×10^{-4}M.

All incubations were carried out for 1 hr at 37°C in flasks flushed with O_2, corked and shaken. At the end of incubation analysis for 5HTP was carried out by assay method II.

Results

TABLE 13

The effect of 5HT, phenylalanine and amethopterin on tryptophan 5-hydroxylation by carcinoid tumour (9)

Additions	5HTP added μmoles	5HT recovered μmoles	Specific activity of 5HT c/min/μmole	mμmoles 5HTP produced /G tissue/hour
None	4	0.17	5.4×10^3	2.44
5HT	4	0.14	4.5×10^3	2.06
L phenylalanine	4	0.13	5.6×10^3	2.62
Amethopterin	4	0.12	6.3×10^3	2.86

The difference between the amount of 5HTP produced in these four incubations is so small that one is forced to say that at the concentrations used these substances had no apparent effect on tryptophan hydroxylation.

THE SUBCELLULAR LOCALISATION OF TRYPTOPHAN 5-HYDROXYLASE IN CARCINOID TUMOURS

As whole homogenates of tumours 4, 5, 6 and 8 were inactive it proved impossible to fractionate the hydroxylase activity. The subcellular localisation of tryptophan 5-hydroxylase in tumour (9) was studied but for technical reasons the experiments were not brought to a final conclusion. On two occasions however there was the suggestion that most of the tryptophan 5-hydroxylase activity in this tumour lay in a 100,000 G particulate fraction prepared from a whole homogenate. No quantitative studies were done however before the supply of this tumour ran out.

Expt. 26 PARTIAL PURIFICATION AND COFACTOR REQUIREMENT OF TRYPTOPHAN 5-HYDROXYLASE IN CARCINOID TUMOUR (7)

Introduction

Although whole homogenates, particulate and supernatant fractions of this tumour were shown to be inactive in respect of tryptophan 5-hydroxylation nevertheless when the partial purification of brain tryptophan 5-hydroxylase was achieved and the cofactor requirement of the enzyme preparation demonstrated (see p.152) an ammonium sulphate precipitate of a supernatant fraction of this tumour was made. This rather blind experiment was done for the following reasons. If in fact one assumed that the tryptophan 5-hydroxylase in the whole homogenate and fractions prepared from it was inactive either

because of lack of cofactors, presence of high levels of 5-hydroxy-indoles, or presence of inhibitory substances, then by making an ammonium sulphate precipitate and adding the necessary cofactor one might remove these possibly inhibitory factors. Unfortunately as the whole homogenate of this tumour and fractions prepared from it were inactive it was impossible to calculate the actual degree of purification but the activity of the ammonium sulphate fraction and its cofactor requirement is of interest.

Preparation of tissue

Metastases of tumour 7 were removed from the surrounding liver and weighed (37 G). These metastases were then homogenised in 0.32 M sucrose + 0.01 M mercaptoethanol 2.1 (v/w) in a Waring blender for 4 min. The homogenate resulting was more finely homogenised in a glass tube with a teflon pestle. An aliquot of the whole homogenate (vol. 60 ccs) was centrifuged at 100,000 g for 1 hour. To an aliquot of the resultant supernatant (40 ccs) was added 12.4 G of $(NH_4)_2SO_4$ to give a 50% saturation with $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ precipitate resulting after standing the mixture at 3°C for 25 mins was removed by centrifugation at 12,000 g for 30 mins and redissolved in 10 ccs of 0.01 M potassium phosphate buffer pH 7 containing 0.001 M mercaptoethanol.

Incubation conditions

$(NH_4)_2SO_4$ precipitate preparation 1cc; NSD 1024 2.5×10^{-4}M

BL tryptophan-3-C^{14} 152 mμmoles/5μc (final concentration 1.09×10^{-4}M)

Potassium phosphate buffer pH 7, 7.15×10^{-2}M; $DMPH_4$ 2×10^{-3}M (see p. 11)

Total volume of incubation, 1.4 ml.

In one sample the enzyme preparation was boiled as a control and from another the $DMPH_4$ was omitted. All samples were shaken in O_2 at 37°C for 1½ hours. At the end of this time the samples were assayed for 5HTP by assay method III (see section on Brain, p. 112).

Results

TABLE 14

The activity of partially purified tryptophan 5-hydroxylase from carcinoid tumour (7)

	DL 5HTP added μmoles	5HT recovered μmoles	Specific activity 5HT c/min/μmole	μμmoles 5HTP formed/ml preparation/hr
Boiled control	2	0.059	8.7×10^3	242
- $DMPH_4$	2	0.089	2×10^3	49
+ $DMPH_4$	2	0.065	1.88×10^4	525

Comments

These results illustrate a difficulty also encountered by Nagatsu et al (1964) in studying tyrosine hydroxylase. They also found that non-enzymatic hydroxylation was sometimes increased by boiling tissue preparations. They overcame this difficulty by using D-tyrosine as a substrate with unboiled tissue, giving a more meaningful index of the non-stereospecific non-enzymatic hydroxylation. In the present experiment the use of D-tryptophan as a substrate with unboiled tissue would have been a more elegant control but as it was not used a more logical control would seem to be the unboiled sample to which no $DMPH_4$ was added. Whether the relatively small amount of hydroxylation seen in this sample is enzymatic or not is unknown but what is striking is the great increase in activity seen when $DMPH_4$ is added.

It is clear therefore that tryptophan 5-hydroxylase has been recovered from a tumour whole homogenate in which no activity could be demonstrated. This is very suggestive that in such whole homogenates there are present influences inhibitory to the activity of tryptophan 5-hydroxylase which are unable to exert themselves when the organisation of the tissue is relatively undisturbed as in the case of incubation carried out with slices. It is also apparent that the carcinoid tumour tryptophan 5-hydroxylase requires a reduced pteridine for full activity, in common with tryptophan hydroxylase prepared from other tissues. This matter will be examined more closely later.

EXPERIMENTAL SECTION II

A. THE BIOSYNTHESIS OF 5-HYDROXYTRYPTAMINE IN INTESTINE

Introduction

There are certain facts which suggest that most of the 5HT formed in the body is synthesised in the gastrointestinal tract.

(1) Removal of the gastrointestinal tract from rats, whilst maintaining tryptophan blood levels, leads to the virtual disappearance of 5HIAA inbthe urine (Bertaccini 1960).

(2) Argentaffinomas (carcinoid tumours) produce large amounts of 5HT and have been shown to possess all the enzymes necessary for its biosynthesis. The majority of these tumours arise in the gastrointestinal tract and are composed of cells having histochemical reactions identical with the argentaffin cells which are normally situated deeply in the crypts of Lieberkühn.

(3) The gastrointestinal tract as a whole contains large amounts of 5HT and though this could be merely a reflection of storage, nevertheless taken together with the preceding facts suggests that intestine synthesises 5HT.

(4) There are the experiments of Cooper and Melcer (1961) which have already been discussed. Their evidence was suggestive, but not conclusive, that preparations of rat gastrointestinal mucosa synthesised 5HT.

Methods having been developed for the demonstration and assay of tryptophan 5-hydroxylation in carcinoid tumours these same methods were applied to the study of tryptophan 5-hydroxylation in the gastrointestinal tract.

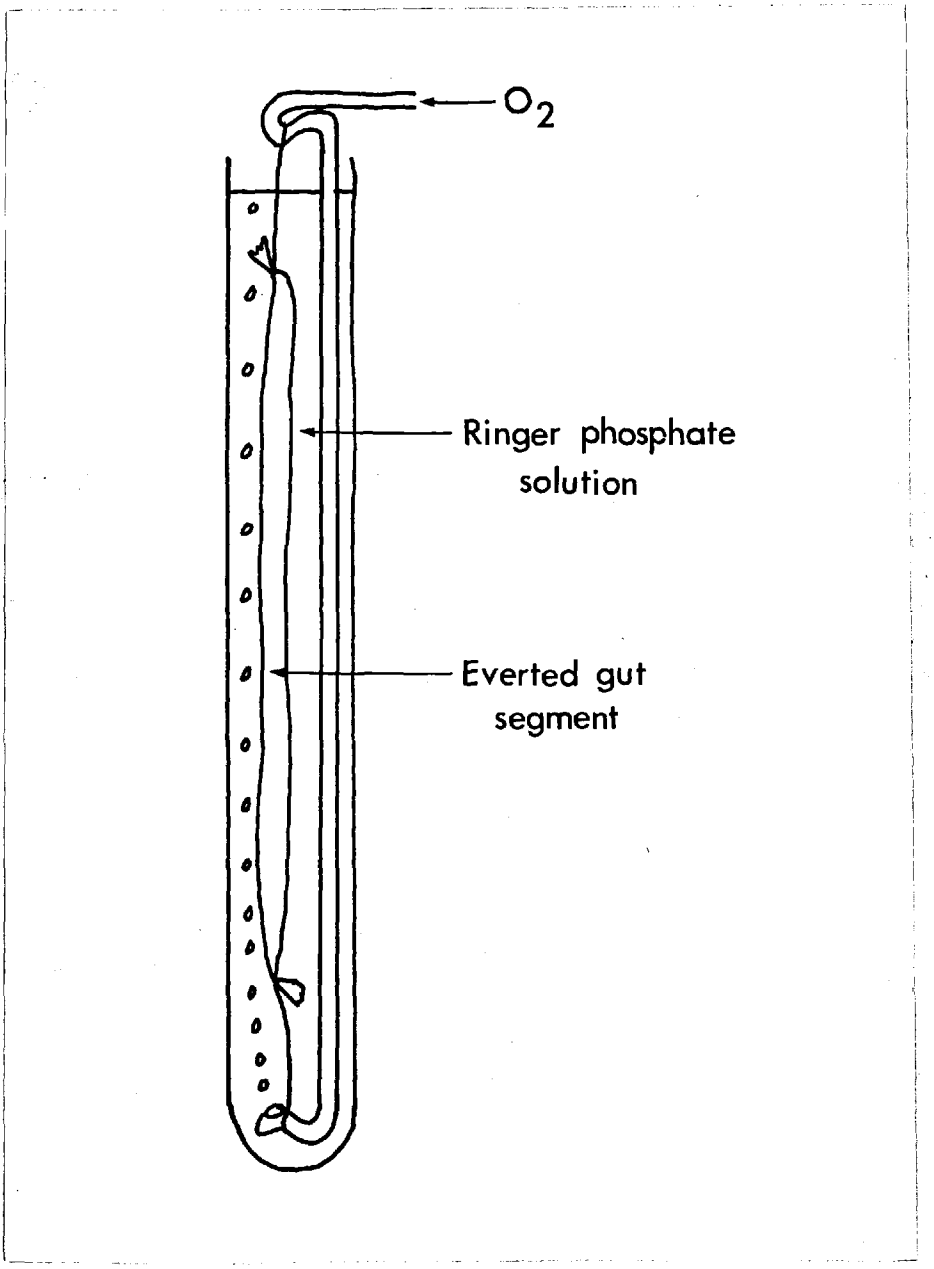


Fig. 25 Diagram to illustrate the method of suspending intestinal segments for incubation.

Preliminary experiments in which intestinal mucosal homogenates prepared by the methods described by Cooper and Melcer (1961) were incubated with DL-tryptophan-3-C^{14} and NSD 1024, and carried through assay method II failed to demonstrate the production of 5HTP. Various additions to the incubation were made (e.g. reduced NAD and NADP, THFA, ascorbic acid and $CuCl_2$) but with none of these could definite enzymatic tryptophan 5-hydroxylation be demonstrated. As this situation had been seen with whole homogenates of carcinoid tumour and was overcome by using slices, experiments were conducted using intestinal segments.

Expt. 1 THE FORMATION OF 5HT FROM TRYPTOPHAN BY INTESTINAL SEGMENTS

Preparation of tissue

Rabbits were killed by a blow on the neck and the abdomen opened. A loose ligature was placed just distal to the pylorus and a 3" segment of intestine distal to this ligature was removed. This was turned inside out over a glass rod pressed against the closed end and the mucosal surface washed clean with ice cold distilled water. The segment of gut was then suspended in Ringer phosphate solution (pH 7.4) in the manner depicted in figure 25.

The gut segment was suspended from a double hooked tube placed in the incubating tube and from the lower hook of the suspending tube oxygen was bubbled. This procedure exposed the maximum internal surface area of the gut to the substrate tryptophan, kept it well oxygenated and at the same time mixing was carried out by the bubbling oxygen. The incubation tube was placed in a water bath at 37°C. The brim of the tube was greased with silicone grease to avoid frothing.

Components of incubation

Ringer phosphate solution, pH 7.4, 10 ml; Gut segment 3" long;

DL tryptophan-3-C^{14} 10 μc/0.5 μmole (4.35×10^{-5}M);

Reduced NAD 5×10^{-4}M; Reduced NADP 5×10^{-4}M

Pyridoxal phosphate 4×10^{-5}M; THFA 5×10^{-3}M

Iproniazid 2.5×10^{-3}M)
) where indicated
5HT 4.5×10^{-5}M)

The samples were preincubated for 10 minutes before the addition of substrate so that in the samples to which iproniazid was added monoamine oxidase was inhibited. The following samples were incubated:

1. Boiled gut segment + full system (control)
2. Gut segment + full system
3. Gut segment - 5HT
4. Gut segment - 5HT and iproniazid

The samples were incubated as described for 1 hour at 37°C. At the end of incubation 0.5 μmoles of 5HT were added to samples 3 and 4. The gut was finely minced with scissors and resuspended in the incubation fluid to which was added 4 vols of 95% ethanol. The denatured protein was removed by centrifugation and the protein-free extract evaporated to dryness at 70°C in a rotary evaporator under vacuum. The dried extract was dissolved in 5 ml of water and the 5HT extracted with butanol and returned to 0.1 N HCl as previously described. The acid extract was evaporated to dryness, dissolved in 0.1 ml of water and subjected to two-way ascending paper chromatography in the solvents described on p. 58 . Radioautograms were prepared from these chromatograms and the latter stained with Ehrlich's reagent to locate the 5HT.

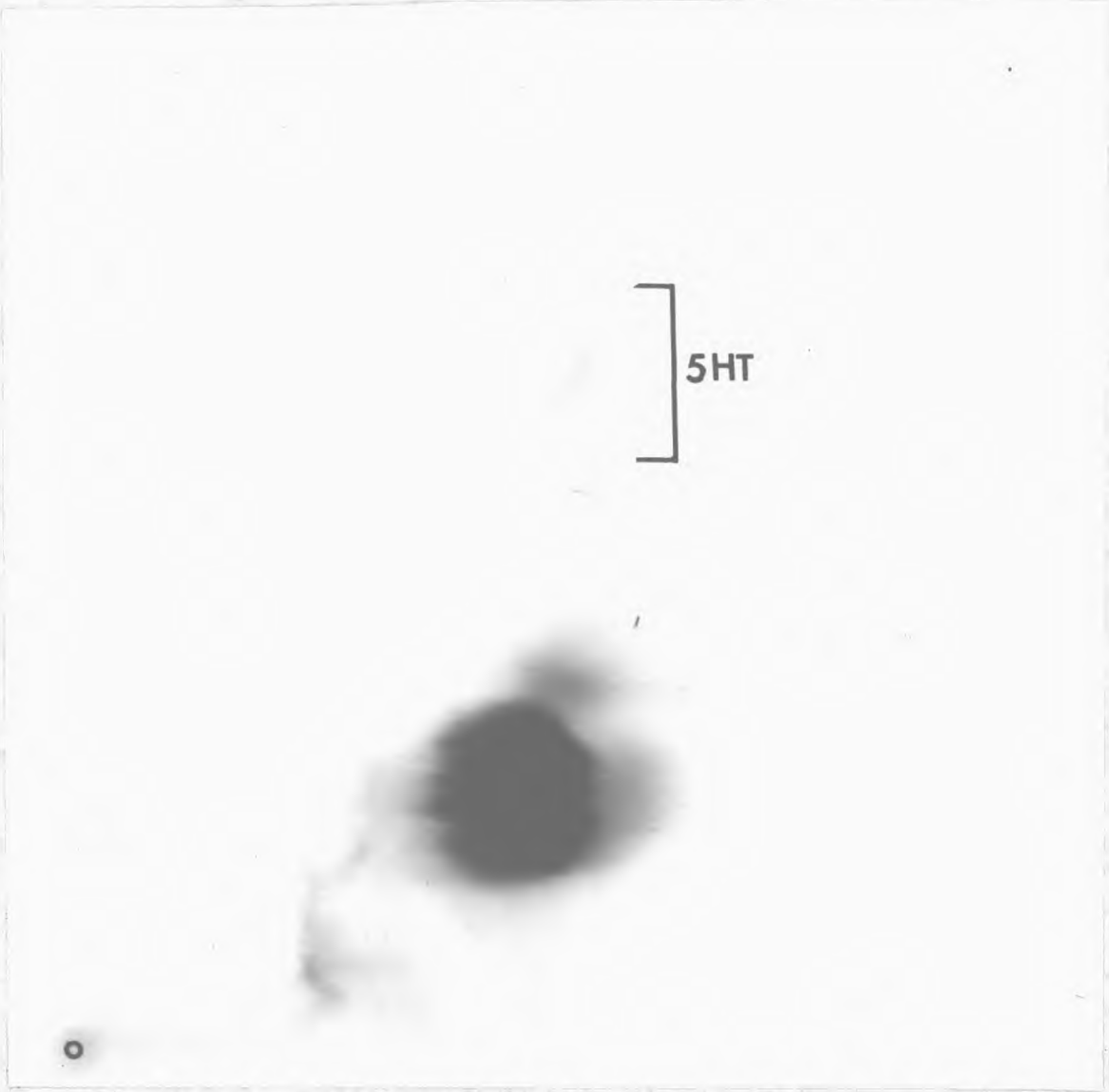


Fig. 26 Expt. 1 Radioautogram of a two way ascending paper chromatogram of a butanol extract prepared from the incubation of an intestinal segment with DL-tryptophan-3-C^{14}. Note the radioactivity associated with 5HT.

Qualitative results

1. No radioactive 5HT could be demonstrated in the boiled control sample.
2. Radioactive 5HT was seen in all the other fractions (see Fig. 26).

Since no quantitative studies were done and the density of the radioautive spots on the radioautograms is an unreliable guide to activity unless recovery of the "Carrier" 5HT is known, no precise estimate of the amount of tryptophan hydroxylated can be given. However the density of the radioactive spots on the radioautograms, in relation to the density of the 5HT spots when stained with Ehrlich's reagent, suggested that the hydroxylation activity of intestine is of the same order as that seen in carcinoid tumour and later in brain.

Expt.2 THE FORMATION OF 5HTP FROM TRYPTOPHAN BY INTESTINAL SEGMENTS

Although experiment 1 appears to confirm that 5HT is synthesised from tryptophan in intestine there is one objection. It is possible that both in the boiled control and experimental samples 5HTP was non-enzymatically formed and that only in the experimental sample was it decarboxylated to 5HT, as only in this sample would the decarboxylase be active.

For this reason a similar experiment was performed but this time a boiled gut segment and a viable gut segment were separately incubated under the same conditions but substituting NSD 1024 (2×10^{-3}M for iproniazid. At the end of incubation the sample was treated by assay method I up to and including the high voltage electrophoresis. It was possible to show that whereas in the control sample no radioactive 5HTP was produced, in the experimental sample, radioactive 5HTP was present on the initial chromatogram and on elution this ran as 5HTP on high voltage paper electrophoresis.

It has not yet been possible to demonstrate enzymatic tryptophan 5-hydroxylation in cell-free extracts of intestinal mucosa using either isotopic methods or non-isotopic methods and because of the difficulty of interpreting any quantitative results resulting from incubation of gut segments, no quantitative studies have been done.

Expt. 3 THE INABILITY OF INTESTINE TO 4-HYDROXYLATE PHENYLALANINE

An attempt was made to see whether intestinal segments incubated with DL-phenylalanine-3-C^{14} (10 μc: 2.9 x 10^{-3}M) would result in the production of radioactive tyrosine. The sample after incubation was treated by the method described on p. 81 and the extract subjected to ascending paper chromatography in the n-butanol: acetic acid: water solvent. Radioautograms of the chromatograms showed no radioactivity present in the areas where "Carrier" tyrosine stained. These results confirm those of Udenfriend and Cooper (1952) and Kaufman (1957) who could find no evidence that phenylalanine 4-hydroxylation took place anywhere but in the liver.

EXPERIMENTAL SECTION III

THE BIOSYNTHESIS OF 5HT IN BRAIN

Introduction

In 1954 Amin, Crawford and Gaddum demonstrated that 5HT was present in brain, and this was confirmed by numerous workers using a variety of chemical and biological techniques. It was found that 5HT was not evenly distributed throughout the brain but concentrated in certain areas (Amin et al 1954, Bogdanski and Udenfriend 1956, Gaddum and Giarman 1956). Although the discrete localisation in various areas of the brain has been studied (for instance by Kuntzman et al 1961), one can generalise and say that whilst the hypothalamus, midbrain and medulla contain fairly large amounts of 5HT, the cerebral cortex and cerebellum have a low concentration.

