THE PHARMACOLOGICAL ACTIVITY OF EXTRACTS OF ANIMAL TISSUES, WITH PARTICULAR REFERENCE TO THEIR CONTENT OF 5-HYDROXYTRYPTAMINE.

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CONTENTS

Results/

PART II/

PAGE

PART II

INTRODUCTION

Since the identification as 5-hydroxy-tryptamine of both enteramine, a constituent of enterochromaffin cells, and serotonin, the vaso-constrictor principle arising in blood when it clots, much interest has been directed to its study.

Enteramine was described by Erspamer (1935) and has been studied by him and his co-workers for twenty years. They have shown its presence in certain tissues of many animal species and in every case these tissues are largely made up of entero-chromaffin (argentaffin) cells. As a result of diuresis measurements in rats, Erspamer believes that enteramine is a hormone concerned with the control of diuresis. In physiological doses enteramine causes a marked reduction of urine flow.

Investigations over many years on vaso-constrictor principles arising in blood were greatly clarified by the work of Rapport, Green and Page, (1948a and b). They isolated a vasoconstrictor from serum/

serum which they called serotonin. It was shown by Rapport (1949) to be 5-hydroxytryptamine creatinine sulphate.

After synthetic 5-hydroxytryptamine salts were prepared both enteramine and serotonin were shown to be identical with one of these - their pharmacological activities being due to the properties of the base.

5-hydroxytryptamine was shown to be present in the tissues of the dog brain.(Amin, Crawford and Gaddum, 1945a and b). Its spasmogenic action on the isolated oestrous uterus of the rat was blocked by lysergic acid diethylamide which has very powerful actions on the brain (Gaddum, 1953b; Stoll, 1947). Also 5-hydroxytryptamine seemed to act on certain preparations through a stimulation of ganglion cells.

For these reasons it seemed worth while that a study of the distribution of 5-hydroxytryptamine throughout the various tissues of a certain species should be undertaken in the hope that some clue of its physiological function might become apparent. The experiments described in Part II sought to clarify its mode of action more locally, viz. on the isolated guinea-pig ileum.

Because/

Because it seemed advisable to exclude the effect of adrenaline from the estimations of tissue extracts (page 59), these methods were tried in turn to achieve this. In the first, a method of paper chromatography was investigated, but a suitable solvent system, causing adequate separation of adrenaline from 5-hydroxytryptamine, was not found among those tested. Second, a method of assay in which the adrenaline effect was specifically antagonised was considered. Here, the isolated perfused ear of the rabbit was used, but the preparation was very tedious and the discrimination tended to be poor. Finally, a method was developed in which an enzyme preparation of mushrooms was incubated with the extracts and this caused a destruction of the adrenaline effect. Assays of the mushroom treated samples were made on the isolated oestrous uterus of the rat.

The 5-hydroxytryptamine used in the experiments and as the reference standard in all the biological assays of tissue extracts was a synthetic preparation of 5-hydroxytryptamine creatinine sulphate - a white crystalline powder, kindly supplied to Professor Gaddum by Messrs. Upjohn Inc. of/

of Kalamazoo, Michigan, U.S.A.

In the text, "5HT-equivalent" means activity equivalent to 5-hydroxytryptamine expressed in terms of the base.

HISTORICAL

Vasotonins

Since the beginning of this century, it has been demonstrated by various workers that a vaso-constrictor principle is released when blood clots.

Brodie (1903) was working on the perfusion of isolated organs. He stated that "serum is by no means a completely inert fluid". By the use of citrate to prevent clotting he found that his preparations were much more satisfactory and he concluded that some 'irritant' was produced by the clotting process.

In similar perfusion systems, Battelli (1905) found that defibrinated blood failed to flow easily through the vessels because of vasoconstriction.

A careful study by Janeway and Park (1912) confirmed the conclusions of these and other workers that a vasoconstrictor arose in blood during clotting and that it was not adrenaline because it constricted coronary, as well as carotid, rings and stimulated intestinal smooth muscle. They stated that its point of action and effects seemed similar to those of barium.

Stewart/

Stewart and Zucker (1913) showed that citrate plasma had no effect on their sheep arterial ring preparation, but that the platelets from citrate blood possessed a high vasoconstrictor activity. They suggested that the clotting process as such, (i.e. fibrin to fibrinogen) was not directly concerned with the liberation of this vasotonin, to which was ascribed a role in haemostasis.

A review on serum vasoconstrictor, supplemented by many experiments, was published by Janeway, Richardson and Park (1918). They confirmed that plasma could be obtained quite free from vasoconstrictor activity, viz. in the absence of clotting or trauma to the platelets. They showed that the vasotonin arising from platelet rupture was not a protein, nor was it adrenaline; it was more readily extracted by water and by alcohol than by ether or chloroform; and it was dialysable and therefore crystalloid. They stated that its appearance was not dependent on the actual formation of the clot, nor was it related to any of the factors concerned in coagulation with the possible exception of thromboplastin.

Both a temporary and a permanent vasotonin were/

were described, in heparinised blood and in defibrinated blood by Bayliss and Ogden (1933). The first was "temporary" because it could be destroyed by passage of the blood through a lung-kidney preparation. The vasoconstrictor effects were antagonised by ergotoxin. Gaddum, Hebb, Silver and Swan (1953) showed recently that the vasoconstrictor and bronchoconstrictor action of serum was removed by passage of the blood through the lungs of a cat. Also, both the broncho-constriction and vasoconstriction were antagonised by ergot alkaloids.

It is interesting to note another observation of an ergot alkaloid effect on serum vasoconstrictor actions. Heymans, Bouckaert and Moraes (1932) showed a reversal of the constriction of rabbit ear vessels caused by defibrinated blood, by previous injection of ergotamine. Landis, Wood and Guerrant (1943) emphasized the necessity to exclude the presence of serum vasotonin (i.e. the occurrence of coagulation) during studies on pressor sub-stances of renal or other origin in the blood.

Further work on platelets was done by Zucker (1944). She confirmed that the origin of/

of serum vasoconstrictor was the platelets and that this vasotonin also stimulated strips of rat intestinal muscle. Studies of a pharmacologically active preparation from human buffy coat showed that this stimulated intestine, uterus, nictitating membrane and carotid artery rings. It constricted vessels of the cat's tail but not those of the rabbit's ear. She demonstrated that the preparation was heat-stable and dialysable, and that it was not histamine, tyramine, a choline-ester nor a sympathomimetic. Most of its actions seem now to be those of 5-hydroxytryptamine with the notable exception of its lack of action on the rabbit ear vessels. This may have been due to the differences in technique of perfusion of the ear vessels, or to a loss in activity during the rather drastic purification of the preparation.

Her later studies showed that the presence of both calcium and plasma were necessary for the liberation of the vasoconstrictor from platelets, (Zucker, 1951).

Serotonin/

Serotonin

A study of the vasoconstrictor activity of blood serum by Rapport, Green and Page (1947; 1948a and b) led to the isolation of a substance which they called serotonin. This substance caused constriction of isolated perfused blood vessels and contraction of intestinal muscle strips. During the purification of serotonin, the perfused ear of the rabbit was used to yield approximately quantitative results, in activity estimations of the various products.

The serum was adjusted to pH 4.5, extracted with various organic solvents and the active principle in the product was crystallised as the 5-nitrobarbituric (dilituric) acid salt. Study of this salt showed serotonin probably to be 5-hydroxytryptamine creatinine sulphate (Rapport, 1949). This material was synthesised by Hamlin and Fisher (1951) and was shortly shown to be the same as the natural serotonin (Page, 1952). Oxidation experiments on serum and on purified serotonin had given evidence favouring the conclusion that serotonin is solely responsible for the vasoconstrictor action of serum (Rapport et al 1948c).

Thrombocytin

Thrombocytin (Thrombotonin).