The problem that concerns us here is how this brain 5HT is produced. Udenfriend et al (1957) showed that 5HT did not freely enter the brain from the circulation. From their findings one can presume that 5HT, be it in the plasma or bound to platelets, does not easily pass the blood brain barrier. On the other hand the same group of workers (Bogdanski et al 1957) showed that the concentration of 5HT in various areas of the brain closely paralleled the activity of 5HTP decarboxylase. This was suggestive that at least the step 5HTP ---→ 5HT, took place within the brain. It was shown that the administration of 5HTP to animals raised the brain 5HT concentration (Udenfriend et al 1957) and that 5HTP is taken up by brain slices by an active transport mechanism (Schanberg and Giarman 1960, Smith 1963). When Cooper and Melcer

(1961) reported tryptophan 5-hydroxylation in intestine they speculated that perhaps 5HTP was synthesised in the intestine, circulated to the brain, entered the brain and was decarboxylated to 5HT. Similar reasoning was also applied after the demonstration of tryptophan 5-hydroxylation in rat liver (Freedland et al 1961a), though this hypothesis was considerably weakened by the demonstration that this activity was due to phenylalanine 4-hydroxylase and probably not a physiological pathway for 5HT biosynthesis (Renson et al 1962).

What are the objections to this concept as the physiological mechanism for the biosynthesis of brain 5HT?

1. 5HTP cannot be detected in circulating blood under physiological conditions (Renson et al 1962).

2. The work of Green and Sawyer (1964) demonstrated that the 5HT in brain derived from exogenously administered 5HTP behaves differently in respect of the kinetics of its appearance and disappearance to 5HT in brain formed from endogenous precursors.

3. Removal of the gastrointestinal tract does not affect the brain concentration of 5HT (Bertaccini 1960).

4. Renson et al (1962) convincingly showed that the inhibition of tryptophan 5-hydroxylation in rat liver caused no decrease in the brain content of 5HT.

5. Let us examine the hypothesis that 5HTP is formed outside the brain. Such a mechanims would involve:

(a) The 5-hydroxylation of tryptophan with the formation of 5HTP at some peripheral site such as intestine or liver;

(b) The escape of this 5HTP into the circulation and its escape

from 5HTP decarboxylase which is widely distributed in tissues;

(c) The circulation of the 5HTP to the brain and its transport into brain cells;

(d) The decarboxylation of 5HTP with the formation of 5HT at or near the site of its action.

When one considers that the 5-hydroxylation of tryptophan is probably the rate-limiting step in the biosynthesis of 5HT, that brain 5HT appears to be discretely localised both at the anatomical and sub-cellular levels and that if 5HT has a function in the central nervous system its action is likely to be at discrete loci, then a mechanism for its biosynthesis such as that outlined above would be very difficult to control.

These objections led one to suspect that 5HT was probably synthesised from tryptophan within the brain. If so, then what supporting evidence was there and what chance of demonstrating this synthesis in vitro with the methods developed for the study of carcinoid tumours and gut? Studies on the turnover of 5HT in brain performed by estimating the increase in brain 5HT after the administration of a monoamine oxidase inhibitor indicated that the half life of 5HT in brain is in the order of 10-30 mins (Udenfriend and Weissbach 1958). Garattini and Valzelli (1961) on the basis of similar experiments calculated that rat brain synthesises about 50 µg of 5HT/day. A rat brain weighs about 1.5 G so that 1 G of rat brain produces about 1.4 µg of 5HT/hour. 1 G of carcinoid tumour tissue in vitro produces up to 1.2 µg of 5HT/hour. If this amount of tryptophan 5-hydroxylation took place in

brain than it seemed reasonable to assume that the methods already developed would be capable of detecting it.

For 5HT synthesis to take place completely in brain there would have to exist an uptake mechanism for tryptophan. Guroff and Udenfriend (1962) demonstrated that indeed a mechanism for the active transport of tryptophan into brain existed, and Gal et al (1963) demonstrated that intracerebral, but not intraperitoneal, injections of DL tryptophan-3-C^{14} produced labelling of brain 5HT.

Expt. 1 THE QUALITATIVE DEMONSTRATION OF TRYPTOPHAN 5-HYDROXYLATION IN RABBIT BRAIN

Preparation of tissue, incubation and assay of tryptophan 5-hydroxylation

Rabbits were always killed by a blow across the mid-cervical spine with a metal bar. The head and upper neck were then cut off. The skull was opened and the whole brain (minus the olfactory bulbs) was removed intact, including the cervical bulb and the upper part of the upper cervical cord. The whole brain was weighed and homogenised in a thick walled glass tube with a teflon pestle (clearance 0.2 mms) in Ringer phosphate solution, pH 7.4 (1cc/G of brain tissue).

To 5ccs of brain whole homogenate were added:
DL-tryptophan-3-C^{14} 20 µc/630 µmoles (final concentration 8.2×10^{-5}M);
NSD 1024 2.5×10^{-4}M; Ringer phosphate solution, pH 7.4, 2ccs.
The total volume of the incubation was 7.7 ccs.
5ccs of boiled brain homogenate were identically incubated as a control.
Incubation was carried out at 37°C for 1 hour in 50 ml flasks flushed with O_2 corked and gently shaken. At the end of the incubation 1 µmole of DL-5HTP was added as carrier and the sample analysed by assay method II.

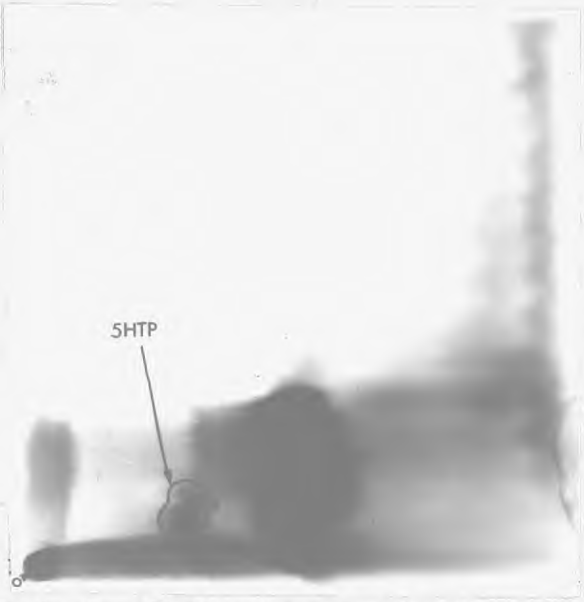


Fig. 27 Expt. 1. Radioautogram of a two way paper chromatogram of an incubation of brain homogenate with DL-tryptophan-3-C^{14}. Note the radioactivity associated with 5HTP.

Fig. 28. Expt. 1. Radioautogram of a two way ascending paper chromatogram of the enzymatically decarboxylated 5HTP eluted from the chromatogram depicted in Fig. 27. Note the radioactivity associated with 5HT.

Results

On the radioautogram of the initial two way paper chromatogram, there was a suggestion of radioactive 5HTP in the unboiled sample (Fig. 27). After decarboxylation of this 5HTP, radioactivity was present on the 5HT spot of the radioautogram of the chromatographed, decarboxylated 5HTP (Fig. 28). No radioactivity was seen associated with either the carrier 5HTP or the 5HT produced from it in the boiled sample.

Expt 2 THE ANATOMICAL DISTRIBUTION OF TRYPTOPHAN 5-HYDROXYLASE IN BRAIN AND ITS QUANTITATIVE ASSAY

Although tryptophan 5-hydroxylation appeared to take place in brain nevertheless if this reaction was going to be quantitatively studied a more active preparation was necessary. In addition the anatomical distribution of the hydroxylase was itself of interest.

Preparation of tissue, incubation and assay of tryptophan 5-hydroxylase

The brain was removed from a rabbit as already described. The cerebral cortex was removed by cutting through the most lateral aspects of the cerebral peduncles and the cerebellum by dissecting off the cerebellum and cutting its connections with the brain stem close to the surface of the latter. This produced three main portions of brain. The cerebral cortex, the cerebellum, and the rest of the brain which is hence forward (when rabbit brainis used) called the hind brain. Each of these portions was homogenised in its own weight of Ringer phosphate solution, pH 7.4.

Whole homogenates of each brain portion (2.5 cc) were incubated with DL tryptophan-3-C 5μc/250 μmoles (4.2×10^{-5}M); NSD 1024 1.5×10^{-3}M

Ringer phosphate solution was added to bring the total volume of the incubation samples to 6 ccs. As control samples, 2.5 ccs of cerebral cortex and hind brain whole homogenates were boiled for 3 mins and incubated in the above system. Incubations were carried out for 1 hour under O_2 at 37°C with shaking. At the end of incubation 2 μmoles of DL-5HTP were added and the samples taken through assay method II.

Results

Cerebral cortex and cerebellum On the radioautogram of the final chromatograms no radioactivity was associated with the 5HT. The radioactivity was not quantitatively assayed as visually none was present, despite dense staining of the carrier 5HT with Ehrlich's reagent.

Hind brain On the radioautogram of the final chromatogram there appeared to be a large amount of radioactivity associated with the 5HT. The 5HT was therefore eluted, its amount assayed by the nitrosonaphthol procedure already described and the radioactivity assayed at infinite thinness in a gas flow counter.

TABLE 15

The anatomical distribution of tryptophan 5-hydroxylase in rabbit brain

Preparation	5HTP added μmoles	5HT recovered μmoles	Specific activity 5HT c/min/μmole.	L 5HTP produced/ hr/G tissue wet wt
Cerebral cortex	2	+ (staining)	0 (radioautogram)	0
Cerebellum	2	+ (staining)	0 (radioautogram)	0
Hind brain	2	0.05	3.3×10^4	1.89 mμmoles

Discussion

These results showed that the bulk of the activity lay in the rabbit hind brain following roughly the distribution of 5HT and 5HTP

decarboxylase activity (see Table 17). Because the rabbit hind brain preparation is too small to allow further dissection and examination of its hydroxylation activity an experiment was done with dog brain.

Expt. 3 ANATOMICAL DISTRIBUTION OF TRYPTOPHAN HYDROXYLASE IN DOG BRAIN

Preparation of tissue, incubation and assay of tryptophan 5-hydroxylase

A dog (mongrel puppy) was anaesthetised by an I.V. injection of nembutal and when unconscious the carotid arteries were cut and the dog bled to death. The skull was opened and the brain dissected free and removed. The cerebral cortex and cerebellum were removed and discarded. The rest of the brain was sectioned by cutting through the cephalic border of the pons dividing the brain, for the purposes of description into "Hypothalamus-thalamus" and "Midbrain-pons-medulla" sections. Whole homogenates in Ringer phosphate solution pH 7.4 (1.1, w/v) were made. Whole homogenates of brain sections (3 cc) were incubated with DL-tryptophan-3-C^{14}, 10 µc/315 mµmoles (final conc. 8.3×10^{-5}M; NSD 1024, 1.5×10^{-3}M. The total volume of the incubation samples was 3.8 ccs. Boiled samples of each section were identically treated as controls. Incubations were carried out at 37°C for 1 hour in an atmosphere of O_2. At the end of incubation 2 µmoles of 5HTP were added to the samples and they were carried through assay method II.

Results

TABLE 16

The anatomical distribution of tryptophan 5-hydroxylase in dog brain

Sections	DL-5HTP added µmoles	5HT recovered µmoles	Specific activity 5HT.c/min/µmole	5HTP produced/1 /G wet wt tissu
"Thalamus-hypothalamus"	2	0.13	2.36×10^4	685 µµmoles
"Midbrain-pons-medulla"	2	0.112	1.34×10^4	378 µµmoles

TABLE 17

The anatomical distribution of 5HT, 5HTP decarboxylase and tryptophan 5-hydroxylase in brain

	5HT * 1.65µg/G tissue = 100%	5HTP decarboxylase ** 117µg 5HT formed/hr/G of tissue = 100%	Tryptophan 5-hydroxylase 685 µmoles 5HTP formed/hr/G of tissue = 100%
Hypothalamus	100%	100%	100%
Thalamus	35%	32%	
Mid brain	61%	83%	
Pons	23%	24%	55%
Medulla	38%	27%	
Cortex grey	16%	6%	
white	0%	-	0%
Cerebellum	0%	0%	0%

* Data of Amin, Crawford and Gaddum (1954) and Bogdanski and Udenfriend (1956)

** Data of Udenfriend, Weissbach and Bogdanski (1957)

Discussion

Already it has been mentioned that the concentration of 5HT in different parts of the brain closely follows the activity of 5HT decarboxylase. In table 17 it will be seen that the activity of tryptophan 5-hydroxylase follows roughly the same distribution. This comparison strongly suggests that 5HT is completely synthesised in the area of the brain in which it is found and that there need be little or no transport of 5HT from one area to another. If 5HT is a substance acting pharmacologically at discrete loci within the brain then this would be the situation one might expect.

If one considers the activity of tryptophan 5-hydroxylase not on the basis of "per G of tissue" but on the basis of the activity of the total brain sections then one finds that whereas the "hypothalamus-thalamus" section produces 9.55 mμmoles of 5HTP/hr, the "midbrain-pons-medulla" section produces only 1.34 mμmoles of 5HTP/hour. This highlights even more the high activity of the "hypothalamus-thalamus" section. It is possible that a large part of this activity is a property of the hypothalamus alone where 5HT is found in very high concentrations.

Expt 4 PROOF OF THE IDENTITY OF THE PRODUCT OF TRYPTOPHAN 5-HYDROXYLATION IN BRAIN AND EXAMINATION OF THE STEREOSPECIFICITY OF THE REACTION

Preparation of tissue, incubation and assay of tryptophan 5-hydroxylase

A whole homogenate of rabbit hind brain was made in Ringer phosphate solution pH 7.4 (1:2 w/v). 1 cc of this whole homogenate was incubated with NSD 1024 (3×10^{-3}M) and DL tryptophan-3-C^{14}, 5μc 158 mμmoles (final conc. 1.5×10^{-5}M). The total volume of the incubation

was 1.5 ml. Boiled tissue identically incubated acted as a control. Samples were incubated for 1 hour at 37°C in tubes flushed with O_2 and corked. Assay method I was performed on the samples after the addition of 4 µmoles of 5HTP.

Results

(a) Identification of 5HTP and 5HT

Radioactive 5HTP was present on the radioautogram of the initial chromatogram of the unboiled sample and on elution this radioactivity ran with the eluted 5HTP on H.V. paper electrophoresis. After decarboxylation the radioactivity now resided in the 5HT area of the final chromatogram. On the radioautograms of the chromatograms no radioactivity was visibly associated with the carrier 5HTP or the 5HT produced from it by decarboxylation, in the boiled control, although as we shall see a small amount of radioactivity could be demonstrated on quantitative assay.

(b) Quantitative assay

The 5HT eluate from the final chromatogram of the decarboxylated 5HTP was colorimetrically assayed for its 5HT content and the radioactivity measured.

Results

TABLE 18

Tryptophan 5-hydroxylation in rabbit hind-brain whole homogenate

	5HTP added µmoles	5HT recovered µmoles	Specific activity 5HT.c/min/µmole	µmoles L-5HTP produced/hr
Control	4	0.11	7.2×10^2	31
Experimental	4	0.025	4.15×10^3	179

These results show that some radioactivity is associated with the 5HT in the control sample, and in fact in all experiments done with brain a small but variable amount of non-enzymatic tryptophan hydroxylation was demonstrated.

(c) Examination of the stereospecificity of tryptophan 5-hydroxylation in brain

It had previously been demonstrated during experiments on carcinoid tumours (p 71), that non-enzymatic tryptophan hydroxylation was not stereospecific whereas the tumour hydroxylation was specific for the L isomer of tryptophan. Using the same reasoning but quantitative techniques the stereospecificity of the hydroxylation in brain was investigated.

In non-enzymatic hydroxylation of DL-tryptophan-3-C^{14}, such as might occur in a boiled control incubation, the specific activities of the D and L isomers of 5HTP isolated after the addition of excess non-radioactive DL 5HTP carrier will be equal. Therefore as only the L isomer of 5HTP is enzymatically decarboxylated the specific activity of the 5HT formed will be equal to that of the D-5HTP remaining.

In the case of an experimental incubation, however, both non-enzymatic and enzymatic hydroxylation will occur and assuming the enzymatic hydroxylation will be specific for the L isomer of tryptophan-3-C^{14}, the total radioactivity of the L-5HTP will be greater than that of the D-5HTP. Therefore after the addition of an excess of non-radioactive DL-5HTP carrier, the specific activity of the L-5HTP will be greater than that of the D-5HTP. So after enzymatic decarboxylation the

specific activity of the 5HT isolated will be greater than that of the D 5HTP remaining.

From the final chromatograms of the decarboxylated 5HTP in the control and experimental samples of the last experiment, the remaining D-5HTP and the 5HT were eluted and their specific activities estimated [illegible].

Results

TABLE 19

The stereospecificity of tryptophan 5-hydroxylation in brain (see text for explanation)

Sample	Specific activity of D-5HTP recovered (c/m/μmole)	Specific activity of 5HT recovered (c/min/μmole)
Control	920	720
Experimental	740	4150

Table 19 shows: (i) In the control sample the non-enzymatic hydroxylation which occurs giving roughly equal specific activities in the 5HT and D-5HTP isolated; (ii) In the experimental sample is shown the non-enzymatic hydroxylation which gives a specific activity of the D-5HTP roughly equal to that in the control sample but a much higher specific activity of the 5HT isolated, signifying an enzymatic stereospecific hydroxylation of the L isomer of tryptophan.

(d) Recrystallisation of 5HT to constant specific activity

The 5HT resulting from the assay of the "Hypothalamus-thalamus" section in Expt. 3 was recrystallised to constant specific activity by the method described on p. 75 . The specific activities of the 5HT picrate on two successive recrystallisations were 19.72 and 20.23 c/min /μmole. The melting point of this recrystallised 5HT picrate was 184-

187°C and of authentic material 186-189°C (reported 185-194°C).

Comments

There seems little doubt therefore that 5HTP is produced by the 5-hydroxylation of tryptophan by brain preparations and that the reaction is stereospecific for the L isomer of tryptophan. Usually boiled control samples were incubated alongside unboiled samples to give an assessment of non-enzymatic hydroxylation. In calculating the results the control non-enzymatic hydroxylation has always been subtracted from the total experimental activity. When controls have not been run this will be stated.

MODIFICATIONS OF THE ASSAY OF TRYPTOPHAN 5-HYDROXYLASE (ASSAY METHOD III & I

Some modifications were now made to the assay methods already described, though fundamentally the technique is the same.

Assay Method III

After incubation DL-5HTP carrier is added to the sample and it is deproteinised and treated with deactivated charcoal as in assay methods I and II. The dried eluate from the charcoal is then subjected to one way ascending paper chromatography on Whatman 3 mm paper (10" x 10") in the n-butanol: glacial acetic acid: water solvent (p. 59). (The sample is applied in a streak 1" long at the origin). This solvent separates the 5HTP from the majority of the radioactive tryptophan, though streaking of the latter does occur and for quantitative work further purification is necessary. This is achieved by eluting the 5HTP from the chromatographic strip after locating it by its fluorescence under Wood's lamp and by comparison with standard strips run at the same time and stained

with Ehrlich's reagent. This eluted 5HTP is then enzymatically decarboxylated as already described and the dried deproteinised extract from the decarboxylation subjected to one-way ascending paper chromatography in the isopropanol: ammonia: water solvent (see p. 58) again applying the sample in a streak at the origin.

The 5HT is located on this chromatogram by its fluorescence and by comparison with a standard strip, eluted and the eluate dried and assayed for the amount (and specific activity) of the 5HT present. Radioautograms are prepared from both chromatograms to ensure reasonable separation of the 5HTP from the radioactive tryptophan in the first and complete separation of the 5HT from any contaminating radioactivity which might finally be present in the last.

Assay Method IV

This is fundamentally the same as assay method III. However, in method III the following procedures are very time consuming:

(i) Perchloric acid deproteinisation,

(ii) Evaporation of the phenolic eluate from the charcoal.

To speed up the assay the following modifications were made:

(i) Deproteinisation was carried out with trichloracetic acid (T.C.A.) (2cc of 5% TCA to a tissue sample) and the TCA extracted from the deproteinised extract with ether (5 ml x 3). The pH of this extract was adjusted to a pH of 4 by adding 2 ml of 2 M sodium acetate buffer, pH 4. The deactivated charcoal was then added to this extract.

(ii) After the charcoal had adsorbed the indoles it was poured, in suspension, into small glass columns 1 cm in diameter. The partially

Fig. 29 Expt. 5 Radioautograms of one-way ascending chromatograms of extracts resulting from the decarboxylation of 5HTP produced by the incubation of brain whole homogenates with DL-tryptophan-3-C^{14} for varying times. Note the increasing intensity of the radioactive 5HT spot with time.

Incubation times: 1 = 0 mins, 2 = 15 mins, 3 = 45 mins, 4 = 75 mins, 5 = 105 mins.

constricted ends of these columns were packed with glass wool by which the charcoal was held back. The thin layer of charcoal was then washed by carefully pouring through the column 20 ml of water and the indoles eluted from the charcoal by slowly passing through the column 10 ml of 1 N acetic acid in 80% ethanol. (In control experiments 75-80% of 2 μmoles of DL-5HTP adsorbed onto 0.25 G of deactivated charcoal was eluted by this method.) The ethanolic eluate was then evaporated to dryness in a vacuum dessicator over P_2O_5, conc. H_2SO_4 and flakes of NaOH at 12 mm Hg. When dry the extract was treated by chromatography etc. as described for the dried "Phenol" eluate in assay method III.

Expt. 5 THE EFFECT OF TIME ON TRYPTOPHAN 5-HYDROXYLATION IN BRAIN

A whole homogenate of rabbit hind brain was made in Ringer phosphate solution pH 7.4 (1:2, w/v). 7ccs of this whole homogenate was incubated with DL-tryptophan-3-C^{14} 20μc/632 mμmoles (final concentration 8.2×10^{-5}M), and NSD 1024 (2.5×10^{-3}M). The total volume of the sample was 7.7 ccs. The incubation was carried out in a flask flushed with O_2 and corked, at 37°C.

1cc aliquots of the incubation were withdrawn at "zero" time, 15 mins, 45 mins, 75 mins and 105 mins. To each of these aliquots was added 4 μmoles of DL-5HTP and the samples subjected to assay method III.

Results

(a) Qualitative In figure 29 are shown the radioautograms of the chromatograms of the extracts from the enzymatic decarboxylation of the 5HTP produced. This illustrates the usefulness of having a visual check on the radioactivity of the 5HT and also shows the increasing radioactivity of the 5HT with time.

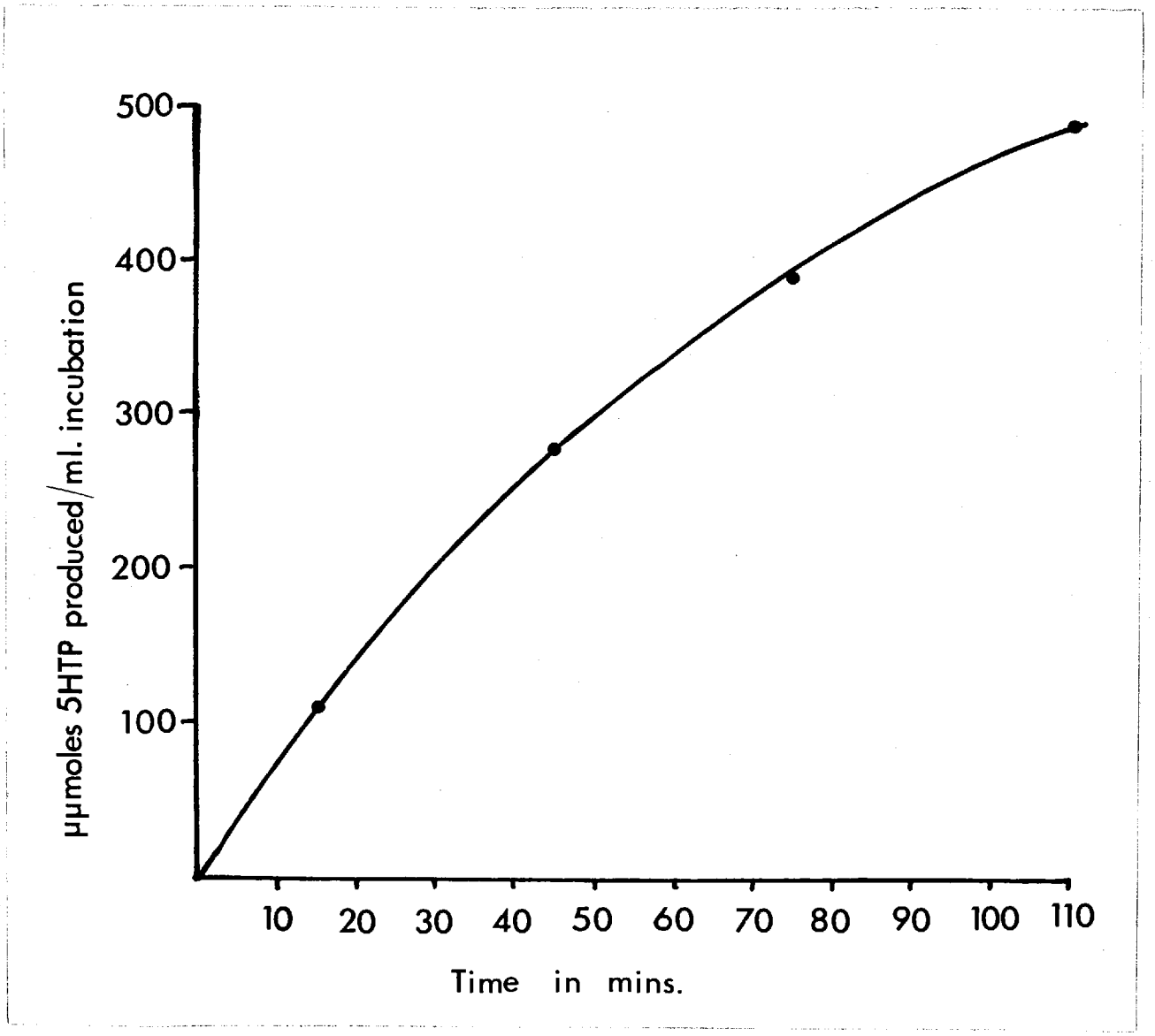


Fig. 30 Expt. 5 The effect of time on the formation of 5HTP in whole homogenates of brain. This figure shows the increasing amount of 5HTP produced with time during the incubation of brain homogenate with DL-tryptpphan-3-C^{14}. (For conditions see text).

(b) Quantitative The 5HT was eluted from the final chromatograms, its recovery estimated colorimetrically and its specific activity assayed. The amount of 5HTP produced in each 1 cc aliquot of the incubation was then calculated.

TABLE 20

The effect of time on tryptophan 5-hydroxylation in brain

Incubation time (mins)	µµmoles L-5HTP present in each 1 cc aliquot
0	0
15	111
45	282
75	386
105	494

For a graphical representation of the results see figure 30.

B THE SUBCELLULAR LOCALISATION OF TRYPTOPHAN 5-HYDROXYLASE IN BRAIN

Introduction

Techniques whereby cells can be disrupted and subcellular structures separated by centrifugation techniques are now in widespread use. Brody and Bain (1952) applied the disruptive and centrifugation techniques which had already been used with liver to separate brain whole homogenates into "Nuclear", "Mitochondrial" and "Supernatant" fractions. When Hebb and Whittaker (1958) and Whittaker (1959) began to study the distribution of acetylcholine in subcellular fractions prepared from brain they found that most of the acetylcholine was bound to particulate matter and was pharmacologically inert until released.

This suggested that a barrier was present which prevented the free diffusion of acetylcholine. These workers realised that the mechanism by which acetylcholine was bound might be of great importance in understanding how acetylcholine is stored and released during synaptic transmission in the central nervous system.

Whittaker (1963) describes how acetylcholine was found to be localised in a crude mitochondrial fraction (15,000 g) and how electron microscopic studies showed that this fraction was unlike similar fractions prepared from liver in containing particles otherthan mitochondria. The crude mitochondrial fraction was further fractionated by equilibrium centrifugation in sucrose gradients and the fractions which were removed were studied in respect of their morphology (by electron microscopy) and content of various substances (e.g. acetylcholine and 5HT). From these studies of the structure and biochemical properties of the fractions obtained and by interpreting the findings in the light of the morphology of undisrupted brain tissue, it became possible to understand the nature of the particles which make up the "Mitochondrial" fraction of brain.

When brain tissue is kept in 0.32 M sucrose solution large extracellular spaces appear, there is swelling and disintegration of cell bodies and their dendrites and shrinkage of nerve endings which appear to be pulling away from their attachments. It is easy to see how homogenisation of such material might cause shearing of the axon and release of the nerve ending as a separate particle.

Gray and Whittaker (1962) examined the heterogeneity of the brain "Mitochondrial" fraction prepared from the cerebral cortex and

resuspended in 0.32 M sucrose. This they did by layering 0.8 M sucrose on top of 1.2 M sucrose and on top of the 0.8 M sucrose they layered an aliquot of the resuspended crude mitochondrial fraction. After equilibrium centrifugation in this discontinuous sucrose gradient they found that three fractions were produced: the lightest (fraction A), floating on the 0.8 M sucrose consisted mainly of myelin fragments and the heaviest fraction (fraction C) as a pellet at the bottom of the tube, consisted mainly of mitochondria. The fraction of intermediate density, floating on the 1.2 M sucrose (fraction B), was composed of particles which had the appearance of nerve endings. They were about 1 μ across, bounded by a thin membrane within which was cytoplasm, one or more mitochondria, and small round bodies (synaptic vesicles) about 0.05 μ in diameter. The appearance of these particles when compared with the morphology of undisrupted brain tissue made it plain that they were separated nerve endings and they have now been given the name of "Synaptosomes", (Whittaker et al, 1964).

The synaptosomes have been shown to contain acetylcholine (Whittaker 1959), choline acetylase and acetylcholine esterase (De Robertis et al, 1963), 5-hydroxytryptamine (Michaelson and Whittaker, 1963) some 5HTP decarboxylase (Rodriguez et al 1964) and substance P (Ryall 1964). The position regarding noradrenaline and histamine is not yet quite clear though it has been suggested that the former has a synaptosomal localisation (Levi and Mayner 1964).

As synaptosomes contain cytoplasm it is obvious that a "Particulate" fraction containing them should be rich in components peculiar to cytoplasm. This is the case. Johnson and Whittaker (1963)

have shown that lactate dehydrogenase (LDH) can be used as a cytoplasmic marker in brain and that the synaptosomal fraction has a high content of this enzyme indicating entrapped cytoplasm. Ryall (1964) has used potassium as a cytoplasmic marker. Because one is separating synaptosomes from mitochondria it has been found that it is useful to employ a mitochondrial marker as a further check and for this reason subcellular fractions are often examined for their succinate dehydrogenase activity (SDH), an enzyme which is mitochondrial in localisation. (Singer and Kearney 1963). To be as clear as one can about the nature of the subcellular fractions prepared from homogenates of brain it is necessary to examine the structure of the particles by electron microscopy and the biochemical properties of the fractions by the enzyme marker techniques just described. The variation in the composition of fractions recovered may vary from area to area of the brain and is also dependent upon the techniques of homogenisation and preparation of the various fractions (Whittaker and Dowe 1965).

Further refinements to the techniques of the subcellular fractionation of brain have been added. Synaptosomes can be disrupted by freezing and thawing (Ryall 1963), hyposmolarity (Michaelson and Whittaker, 1963), and ultrasonic disintegration (De Lorenzo et al 1964). The properties of the synaptic vesicles, synaptosomal membrane and synaptosomal mitochondria can then be separately studied after these components have been further separated by centrifugation in sucrose density gradients. It is of interest to note that acetylcholine is bound to the synaptic vesicles (Whittaker 1959) and that

though some 5HT is bound to synaptic vesicles, this binding is more labile than that of acetylcholine (Michaelson and Whittaker 1963).

The ability to isolate synaptosomes and study their properties has led to a much greater understanding of the biochemical and pharmacological properties of the nerve ending. It is apparent however that a synaptosomal fraction must contain various types of nerve ending differing not only in structure (Whittaker and Gray 1962) but also in function. Indeed De Robertis et al (1962) have attempted to separate nerve endings containing acetylcholine from those which do not by density gradient centrifugation.

The demonstration that 5HT and 5HTP decarboxylase both have a significant synaptosomal localisation raised the question of where tryptophan 5-hydroxylase was localised. Though it is not impossible for 5HTP to be synthesised in the cell body and transported to the nerve ending where decarboxylation to 5HT takes place, it seemed quite likely that the total synthesis of 5HT took place in the nerve ending and this of course would require a synaptosomal localisation for tryptophan 5-hydroxylase. Subcellular fractionation of brain to define the localisation of tryptophan 5-hydroxylase has therefore been undertaken.

Expt. 6 THE DISTRIBUTION OF TRYPTOPHAN 5-HYDROXYLASE ACTIVITY BETWEEN THE TOTAL PARTICULATE AND SUPERNATANT MATTER OF RABBIT HIND BRAIN

Preparation of tissue, incubation and assay of tryptophan 5-hydroxylase

A rabbit hind brain was homogenised in Ringer phosphate solution pH 7.4 (1:1, w/v) in a glass tube with a teflon pestle (15 passes, 1240 r.p.m., 0.2 mms clearance). An aliquot of this whole homogenate was then centrifuged at 100,000 g for 60 mins. The resultant supernatant fraction

was decanted and kept and the particulate fraction resuspended to the original volume in Ringer phosphate solution pH 7.4. The following fractions were then incubated under the conditions stated: whole homogenate 1cc, particulate fraction 1cc, supernatant fraction 1cc, particulate fraction 0.5 cc + supernatant fraction 0.5cc. 1cc of the tissue samples was incubated with DL-tryptophan-3-C^{14} 5μc/315 mμmoles (final concentration 2.42 x 10^{-4}M) and NSD 1024 (1.5 x 10^{-3}M). The samples were incubated, gently shaking, for 1 hour at 37°C in tubes flushed with O_2 and corked. At the end of the incubation 1 μmole of DL-5HTP was added and the samples passed through assay method II up to but not including quantitative assay. The final radioautograms were examined togethr with the chromatograms stained with Ehrlich's reagent.

Results

The radioautograms of the chromatograms of the decarboxylated 5HTP are shown in figure 31. These demonstrate that radioactive 5HT is present in the whole homogenate (a), the particulate fraction (b) and in the particulate + supernatant sample (c). No radioactive 5HT was seen in the supernatant sample despite good recovery of the carrier 5HT,

Comments

Although under the conditions used here [i.e. suspension in Ringer phosphate solution and at a 1:1 (w/v) dilution) tryptophan 5-hydroxylase appeared to be wholly particulate in distribution, later experiments using more dilute suspensions of tissue in sucrose media demonstrated that some tryptophan 5-hydroxylase can be found in the

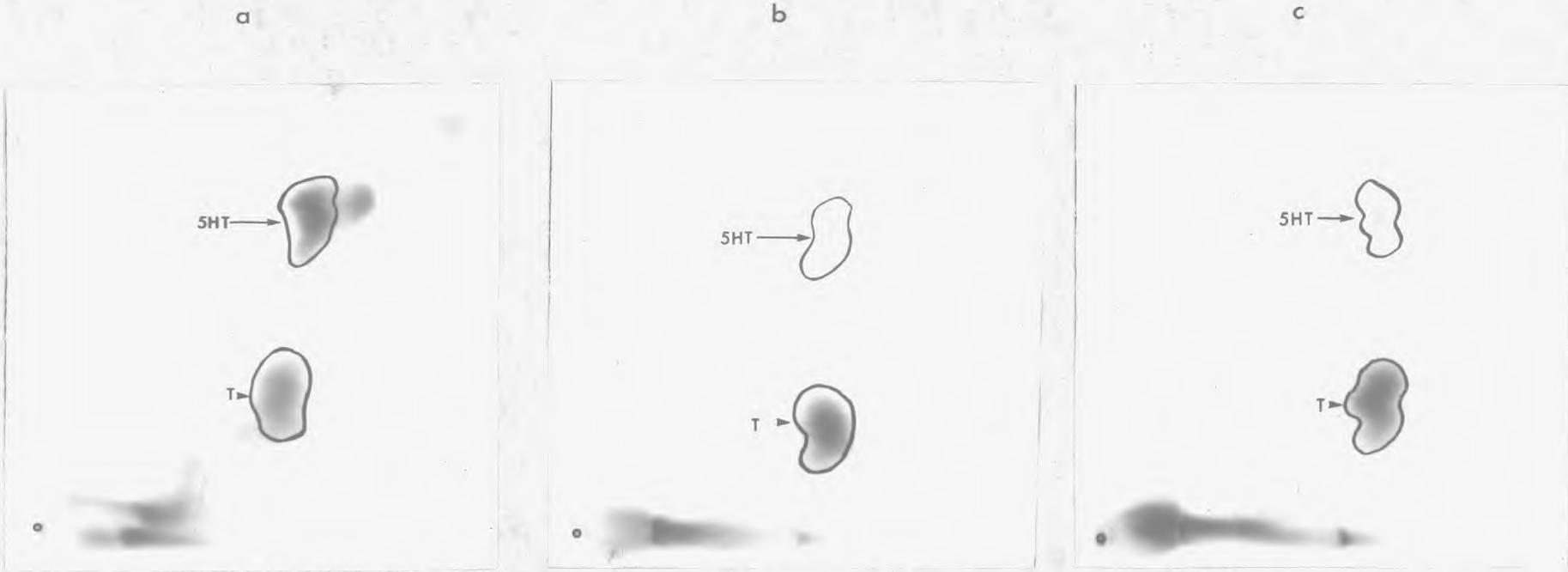


Fig. 31 Expt. 6 Radioautograms of two way paper chromatograms of extracts from the enzymatic decarboxylation of 5HTP produced by the incubation of DL-tryptophan-3-C^{14} with whole homogenate (a), 100,000 g particulate (b), and 100,000 g particulate + 100,000 g supernatant (c), fractions prepared from rabbit hind brain. Note the presence of radioactive 5HT in all three samples.

supernatant fraction. This apparent difference in the distribution of the enzyme with changing conditions of preparation will be discussed later.

Expt. 7 THE SUBCELLULAR LOCALISATION OF TRYPTOPHAN 5-HYDROXYLASE IN RABBIT HIND BRAIN

It was obvious that if the sort of precise subcellular fractionation outlined in the introduction to this section was to be carried out then homogenates of brain in sucrose media would have to be made.

Several whole homogenates made in 0.25 M and 0.32 M sucrose were inactive in respect of tryptophan 5-hydroxylase activity so that initially further examination of this problem was impossible. However Nagatsu et al (1964) reported their work on tyrosine hydroxylase, the enzyme responsible for the initial step in noradrenaline biosynthesis and they found that during purification of this enzyme from brain and beef adrenal homogenates made in 0.32 M sucrose that enzyme activity was lost but that by the addition of mercaptoethanol to such preparations enzyme activity could be restored. As it seemed quite likely that tyrosine hydroxylase and tryptophan 5-hydroxylase had similar properties, 0.001 M mercaptoethanol was added to the sucrose media in which whole homogenates of brain were made. It was then found that tryptophan 5-hydroxylase activity was restored and so further subcellular fractionation was attempted.

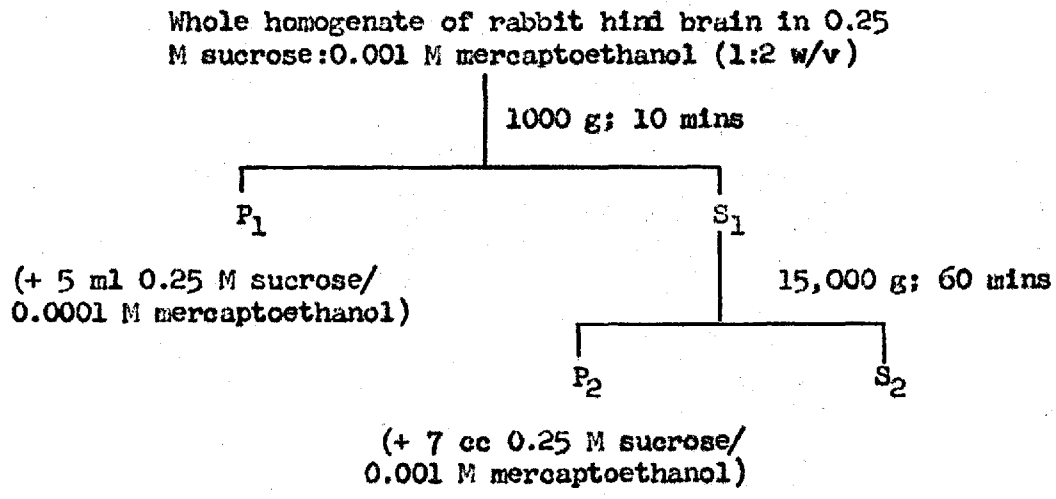
In this experiment the aim was to prepare from a whole homogenate of rabbit hind brain a "Crude mitochondrial" fraction and corresponding supernatant fraction and compare the tryptophan hydroxylase

activity.

Preparation of tissue, incubation and assay of tryptophan 5-hydroxylase

Hind brain preparations of rabbits were prepared as previously described and weighed. They were then homogenised in a glass tube with a teflon pestle (1240 r.p.m., 12 passes, clearance 0.2 mms) in 0.25 M sucrose and 0.001 M mercaptoethanol (1:2, w/v). An aliquot of the whole homogenate was then centrifuged at 1000 g for 10 minutes. The supernatant was removed with a pipette and the 1000 g particulate resuspended in 5 ml of 0.25 sucrose and 0.001 M mercaptoethanol. The 1000 g supernatant was then centrifuged at 15,000 g for 60 minutes. The supernatant resulting was removed with a pipette and the particulate fraction resuspended in 0.25 M sucrose and 0.001 M mercaptoethanol (7 ccs). Aliquots of the whole homogenate, 1000 g particulate (P_1), 1000 g supernatant (S_1), 15000 g particulate (P_2), and 15,000 g supernatant (S_2), were taken for the assay of tryptophan 5-hydroxylase activity. The total volume of each of these preparations was measured. In fig. 32 is shown the centrifugation procedure for this experiment.