Thrombocytin is a vasoconstrictor principle found in serum only when platelets are present in the plasma at the time of clotting. Using the method of Rapport et al (1949a) for purification of the serum activity as the nitro-barbiturate complex, Reid and Rand studied the pharmacological properties of serotonin. They determined its actions on various test organs and intact animals and they found that these were all shared by serum itself. They discovered that the complex prepared from the serum from "platelet free" plasma was almost devoid of activity, whereas that prepared from serum of "platelet rich" plasma had a high activity (Reid and Rand, 1951). Thrombocytin was thus shown to be identical with serotonin - a series of comparisons on four different test organs was made (Rand and Reid, 1951).

When the synthetic compound, 5-hydroxy-tryptamine creatinine sulphate, was available, Reid (1952) made a careful study of its circulatory effects; while Reid and Rand (1952) detailed its pharmacological actions and showed these to conform to those of partially purified serum vasoconstrictor-serotonin or thrombocytin.

Enteramine/

Enteramine

In 1933, Vialli and Erspamer described the enterochromaffin argentaffin cells, which occur in the mucosa of the gut of vertebrates. They distinguished these cells from acidophil basal-granular cells, by the ability of the former to stain with diazotised p-nitraniline and to give positive chromaffin and argentaffin reactions (Vialli and Erspamer, 1933). The mucosa of the fundus region of the rabbit's stomach was found to be very rich in these cells (Erspamer, 1935). The component of the enterochromaffin cells which Erspamer found to be responsible for their staining reactions he called "enteramine". He began to investigate more fully its chemical and pharmacological properties in acetone and alcohol extracts of tissues rich in these cells. Enteramine seemed to be a di- or poly- phenolic amine (Erspamer, 1940). Soon Vialli and Erspamer (1942) recognised the presence of a second substance in the extracts. This gave the same colour reactions as enteramine but was not pharmacologically active. They found that it could be made so, by heating it in solution at pH 7 - at 100°C for 10 minutes. They called it Enteramine I (or inactive enteramine),(also, Erspamer, 1942b).

Extracts/

Extracts containing enteramine stimulated the atropinised oestrous uterus of the rat and the mouse; stimulated the isolated atropinised duodenum of the rat, and the urinary bladder of the dog, both in vitro and in vivo; and had a hypotensive action in the rabbit (Erspamer, 1940).

The presence of entermine in the human spleen was demonstrated by Erspamer (1943) in a study of acetone extracts of normal and pathological organs. A series of papers followed in which details are given of the finding of enteramine, or an enteramine-like substance, in extracts of various organs of vertebrates and invertebrates. Viz. -

extracts of gastro-intestinal tract and spleen of fishes (except Teleostei and Cyclostoma);

extracts of gastrointestinal tract of Ascidia;

extracts of posterior salivary glands of Octopus vulgaris and Eledone;

extracts of hypobranchial body of Murex;

extracts of amphibian skin.

In 1946, the indolalkylamines in the venom of the toad, known as Bufotenine and Bufotine were shown by Erspamer to have chemical and pharmacological properties, very similar to those of enteramine (Erspamer, 1946a). He thus concluded that enteramine was/

was probably an indolalkylamine and not a polyphenolic amine. Later he suggested that it was a derivative of indole having a phenolic-OH as well as a side chain with a primary or secondary amino grouping (Erspamer, 1948a).

Erspamer and Ottolenghi (1950) showed such a parallel distribution of enteramine (and the enteramine-like substances) and an anti-diuretic principle in acetone extracts of tissues, that it seemed almost certain that enteramine itself possessed this anti-diuretic property. Not only was the distribution similar but with care the anti-diuretic property of an extract could be shown to be roughly proportional to the enteramine activity as measured colorimetrically and pharmacologically. Then, any treatment destroying the enteramine activity also destroyed the anti-diuretic activity. Thus it was found that enteramine inhibits normal and water diuresis, as well as that due to xanthines, mercurials, salts and urea.

At about the same time, Erspamer and Ghiretti (1951) found that enteramine-containing extracts had a positive inotropic, chronotropic and tonotropic action on the molluscan heart, particularly on the heart of Helix pomata.

The/

The chemical and physical characterization of enteramine became clearer, and, particularly with the aid of paper chromatography, Erspamer and Boretti (1951) were able to confirm the presence of enteramine in all tissues in extracts of which enteramine-like substances had been demonstrated biologically and chemically. Enteramine seemed a single entity no matter what species or organ was its source.

Meanwhile, the pharmacological studies of enteramine effects on diuresis were continued by Erspamer and Ottolenghi (1950, 1951 and 1952), so that Erspamer was soon able to conclude that enteramine in the animal body is the specific hormone of enterochromaffin cells. Also, it is concerned in certain mammals with anti-diuresis by the control of intraglomerular pressures, i.e. by constriction of the afferent vascular bed of the glomerulus.

Then enteramine was isolated as the pure picrate from extracts of the posterior salivary glands of Octopus vulgaris and also from extracts of the skin of Discoglossus pictus. These were both identified with the picrate of 5-hydroxy-tryptamine which was synthesised at about the same time/

time (Erspamer and Asero, 1951 and 1952; and Asero, Colo, Erspamer and Vercellone, 1952).

The 'stable antidiuretic substance' (Stable ADS) described at this time in serum by Ginsburg and Heller (1951 and 1953) was later identified by Erspamer and Sala (1954) as 5-hydroxytryptamine.

In experiments on dogs, Barac (1953) was unable to find any reduction in blood flow through the kidney, after the administration of 5-hydroxytryptamine. He concluded that the antidiuresis here was not attributable to renal vasoconstriction and was therefore caused by some other mechanism than that demonstrated by Erspamer and Ottolenghi (1952) in the rat.

Other studies of 5-hydroxytryptamine distribution.

After synthetic 5-hydroxytryptamine became available for comparison, its distribution in tissues was studied by a number of workers.

Erspamer and Faustini (1953) found that 5-hydroxytryptamine occurred in the serum and haemolymph as well as the spleens of many species. Erspamer could now make more accurate estimates of 5-hydroxytryptamine (enteramine) activity and he studied/

studied the concentration of 5-hydroxytryptamine in the serum of patients suffering from various diseases (Erspamer 1954a and b).

Udenfriend, Clark and Titus (1952) were aware of the high concentrations of N-methyl-indolalkylamines in toad venom (Bufo marinus), so they suspected the presence of 5-hydroxytryptamine there also. Isotopic and chromatographic techniques made it possible for them to demonstrate 5-hydroxytryptamine to the extent of about 0.1% of the dry weight of the venom. This discovery made it possible for them to use B. marinus in their further studies of 5-hydroxytryptamine metabolism (see p.34).

Acetone extracts of the wall of the digestive tract of dogs and rabbits were estimated for 5-hydroxytryptamine activity by Feldberg and Toh (1953) on the atropinised rat colon. They found much more activity in the mucosa than in the muscularis externa. In the dog, the pyloric mucosa contained most 5-hydroxytryptamine whereas in the rabbit, the stomach fundus mucosa contained more than the pyloric mucosa. The extracts were then assayed for substance P (cf. von Euler and Gaddum/

Gaddum, 1931), on guinea pig ileum. This preparation was first treated with atropine and mepyramine and then desensitised to 5-hydroxy-tryptamine by a large dose of tryptamine (Gaddum, 1953). (This tryptamine treatment sensitised the tissue to substance P.)

Amin, Crawford and Gaddum (1952 and 1954) have developed a method for the separate extraction of 5-hydroxytryptamine and substance P from tissues. By treating the samples of tissue with 20 volumes of acetone they obtained quantitative extraction of the 5-hydroxytryptamine in the acetone and left the substance P in the insoluble residue. If the latter was treated with acid, (N hydrochloric acid), the substance P passed into solution.

By the application of this method, they obtained estimates of 5-hydroxytryptamine distribution in the central nervous system of dogs. Assays were made on the atropinised oestrous uterus of the rat. In all cases, a specificity test of the extract activity was made with L.S.D. This compound was found by Gaddum (1953) to be a specific antagonist of 5-hydroxytryptamine contractions of rat oestrous uterus. The results of this/

this test together with chromatography and parallel assay on the perfused ear of the rabbit showed that the active principle of the acetone extracts was 5-hydroxytryptamine.

The areas containing most 5-hydroxy-tryptamine were the hypothalamus, area postrema, mid brain, colliculi and nuclei cuneatus and gracilis. There appeared to be no correlation between the amount of substance P and the amount of 5-hydroxytryptamine in the nervous tissues studied, (Amin, Crawford and Gaddum, 1954).

In studies of the vasoconstrictor action of rabbit plasma, Holgate (1953) showed a high histamine equivalent in the plasma of heparinised blood. Assays were made on the perfused ear of the rabbit. Though responses to standard histamine were abolished by mepyramine, the extract responses were little affected, so he re-estimated the activity in terms of standard 5-hydroxytryptamine. This gave the value 0.6μg/ml plasma. Plasma from citrated blood gave a much lower histamine equivalent and this activity was almost completely abolished by mepyramine.

Humphrey and Jaques (1953 and 1954) studied the release of histamine and 5-hydroxytryptamine from/

from platelets by antigen-antibody reactions. This work was done 'in vitro' with carefully collected and washed 'buffy layer' of rabbit blood, partially purified antibody and plasma. The 5-hydroxy-tryptamine was assayed on rat colon or rat oestrous uterus and a specificity test was made by the 5-hydroxytryptamine specific antagonist, 2-methyl, 3-ethyl, 5-amino indole. Citrated plasma did not have this 'releasing' activity.

5-hydroxytryptamine and histamine were liberated in parallel, though the actual quantity of 5-hydroxytryptamine was about 3 times that of histamine.

With a fluorimetric method of estimation Udenfriend and Weissbach (1954) have been able to confirm that the platelets contain all the 5-hydroxytryptamine which can be shown to be present in whole blood. The plasma contains none. Estimates of 5-hydroxytryptamine-equivalent were quoted for a few animals. Other studies, with C_{14} labelled tryptophan showed the half-life of platelet 5-hydroxytryptamine to be 2-5 days, which is in close agreement with the reported half-life of the platelet. 5-hydroxytryptamine isolated from the intestinal/

intestinal tract showed a more rapid turnover. They found no evidence for a concentrating mechanism for 5-hydroxytryptamine within the platelets nor for its formation by the platelets from 5-hydroxytryptophan. They therefore suggested that 5-hydroxytryptamine is synthesised and incorporated at the site of platelet formation and that it is metabolically inert until the platelet disintegrates (Udenfriend and Weissbach, 1954).

But Humphrey and Toh (1954) contended that since 'in vitro' dog platelets were able to adsorb 5-hydroxytryptamine from their surrounding fluid, the platelets were likely to have such a power within the animal body. They suggested that this property prevents the 5-hydroxtryptamine concentration in plasma from rising above a very low threshold.

The heart of Venus mercenaria was used by Twarog and Page (1953) in conjunction with paper chromatography for investigations of the 5-hydroxytryptamine content of various tissues of dogs, brains of rats and rabbits, as well as blood and serum. This molluscan heart is very sensitive to/

to stimulation by 5-hydroxytryptamine-in concentrations as low as 10^{-9} - and to inhibition by acetylcholine (p. 57). The latter effect can be blocked by mytolon[+]. The authors found 5-hydroxytryptamine activity in all the samples of brain tissue, but not in the nerve and muscle samples. Variable responses to kidney and left ventricle samples were recorded. Saline dilutions of blood and acetone extracts of serum (both from normal dogs), yielded similar concentrations of 5-hydroxytryptamine/ml serum: also, the activity in dog urine showed an excretion of 0.06-4 µg/ml which was increased after intravenous infusion of 5-hydroxytryptamine solution.

Florey and Florey (1953) studied aqueous extracts of the nerves and ganglia of crayfish and cuttlefish for their excitor action on the hearts of the same species. They showed that the activity of the extracts, relative to 5-hydroxytryptamine was equal on two test organs and that various chemical and physical tests gave similar results. Thus they state that the nervous tissue of these crayfish contained 30-66 µg 5-hydroxytryptamine/g dry weight.

Bacq/

[+]Mytolon - 2;5-Bis (3-diethylamino propyl amino) benzoquinone-bis-benzyl chloride.

Bacq, Fischer and Ghiretti (1952) showed that on nerve stimulation the perfused salivary glands of O. Vulgaris secrete 5-hydroxytryptamine in their saliva; also 5-hydroxytryptamine itself has a secretory effect on these glands. The glands of O. macropus did not respond in this way.

Histological studies by Barter and Everson Pearse (1953) led them to conclude that they have detected 5-hydroxytryptamine in the enterochromaffin cells. They showed that the granules, typical of these cells, were an artefact of the fixing process, but were derived from 5-hydroxytryptamine.

A carcinoid tumour is a local hypertrophy of enterochromaffin cells. It was because of this that Lembeck was anxious to estimate the enteramine (5-hydroxytryptamine) content of such a tumour. After some considerable time he obtained a specimen and was able by biological methods, with the aid of paper chromatography, to estimate that the tumour contained 2·5 mgm 5-hydroxytryptamine/g fresh tumour. Thus he was able to agree with Erspamer that the origin of 5-hydroxytryptamine in the animal body is the enterochromaffin cell system (Lembeck, 1953 and 1954).

Recently/

Recently the "carcinoid syndrome" has been described, particularly in a series of seven carcinoid cases detailed by Thorson, Biörck, Björkman and Waldenström (1954). The syndrome consists of carcinoid of the ileum, valvular disease of the right side of the heart, flushing of the skin with an unusual type of patchily distributed changing cyanosis and 'asthmatic' attacks. These authors believed the cause to be a malignant carcinoid having hormonal properties. They noted that 5-hydroxytryptamine seemed the most likely of the vaso-active substances known to have effects corresponding with the signs and symptoms of the syndrome. The finding of a considerable concentration of 5-hydroxytryptamine in a carcinoid, by Lembeck (1953 and 1954), supported this view.

Patients suffering from carcinoids experience sudden flushing attacks. During such attacks, Pernow and Waldenström (1954) sampled blood from two patients. They prepared acetone extracts of the serum and found a high 5-hydroxytryptamine equivalent in both cases:-

5.2 µg and 6.5 µg hydroxytryptamine/ml serum compared with the figures of 0.03-0.20 µg/ml given/

given by Erspamer and Faustini (1953) for normal humans.

A case of carcinoid was diagnosed after operational removal of a malignant tumour which was causing obstruction of the ileum. Ten days from the operation, a blood sample was taken. The serum was extracted and estimated by the method described in detail on p. 50. This sample had a 5-hydroxytryptamine eqivalent of 2.8-3.2 µg/ml serum, which is about ten times greater than the equivalent of serum from normal persons estimated in the same manner. A full description of this case and the details of the investigations made are given by Duncan, Garven and Gibbons,(1955).

Pharmacological properties of 5-hydroxy-tryptamine.

Shortly after the synthesis of 5-hydroxytryptamine creatinine sulphate, surveys of its pharmacological properties were made in Australia by Reid and Rand (1952) and by Reid (1952) and in America by Freyburger, Graham, Rapport, Seay, Govier and Vander Brook (1952).

Their findings can be summarised as follows:

a)/

a) Intravenous injection of 5-hydroxytryptamine into chloralose cats caused -

an initial fall of systemic arterial pressure associated with increased resistance in the pulmonary circulation:

followed by a rise, due to vasoconstriction, (demonstrable in hind-limb, intestines and kidney):

and then by a more prolonged fall.