Fig. 32 Partial subcellular fractionation of brain whole homogenate



In this experiment THFA, $FeSO_4$ and mercaptoethanol were added. This was done because Nagatsu et al (1964) had reported that tyrosine hydroxylase required these additions for full activity. It is doubtful whether these additions materially affected the amount of tryptophan hydroxylation occurring under these conditions. (See section on cofactor requirements, p. 146).

1 cc aliquots of whole homogenate, P_1, S_1, P_2 and S_2 were incubated with: DL tryptophan-3-C^{14} 5μc/155 mmoles (final concentration 8.5×10^{-5}M); NSD 1024 (1.5×10^{-3}M), THFA (4.1×10^{-3}M), $FeSO_4$ (2.46×10^{-4}M and mercaptoethanol (5.5×10^{-3}M). Boiled 1cc portions of whole homogenate and P_2 were identically incubated as controls.

In all of the experiments to be described incubations were carried out at 37°C in tubes flushed with O_2, corked and shaken. In this experiment the incubation was carried out for 2 hours. At the end of the incubation 4 μmoles of DL-5HTP were added and assay method III carried out.

Index of amount of tissue in each incubation sample

Protein concentrations of tissue aliquots were not estimated until later in this series of experiments. However an index of the amount of tissue in each fraction used in this experiment the dry weight of each sample was estimated. After deproteinisation the resultant precipitate was dried in a vacuum dessicator at 10 mm Hg over P_2O_5, conc. H_2SO_4 and NaOH flakes for 4 days. The tubes containing the dried precipitate were then weighed. After this the precipitate was removed from the tubes, and the tubes were carefully dried and reweighed. The differences between the initial and final

weights is taken as the dry weight of the particular sample.

Results

TABLE 21

The production of 5HTP in subcellular fractions of rabbit hind brain

Fraction	µµmoles 5HTP formed /hr/cc of fraction	µµmoles 5HTP formed /hr/mgm dry wt.	µµmoles 5HTP formed /hr/total vol.of fraction
WH	228	2.5	4560
P_1	274	1.97	2190
S_1	20	1.26	273
P_2	102	7.85	816
S_2	47	2.62	502

(WH = whole homogenate)

TABLE 22

The percentage distribution of recovered tryptophan 5-hydroxylase activity in brain subfractions and its absolute recovery from primary fractions

Primary fraction	Subfraction	% distribution of recovered tryptophan 5-hydroxylase	% recovery of tryptophan 5-hydroxylase
WH	P_1	89%	$P_1 + S_1$ = 74% WH
	S_1	11%	
S_1	P_2	62%	$P_2 + S_2$ = 356% S_1
	S_2	38%	

TABLE 23

The relative specific (RSA) of tryptophan 5-hydroxylase in each subfraction of brain

Subfraction	RSA tryptophan 5-hydroxylase
P_1	1.06
S_1	0.69
P_2	1.72
S_2	0.52

Interpretation of results

The results of all the experiments on the subcellular localisation of tryptophan 5-hydroxylase in brain have been expressed in a number of ways.

1. In Table 21 the amounts of 5HTP produced per cc of the fractions, per mg. of dry wt and by the total volume of the fractions are shown. On the basis of activity per mg of dry wt, fraction P_2 has by far the highest activity and on the basis of activity of the total volume has a higher activity than S_1. However the highest activity on the basis of total volume is seen in fraction P_1.

2. In Table 22 the absolute recovery of tryptophan 5-hydroxylase activity from the primary fractions and the percentage distribution of recovered activity in the subfractions are shown.

It is striking that the combined activity of P_2 and S_2 is 356% that of the S_1 activity. This changing activity with subcellular fractionation has been a variable finding but one suspects that in a whole homogenate of brain inhibitors of tryptophan 5-hydroxylation are present which are variably removed during fractionation. This changing activity makes the interpretation of the results of subcellular fractionation difficult because one cannot be sure whether the apparent localization of tryptophan 5-hydroxylase activity to a fraction is exaggerated by an artefactual increase in enzyme activity and of course the converse may also apply. However in these experiments the results have been taken at face value as indicating at least the qualitative subcellular localisation of tryptophan 5-hydroxylase.

To express the distribution of tryptophan 5-hydroxylase in the

various fractions as a percentage the following procedure has been adopted to overcome the changing activity during fractionation. The activities of related subfractions had been combined and taken as representing the activity of the primary fraction. The activity of the subfractions has then been expressed as a percentage of this and gives the percentage distribution of recovered tryptophan 5-hydroxylase activity.

Again, by this method of interpretation fraction P_1 is more active than S_1 and fraction P_2 more active than S_2.

3. In table 23 is shown the relative specific activity (R.S.A.) of tryptophan 5-hydroxylase in each subfraction. This is calculated in the following way:

$$\text{R.S.A.} = \frac{\%\ \text{recovered tryptophan 5-hydroxylase activity}}{\%\ \text{recovered dry weight (in expt. 9 protein recovered is used)}}$$

A relative specific activity greater than one reflects the relative concentration of activity in the particular fraction.

It is evident that fraction P_2 has the highest R.S.A. followed by P_1, S_1 and S_2 in order of decreasing activity.

Conclusions

The important point to emerge from this experiment was that a significant amount of tryptophan 5-hydroxylase activity was localised in the crude mitochondrial fraction (P_2) sedimenting at 15,000 g. In order to clarify the situation more complex centrifugation procedures were applied to the problem.

It is likely that the high activity of fraction P_1 was caused by three factors:-

(a) The initial high concentrations of the whole homogenate (1:2 w/v) protecting whole cells from rupture;

(b) The bulky particulate matter of the concentrated whole homogenate dragging down particles at1000 g which would otherwise have appeared in fraction P_2;

(c) The omission of the washing of fraction P_1.

Expts. 8 and 9 THE SUBCELLULAR LOCALISATION OF TRYPTOPHAN 5-HYDROXYLASE ACTIVITY IN RABBIT HIND BRAIN

Although there are certain methodological differences between these two experiments they will be described and interpreted together as they are designed to investigate the same problem and fundamentally they lead to the same conclusions. Briefly the differences between the two experiments are as follows:-

(a) the homogenisation technique was slightly different;

(b) the index of the amount of tissue used was mg. of dry wt. in expt. 8 and protein concentration in expt. 9;

(c) fraction S_2 was fractionated further in expt. 8;

(d) in expt. 9 the subcellular distribution of the cytoplasmic marker lactate dehydrogenase (L.D.H.) and the mitochondrial marker succinate dehydrogenase (S.D.H.) was studied;

(e) in expt. 9 electron microscopic studies were carried out.

Methods

1. Preparation of tissue In both experiments a whole homogenate of rabbit hind brain in 0.32 M sucrose + 0.001 M mercaptoethanol (1:8, w/v) was prepared in a glass tube with teflon pestle (clearance 0.2 mms). In expt. 8 the pestle was turned at 1240 r.p.m. and 12 passes of the pestle were made. In expt. 9 the pestle was

turned at 840 r.p.m. and 12 passes of the pestle were made.

2. Centrifugation techniques These follow the procedure described by Gray and Whittaker (1962) and the fractionation scheme is depicted in figure 33. Certain points must be discussed. Note that the P_1 fraction was washed twice with the sucrose medium and the washings combined with fraction S_1. Fraction P_2 (17,000 g particulate) was resuspended in 17 ccs of 0.32 M sucrose and 0.001 M mercaptoethanol and 5 cc aliquots layered on top of 10 ml of 0.8 M sucrose which ½-1 hr before had been layered on top of 1.2 M sucrose (10 ml). The lusteroid tubes in which the discontinuous sucrose gradient fractionation was carried out were centrifuged at 53,500 g for 2 hrs in a swing out head (swinging bucket rotor SW 25.1) in a spinco ultracentrifuge (Spinco Model L). At the end of centrifugation the tubes had the appearance seen in figure 34.

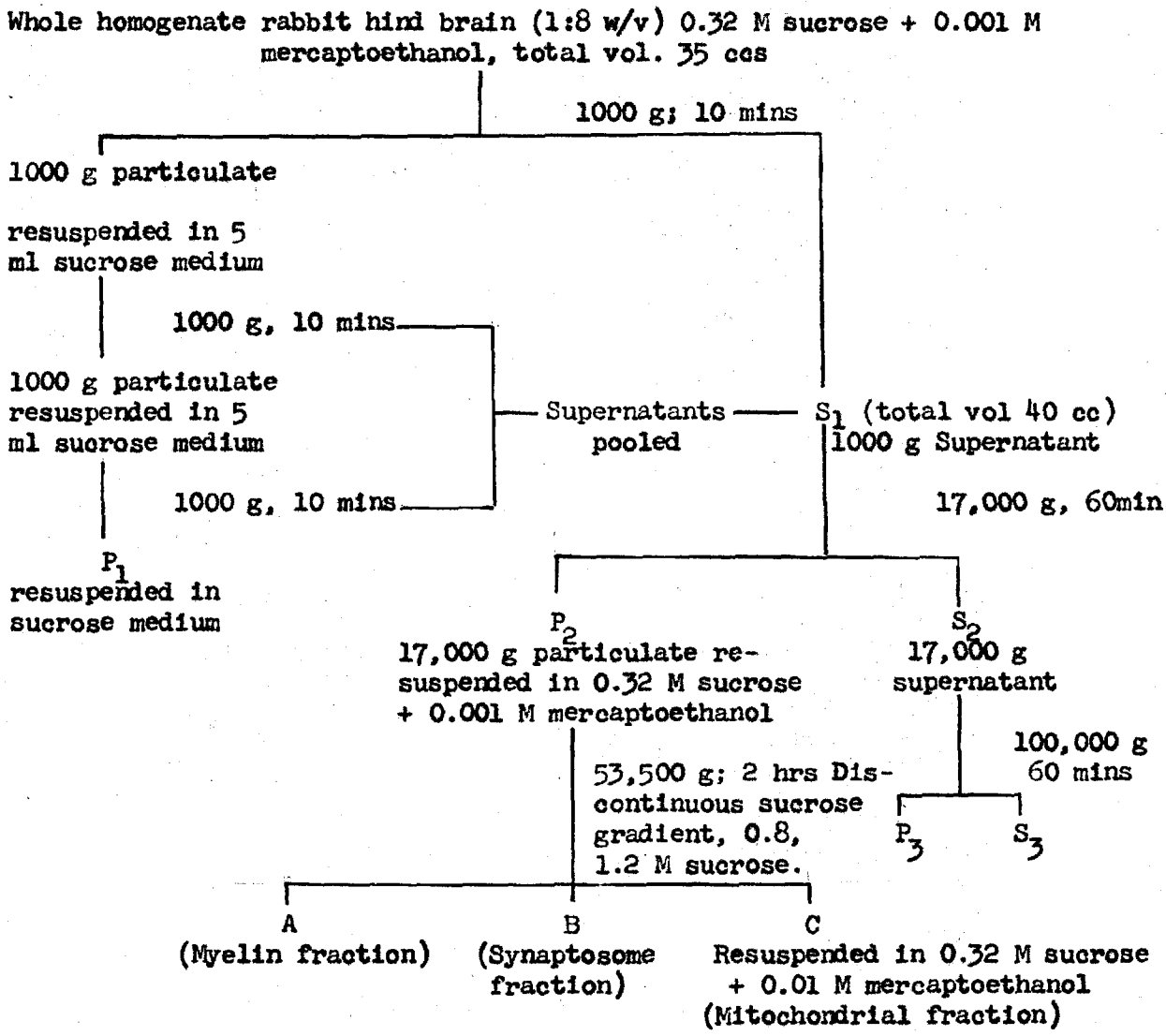
In expt. 8 the layers A and B were separately and carefully removed with a Pasteur pipette, the clear fluid above and below these layers being discarded. The A and B layers from each tube were respectively pooled and their volumes measured. In expt. 9 the layers A and B were separated by cutting the tube in a tube cutter.

The "Mitochondrial" pellet C was resuspended in 5 ccs of 0.32 M sucrose + 0.001 M mercaptoethanol. In expt. 9 fraction S_2 (17,000 g supernatant) was centrifuged at 100,000 g for 60 mins to produce a supernatant fraction (S_3) and a "Microsomal" fraction (P_3) which was resuspended in 5 cc of 0.32 M sucrose + 0.001 Mmercaptoethanol.

3. The assay of tryptophan 5-hydroxylase activity In expt. 8 the whole homogenate (W.H.) and fractions P_1, S_1, P_2, S_2, A, B and C

FIGURE 33

Scheme of subcellular fractionation of brain, expts 8 and 9



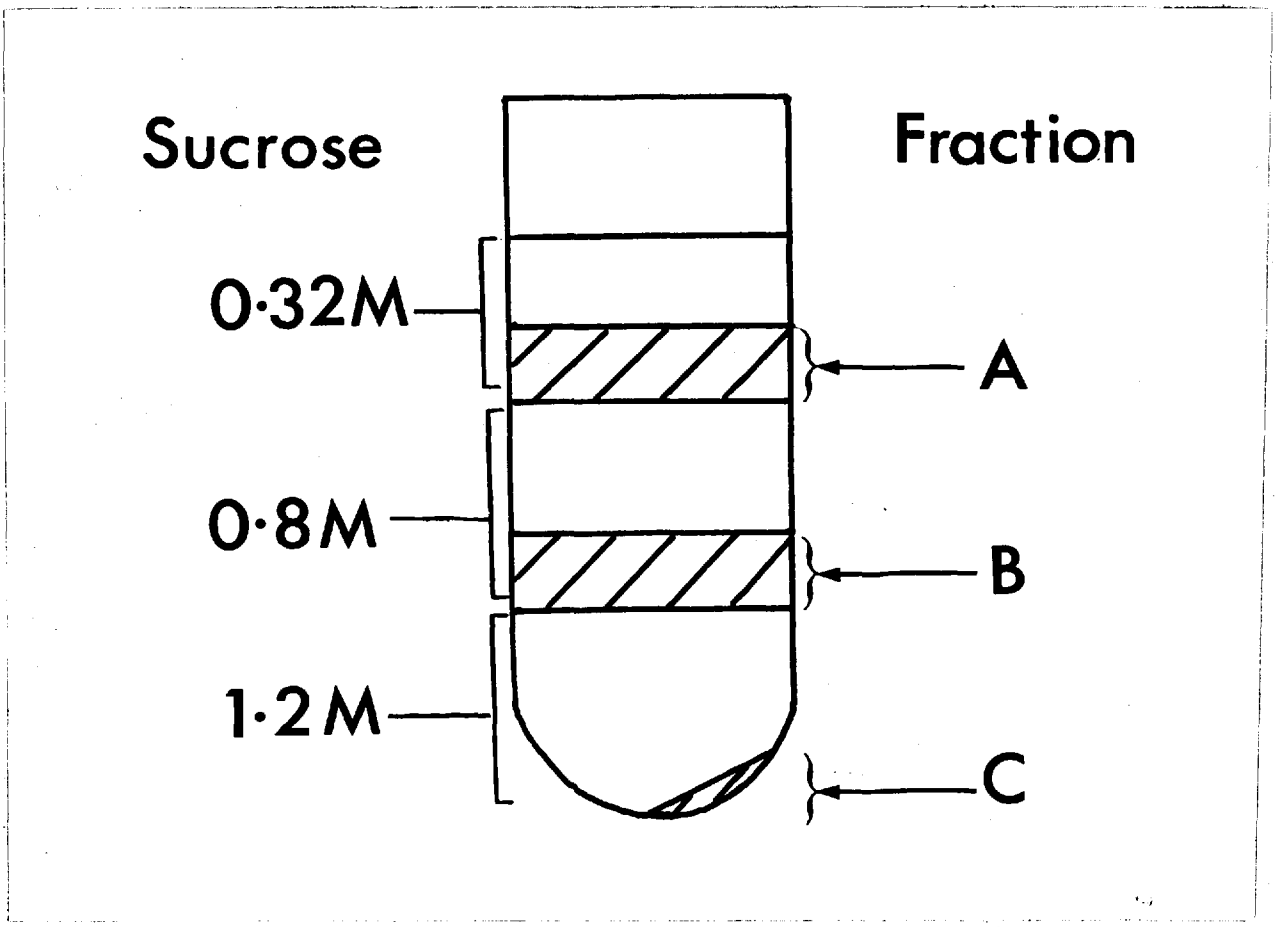


Fig. 34 Diagram illustrating the appearance of the centrifuge tube after the centrifugation of fraction P_2 on a discontinuous sucrose gradient at 53,500 g for 60 mins. Fraction A = "Myelin fraction". Fraction B = "Synaptosome fraction". Fraction C = "Mitochondrial fraction".

and in addition in expt 9 P_3 and S_3 also were all assayed for tryptophan 5-hydroxylase activity.

A 1 cc aliquot of each fraction was incubated with DL-tryptophan-3-C^{14} (5 μc/155 mμmoles, final conc. 1.19×10^{-4}M); NSD 1024 (1.5×10^{-3}M); and potassium phosphate buffer pH 7 (7.7×10^{-3}M). The samples were incubated for 1 hr at 37°C in an atmosphere of O_2 in a metabolic shaker. At the end of incubation 4 μmoles of DL-5HTP were added and the samples carried through assay method III (for expt. 8) and assay method IV (for expt. 9).

Index of amount of tissue used for assays In expt. 8 the dry wt of each sample was estimated as described in expt. 7. In expt. 9 the protein concentration of suitably diluted aliquots of each fraction was estimated by the method described by Lowry et al (1951), using a fresh solution of bovine serum albumin as a standard.

The assay of Lactate dehydrogenase (LDH) activity In expt. 9 each subfraction was assayed for IDH activity by the method described by Kornberg (1955). Known aliquots of each fraction were taken and suitably diluted for assay. Three concentrations of tissue from each fraction were assayed and the average activity calculated. The change in optical density at 340 mμ which occurs as NADH is oxidised to NAD during the reduction of pyruvate to lactate by lactate dehydrogenase was measured over an interval of 3 mins in a recording spectrophotometer. Assays were carried out at room temperature. Results are calculated on the basis of units of optical density change (Δ O.D.) in 100 secs/unit of tissue. Controls without the addition of pyruvate acted as the blank.

The assay of succinate dehydrogenase (SDH) activity (succinate-tetrazolium reductase). In expt. 9 each subfraction was assayed for SDH activity by the method described by Pennington (1961). This assay depends upon the reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) with the production of a coloured formazan compound as succinate is oxidised by succinic dehydrogenase, INT acting as an electron acceptor. The reduction of INT is followed by extracting the formazan dye into ethyl acetate and measuring the optical density of the extract at 490 mμ. Aliquots of the fractions were suitably diluted for the assay and three different concentrations of tissue assayed from which an average value for SDH activity was calculated. The results were calculated on the basis of the optical density of the formazan extract at 490 mμ produced after 15 mins incubation at 37°C. Controls without the addition of succinate acted as blanks.

Electron microscopic studies Electron microscopic examination of fractions A, B and C were performed by negative staining techniques (Horne and Whittaker) and tissue sectioning

Results

(see Tables 24-27)

TABLE 24

1. The production of 5HTP in subcellular fractions of rabbit hind brain

Fraction	μmoles 5HTP produced/hr/ unit of tissue*		μmoles 5HTP produced/hr/total vol. of fraction	
	Expt. 8	Expt. 9	Expt. 8	Expt. 9
WH	10.9	116	16,300	4320
P_1	0.9	35	2,140	464
S_1	25.8	194	10,320	2688
P_2	17.6	148	5,720	1125
S_2	23,2	102	5,470	1470
A	55.5	244	6,987	280
B	112	410	7,775	315
C	32	155	1,440	130
P_3	-	27	-	82
S_3	-	88	-	1050

* Unit of tissue - Expt 8 = mg dry wt; Expt 9 = mg protein.

TABLE 25

2. The percentage distribution of recovered tryptophan 5-hydroxylase activity in brain subfractions and its absolute recovery from primary fractions

Primary fraction	Subfraction	% distribution of recovered tryptophan 5-hydroxylase		% recovery of tryptophan 5-hydroxylase	
		Expt. 8	Expt. 9	Expt. 8	Expt. 9
WH	P_1	17	15	77	73
	S_1	83	85		
S_1	P_2	51	44	108	97
	S_2	49	56		
P_2	A	43	38	301	65
	B	48	44		
	C	9	18		
S_2	P_3	-	7	-	77
	S_3	-	93		

TABLE 26

3. The relative specific activity (R.S.A.) of tryptophan 5-hydroxylase in each subfraction of brain

Subfraction	R.S.A. tryptophan 5-hydroxylase Expt. 8	Expt. 9
P_1	0.2	0.31
S_1	5.5	1.67
P_2	0.88	1.26
S_2	1.2	0.86
A	0.83	0.93
B	1.7	1.57
C	0.47	0.58
P_3	-	0.28
S_3	-	1.27

TABLE 27

The percentage distribution of recovered LDH and SDH activity and the R.S.A.s of LDH and SDH in brain subfractions (Expt. 9)

Primary fraction	Subfraction	% distribution of recovered LDH	SDH	R.S.A LDH	SDH
WH	P_1	18	57	0.36	1.16
	S_1	82	43	1.6	0.85
S_1	P_2	24	100	0.69	2.86
	S_2	76	0	1.17	0
P_2	A	22	6	0.54	0.16
	B	45	10	1.61	0.34
	C	33	84	1.06	2.7

Interpretation of results

1. Primary fraction WH and subfractions P_1 and S_1

(a) Tryptophan 5-hydroxylase activity The recovery of tryptophan 5-hydroxylase in the combined fractions P_1 and S_1 is 77% (Expt.8) and 73% (Expt.9) of the whole homogenate activities (Table 25). Examination of the distribution of the recovered tryptophan 5-hydroxylase activity shows that an average of 16% (±1) is recovered in fraction P_1 and 84% (±1) in fraction S_1 (Table 25). A similar distribution of activity is also seen in table 24 where the absolute amounts of 5HTP produced are shown.