Apnoea and brief bronchoconstriction occurred also.

b) Dogs presented a varied response to intravenous injection of 5-hydroxytryptamine - sometimes pressor, sometimes depressor. Pithing of the dogs caused a more regularly pressor response.

c) The nictitating membrane of the cat was constricted, as was the pupil, in response to intracarotid injection of 5-hydroxytryptamine.

d) Injection of 5-hydroxytryptamine into the superior mesenteric artery of the eviserate cat caused the liberation of adrenaline.

e) The 'in situ' uterus of the non-gravid adrenalectomised cat relaxed on intravenous injection/

injection of 5-hydroxytryptamine.

f) The uterus of the rat was stimulated by 5-hydroxytryptamine.

g) The bladder of the dog was stimulated by 5-hydroxytryptamine.

h) The jejunum of the guinea-pig was stimulated by 5-hydroxytryptamine.

i) A spiral strip of the sheep's carotid artery was contracted by 5-hydroxytryptamine.

j) Intradermal or subcutaneous injection of 5-hydroxytryptamine in the human skin caused a slight red reaction without itching or wealing and a local vasoconstriction.

Toxicity - Intravenous injection.

In mice - L.D.50 = 160 mgm 5-hydroxytryptamine/kg
In rats - L.D.50 = 30 mgm 5-hydroxytryptamine/kg

Since the above results were reported, observations on the pharmacology of 5-hydroxytryptamine have been made by many other workers.

Gaddum et al (1953) studied the bronchial effects/

effects of 5-hydroxytryptamine in the isolated perfused lungs of the cat. 5-hydroxytryptamine caused a distinct bronchoconstriction and a constriction of the pulmonary blood vessels. Both these effects were antagonised by lysergic acid diethylamide and by dihydroergotamine.

In the cat, Schneider and Yonkman (1953) recorded an increase in frequency of vagal impulses from the stretch receptors in the lung, after injection of 5-hydroxytryptamine. Respiration ceased after an expiration but later tachypnoea developed, probably because of central stimulation, for the vagal activity was still increased.

Comroe, van Lingen, Stroud and Roncoroni (1953) showed well defined apnoea in chloralose cats with very small doses of 5-hydroxytryptamine, (10 µg/kg), and suggested that a stimulation of chemo-receptors occurred. Pulmonary vasoconstriction was recorded also.

"5-hydroxytryptamine is by no means a pure hypertensive substance, in as much as it may possess, according to circumstances, hypertensive, hypotensive and biphasic pressure actions". This statement was made by Erspamer (1952c) and supported the/

the conclusions of other workers (Page, 1952; Freyburger et al. 1952; Page and McCubbin, 1953a).

Although 5-hydroxytryptamine had no effect on 'in vitro' blood-clotting systems, it caused immediate haemostasis when given intravenously to animals with peripheral wounds,(Correll, Lyth, Long and Vanderpoel, 1952). A similar response occurred in heparinised rats, and though the effect of an intravenous dose is of limited duration this can be extended by further injections. Correale (1954) recorded the results of similar experiments. He found that subcutaneous injections of 5-hydroxytryptamine reduced the bleeding time of the cut tail veins of the rat. Page (1952) also suggested that 5-hydroxytryptamine had a haemostatic property due to local vasoconstriction.

The responses of the heart of Venus mercenaria to 5-hydroxytryptamine were first described by Welsh (1953). He had previously reported that this heart was very sensitive to inhibition by acetylcholine and that it was relatively much less sensitive to stimulation by adrenaline, noradrenaline, tyramine and histamine (Welsh and Taub, 1948). 5-hydroxytryptamine, however, excited the heart at concentrations as low as 0.0002 μg/ml. He found that/

that electrical stimulation of the visceral ganglion, after block of cholinergic inhibition, caused a stimulation very similar to that elicited by 5-hydroxytryptamine. Ergot and lysergic acid diethylamide, which blocked 5-hydroxytryptamine excitation, also abolished the effect of direct electrical stimulation (Welsh, 1954). Since 5-hydroxytryptamine was found to be present in the ganglia of Venus mercenaria, Welsh concluded that it was the normal mediator of excitatory nerves in this mollusc.

The stimulating effect of 5-hydroxytryptamine (enteramine, serotonin) on the Helix heart, and on the vessels of the perfused ear of the rabbit have already been mentioned (see p.11 and p.5 respectively).

On the exposed base of a blister caused by cantharidin, 5-hydroxytryptamine and tryptamine both caused pain which was indistinguishable from that produced by serum. Plasma produced no pain. There was a specific refractory period after the pain stimulation, which in the case of 5-hydroxytryptamine and serum was a reciprocal block, but was not so for acetylcholine (Armstrong, Dry, Keele and Markham, 1952; 1953).

Antagonism/

Antagonism of responses to 5-hydroxy-tryptamine.

For some time it has been clear that a drug having a specific antagonistic action to responses caused by 5-hydroxytryptamine would be most useful. Such a drug might help us to understand the physiological function of 5-hydroxytryptamine; it might help in the identification of 5-hydroxytryptamine in tissue extracts; it might be useful also in therapy.

1) Ergot alkaloids and lysergic acid diethylamide.

Fingl and Gaddum (1953) made a study of the activity of ergot alkaloids, with the above ideas in mind. They found that dihydroergotamine gave a highly specific irreversible blockade of 5-hydroxytryptamine and tryptamine responses in the isolated rat uterus. It also blocked both adrenaline and 5-hydroxytryptamine constrictions of rabbit ear vessels, but was more effective against the 5-hydroxytryptamine response. Ergotamine gave similar results (also Gaddum and Hameed, 1954).

Gaddum (1953b) found that lysergic acid diethylamide (L.S.D.) had also a highly specific antagonistic power, with reference to 5-hydroxy-tryptamine/

tryptamine responses on both the rat uterus and the rabbit ear vessels. (Lysergic acid diethylamide is related structurally to the ergot alkaloids; it has powerful effects on the human brain even in doses of 1 µg/kg).

The ergot alkaloids were studied too by Erspamer (1953). He showed that dihydroergotamine and hydergin, blocked the antidiuretic response of rats to subcutaneous injection of 5-hydroxytryptamine. (Hydergin is a mixture of hydrogenated alkaloids of ergot).

Ergotoxine was reported by Woolley and Shaw (1953) to have a reversible but incomplete antagonistic effect on 5-hydroxytryptamine stimulation of isolated carotid rings.

On isolated guinea-pig ileum the antagonism between ergot alkaloids and 5-hydroxytryptamine is feeble and less clearly specific (Gaddum and Hameed, 1954).

2) Adrenolytic drugs.

Various other adrenolytic drugs have been tested for antagonistic effects on 5-hydroxytryptamine responses in various tissues.

Dibenamine was found, by Erspamer (1952b) to be a powerful inhibitor of the spasm induced by 5-hydroxytryptamine/

5-hydroxytryptamine on the isolated rat uterus; this action was quite specific and was suggested to be a case of substrate competition.

In the isolated ear of the rabbit, adrenaline responses were more readily blocked by dibenamine than those of 5-hydroxytryptamine, but the latter were also affected (Fingl and Gaddum, 1953; Gaddum and Hameed, 1954). On the isolated guinea-pig ileum, 5-hydroxytryptamine and histamine responses were reduced about equally with recovery only of the 5-hydroxytryptamine effect, after the dibenamine was washed out (Gaddum and Hameed, 1954).

Piperoxane (10^{-6}) abolished the power of adrenaline to cause vasoconstriction in the perfused rabbit ear without causing any change in the response to 5-hydroxytryptamine (Fingl and Gaddum, 1953; Gaddum, 1954; Gaddum and Hameed, 1954).

Regitine in the same concentration (10^{-6}) also has a very marked anti-adrenaline effect. Page and McCubbin (1953b) noted that piperoxane gave a transient reversal of the 5-hydroxytryptamine pressor response in normal dogs.

In studies of adrenergic blocking drugs, Leitch, Leibig and Haley (1954) measured effects on the response of the rat seminal vesicle to adrenaline./

adrenaline. Dibenzyline had the highest blocking potency with also a very high degree of fixation to the tissue. Dibozane was next in blocking potency to dibenzyline (being about 10× as active as piperoxane), but unlike the latter, dibozane was easily washed out of the tissue and a recovery of the adrenaline response was recorded. These compounds have been studied here for anti-adrenaline and anti-5-hydroxytryptamine effects on the isolated perfused ear of the rabbit. The results are reported on p. 88.