(b) LDH activity (Expt.9) Examination of the distributions of LDH in fractions P_1 and S_1 (Table 27) shows that 18% of the LDH activity lies in fraction P_1 and 82% in fraction S_1. The R.S.A.s of LDH and tryptophan 5-hydroxylase are likewise closely related in these two fractions (Tables 26 and 27, expt. 9).

(c) SDH activity (Expt.9) The percentage distribution of SDH activity (Table 27) and the R.S.A. of SDH are quite different from those of tryptophan 5-hydroxylase and LDH, both being much higher in fraction P_1 than S_1.

2. Primary fraction S_1 and subfractions P_2 and S_2

(a) Tryptophan 5-hydroxylase activity Although there is some difference in the distribution of tryptophan 5-hydroxylase between fractions P_2 and S_2 in the two experiments nevertheless by all the methods of expressing the results we see that tryptophan 5-hydroxylase activity is fairly evenly split between these two fractions, P_2 possessing an average of 48.5% (±2.5%) of the activity and S_2, 52.5% (±3.5%).

(b) IDH activity (Expt.9) As might be expected in the case of a cytoplasmic marker, most of the IDH activity lies in fraction S_2, but it is important to note that 24% of the activity lies in fraction P_2, representing entrapped cytoplasm in this fraction (Table 27). It is interesting also that the R.S.A. of tryptophan 5-hydroxylase in fraction P_2 is greater than that of IDH, denoting a greater concentration of the hydroxylase in the entrapped cytoplasm of fraction P_2, than in the free cytoplasm of fraction S_2.

(c) SDH activity When the distribution of SDH activity and the R.S.A. of SDH in the various fractions is examined (Table 27), a striking fact emerges. Whereas tryptophan 5-hydroxylase activity is fairly evenly spread between fractions P_2 and S_2, SDH activity is solely localised to fraction P_2. If we assume for the moment only one subcellular localisation for tryptophan 5-hydroxylase then this dissociation between the activity of SDH and tryptophan 5-hydroxylase in fractions P_2 and S_2 is evidence against the tryptophan 5-hydroxylase activity of fraction P_2 being located in the mitochondria of that fraction.

3. Primary fraction P_2 and subfractions A, B and C

(a) Tryptophan 5-hydroxylase activity In tables 24, 25 and 26 we see that by all the methods of expression a consistent pattern emerges. Fraction A ("Myelin fraction") contains an average of 40.5% (±2.5%) of the activity, fraction B ("Synaptosome fraction") 46% (±2%) and fraction C ("Mitochondrial fraction") 13.5% (±4.5%). The average R.S.A. of tryptophan 5-hydroxylase in fraction A is 0.88 (±0.05), in fraction B 1.635 (± 0.065) and in fraction C 0.525 (±0.055).

These results show that tryptophan 5-hydroxylase is mainly localised in the synaptosomal fraction B prepared from the crude mitochondrial fraction P_2.

(b) IDH activity (Expt. 9) Table 27 shows that fraction B has the highest concentration and R.S.A. of IDH. It should be noted however that the activity of IDH is lower in fraction A and higher in fraction C than tryptophan 5-hydroxylase activity (Table 25). Nevertheless there is no doubt that the distribution of tryptophan 5-hydroxylase in these three fractions fits the distribution of IDH far better than it fits that of SDH.

(c) SDH activity There is little activity of SDH in fractions A and B, 84% of the activity being located in fraction C ("Mitochondrial fraction") (Table 27). It is apparent again that tryptophan 5-hydroxylase activity and SDH are quite dissociated in these subcellular fractions.

4. Primary fraction S_2 and subfractions P_3 and S_3

(a) Tryptophan 5-hydroxylase activity Tables 24, 25 and 26 show that very little tryptophan 5-hydroxylase activity resides in fraction P_3 ("Microsomal fraction"), 93% of the activity being present in the high speed supernatant fraction, S_3

5. Electron microscopic studies

Fraction A (Fig. 35) This fraction consisted mainly of myelin fragments and its appearance was similar to that which might have been expected had the fraction been prepared from cortical tissue, i.e. a typical "Myelin" fraction.

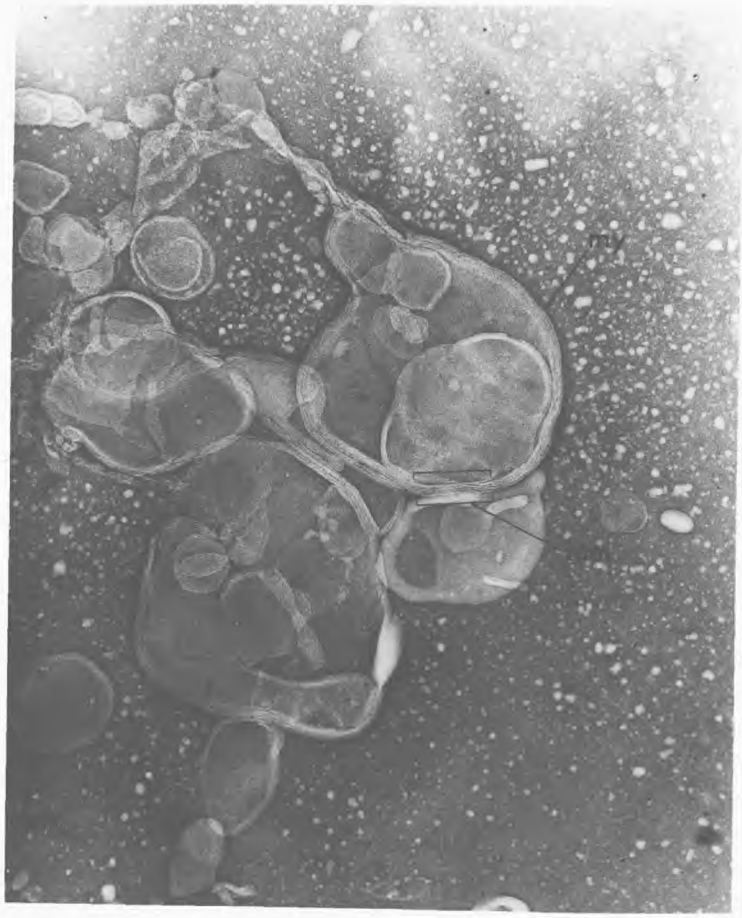


Fig. 35 Electron micrograph of negatively stained preparation of fraction A. Note the myelin fragments (My) and the laminated appearance of the myelin (lam).

Magnification = x 3,500

Fraction B Unfortunately the electron micrographs of this fraction did not show good preservation, probably due to damage during processing. From the appearances of fraction A where myelin fragments predominated and the appearances of fraction C where mitochondria predominated one would suspect that this fraction B was a fairly average preparation containing a predominance of synaptosomes. In addition the high LDH activity and low SDH activity of this fraction is supportive evidence for this view.

Fraction C (Fig. 36) Although this fraction contained a predominance of mitochondria both on the basis of its electron microscopic examination and high SDH activity, nevertheless the fraction did contain a few very large synaptosomes, not normally seen in fraction C prepared from cortical tissue. These large synaptosomes may account for the rather high IDH activity of this fraction which is higher than one would expect for a C fraction prepared from cortical tissue. On the other hand the tryptophan 5-hydroxylase activity of this fraction was quite low and it is tempting therefore to speculate that perhaps these large synaptosomes do not contain tryptophan 5-hydroxylase.

Conclusions

1. It is apparent that tryptophan 5-hydroxylase activity is not associated with the mitochondria in any of the fractions studied.

2. However tryptophan 5-hydroxylase activity is located in the crude mitochondrial fraction P_2, (17,000 g particulate fraction). Fractionation of this material on a discontinuous sucrose gradient has shown that the synaptosomal fraction contains most of the tryptophan 5-

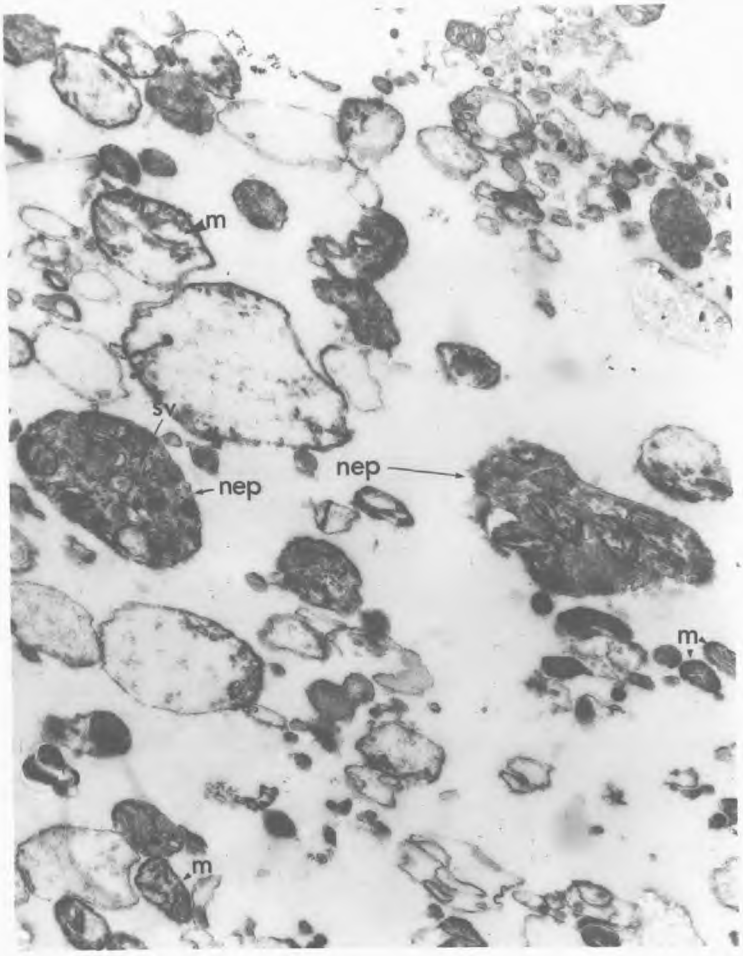


Fig. 36 **Electron micrograph of tissue section of a pellet of fraction C. Note the presence of mitochondria (m) and the large synaptosomes (N.E.P) and the synaptic vesicles (sv) and mitochondria within the synaptosomes.**

Magnification = x 17,500

Preparative method Pellet of tissue fixed in potassium permanganate solution by the method of Lufts (1956). Dehydration and embedding carried out as described by Robertson et al (1963). Embedded reactions stained with lead hydroxide solution as described by Millonig (1961)

Lufts J.H. (1956) J. Biophys. Biochem. Cytol. 2, 799.
Robertson J.D., Bodenheimer T.S. and Stage D.E. (1963) J.Cell Biol.19, 159
Millonig G. (1961) J. Biophys. Biochem. Cytol. 11, 736

hydroxylase activity and suggests therefore that tryptophan 5-hydroxylase is present in some nerve endings in the rabbit hind brain. 3. One cannot be certain however that all the tryptophan 5-hydroxylase in the rabbit hind brain is located in nerve endings. Although it is tempting to speculate that the tryptophan 5-hydroxylase activity seen in the high speed supernatant fraction has been released from synaptosomes ruptured during preparations one cannot exclude that it may have come from the cytoplasm of ruptured nerve cell bodies. If one could show that as the yield of synaptosomes increased, supernatant tryptophan 5-hydroxylase activity diminished and that with very high synaptosomal yields there was very little tryptophan 5-hydroxylase activity in the supernatant, then there might be some justification in assuming that the synaptosome was the sole localisation of tryptophan 5-hydroxylase. There is some indirect evidence on this point. In expt. 6, no tryptophan 5-hydroxylase activity was found in the supernatant fraction (100,000 g), in expt. 7 about 25% of the activity was in the supernatant fraction and in expts 8 and 9 about 40%. The initial concentration of the whole homogenate in expt. 6 was 1:1 w/v, in expt. 7 1:2 w/v, and in expts 8 and 9 1:8, w/v. It therefore appears that as the initial concentration of the whole homogenate diminishes so the amount of tryptophan 5-hydroxylase in the supernatant rises. This suggests that some property of very concentrated whole homogenates is protecting some part of the nerve cell from rupture, and one suspects that the nerve endings are more likely to escape rupture than the large cell bodies. If one accepts this it would be evidence for the nerve ending particle being the sole localisation for tryptophan 5-hydroxylase.

4. No studies have yet been done on the localisation of tryptophan 5-hydroxylase within the nerve ending particle but the finding of about 50% of the activity in the supernatant fraction under isosmolar conditions is suggestive that tryptophan 5-hydroxylase is situated in the cytoplasm of the nerve ending and not bound to the intraterminal mitochondria as was previously suggested (Grahame-Smith and Moloney 1965).

These studies have therefore demonstrated that some nerve endings contain tryptophan 5-hydroxylase and as 5-hydroxytryptamine (Michaelson and Whittaker 1963) and 5-hydroxytryptophan decarboxylase (Rodriguez et al 1964) are also found in the nerve ending particle it is suggested that certain nerve endings in certain areas of the brain possess the enzymes necessary for the complete biosynthesis of 5HT. The significance of this will be discussed in the final discussion.

C THE PARTIAL PURIFICATION AND PROPERTIES OF TRYPTOPHAN 5-HYDROXYLASE IN BRAIN

Expt. 10 and 11 The effect of tetrahydropteridines on tryptophan 5-hydroxylation in whole homogenates of rabbit hind brain

The requirement for full activity of both phenylalanine 4-hydroxylase (Kaufman 1959) and tyrosine hydroxylase (Nagatsu et al 1964) has been shown to be a reduced pteridine. It seemed possible that tryptophan 5-hydroxylase would also require a similar cofactor and so estimations of tryptophan 5-hydroxylase activity in whole homogenates of rabbit brain with and without the addition of reduced pteridines was carried out.

Methods

In expt. 10 the effect of tetrahydrofolic acid was studied and in expt. 11 the effect of 6,7-dimethyl 5,6,7,8-tetrahydropteridine ($DMPH_4$). In both experiments whole homogenates of rabbit hind brain were made in 0.25 M sucrose and 0.001 mercaptoethanol, 1:2, w/v in expt. 10 and 1:3, w/v, in expt. 11. 1 cc of these whole homogenates was incubated with DL-tryptophan-3-C^{14} (5μc/155 mμmoles:final conc 8.5×10^{-5}M) and NSD 1024 (1.5×10^{-3}M) and potassium phosphate buffer pH 7.4, (5×10^{-2}M). When tetrahydrofolic acid was added the final concentration was 4.1×10^{-3}M and for $DMPH_4$ 3.54×10^{-3}M. All incubations were carried out for 1 hr at 37°C in O_2 and at the end of the incubation 2 μmoles of DL-5HTP were added and the tryptophan 5-hydroxylase activity estimated by assay method IV.

TABLE 28

The effect of tetrahydropteridines on tryptophan 5-hydroxylation in whole homogenates of rabbit hind brain (Expts.10 and 11)

Expt.	Conditions	μμmoles 5HTP produced/ hr/cc of whole homogenate
10	-THFA	459
	+THFA	228
11	-$DMPH_4$	70
	+$DMPH_4$	69

Discussion

These experiments were repeated several times and on no occasion was an increase of tryptophan 5-hydroxylase activity observed when either THFA or $DMPH_4$ was added to whole homogenates of brain, nor was an increase in activity seen when in addition to these pteridines, Fe^{++}, mercaptoethanol, NADPH and NADPH regenerating systems were added. As this series of experiments was carried out with concentrated whole homogenates (i.e. 1:1 - 1:3 w/v) and as the subcellular fractionation studies showed that under these conditions nearly all of the tryptophan 5-hydroxylase activity is particulate bound probably within the nerve ending particle, then if the pteridines were in fact cofactors two possibilities arose: (1) That within the nerve ending particle there was an optimum concentration of the pteridine cofactor or (2) That the pteridine cofactor under these conditions did not easily crosss the nerve ending membranes.

Because of this various methods were used to disrupt the synaptosomes after which the tryptophan 5-hydroxylase activity of such preparations was assayed with and without the addition of reduced pteridines. Johnson and Whittaker (1963) demonstrated that hypoosmolar conditions disrupt the synaptosomes. Feldberg (1945) showed that bound choline acetylase was activated by ether treatment of brain preparations and Hebb and Smallman (1956) that choline acetylase is present in isoosmolar homogenates of brain in an occluded form. One presumes that ether treatment of brain preparations breaks the occluding membrane of the synaptosome and allows the precursors of acetylcholine more ready access to choline acetylase resulting in an increased rate of acetylcholine synthesis.

Expt. 12 The effect of "Hypoosmolarity", ether treatment, and ultrasonication upon the tryptophan 5-hydroxylation activity of brain whole homogenates with and without the addition of $DMPH_4$

Methods

1. Hypoosmolarity A rabbit hind brain was homogenised in 0.01 M potassium phosphate buffer (pH 7) and the homogenate stood for 20 mins at 5°C.

2. Ether treatment An aliquot of the above preparation was then added to peroxide free ether (0.8 cc ether/1 cc homogenate), shaken for 3 mins and stored at 5°C for 10-15 mins after which air was bubbled through the mixture at 4°C for 1 min to remove the ether.

3. Ultrasonication A whole homogenate of brain was made in isotonic Ringer phosphate solution, PH 7.4, + 0.002 M mercaptoethanol. An aliquot was subjected to ultrasonication for 2 mins at 20 kc/s in an

M.S.E. ultrasonic disintegrator (Model 60 W). The tryptophan 5-hydroxylase activity of the "Hypoosmolar" whole homogenate, the ether treated whole homogenate and the whole homogenate before and after ultrasonication was assayed with and without the addition of $DMPH_4$ as indicated in the results. The basic incubation mixture contained the following: 1 cc of the sample to be studied, DL-tryptophan-3-C^{14}, (5μc/151 mμmoles, final conc. 5×10^{-5}M,) NSD 1024 (1.5×10^{-3}M) and potassium phosphate buffer (3.3×10^{-2}M).

Additions When $DMPH_4$ was added it was added together with the system shown for the reduction of NADP: $DMPH_4$ (1×10^{-3}M), NADPH (3.3×10^{-4}M), glucose 6-phosphate (1.3×10^{-6}M), glucose-6-phosphate dehydrogenase (I.E.U.), nicotinamide (5×10^{-3}M) and $MgCl_2$ (3.3×10^{-4}M). All incubations were carried out at 37°C for 1 hr in an atmosphere of O_2. Tryptophan 5-hydroxylase activity was estimated by assay method IV.