Ephedrine effects on the responses to adrenaline of the perfused ear of the rabbit were described by Gaddum and Kwiatkowski (1938). They showed that low concentrations (10^{-6}) potentiated adrenaline vasoconstrictions, whereas higher concentrations antagonised them. The potentiation effect was ascribed to an inhibition by the ephedrine of tissue amine-oxidase, and the antagonistic effect to a blocking of the adrenaline receptors.

5-hydroxytryptamine is also destroyed by amine-oxidase (Blaschko, 1952; Freyburger et al. 1952). So Gaddum and Hameed (1954) tested the effect of ephedrine on 5-hydroxytryptamine responses in/

in the rabbit ear. In these experiments, 10^{-5} ephedrine was found to be already inhibiting adrenaline but greatly increasing the response to 5-hydroxytryptamine. This was explained as the blockade of adrenaline receptors, the persistence of inhibition of amine-oxidase and the absence of any block of tryptamine receptors.

3) Local anaesthetics.

Cocaine, as well as being a local anaesthetic, is an inhibitor of amine-oxidase (Philpot, 1940).

The constrictor effect of 5-hydroxytryptamine on sheep carotid artery strips was increased by cocaine, (Reid and Rand, 1952) but the authors gave no indication of the specificity of this effect. On the isolated rat uterus, cocaine (10^{-5}) was shown to potentiate the responses to both 5-hydroxy-tryptamine and acetylcholine. Higher concentrations gave a temporary inhibition of the responses to both drugs. Reid and Rand, therefore concluded that there was no support here for the 'inhibition of amine-oxidase' theory. But in the rabbit ear, 10^{-5} cocaine potentiated the adrenaline vasoconstriction but not the 5-hydroxytryptamine one (Gaddum and Hameed, 1954).

On/

On the guinea-pig ileum, cocaine caused a reversible inhibition of the spasm induced by 5-hydroxytryptamine, without much effect on histamine or acetylcholine responses (Sinha and West, 1953; Robertson, 1953; Rocha e Silva, Valle and Picarelli, 1953; Gaddum and Hameed, 1954). This was explained by the various workers on the theory that 5-hydroxytryptamine acts through the nerves of the intestine and cocaine prevents its action by paralysing these nerves.

A number of other local anaesthetics, including procaine and cinchocaine, were studied by Sinha and West (1953) for their antagonism of 5-hydroxytryptamine responses. They found a fair degree of agreement between the antagonistic potencies of some, against 5-hydroxytryptamine stimulation of rabbit ileum and rabbit auricles and the local anaesthetic properties of these drugs. Others were shown to antagonise the action of 5-hydroxytryptamine on guinea-pig ileum, cat trachea, and rat uterus preparations.

4) Antihistamines.

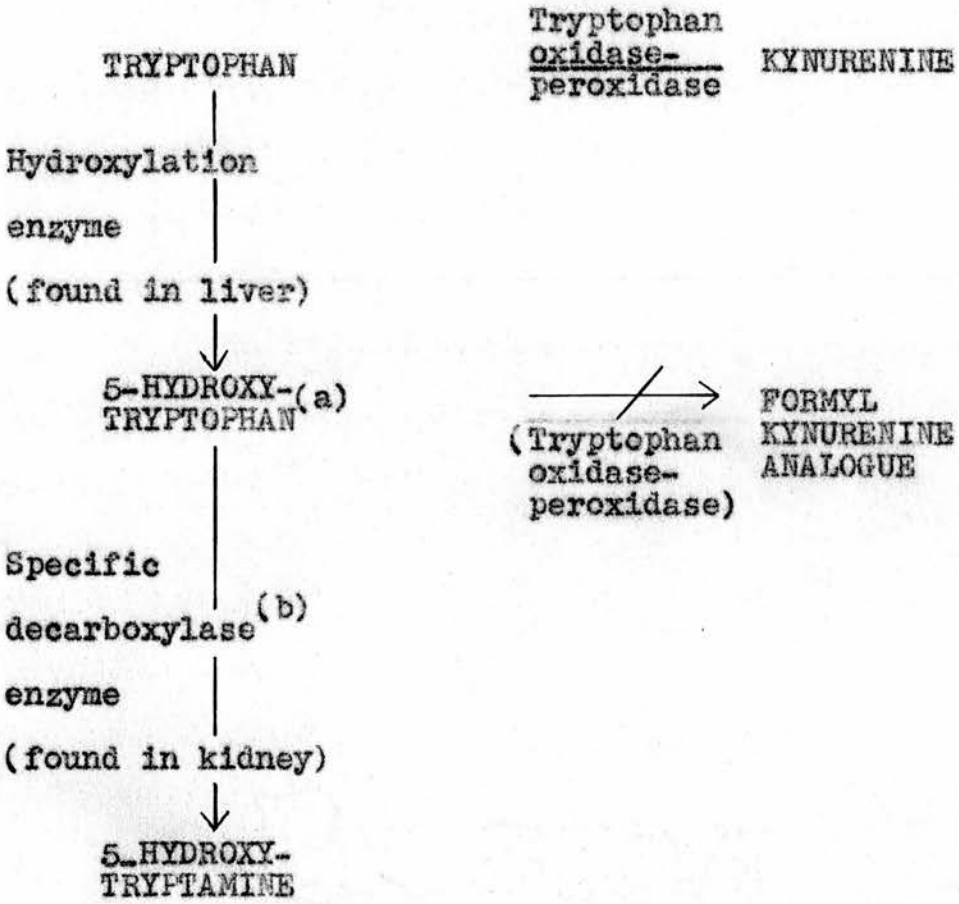
The antihistamine, tripelennamine, was found to be less effective in abolishing 5-hydroxytryptamine spasm of guinea-pig ileum than in/

in abolishing histamine spasm, and still less effective in abolishing acetylcholine spasm (Rapport and Koelle, 1953). Mepyramine in a dose which protects guinea-pigs completely against dyspnoea caused by histamine, has no effect against a similar condition produced by 5-hydroxytryptamine (Herxheimer, 1953). Other antihistamines were tested by Page and McCubbin for anti-5-hydroxytryptamine effects, but such effects as were recorded were not clear-cut,(Page and McCubbin, 1953b).

Metabolism of 5-hydroxytryptamine.

It has been known for some time that tissue homogenates destroy the activity of extracts containing 5-hydroxytryptamine (Erspamer, 1948b) and that amine-oxidase destroys 5-hydroxytryptamine (Blaschko, 1952). Recently, the work of Ek and Witkop (1953) and of Udenfriend, Clark and Titus (1953) enable the following table of 5-hydroxy-tryptamine/

tryptamine synthesis to be drawn.



(a) Ek and Witkop found 5-hydroxy-tryptophan to be quite stable to kynurenine-forming enzymes.

(b) This decarboxylase would not decarboxylate tryptophan; thus tryptophan ⟶ tryptamine ⟶ 5-hydroxytryptamine is very unlikely.

The product of amine-oxidase action on

5-hydroxytryptamine/

5-hydroxytryptamine is 5-hydroxyindole-acetic acid. This was shown to be a normal constituent of the urine of carnivorous and omnivorous animals, (Titus and Udenfriend, 1954; Erspamer, 1955).

Chemical properties of 5-hydroxytryptamine.

Since Erspamer studied the staining reactions of the enterochromaffin cells, he has made frequent use of colour reactions in his work on enteramine (5-hydroxytryptamine) in solutions and on chromatograms (Erspamer and Boretti, 1951; Vialli and Erspamer, 1940, 1942).

Two of his tests are the following -

1) coupling reaction in acid medium.

Reagent - 20 vols. saturated frozen solution of p-nitraniline in 0.05 N hydrochloric acid and 1 vol. of 1% sodium nitrite. If this is used to spray a chromatogram enteramine A and enteramine I spots will give a yellow-red colour, becoming peach-red later and being very persistent. If the reagent is added to a solution containing either/

either enteramine A or enteramine I, a wine-red colour can be extracted from the mixture, by shaking with amyl or butyl alcohol. The intensity of the colour in this alcohol layer is proportional to the concentration of enteramine.

2) Enteramine in alkaline media gives a yellow or gold-yellow fluorescence in Wood's light.

This latter ability of 5-hydroxytryptamine to cause fluorescence under certain conditions led Shepherd, West and Erspamer (1953) to quantitative detection of 5-hydroxytryptamine on chromatograms. They used butanol, acetic acid and water for their chromatography solvent, and sprayed the dried papers with a mixture of potassium dichromate solution and formaldehyde. After heating the papers at 100-110°C for 5 minutes, the spots caused by 5-hydroxytryptamine showed a golden-yellow fluorescence when viewed under ultra violet light. This method is

quantitative/

quantitative as low as 0.2 µg 5-hydroxytryptamine base. Bufotenine and bufotenidine behave quite similarly; but the test is much less sensitive to tryptamine.

Jepson and Stevens (1953) published a fluorimetric test which is specific for certain tryptamines, including 5-hydroxytryptamine and tryptamine itself.

A chemical method for the estimation of 5-hydroxytryptamine in platelets was developed by Udenfriend and Weissbach (1954). This method is based on the fluorescence intensity arising when 5-hydroxytryptamine is activated at 295 mµ and fluoresces at 330 mµ (Udenfriend, 1954).

Synthesis of 5-hydroxytryptamine.

Asero, Colo, Erspamer and Vercellone (1952) synthesised 5-hydroxytryptamine and isolated it as the picrate. The outline of their method is as follows -

m-cresol ⟶ 2-nitro 5-oxytoluol ⟶ 2-nitro 5-methoxytoluol ⟶ 2-nitro 5-methoxyphenyl pyro-racemic acid ⟶ 5-methoxyindole carbonic acid ⟶ 5-methoxyindole ⟶ 5-methoxyindole β-aceto-nitrile ⟶ 5 methoxytryptamine HCl ⟶ 5-hydroxytryptamine HCl.

Hamlin/

Hamlin and Fischer made their synthesis in 1951, thus -

5-benzyloxyindole ⟶ 5-benzyloxygramine 5-benzyloxyindole 3-acetamide ⟶ 5-benzyloxytryptamine ⟶ 5-hydroxytryptamine HCl. This product was described as a light sensitive hygroscopic salt.

At the same time Speeter, Heinzelmann and Weisblat (1951) synthesised 5-hydroxytryptamine creatinine sulphate, also from 5-benzyloxyindole, in this way, -

5-benzyloxyindole ⟶ 5-benzyloxyindole β-aceto-nitrile ⟶ 5-benzyloxytryptamine HCl ⟶ 5-hydroxytryptamine creatinine sulphate.

Speeter and Anthony (1954) have developed another method of synthesis of 5-hydroxytryptamine and related compounds.

METHODS

METHODS

Chromatographic techniques.

Paper chromatography as we understand it to-day, was first used by Consden, Gordon and Martin (1944). It is thought to depend on differential partition between two solvents, which on paper means a partition between the stationary phase of the cellulose and adsorbed water and the mobile phase of the organic solvent.

Here, the ascending method of paper chromatography was used. The distances moved by adrenaline and 5-hydroxytryptamine under the influence of various solvent systems was studied.

The components of the chosen solvent were mixed 12-16 hours before required and placed in a separating funnel. If there was a separation into layers, the aqueous layer was rejected and the solvent, (i.e. organic) layer was placed in the tank. Large rectangular glass tanks, (20 cm. x 30 cm. x 40 cm.) with plate glass covers were used.

A/

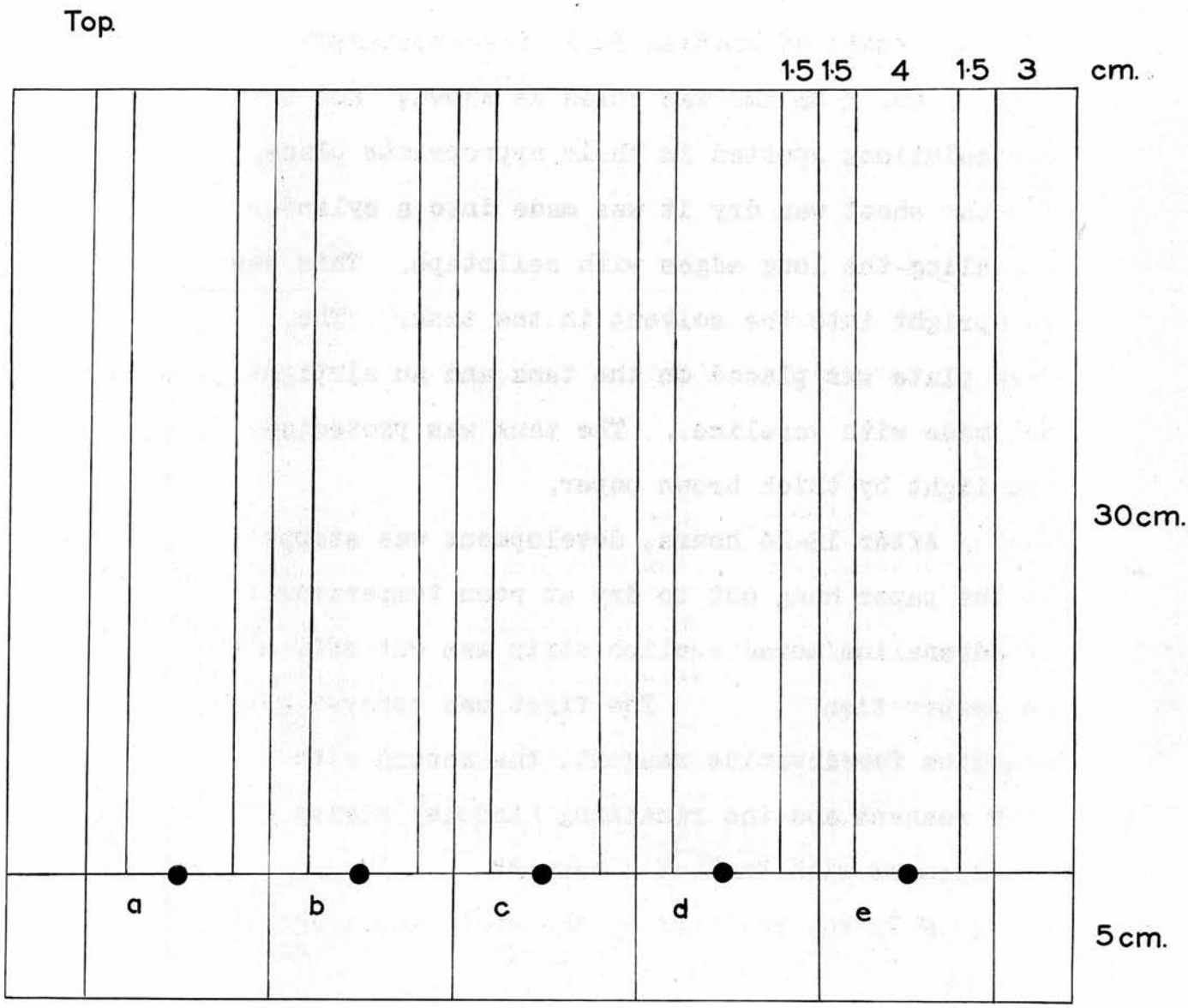


Diagram of method of ruling a sheet of chromatography paper for determination of R_F values of five test solutions (a-e).

A sheet of Whatman No.1 (Chromatography) Paper 39 cm. x 35 cm. was ruled as shown; and the test solutions[+] spotted in their appropriate place. When the sheet was dry it was made into a cylinder by sealing the long edges with cellotape. This was set upright into the solvent in the tank. The glass plate was placed on the tank and an airtight seal made with vaseline. The tank was protected from light by thick brown paper.