Results

TABLE 29

The effect of various treatments of brain homogenate upon its tryptophan 5-hydroxylase activity and the influence of $DMPH_4$

Preparation	Additions	μμmoles 5HTP produced/ hr/cc of preparation
Hypoosmolar whole homogenate	None	0
Hypoosmolar whole homogenate	+ $DMPH_4$	78
Ether treated whole homogenate	+ $DMPH_4$	39
Untreated whole homogenate	None	80
Untreated whole homogenate	+ $DMPH_4$	90
Ultrasonicated whole homogenate	+ $DMPH_4$	123

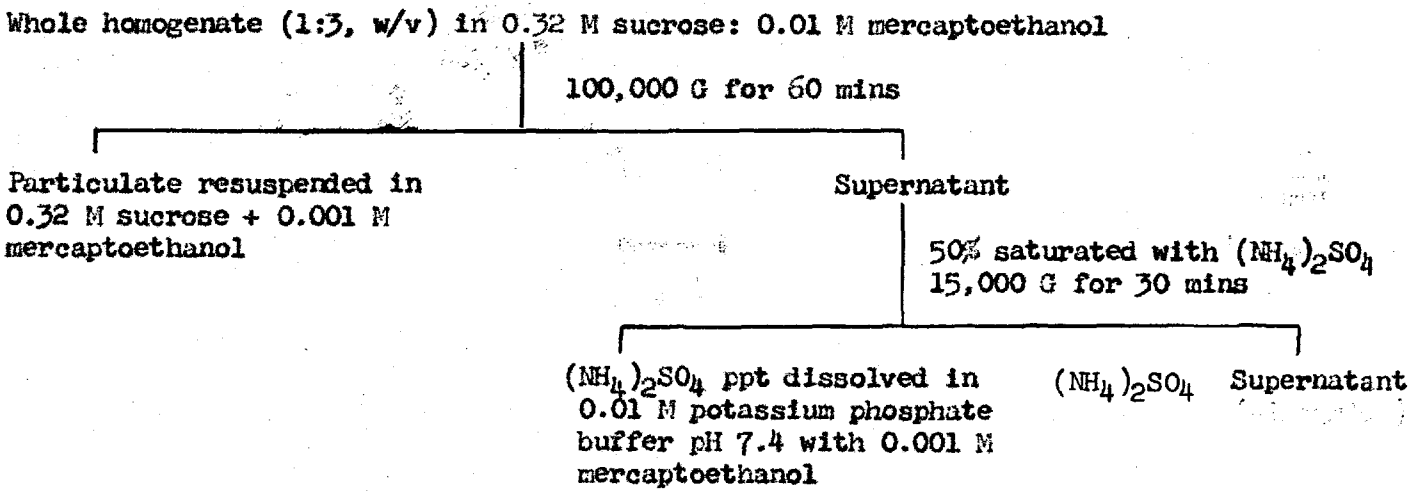
Discussion

Although the hypoosmolar whole homogenate to which no $DMPH_4$ has been added has no activity, this is probably due to the fact that no mercaptoethanol has been added to this preparation which Huennekens et al (1964) have shown stabilises tetrahydropteridines. Therefore any endogenous tetrahydropteridine would be unstable under these experimental conditions. We can see however that the addition of $DMPH_4$ to both hypoosmolar and ether treated homogenates increased the tryptophan hydroxylase activity. The isotonic whole homogenate (to which mercaptoethanol was in fact added) has activity which is only slightly increased by $DMPH_4$, however after ultrasonication a larger increase in activity is seen upon the addition of $DMPH_4$. These results show that under conditions in which the nerve endings would be expected to be intact little stimulation of activity is seen upon the addition of $DMPH_4$. After treatment calculated to rupture the membranes of the nerve ending particles however, $DMPH_4$ is seen to stimulate tryptophan 5-hydroxylation. These findings are compatable with the hypothesis that tryptophan 5-hydroxylase is situated within the nerve ending particle where either it is surrounded by an optimal concentration of pteridine cofactor or occluded from added cofactor by the membrane of the nerve ending.

Although these experiments suggested that tryptophan 5-hydroxylase required a reduced pteridine for full activity the results using treated whole homogenates were not very clear cut and so an attempt was made to partially purify tryptophan 5-hydroxylase from brain tissue in the hope that it would be possible to completely dissociate it from its cofactor and show more marked stimulation of activity on adding back the cofactor

Figure 37

Scheme of partial purification of tryptophan 5-hydroxylase from brain



or a substitute (i.e. $DMPH_4$)

Expt.13 THE PARTIAL PURIFICATION OF TRYPTOPHAN 5-HYDROXYLASE FROM BRAIN

Purification procedure, incubation and assay of tryptophan 5-hydroxylase

Two rabbit hind brains were homogenised in 0.32 M sucrose + 0.001 M mercaptoethanol (1:3, w/v). This whole homogenate was then subjected to ultrasonication for 3 mins (20 kc/s). The ultrasonicated whole homogenate was centrifuged at 100,000 g for 60 mins. The particulate material resulting was suspended in 0.3 M sucrose + 0.001 M mercaptoethanol. Ammonium sulphate was added to the 100,000 g supernatant to give 50% saturation (Dixon and Webb 1960). This solution was allowed to stand with an occasional shake for 20 mins and then it was centrifuged at 15,000 g for 30 mins. The supernatant material was decanted. The ammonium sulphate precipitate was dissolved in 10 cc of 0.01 M potassium phosphate buffer pH 7.4 + 0.001 M mercaptoethanol. Dialysis of an aliquot was carried out against 0.001 M potassium phosphate buffer pH 7.4 + 0.001 M mercaptoethanol for 12 hrs at 4°C. (See figure 37 for purification scheme). The volume of each fraction was measured during this process. Protein concentrations of each fraction were estimated by the method described by Lowry et al (1951).

1 cc of each preparation was incubated with DL-tryptophan-3-C^{14} (5μc/151 mμmoles), NSD 1024 (1.5×10^{-3}M), potassium phosphate buffer (7.7×10^{-2}M). The total volume of the incubation was 1.3 ml. $DMPH_4$ was added as indicated in table 30. Incubations were carried out in O_2 at 37°C for 1 hr and tryptophan 5-hydroxylase activity was estimated by assay method III.

Results These are tabulated in Table 30

TABLE 30

The partial purification of tryptophan 5-hydroxylase from brain

Preparation	Addition	Vol ccs	Activity* /ml/hr	Total activity/hr†	Protein conc/ml	Activity+/mg protein/hr		Yield	Purification
Whole homogenate	-	28	288	8050	2.9	99.5		100	1
100,000 g supernatant	-	14	41.6	583	2.6	16		7.25	0.16
100,000 g particulate	-	20	226	4520	1.2	188		56	1.89
$(NH_4)_2SO_4$ precipitate	-	10	0	0	0.6	0		0	0
$(NH_4)_2SO_4$ supernatant	-	20	0	0	0.2	0		0	0
$(NH_4)_2SO_4$ precipitate	$+DMPH_4$	10	101	1010	0.6	168	(WH) (S)	12.5^{α} 174^{α}	1.69^{α} 10.5^{α}

* μμmoles 5HTP produced/ml/hr † μμmoles 5HTP produced whole fraction/hr

\+ μμmoles 5HTP produced/mg protein/hr

α The yields and purifications of this fraction are expressed here in two ways: (a) In respect of the whole homogenate (WH); (b) in respect of the 100,000 g supernatant (s)

After storage for 16 days at -10°C the $(NH_4)_2SO_4$ precipitate solution was dialysed and its activity compared with the undialysed material, with and without the addition of $DMPH_4$. The results are shown in table 31.

Expts. 14 and 15

Two further partial purifications of tryptophan 5-hydroxylase from brain were performed and the activity of various fractions assayed. The activity of these fractions and the effect of $DMPH_4$ are shown in table 32 where the results of these experiments are compared with those of expt. 13.

Comments

1. In expt. 13 (Table 30) ultrasonication appeared to be relatively inefficient in releasing particulate bound tryptophan 5-hydroxylase since only 13% of the recovered activity was found in the 100,000 g supernatant. This apparent low activity of the supernatant may have been caused by the ommission of $DMPH_4$ to the assay mixture. In Expt. 14 (Table 32) where activities are throughout rather low, ultrasonication appears to have released all the particulate bound tryptophan 5-hydroxylase activity, the 100,000 g supernatant on this occasion being assayed with the addition of $DMPH_4$.

2. No activity was found in the $(NH_4)_2SO_4$ supernatant irrespective of whether $DMPH_4$ was added or not.(Table 32).

3. The striking finding in these experiments was the tryptophan 5-hydroxylase activity of the $(NH_4)_2SO_4$ precipitate and the marked stimulation of this activity by $DMPH_4$ (Table 30 and 32). This stimulation of activity was not seen in boiled samples of the $(NH_4)_2SO_4$ precipitate

TABLE 31

The effect of dialysis on tryptophan 5-hydroxylase partially purified from brain

Preparation	Addition	μμmoles 5HTP produced /hr/mgm protein
Undialysed	-	0
Undialysed	$DMPH_4$	285
Dialysed	-	0
Dialysed	$DMPH_4$	143

TABLE 32

Comparison of the results of the partial purification of tryptophan 5-hydroxylase from brain

Preparation	Tryptophan 5-hydroxylase activity μμmoles 5HTP produced/mg protein/hr				
	Expt 13	Expt 13	Expt 14	Expt 15	Expt 15
Whole homogenate					
$-DMPH_4$	99	-	-	-	-
$+DMPH_4$	-	-	8.6	-	-
100,000 g particulate					
$-DMPH_4$	188	-	-	-	-
$+DMPH_4$	-	-	0	-	-
100,000 g supernatant					
$-DMPH_4$	16	-	-	-	-
$+DMPH_4$	-	-	49	-	-
$(NH_4)_2SO_4$ precipitate					
$-DMPH_4$	0	-	0	-	-
$+DMPH_4$	168	285	51.8	-	31 (stored 1 month)
$(NH_4)_2SO_4$ precipitate (Dialysed) $-DMPH_4$	-	0	-	94	-
$+DMPH_4$	-	143	-	333	76 (stored 1 month)
$(NH_4)_2SO_4$ supernatant					
$-DMPH_4$	0	-	-	-	-
$+DMPH_4$	-	-	0	-	-

Final conc. $DMPH_4$ Expt 13 = 2.4×10^{-4}M, Expt 14 = 1.84×10^{-4}M, Expt 15 = 3.7×10^{-3}M.

- = Not estimated

solution and is strongly suggestive that $DMPH_4$ is acting as a co-factor in the enzymatic 5-hydroxylation of tryptophan. In expt. 13 (Table 30) it will be seen that the $(NH_4)_2SO_4$ precipitate was completely inactive until $DMPH_4$ was added suggesting that the enzyme may be completely dependent upon a reduced pteridine cofactor for activity. The role of reduced pteridine cofactors in tryptophan 5-hydroxylase is further considered in the final discussion.

4. Dialysis of the $(NH_4)_2SO_4$ precipitate solution in Expt. 13 appeared to decrease the activity by 47%, though the dependence of activity upon $DMPH_4$ is again well illustrated (Table 31). However dialysis of stored material in expt. 14 increased activity by 145%.

5. Storage for 16 days at -10°C did not appear to greatly affect the activity of the $(NH_4)_2SO_4$ precipitate but storage for 1 month at -10°C led to a 77% decrease in activity (Table 32).

6. The yield of tryptophan 5-hydroxylase activity from the whole homogenate in Expt. 13 was rather poor (12.5) but better in respect of the 100,000 g supernatant fraction (174). The purification in terms of the 100,000 g supernatant was 10.5. This is a poor degree of purification but it has clearly revealed that the full activity of tryptophan 5-hydroxylase is dependent, at least in part, upon a reduced pteridine.

Expt. 16 THE EFFECT OF AMETHOPTERIN, 5HTP, AND ASCORBIC ACID UPON TRYPTOPHAN 5-HYDROXYLATION BY BRAIN WHOLE HOMOGENATES

Introduction

(a) Amethopterin has been shown to inhibit phenylalanine and tryptophan hydroxylation by rat liver both in vivo and in vitro (Renson et al 1962, Kaufman 1958). If tryptophan hydroxylation in brain occurred

via a similar reaction then it was possible that amethopterin might be an effective inhibitor. This did seem unlikely however as Renson et al (1962) had shown that the brain levels of 5HT were unaffected in animals treated with amethopterin whilst phenylalanine hydroxylation was inhibited. The point was however tested.

(b) The effect of 5HTP was studied as this is the immediate product of tryptophan 5-hydroxylation and product inhibition of enzyme reactions is a fairly common phenomenon.

(c) The effect of ascorbic acid was also tested because Cooper and Melcer (1961) had cited this substance as a cofactor in the hydroxylation of tryptophan by intestinal mucosa.

Tissue preparation, incubation and assay of tryptophan 5-hydroxylase

A whole homogenate of the "Hypothalamus-Thalamus" section (see p. 104) of a dog's brain was made in Ringer phosphate solution, pH 7.3 (1:2 w/v). The whole homogenate (4 ccs) was incubated with DL-tryptophan -3-C^{14} 10 μc/315 mμmoles (final conc. 6.3×10^{-5}M) and NSD 1024 (1.5×10^{-3}M). Total volume of all samples incubated was 5 ccs.

When the following additions were made their final concentrations were: amethopterin 2×10^{-4}M, DL-5HTP 4×10^{-4}M, ascorbic acid 2×10^{-3}M, Incubation was carried out for 1 hr at 37°C in O_2. At the end of incubation the amount of 5HTP produced was assayed by method III.

Results

TABLE 33

The effect of amethopterin, 5HTP and ascorbic acid on tryptophan 5-hydroxylation in whole homogenates of brain

Additions	µµmoles 5HTP produced/hr /4 cc of whole homogenate
None	450
Amethopterin	775
DL-5HTP	486
Ascorbic acid	510

Comments

Amethopterin appeared to increase the hydroxylase activity but this was not repeated and it significance remains unknown. What is important, is that amethopterin does not inhibit tryptophan hydroxylation in brain. The possible significance of this finding is discussed later.

DL-5HTP, at the concentration used, did not inhibit tryptophan hydroxylation.

Ascorbic acid caused a slight increase in activity but as reduced pteridines were shown to be the essential cofactors, this point was not pursued.

D (Expt. 17) THE EFFECT OF L-PHENYLALANINE UPON TRYPTOPHAN 5-HYDROXYLATION IN BRAIN

Introduction

Apart from the carcinoid syndrome one of the diseases in which, it has been speculated, changes in 5HT synthesis and metabolism might play a part is phenylketonuria. This disease was first described by

Fölling in 1934, and presents as mental deficiency but besides this there are also disturbances in growth and pigmentation (Penrose 1946). The "Inborn error of metabolism" (Garrod 1909) in this disease is a defect in phenylalanine hydroxylation (Jervis 1953). Because phenylalanine cannot be hydroxylated to form tyrosine it accumulates in the blood and is metabolised via alternate pathways to phenylpyruvic acid, phenyllactic acid and phenylacetic acid. The detection of abnormal amounts of phenylpyruvic acid in the urine is the accepted diagnostic test for the disease.

Pare et al (1957, 1958, 1959) found that in the blood of patients with phenylketonuria there was a decreased amount of 5HT and also a decreased urinary excretion of 5HIAA. These findings suggested that there might be an association between phenylalanine and tryptophan metabolism.

On the assumption that the main defect in this disease was the accumulation of phenylalanine Yuwiler and Louttit (1961) fed increased amounts of phenylalanine to animals to overload the phenylalanine hydroxylating system. They found that under such conditions the brain content of 5HT fell significantly. This experimental situation is not entirely the same as the natural condition because of course in the experimental condition, large amounts of tyrosine are also formed, nevertheless the finding of a lowered brain 5HT concentration required explanation.

Davidson and Sandler (1958) showed that 5HTP decarboxylase prepared from guinea pig kidney was inhibited by the addition of phenylalanine and its metabolites. Yuwiler et al (1965) could not

however find any inhibition of 5HTP decarboxylase in the brains removed from animals with experimental phenylketonuria in which the 5HT content was lowered. They also calculated from certain assumptions, that would maximisethe effect of any inhibitor of 5HTP decarboxylase under the experimental conditions they describe, that only a 0.4% decrease in 5HTP decarboxylase activity would result. Yet they showed that the brain 5HT is lowered by 22%. Their arguments are very convincing against the inhibition of 5HTP decarboxylase being the main cause of the lowered brain 5HT. They also present evidence to show that there is unlikely to be any increased activity of monoamine oxidase activity leading to a fall in brain 5HT.

When Freeland et al (1961a) demonstrated that rat liver 5-hydroxylated tryptophan and that this was inhibited by phenylalanine, it was suggested that the cause of the lowered brain 5HT in experimental phenylketonuria was an inhibition of tryptophan 5-hydroxylation. But when Renson et al (1962) were able to show not only that hepatic tryptophan 5-hydroxylation was due to phenylalanine 4-hydroxylase and unlikely to be of physiological importance in the biosynthesis of 5HT, but also that under conditions in vivo where phenylalanine hydroxylation was inhibited, brain 5HT was not decreased this explanation fell down.

When the 5-hydroxylation of tryptophan in brain was demonstrated it became of some interest to see whether phenylalanine would have any effect on the reaction.

Tissue preparation, incubation and assay of tryptophan 5-hydroxylase

A whole homogenate of rabbit hind brain was made in 0.25 M sucrose + 0.001 M mercaptoethanol (1:2 w/v). Aliquots of the whole

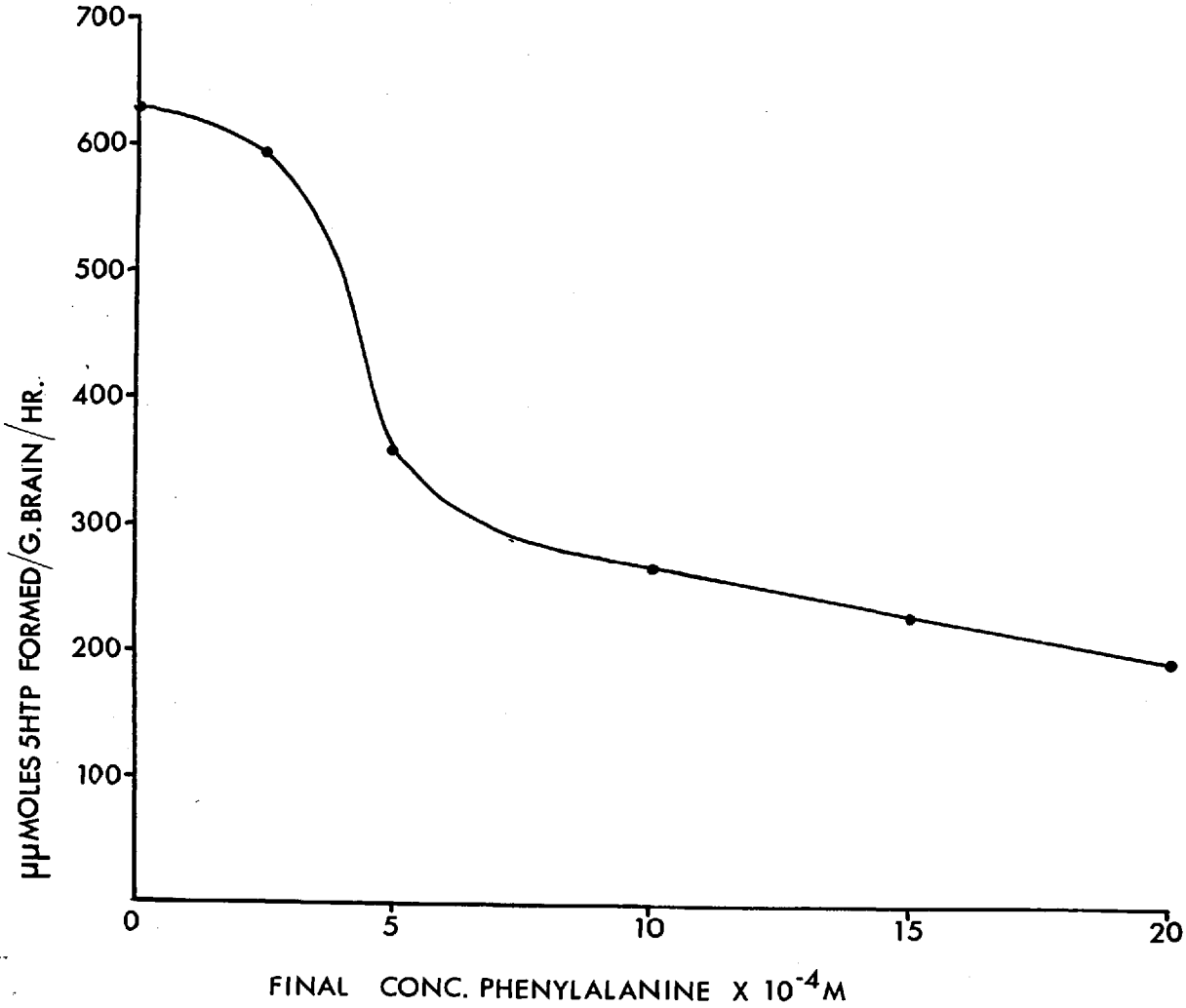


Fig. 38 The inhibitory effect of L-phenylalanine upon the production of 5HTP by brain homogenates. Concentration of added DL-tryptophan-3-C^{14} = 7.55 x 10^{-5}M.

homogenate (1 cc) were incubated with: DL tryptophan-3-C^{14} 5 µc/ 151 mµmoles (final conc. 7.55×10^{-5}M), NSD 1024 (1.5×10^{-3}M and potassium phosphate buffer pH 7.4 (5×10^{-2}M). To this mixture were added various amounts of L-phenylalanine as shown in table 34. The samples were then incubated for one hour at 37°C in O_2. At the end of incubation the amount of 5HTP produced was estimated by assay method III.

Results (see also fig. 38) TABLE 34

The effect of L-phenylalanine on tryptophan 5-hydroxylation by whole homogenates of brain

Final concentration of added L-phenylalanine	µµmoles of 5HTP produced /G brain (wet wt.)/hr
0	627
2.5×10^{-4}M	597
5×10^{-4}M	362
10×10^{-4}M	274
15×10^{-4}M	229
20×10^{-4}M	197

Discussion

It should be noted that the final concentration of added tryptophan in terms of the L isomer is 3.8×10^{-5}M but this does not include the free endogenous tryptophan which from the data of Schurr et al (1950) we can assume is in the region of 4.6 - 5.8 µg/G of brain.

These results show that phenylalanine inhibits tryptophan hydroxylation by whole homogenates of brain. Nakamura et al (1965) have recently reported though that partially purified tryptophan hydroxylase from brain is not inhibited by phenylalanine. If this

is correct how can these two facts be reconciled?