After 18-24 hours, development was stopped and the paper hung out to dry at room temperature. The adrenaline/noradrenaline strip was cut off, also the preparation P. strip. The first was sprayed with potassium ferricyanide reagent, the second with Pauly reagent and the remaining (indole) strips were sprayed with Ehrlich's reagent.

When the position of the spots was clear the distance/

[+] Test solutions	Concn.	Vol. used
Adrenaline and noradrenaline	aa 5 mgm/ml in 0.01N HCl	0.005 ml.
Tryptamine HCl.	5 mgm/ml in water	0.005 ml.
5-hydroxytryptamine	5 mgm/ml in 0.01N HCl	0.005 ml.
A preparation of substance P	20 mgm/ml in water	2x0.005 ml.
Tryptophan	5 mgm/ml in 70%	0.005 ml

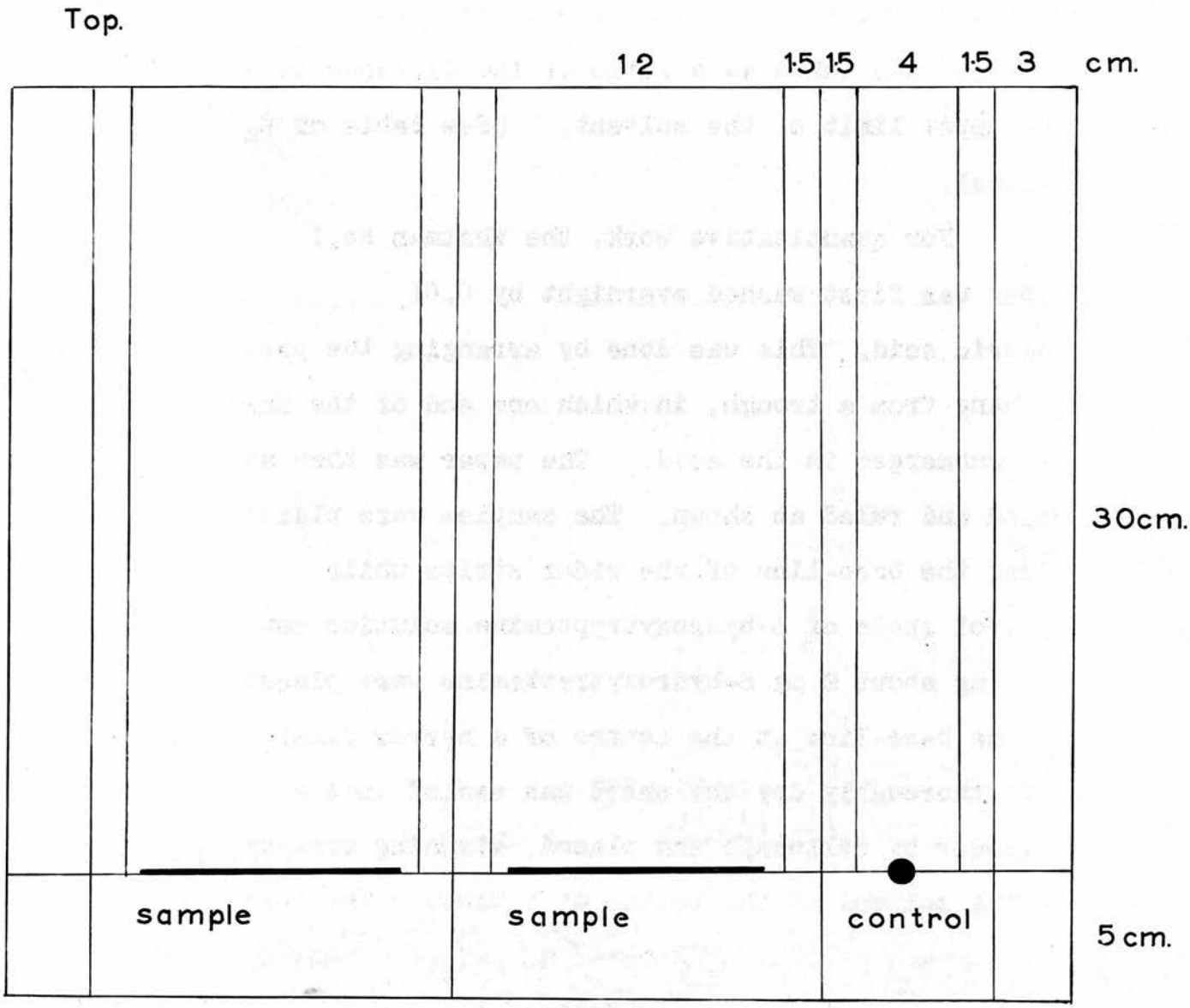


Diagram of method of ruling a sheet of paper for chromatography of two samples of serum with a control spot of 5-hydroxytryptamine.

distance from the starting line to each was measured and taken as a ratio of the distance to the upper limit of the solvent. (See table of R_F values).

For quantitative work, the Whatman No.1 paper was first washed overnight by 0.01N hydrochloric acid. This was done by arranging the paper to hang from a trough, in which one end of the sheet was submerged in the acid. The paper was then air-dried and ruled as shown. The samples were placed along the base-line of the wider strips while control spots of 5-hydroxytryptamine solution containing about 2 µg 5-hydroxytryptamine were placed on the base-line at the centre of a narrow panel. When thoroughly dry the sheet was sealed into a cylinder by cellotape and placed, standing upright, in the solvent at the bottom of a tank. The tank was closed, sealed and covered as before. After 18-24 hours, the paper was taken out and hung up to dry - in air at room temperature. When completely dry, the narrow (or control) strips were cut off and sprayed with Ehrlich's reagent. A purple-blue colour indicated the position to which 5-hydroxytryptamine had travelled. A strip 6 cm. wide was cut/

cut from each sample strip, in such a way that its centre was the same distance from the base-line as was the control purple spot.

These strips were suspended from small celluloid troughs - one end of the paper being held between two glass microscope slides by a rubber band. These slides were held within the trough by a third slide. The trough was filled with eluting fluid which slowly seeped down the paper and was collected in a suitably placed glass tube. All the troughs and tubes were arranged within a small cabinet in which a beaker of water kept the atmosphere moist.

In some cases, samples of 5-hydroxytryptamine were placed on paper in imitation of a chromatogram, where study of the efficacy of elution methods was being tested. Here, portions of acid-washed paper were cut 6 cm. x 12 cm. and a solution containing 1 µg or 2 µg of 5-hydroxytryptamine in 1 ml saline was carefully pipetted on to each portion. These were allowed to dry, in air at room temperature.

Amberlite resin, IRC 50/

Amberlite resin, IRC 50

The cation exchange resin, IRC 50, was ground to a fairly fine powder. Any very fine particles which did not settle within 5 minutes of suspending the resin in water and stirring briskly, were discarded.

100 mgm. portions of this fairly fine powder were taken and suspended in N/10 sodium hydroxide. Then they were transferred to fine glass tubes - 3mm internal diameter and 15 cm. long, (column height, 2 cm.)-one end of which had been constricted and drawn to a short tip. This tip was plugged with cotton wool and the resin allowed to settle above the wool. The tightness of the packing of the wool, together with the height of fluid in the tube determined the rate of flow of the solution. The tubes, already containing the resin were generally placed in phosphate buffer, pH 7, overnight.

During the use of the columns, the rate of flow was adjusted as far as possible by the height of the column of fluid above the resin. The columns were generally used at a flow rate of 0.2ml/ minute.

Freeze-drying/

Freeze-drying.

The apparatus consisted of a large desiccator joined by a wide bore (1.5 cm) glass tube to two glass traps in series. The second of these was attached to a vacuum pump and motor by a similar tube. Both the glass traps were suspended in thermos jars and packed into these with a freezing mixture of solid carbon dioxide and acetone. Cotton wool was used to cover this mixture and the tops of the traps which projected above it.