The investigations of the subcellular localisation of tryptophan 5-hydroxylase in brain have shown the enzyme to be localised in the synaptosome and of course it would be so localised in the concentrated whole homogenate (1:2 w/v) prepared in isotonic media and used in this experiment.

The inhibition by phenylalanine of hydroxylation in whole homogenates but not of the purified enzyme could therefore be explained by supposing that phenylalanine inhibits the transport of tryptophan across the wall of the synaptosome thereby depriving the hydroxylase of its substrate. This mechanism would obviously not apply when a purified preparation of tryptophan 5-hydroxylase was studied.

Expt. 18 THE INHIBITION OF THE UPTAKE OF L-TRYPTOPHAN-3-C^{14} INTO BRAIN SLICES BY L-PHENYLALANINE

Introduction

Is there any evidence to back up the hypothesis that phenylalanine inhibits 5HT biosynthesis in brain, not by inhibiting tryptophan 5-hydroxylase, but by inhibiting the uptake of tryptophan by the brain. A few studies have been done on the transport of tyrosine, phenylalanine, 5HTP and tryptophan into brain and from these certain speculations can be made.

McKean et al (1962) have shown that 5-hydroxytryptophan uptake by brain is inhibited by phenylalanine and also by L-tryptophan. Smith (1963) confirmed this in vitro and also found that α-methyldopa inhibited the uptake of 5HTP by brain. Udenfriend and his colleagues (Chiragos et al 1960, Guroff et al 1962, and Guroff and Udenfriend 1962) studied the uptake of tyrosine by brain both in vivo and in vitro. They found both in

vivo and in vitro that brain possesses a mechanism for the uptake of tyrosine against a concentration gradient. They found that this uptake is inhibited by aliphatic monoamine carboxylic acids but not by their related nitrogen free acids. The uptake is also inhibited by substances closely related to tyrosine and also by tryptophan and phenylalanine. However certain differences between the uptakes observed in vivo and in vitro led them to suppose that two mechanisms were involved in the uptake of tyrosine by brain in vivo. The first involved transport across the capillary wall and the second passage across the nerve cell membrane. Overall though they concluded that the uptake of tyrosine depended upon a catalytic process. They also demonstrated that the uptake of L-tryptophan is probably dependent upon similar mechanisms. Although this line of work has not yet been taken very far the experiment now to be described demonstrates that the uptake of L-tryptophan by brain slices is indeed inhibited by L-phenylalanine.

Methods

Preparation of brain slices Because Smith (1963) showed that there were quantitative though not qualitative differences in the uptake of 5HTP by slices prepared from different areas of the dog's hind brain the present studies have been done on rabbit cortical tissue in order to achieve a structurally more homogeneous population of slices.

A rabbit was killed by a blow onthe neck and the whole brain removed, minus the olfactorylobes, and chilled on ice. Slices of cerebral cortex were cut by hand and between 100-150 mgms of these slices roughly and quickly weighed out for each incubation. Each 100-

150 mg portion of slices was suspended in 2.5 ccs of Ringer phosphate solution pH 7.4 in 25 ml flasks. All these operations were carried out at 0-4°C as quickly as possible and the Ringer phosphate solution was cooled to 4°C before the slices were suspended in it.

Incubation conditions To each flask at 0-4°C was then added 0.25 ml of an aqueous solution of L-tryptophan-3-C^{14} (10 µmole/ml: specific activity 0.84 µc/µmole or 4.2×10^{-3} µc/µg). With the efficiency of the gas flow counter at 29% the counts/min/µg of the L-tryptophan-3-C^{14} were 2700. This addition gave a final concentration of added L-tryptophan of 1×10^{-3}M. When L-phenylalanine was added the final concentration in each case was 1.67×10^{-3}M.

Uptake of L-tryptophan into the slices was measured at 37°C with and without the addition of L-phenylalanine in flasks gassed for one minute with O_2 and then corked. An attempt was made also to study the uptake of L-tryptophan in an atmosphere of N_2 at 37°, but it is likely that for technical reasons the percentage of N_2 in the atmosphere above the incubation was not 100% and also that O_2 was not completely removed from the incubation medium though, as will be shown in the results, N_2 was found to have some effect on uptake.

The uptake of tryptophan was also studied at 0°C in an atmosphere of O_2, by placing the incubation flasks in ice and intermittently shaking these by hand. In all cases the uptake over predetermined periods of time was studied.

Estimation of the amount of L-tryptophan taken up by brain slices At the end of the chosen time interval the slices were removed from the suspending medium, drained and blotted for 30 secs on Whatman No. 4 filter

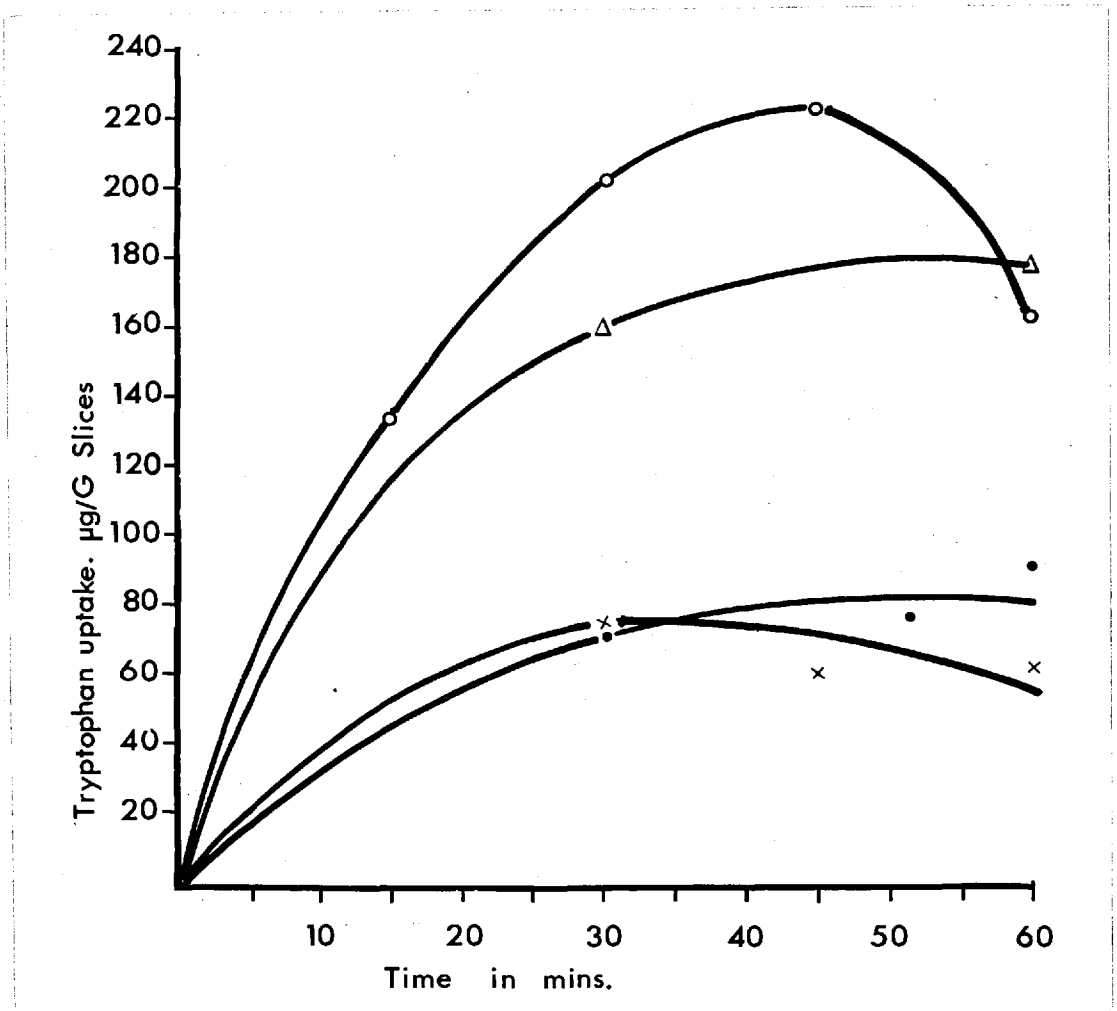


Fig 39 The uptake of L-tryptophan by slices of rabbit cerebral cortex. For conditions see text. Concentration of added L-tryptophan-3-C^{14} = 1 x 10^{-3}M.

× = at 0°C + O_2. O = at 37°C + O_2 Δ = at 37°C + N_2
● = at 37°C + L-phenylalanine (1.67 x 10^{-3}M)

paper. The final wet weight of the slices was then determined to the nearest mg. The slices were then homogenised in 5 ml of 70% ethanol and the denatured protein removed by centrifugation. 0.5 ml of the deproteinised extract was then plated out on aluminium planchettes and assayed after drying at infinite thinness in a gas flow counter (efficiency 29%). Control studies showed that the recovery of radioactivity under these conditions was 99% and that recovery is quantitative and not dependent upon the amount of tissue used (i.e. there is no appreciable non-specific absorption of tryptophan onto the tissue).

The results are calculated in two ways: (1) As the amount (in µg) of L-tryptophan-3-C^{14} taken up per G of wet slice tissue in a given time, (2) As the ratio between the intracellular concentration (µg L-tryptophan-3-C^{14} per G wet tissue) and the concentration of L-tryptophan -3-C^{14} in the starting medium (µg/ml).

Results

TABLE 35

The uptake of L-tryptophan by brain slices and the inhibiting effect of L-phenylalanine

Time mins.	µg L-tryptophan/G tissue				Intracellular conc (µg/G tissue) Medium conc. (µg/ml)			
	0°C +O_2	37°C +O_2	37°C + N_2	37°C + ph	0°C +O_2	37°C + O_2	37°C + N_2	37°C + ph
0	0	0	0	0	0	0	0	0
15	55	154	-	-	0.296	0.83	-	-
30	77	203	161	72	0.415	1	0.87	0.423
45	61	224	-	-	0.328	1.2	-	-
60	63	163	178	93	0.338	0.88	0.96	0.553

ph - phenylalanine.

These results are graphically represented in fig. 39

Discussion

The difference in the uptake of L-tryptophan by brain slices at 37°C and 0°C is very striking and the following facts suggest that this is not merely due to an effect of temperature on diffusion rates.

1. The concentration of L-tryptophan in the slices reached at 45 mins at 37°C is greater than that in the medium (ratio = 1.2). This suggests not only an active uptake mechanism but also a concentration mechanism. In this experiment the endogenous concentration of L-tryptophan in the brain slices has been ignored, so that the intracellular conc/medium conc ratio may have been slightly higher than this and I suspect that if the medium concentration had not been so high then the ratio would have been higher than this.

2. An atmosphere of N_2 (for qualifications see Methods) inhibited the uptake of tryptophan by about 20% at 30 mins which would not be expected if uptake were merely due to diffusion.

3. Phenylalanine is shown to reduce the uptake of tryptophan to levels seen at 0°C. This is perhaps the most telling point in favour of the uptake at 0°C being mostly due to diffusion and that at 37°C (minus phenylalanine) being due to an active uptake mechanism.

4. The curves of uptake at 0°C and 37°C have different forms. Uptake at 0°C ceases at about 30 mins whilst at 37°C uptake continues for another 15 mins. Had the uptake at 37°C been due to diffusion one would have expected the time relationships to have been similar.

If then we assume that the uptake at 0°C represents only the diffusion of L-tryptophan into brain slices, then we see that L-phenyl-

alanine at a concentration of 1.67×10^{-3}M with a medium concentration of L-tryptophan of 1×10^{-3}M, more or less completely inhibits the active uptake of tryptophan by the brain slices.

At the present time the loss of tryptophan by the brain slices after 45 mins at 37°C and about 30 mins at 0°C in oxygen and the apparent protection against this loss by N_2 and phenylalanine are unexplained.

Although more studies are needed on the uptake of tryptophan by brain this experiment has been included because it does show that an active uptake mechanism for tryptophan does exist and that this is inhibited by phenylalanine. These results reconcile the points previously made, that whereas phenylalanine appears to inhibit tryptophan 5-hydroxylase by whole homogenates of brain made in isotonic media, in which synaptosomes will be largely intact, it does not inhibit partially purified tryptophan hydroxylase. The evidence is now strongly in favour of the whole homogenate inhibition being due to the inhibition of tryptophan uptake by the synaptosomes and the lowered 5HT content of brain in experimental phenylketonuria being due to the inhibition of tryptophan uptake by brain, by the high plasma levels of phenylalanine.

If this explanation is correct then some exciting possibilities arise. If transmitter substances are synthesised in nerve endings (as seems likely) then with the techniques now available it should be possible to study their biosynthesis in purified synaptosome fractions and investigate the effects of various substances upon the transport of their precursors across the synaptosomal membranes, since this may be an important controlling step in the synthesis of these substances and subject both pathological disturbances and pharmacological attack.

GENERAL DISCUSSION

The studies reported here have demonstrated that enzymatic tryptophan 5-hydroxylation with the formation of 5HTP is the first step in the biosynthesis of 5HT in carcinoid tumour, brain and intestinal tissue. The list of tissues in which this reaction has been described lengthens and at present stands as follows:

(1) Chromobacterium Violaceum (Mitoma et al 1956)

(2) Mast cell tumours (Schindler 1958)

(3) Rat liver (Freedland et al 1961a)

(4) Intestine (Cooper and Melcer 1961; Grahame-Smith 1964b)

(5) Carcinoid tumour (Grahame-Smith 1964a)

(6) Brain (Grahame-Smith 1964c; Nakamura et al 1965; Gal et al 1965; Green and Sawyer 1965)

The work of Renson et al (1962) suggested though that liver was not responsible for the biosynthesis of 5HT under physiological conditions. In mammals it seems very likely that the main site of 5HT synthesis is the intestine and that the argentaffin cells of the intestinal mucosa are responsible, in view of the histochemical properties and apparent origin of the majority of carcinoid tumours. One presumes too that the enzymatic properties of these tumours are in many respects similar to those of the intestinal argentaffin cells. If one accepts this presumption then it is likely that the tryptophan 5-hydroxylase in argentaffin cells of normal intestine requires a reduced pteridine as a cofactor. So far the tryptophan 5-hydroxylases shown to require such a cofactor are those prepared from mast cell tumours ((Lovenberg et al

1965), carcinoid tumours and brain. One point is puzzling here. Whereas phenylalanine hydroxylation, a reaction also requiring a reduced pteridine for full activity, is markedly inhibited both in vivo and in vitro by amethopterin, 5HT synthesis appears to be little affected by this substance either in vivo (Renson et al 1962) or in vitro. If phenylalanine hydroxylation and tryptophan hydroxylation depended upon the same electron donor and the oxidised form of this donor was reduced by the same dihydropteridine reductase one would have expected tyrosine and 5HT biosynthesis to be equally diminished by amethopterin in vivo and in vitro. That they are not so affected suggests some difference between the two reactions. Although at present one can only speculate about the mechanism of tryptophan 5-hydroxylation it seems quite likely to proceed in the way described for phenylalanine hydroxylation, i.e.

L-tryptophan + reduced pteridine

$\downarrow$ tryptophan hydroxylase

L-5HTP + H_2O + "oxidised pteridine"

That a reduced pteridine is required for this reaction is of interest as Jacobson and Simpson (1965) has extracted material from carcinoid tumours which has the properties of a pteridine and which he thought was Xanthopterin. It would be of interest to know the precise nature of the natural cofactor as this might explain the different behaviour of phenylalanine and tryptophan hydroxylation in respect of amethopterin. It is also of importance to further purify tryptophan hydroxylase and study in detail its precise requirements, for in purified systems it

may turn out that amethopterin does inhibit tryptophan hydroxylation via the cofactor system whereas factors, as yet unknown, may negate its inhibiting influence in crude isolated preparations and in vivo.

Although detailed studies of the specificity of the tryptophan 5-hydroxylase investigated have not been carried out, nevertheless it is of importance to note that carcinoid tumour intestine and brain tissue do not hydroxylate phenylalanine. Table 36 compares the hydroxylating activity of various tissues for phenylalanine and tryptophan.

TABLE 36

Comparison of the tryptophan 5-hydroxylase and phenylalanine 4-hydroxylase activity in various tissues and organisms

	Phenylalanine Hydroxylase	Tryptophan Hydroxylase
Rat liver (Renson et al 1962)	++++	++++
Mast cells (Lovenberg et al 1965)	++	++
Carcinoid tumour	-	++
Brain	-	++
Intestine	-	++
Chromobacterium Violaceum (Mitoma et al 1956)	-	++
Pseudomonas species ATCC 11299a (Guroff & Ito 1965)	++	-

It would be of great interest to know the factors involved which cause these differences in specificity. Although Nakamura et al (1965) have shown that tryptophan 5-hydroxylase prepared from brain will not hydroxylate tyrosine and Nagatsu et al (1964) that tyrosine hydroxylase will not hydroxylate tryptophan one wonders whether sometime during evolution there was not a common aromatic amino acid hydroxylase. The

similarity of the hydroxylating and decarboxylating steps in the biosynthesis of 5HT and catecholamines and the wide specificity of the decarboxylase is somewhat suggestive that this may have been so. It would be of interest here to investigate the specificity of the hydroxylases responsible for catecholamine and 5HT biosynthesis in plants.

Many observations suggest that the biosynthesis of 5HT, like that of dopamine and noradrenaline is limited in rate by the initial enzymatic hydroxylation of tryptophan and tyrosine respectively. The work done in vivo on this point was discussed in the introduction. Although there have been no detailed studies done on the kinetics of tryptophan 5-hydroxylation in isolated mammalian tissues (other than mast cells) the observations reported here make it clear that in intestinal carcinoid tumour and brain tissue tryptophan 5-hydroxylation is a much less active reaction than 5HTP decarboxylation. If tryptophan hydroxylation is the rate-limiting step in the biosynthesis of 5HT then its inhibition may effectively lower tissue 5HT concentrations and give valuable information about the true physiological functions of 5HT. However enzymatic reactions are not the only limiting factors in the synthesis of biological substances in vivo. We have seen for instance that phenylalanine may inhibit 5HT biosynthesis in brain by blocking the transport of tryptophan across nerve cell membranes.

A consideration of the quantitative aspects of tryptophan 5-hydroxylation in isolated carcinoid tumour and brain throws light upon two points. Firstly a rough comparison between the rates of synthesis of 5HT in a patient with metastasising carcinoid tumour (tumour 9) shows that <u>in vivo</u> the rate of 5HT synthesis was in the region of 1-2 µgm/ G tumour tissue/hr (Tumour tissue estimated at 2.5 Kg and urinary 5HIAA excretion

100 mgms/day) and *in vitro* 0.2 - 0.3 µgm 5HT/G tumour tissue/hr). Considering the highly artificial conditions of the *in vitro* experiments and probable deterioration in enzyme activity this is fair agreement. Unfortunately it is not yet possible to assess the rate of 5HT biosynthesis per gram of argentaffin cells in the normal intestine but if we compare the activity of the carcinoid tumour *in vitro* (0.2-0.3 µgm of 5HT/G of tumour/hr) with the activity of rabbit brain stem (approx 0.4 µg 5HT/G of brain/hr), we see that the tumour tissue has a fairly physiological rate of 5HT biosynthesis. This means that the increased 5HT biosynthesis seen in the carcinoid syndrome is not due to a greatly increased biosynthetic activity in each tumour cell but to an increase in the number of such cells. This suggests that the tumour cells are probably functioning normally when compared with their non-neoplastic counterpart, the argentaffin cell of the intestinal mucosa.

The second point of importance is to consider whether the activity of tryptophan hydroxylation observed in isolated brain tissue can account for all the 5HT synthesised in vivo by brain, for if it doesn't, it would indicate that brain tissue relies upon a supply of 5HTP from elsewhere in the body. The actual rate of 5HT synthesis is difficult to measure in vivo but Garattini and Valzelli (1961) have estimated that rat brain probably turns over about 1 µgm 5HT/G of brain/hr and this presumes a similar rate of synthesis. The in vitro rate of synthesis observed during these studies has been in the region of 0.4 µg 5HT /G/hr so that there is fair agreement, again making allowances for the

artificial and probably as yet not ideal conditions of in vitro study. These findings along with those discussed in the introduction to section III make it almost certain that the brain independently synthesises all the 5HT which it needs.

One is forced to speculate as to how far the biosynthesis of 5HT in brain and intestine is dissociated and each one independent of the other. One of the properties of the "Blood brain barrier" may be to protect the brain from the extracerebral influences which would otherwise affect the synthesis of probable synaptic transmitters. Many of the putative transmitters, noradrenaline, acetylcholine, 5HT, histamine and substance P are found to have a function outside the brain and there would obviously be cerebral chaos if the factors influencing the peripheral biosynthesis and metabolism of these substances were allowed to exert themselves upon the same processes in the brain.

This brings us to a consideration of the physiological and pathological functions of 5HT in the brain. Several times during this report the supposition that 5HT is a synaptic transmitter substance has been made. What is the evidence for this?

Three criteria have been suggested for the identification of a substance as a synaptic transmitter, (Salmoiraghi et al 1965). (1) The demonstration that the substance is localised at the nerve ending; (2) The demonstration of an identical action for the natural transmitter and its synthetic analogue; (3) The demonstration that the suspected transmitter is released during stimulation of the relevent synapse. How far have these criteria been fulfilled for 5HT?

The subcellular fractionation of brain tissue has shown that 5HT, 5HTP decarboxylase and tryptophan 5-hydroxylase are all localised at the presynaptic nerve ending. Carlsson et al (1964) have observed, using fluorescent microscopy, that some nerve endings in the central nervous system appear to contain 5HT. Criterion one is therefore fulfilled.

The second criterion has not been fully fulfilled. Various workers have utilised the method whereby small amounts of various substances have been placed in the vicinity of single neurones by inotophoresis with multibarrelled micropipettes. The activity of these neurones can be continuously recorded. The trouble is that with the mammalian central nervous system it is virtually impossible to exclude the action of these substances on neighbouring structures. Bradley and Wolstencroft (1965) have tested many neurones in the pons and medulla with acetylcholine, noradrenaline and 5HT and all sorts of combinations of excitation and inhibition were seen, which makes the interpretation of their results difficult.

Krnjevic (1965) found that 5HT had a quick depressant action on several tested cortical neurones, though its depressant action was weak compared with γ-aminobutyric acid and Krnjevic concludes that 5HT is unlikely to play much part in intracortical transmission. Curtis (1965) described his investigations into the action of 5HT on the excitation of lateral geniculate neurones by optic tract impulses. He found that such excitation was depressed by 5HT, though other substances such as 4-hydroxytryptamine, 7-hydroxytryptamine and certain lysergic acid and phenylethylamine derivatives had a similar action. It is

thought that this inhibition occurs via the blocking of the access of the excitatory transmitter to the sub-synaptic receptors of the lateral geniculate neurones or inhibition of the release of a transmitter the nature of which remains unknown. This hypothesis raises the point as to whether certain substances such as 5HT, thought at present to be direct synaptic transmitter substances, may in fact function indirectly by controlling the release of actual synaptic transmitter substances or by interfering with their action on synaptic receptor sites.

Florey (1965) reviewing the pharmacology of 5HT in invertebrates, is very cautions about concluding that 5HT acts as a synaptic transmitter in these organisms. However the evidence that he cites for this role, especially in the lamellibranch cardioaccelerator neurones is very impressive. Feldberg and his co-workers (Feldberg and Fleischhauer 1965) in a series of reports, have implicated nor-adrenaline and 5HT in the control of body temperature via their action on the hypothalamus. By techniques of intraventricular infusion they have administered 5HT and noradrenaline and have shown a hyperthermic effect for 5HT and a hypothermic effect for noradrenaline. The area sensitive to these amines in producing these effects is the anterior hypothalamus and Feldberg and Myers (1965) extended these studies by showing that microinjections of these amines into the anterior hypothalamus produced the same effects as their intraventricular infusion. Feldberg (1965) has also demonstrated that during hypothermia 5HT appears in the C.S.F. of the cerebral ventricles suggesting hypothalamic release of this substance. It is dangerous to speculate too far on these findings but they do suggest a role for 5HT (and noradrenaline) in body temperature control by the hypothalamus.

Whether these amines act at synapses within the anterior hypothalamus is unknown. Although the second criterion for the identification of 5HT as a synaptic transmitter is not completely fulfilled, nevertheless the evidence available suggests that it may be so, for certain neuronal pathways.

On the third criterion, the evidence is somewhat indirect. Anden et al (1964) have shown that the in vitro stimulation of the spinal cord results in a release of 5HT into the incubation bath and Dahlström et al (1965) that electrical stimulation of the medulla oblongata in vivo results in the depletion of 5HT stores in the varicosities of the nerve endings (as judged by fluorescent microscopy). Although,suggestive, these studies do not prove that 5HT is actually released from the presynaptic nerve endings during synaptic transmission.

Overall then one can say that although the case for 5HT being a synaptic transmitter or a substance indirectly controlling synaptic transmission in certain areas of the central nervous system is not proved, there is a good deal of evidence for this being the case.

Dahlström and Fuxe (1964 and 1965) have used histochemical methods to show up neurones containing the monoamines dopamine, noradrenaline and 5HT. They claim that the methods which they use distinguish between the fluorescence produced by cells containing the 5HT on the one hand and the catecholamines on the other. Using these methods they have charted the localisation of neurones containing the monoamines in the central nervous system. It is of interest to note that although

the synaptic terminals of neurones show a very high fluorescence, (which is presumed to indicate a high concentration of the monoamine at this site), the cell body and its processes do whow a low fluorescence. These findings suggest that although 5HT is mainly localised to the presynaptic nerve ending some is spread throughout the nerve cell. What this means in terms of the locus of synthesis of this amine is unknown but it may be that the nerve cell body itself can synthesise 5HT and this might account for the localisation of some of the tryptophan 5-hydroxylase in the high-speed supernatant fraction of brain preparations.

These workers have also shown that the monoamine containing neurones are distributed throughout the central nervous system in a definite way. They have characterised three neuronal systems: one large nigro-neostriatal system containing dopamine and two bulbospinal systems containing noradrenaline and 5HT respectively. Lewis (1965) has studied the histochemical localisation of cholinesterase, assuming that the presence of a high concentration of cholinesterase in neurones indicates that such neurones rely upon innervation by acetylcholine. He and his coworkers have demonstrated a cholinergic pathway arising from the reticular formation and supplying the whole of the cortex and many subcortical structures. These findings that putative synaptic transmitters are localised to various well-defined tracts and areas of the central nervous system suggest that functional differentiation within the central nervous system may be dependent partially upon having several synaptic transmitters or regulators of synaptic transmission each of which may serve an independent neuronal pathway to which other pathways

dependent upon other transmitter substances are insensitive. This hypothesis coupled with the fact that transmitter substances are likely to be destroyed or inactivated very quickly may explain how such discrete function can be maintained within the central nervous system.

There are certain properties in relation to carcinoid and related tumours which I should like to discuss.

Campbell (1959) has considered the association between the property of certain normal and neoplastic cells to stain with silver stains and their ability to synthesise or store 5HT. There appears to be two types of cell in this respect; those which contain granules which both take up and reduce the silver stain, and those which take up the silver stain but do not reduce it, so that an external reducing agent must be applied before the staining becomes obvious. The former are known as "Argentaffin" cells and the latter "Argyrophil" cells. A good deal of controversy has taken place about the significance of these staining reactions and the problem remains unsolved. There is no doubt that there is some relationship between the argentaffinity of the granules of the argentaffin cell and their 5HT content but it does not appear to be a direct one. Jacobson (1958) and others have shown that the degree of argentaffinity of carcinoid tumours bears little relationship to their content of 5HT, though the content of 5HT per cell may be the important factor and this will vary from place to place within the tumour and from time to time depending upon the local rates of synthesis, storage, release and destruction of 5HT. On the other hand the release of 5HT by reserpine (Pletscher 1958) has been shown to correlate well with changes in the argentaffinity of the tissues studied. In addition

formalin-fixed gelatin models of 5HT have been shown to have most of the relevant histochemical properties of argentaffin granules (Barter and Pearse 1955).

When the property of Argyrophilia is considered the problem is even more complex and as the basis of neither direct or indirect silver staining is known any significance we attach to them must be conjectural. Whereas in vertebrates the argentaffin cells are distributed in the gastrointestinal mucosa, biliary tract and pancreas, the argyrophil cells are much more widely distributed in the same organs and also in the prostate, adrenal medulla, organ of Zuckerkandl, carotid body and in the epithelial nests found in bronchiectatic bronchi, (though not in normal bronchi). The property of argyrophilia may be "non-specific" and would not warrant consideration here were it not for the fact that many carcinoid and related tumours show this property, suggesting that argyrophilia may in some way be related to 5HT production or storage. Erspamer (1939) originally suggested that argyrophil cells were biochemically and morphologically immature argentaffin cells. Sandler and Snow (1958) followed up this line of thought when they reviewed three atypical cases of the carcinoid syndrome. The causative tumours were argyrophilic and the patients excreted 5HTP, 5HT and 5HIAA in the urine. They suggested that argyrophilia might be related to the ability of the cell to synthesise 5HTP but not to decarboxylate it to 5HT or store the latter. A sort of "half-way house" toward the full functional and morphological differentiation of the argentaffin cell.

In the present state of our knowledge a cautious overall interpretation of these findings might be made. It seems likely that

the argentaffinity of argentaffin cells is due to their 5HT content or to some property of the granular material which stores the 5HT in these cells. Argyrophilia on the other hand does not appear to be related so closely to the ability of the cell to synthesise or store 5HT but the frequent occurrence of argyrophilic cells in tumours shown to synthesise 5HT does suggest some relationship. The suggestion though that argyrophil cells are functional and morphological precursors of argentaffin cells is largely speculative.

Williams and Sandler (1963) attempted to classify carcinoid and related tumours by consideration of their histochemical and biochemical properties, metastatic characteristics, histological structure and embryological origin. As their reference point they took the commonest and most easily recognised ileocaecal carcinoid tumour. Histologically this type of tumour is made up of nests of regular cells separated by a delicate connective tissue framework, the cytoplasm of the cells contains argentaffin granules. Sometimes the structure has a slight trabecular appearance. This typical type of tumour contains usually a high concentration of 5-hydroxytryptamine, metastasis to the liver only, and produces the carcinoid syndrome. Patients with this type of tumour excrete mainly 5HIAA in the urine.

Bronchial, pancreatic and gastric tumours associated with the carcinoid syndrome frequently differ from the more typical ileo-caecal tumours in many respects. In general the prognosis is worse, metastases to bone and skin frequently occur and the patients often excrete 5HTP, 5HT and 5HIAA in the urine. These tumours on the whole contain a smaller concentration of 5HT than the ileo-caecal tumours. The structure

of these tumours is frequently atypical and the argentaffin reaction is usually negative.

Carcinoid tumours arising in the rectum are usually more trabecular in histological appearance, the argentaffin reaction is usually negative and the carcinoid syndrome does not occur. The tumours do not contain abnormal amounts of 5HT and nor do the patients excrete abnormal quantities of 5HIAA in the urine. These rectal carcinoids frequently metastasise to skin and bone.

Williams and Sandler saw some pattern in these phenomena and proposed that the developmental origin of the tissue from which the tumour arose might in some way be involved in these differences. All the tissues from which these tumours arise are of endodermal origin. The bronchial, pancreatic and gastric tumours arise from the embryological foregut, the ileocaecal tumours from the midgut and the rectal from the hindgut. Although this classification in no way explains the biochemical and structural differences observed, nevertheless it does raise an interesting point. It infers that in the three areas of the developing gut there is distributed a system of cells perhaps potentially having similar functions which biochemically differentiate in different ways during development of the embryo. It is important to note that some of the bronchial and pancreatic tumours show none of the histological characteristics of carcinoid tumours whilst possessing the ability to synthesise 5HT and bradykinin. There is no evidence as yet that normal bronchial and pancreatic tissue synthesises 5HT, though the problem is as yet largely unexplored.

There are now many reports describing humoral syndromes in

association with tumours originating in tissues not known to have the property of producing the humoral substances thought to be involved. It is important to note that the precise structure of many of the humoral substances produced by these tumours is unknown so that one cannot be certain whether they are the same as the physiological and natural hormone. It is evident from clinical and physiological studies however that many of these substances appear to be acting in the same way as their purported natural counterparts, though their production is not susceptible to normal controlling mechanisms. In table 37 are shown the humoral syndromes associated with non-endocrine tumours, the humoral substances thought to be involved, and an indication as to the acceptability of the evidence for the identification of these substances.

Consideration of these tumours and their peculiar properties leads one to ask whether a true change in cellular function is occurring during the process of carcinogenesis. It could be that cells are present in tissues which have the property of synthesising hormonal substances but that their number is normally so small or activity so low that it is only when a large mass of such cells is present, as in a tumour or its metastases that this property becomes obvious though its clinical effects.

Another possibility is that scattered throughout the body are a number of morphologically and biochemically undifferentiated cells which upon becoming cancerous differentiate functionally to produce substances at variance with the tissue from which the tumour appears to rise. This is a return to the embryonal cell theory of Cohnheim (1877), (see also Cowdry 1955) which at present is rather unfashionable!

TABLE 37.

Humoral syndromes associated with "Non-endocrine" tumours

Humoral syndrome	Suggested hormone involved	Tissue of origin of tumours
Cushing's syndrome	A.C.T.H. (structure unknown, physiological evidence good)	Bronchus, pancreas, thymus ovary, thyroid, misc.
Hypercalcaemia (with -out bone metastases)	Parathormone (evidence for nature of hormone poor)	Kidney, bronchus, pancreas, ovary, uterus, misc.
Hypoglycaemia	Insulin-like activity recovered from a few tumours only	Mesenchymal tissues, liver, adrenal cortex, misc.
Hyponatraemia	A.D.H. (probably arginine vasopressin)	Bronchus
Carcinoid syndrome	5HT and lysyl-bradykinin (evidence certain)	Bronchus, pancreas
Polycythaemia	Erythropoietin (physiologically and immunologically like natural hormone.)	Cerebellum, uterus, liver adrenal medulla
Hyperthyroidism	Thyrotrophic hormine. (Physiological activity recovered from some tumours.)	Trophoblast, gastrointestinal, bronchus, prostate.
Precocious puberty	Gonadotrophic hormone. (Physiological evidence good)	Liver

Data compiled from review by Lipsett et al (1964)

Perhaps the most attractive explanation is that during the process of carcinogenesis something happens to the fully differentiated non-hormone producing cell to de-repress the mechanisms for the synthesis of the relevant hormone. Although in the present state of knowledge it is difficult to be precise nevertheless the possibility that disturbances in the control of cell multiplication and growth may be associated with dramatic qualitative disturbances in cellular function is of great importance. It does suggest that these two abnormalities may be linked through a common mechanism presumably at the level of the cell's genetic material. Investigation into the processes by which these tumours synthesise substances not apparently synthesised by the tissues from which the tumour arises may provide a clue to the mechanism of carcinogenesis itself.

APPENDIX

DETAILS OF TUMOURS

Number in text	1	2	3	4	5	6	7	8	9
Age and Sex	48, F	51, F	50, M	57, F	60, F	54, F	52, M	38, M	52, M
Interval stored at -10°C to -15°C before first study	2 months	1 year	3 days	1 day	1 day	4 hours	4 days	2 hours	8 hours
Primary site	Pancreas	Ileum	Bronchus	Ileum	Ileum	Ileum	Ileum	Ileum	Bronchus
Metastases	liver, bone, lung, lymph nodes.	Liver	Liver, ovaries	Liver, ovaries	Liver	Liver, ovaries	Liver	Liver	Liver
Clinical Features: Flushing	+	+	+	+,-	+	+	+	+	+
Clinical Features: Diarrhoea	+	+	+,-	-	+	+	+	+	+
Clinical Features: Valvular lesions	+	+	?	-	+	+	+	-	+(P.M.)
Site of tumour studied	liver metastases	liver metastases	liver metastases	Ovarian metastases	Liver metastases	Liver metastases	Liver metastases	Ileal primary	Liver metastases
Tumour 5-hydroxyindoles	5HTP 40µg/G 5HT 32µg/G 5HTP 25mg/day	not examined	not examined	5HT 150µg/G C.5HT++,5HIAA±	C.5HT ++	C. 5HT ++	C.5HT +++ little 5HIAA	C.5HT +	C.5HT ±
Urinary 5-hydroxyindoles	5HT 20mg/day 5HIAA 430mg/day	5HIAA 30-100mg/day	5HIAA raised on screening test	5HIAA raised on screening test	C.5HIAA ++ No 5HTP or 5HT on C.	C. 5HIAA only 65 mgms/day	C.5HIAA only. 3o50 mgm/G creatinine	C.5HIAA only 62 mgms/day	C.5HIAA 74 mg/day
Histology	Pancreatic Adenocarcinoma	Carcinoid	Bronchial Carcinoid	Carcinoid	Carcinoid	Carcinoid	Carcinoid	Carcinoid	Bronchial "Round-cell" Carcinoma

C = two way ascending paper chromatography in solvents described on p.60.

Stained with Ehrlich's reagent.

ADDENDUM

5-hydroxytryptamine creatinine sulphate, L-tryptophan, L-phenylalanine, pyridine nucleotides, ascorbic acid and nicotinamide were purchased from British Drug Houses Ltd. Tetrahydrofolic acid was prepared by the method described by Heuennekens et al (1963)[1]. 6-7 dimethyl-5,6,7,8-tetrahydropteridine HCl was obtained from Calbiochem Ltd. Glucose-6-phosphate dehydrogenase (100 Kornberg Units/mg protein)[2], prepared from yeast, was bought from British Drug Houses Ltd, as was the barium salt of glucose-6-phosphate which was converted to the potassium salt in the way described by Kornberg and Horecker (1955)[2]. 5-hydroxytryptophan, 5-hydroxyindole acetic acid (diethylammonium salt) and iproniazid phosphate were donated by Roche Products Ltd. α-Methyl-dihydroxyphenylalanine (Aldomet) was provided by Merck Sharp and Dohme Ltd, and amethopterin by Lederle Ltd. Sephadex G 25 was purchased from Pharmacia, Sweden; Zeocarb 225 and activated charcoal from B.D.H. Ltd. 3-Hydroxybenzyloxyamine (NSD 1024) was provided by Dr. Drain of Smith & Nephews Ltd. All radioactive chemicals were obtained from the Radiochemical Centre, Amersham. All other substances used in enzyme incubations were of analytical grade.

1. Heuennekens, F.M., Mathews, C.K. and Scrimgeour, K.G. (1963) Methods in Enzymology, Ed. Colowick, S.P. and Kaplan, N.O., Vol IV. p. 803, Academic Press Inc., New York.

2. Kornberg, A., and Horecker, B.L. (1955) ibid, Vol I, p. 323.

SUMMARY

The enzymatic 5-hydroxylation of tryptophan with the formation of 5-hydroxytryptophan, as the initial step in the biosynthesis of 5-hydroxytryptamine, has beendemonstrated in isolated preparations of carcinoid tumour, intestine and brain. The method developed to assay this reaction involves the incubation in vitro of the substrate tryptophan-3-C^{14} with the tissue preparation under study, inhibition of 5-hydroxytryptophan decarboxylation, isolation of the 5-hydroxytryptophan produced and its controlled enzymatic decarboxylation to 5-hydroxytryptamine which is then isolated and assayed.

Carcinoid tumour, intestine and brain whilst 5-hydroxylating tryptophan will not hydroxylate phenylalanine, nor is tryptophan hydroxylation in these tissues inhibited by amethopterin. This distinguishes the tryptophan 5-hydroxylation occurring in these tissues from that occurring in rat liver which appears to be due to the non-specific activity of phenylalanine 4-hydroxylase. These findings add weight to the argument that liver is normally not a site of 5-hydroxytryptamine biosynthesis.

Tryptophan 5-hydroxylase appears to be stereospecific for the L isomer of tryptophan and has been partially purified by ammonium sulphate precipitation from preparations of carcinoid tumour and brain. After purification it is possible to show that the enzyme requires a reduced pteridine cofactor for full activity, suggesting a similarity with phenylalanine 4-hydroxylase and tyrosine hydroxylase.

Studies of tryptophan 5-hydroxylation in brain have shown that this organ is capable of synthesising independently all the 5-hydroxy-

tryptamine it needs and that there need be no reliance upon the synthesis of 5-hydroxytryptophan or 5-hydroxytryptamine in other areas of the body. Anatomically tryptophan 5-hydroxylation is most active in the phylogenetically older parts of the brain and at the subcellular level a large proportion of the enzyme is localised in the presynaptic nerve endings. The anatomical and subcellular distribution of tryptophan 5-hydroxylase in brain broadly follows that of 5-hydroxytryptophan decarboxylase activity and 5-hydroxytryptamine concentration, and these findings are compatible with the hypothesis that 5-hydroxytryptamine is a substance directly or indirectly involved in the chemical transmission of nervous impulses in certain areas of the nervous system. The study of tryptophan 5-hydroxylation by brain homogenates prepared so that nerve endings remain intact has suggested that the membrane envelope of these nerve endings either encloses an optimal environmental level of the reduced pteridine cofactor for tryptophan 5-hydroxylase or that the pteridine does not easily cross the nerve cell membrane.

The phenomenon of a lowered brain 5-hydroxytryptamine concentration in experimental phenylketonuria has been investigated. Although phenylalanine inhibits tryptophan 5-hydroxylation in preparations of brain in which nerve endings remain intact it has been reported that it does not inhibit purified tryptophan hydroxylase. Experiments have been done which reconcile these findings and suggest that phenylalanine lowers brain 5-hydroxytryptamine by inhibiting the transport of tryptophan across the nerve cell membrane.

Quantitative data have shown that carcinoid tumours synthesise 5-hydroxytryptamine at a physiological rate and that the overall increased

biosynthesis of 5-hydroxyindoles in the carcinoid syndrome is due to the greatly increased number of functioning cells and not due to a greatly increased function of individual cells.

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