Samples of solutions to be freeze-dried were run into MacCartney bottles which had been cooled in a solid carbon dioxide and acetone mixture. The tubes were rotated as the sample was run in so that the latter froze in a thin film over the wall. The tubes were then covered with a gauze and placed in the desiccator. All joints in the apparatus were sealed with vaseline. The pump was started and after the air was expelled the system was closed. When the samples were quite dry, air was let into the system slowly and the desiccator opened. Screw caps were placed at once on the tubes and these were stored at -17° until required.

Extraction/

Extraction of 5-hydroxytryptamine from tissues.

Amin (1954) and Amin, Crawford and Gaddum (1954) have studied the quantitative extraction of 5-hydroxytryptamine from the brain of the dog. They found the best results with a double extraction of the tissues with 95% acetone: i.e. first extraction: 20 ml A.R. acetone/g tissue; second extraction: 20 ml 95% acetone/g tissue. This removed all the extractable 5-hydroxytryptamine in solution in the acetone and left the substance P in the insoluble residue.

In detail the method was as follows - A dog was anaesthetised with chloroform and bled out. Samples from the brain were placed in weighing bottles and the weight of tissue determined. The extraction of the smaller samples was made in these bottles but the larger were transferred to beakers. 20 vols. A.R. acetone was added to each and the tissue finely divided with scissors and pressed and stirred occasionally with a flattened glass rod for one hour. Each supernatant was filtered through Whatman No.1 paper, which had been washed with 5 ml 95% acetone. When the filtration of the extracts was complete, the papers were each washed with 2 ml 95%/

95% acetone. To each of the residues was added 20 vols. of 95% acetone; these were again stirred and pressed, for half-an-hour. The supernatants were filtered, each through the same paper as before and the precipitates were transferred to their respective paper. The bottle, or beaker, and precipitate were washed with 2x5 ml 95% acetone. The combined acetone filtrates were evaporated to dryness, under reduced pressure at 30-35°C.(2 ml ethanol was added when necessary to reduce frothing).

One ml water was added to each residue and this was extracted twice with 10-15 ml petroleum ether (b.p. 40-60°C), to remove lipids. The aqueous residue was again evaporated to dryness under reduced pressure, at 30-35°C. The extracts were stored overnight at -17°C, and assayed, if possible, on the next day, occasionally not until the following day.

1) Extraction of rabbit tissues.

Since clotted rabbit blood has a high 5-hydroxytryptamine equivalent,(i.e. high serum - 5-hydroxytryptamine content compared with other animals - Erspamer, 1954a and b), efforts were made/

made to remove as much blood as possible from the tissue before sampling.

A rabbit was anaesthetised with ether and its chest was opened. The aorta was cannulated and perfusion of about 1 l. of saline was made at about 60 cm. water pressure. Blood and perfusate were allowed to escape by an opening in the inferior vena cava.

When samples from the head and neck were required, the carotid arteries were cannulated and the saline perfusion allowed to escape by the jugular veins.

The pulmonary artery was cannulated when lung samples were required. In one case where this was done a series of samples of perfusate was collected throughout the perfusion. They were later calibrated colorimetrically in terms of the first sample - this showed that when the tissue appeared creamy-white and bloodless, and the perfusate was almost colourless, this perfusate contained less than 1% of blood, therefore the tissue now contained less than 1% of blood. No oedema was apparent.

Thus/

Thus when the perfusate was clear, samples of tissue were taken, weighed and extracted. If there was any delay in dissection, e.g. while the skull was being opened for the brain, any other tissue samples already collected were kept at -17°C.

The extraction method was as for the dog brain samples, but tough, or dense, tissues, e.g. skeletal muscle, diaphragm, heart, were cut up under acetone and then rubbed in a glass mortar with silver sand. Extraction was completed as before. (See p.46). On two occasions, after the perfusion with saline was complete, electrodes were placed on the spleen and stimulation was applied until the organ was quite contracted. It was then removed, weighed and extracted with acetone.

In sampling the gut mucosa, the whole stomach and intestines were removed, and separated into stomach, upper half of the small intestine, lower half of the small intestine, and large intestine. Each portion was slit open throughout its length and washed under running tap water. When clean it was blotted dry with filter paper. The whole mucosa of each area was scraped gently off with a blunt scalpel, and extracted; then

an/

an aliquot of the acetone extract was evaporated to dryness, and the treatment completed as before.

2) Extraction of serum.

Blood was obtained from the leg vein of a trained unanaesthetised dog. About 60 ml was collected in each experiment. It was allowed to run directly from the intravenous needle into a clean dry measuring cylinder. In one case, blood was taken from a chloroform anaesthetised dog, just as it was being prepared for another experiment.

Clots were broken up with a glass rod, and, 10-20 minutes from the end of collection, the blood was placed in a centrifuge tube and spun at 3,500 r.p.m. for 10 minutes.

Samples of 2.5 ml or 5 ml serum were measured into dry flasks and to some was added a small volume of saline containing either 1 µg or 2 µg 5-hydroxytryptamine, and to the others a similar volume of saline. 20 volumes of acetone were added and after being thoroughly shaken the flasks were stored at 5°C for half-an-hour. The extracts were filtered off through acetone-washed papers and the flasks and precipitates were washed

with/

with 2 x 10 ml 95% acetone. (Extraction of the residue with 95% acetone was considered unnecessary for serum, blood or bile). After evaporation of the extracts to dryness, the residues were taken up in water and the lipids removed as before. The extracts were again dried, and stored at -17°C.

A sample of rabbit serum obtained from blood removed under ether anaesthesia, was similarly extracted, but without the addition of standard 5-hydroxytryptamine.

3) Extraction of blood.

Two rabbits were used; one was anaesthetised with ether (I) and the other with urethane (II). A polythene cannula was inserted in the carotid artery and the free-flowing blood collected into a siliconed tube.

(I) The blood was immediately measured in 1 ml portions by means of a siliconed pipette into each of two siliconed flasks (A and B) already containing 20 ml A.R. acetone, two dry siliconed flasks (C and D) and two dry glass (i.e. not siliconed) flasks (E and F).

The blood in A and B never clotted and extraction was continued at once by shaking and storing at 5°C for one hour, followed by filtration etc./

etc. as in the serum method (p. 50).

The samples in E and F clotted almost immediately but C and D stood for about five hours at room temperature before the clotting was complete. These four samples were then extracted with 20 ml A.R. acetone each, stored at 5°C for an hour..... Subsequent treatment followed the technique already described for extraction of serum (see p. 50).

(II) Three samples of 1 ml blood were each measured directly into 20 ml A.R. acetone, and two into dry non-siliconed glass flasks. The latter samples clotted and after one hour 20 ml A.R. acetone was added to each of these. All the samples were stored at 5°C for an hour and subsequent treatment was as for serum extraction (see p. 50).

Biological preparations

1) The isolated rat uterus - Method for assay.

During the past few years it has been apparent that the isolated oestrous uterus of the rat can be a sensitive, regular test organ for 5-hydroxytryptamine.

Erspamer first discovered the suitability of this/

this test organ and prepared his rats in oestrous by ovariectomy and, 20-30 days later, injection of 50-100 µg oestradiol dipropionate as one dose or as two divided doses. The rats were best used 5-7 days from injection (Erspamer, 1942a; 1952a).

In this laboratory, virgin rats of 160-200 g were injected subcutaneously with 100 µg/kg of stilboestrol (solution for injection contained 100 µg stilboestrol/ml in arachis oil), 20-24 hours before being required for the experiment,(Amin, Crawford and Gaddum, 1954).

Next day, the rat was killed by a blow on the head and its throat was cut. The uterus, with adhering adipose tissue was carefully removed. The latter was trimmed off and most of one horn (i.e. about 2 cm relaxed length), was ligatured at both ends, attached to a hook and suspended in a 2ml bath.

It was immersed in de Jalon solution+, containing 10^{-6} atropine, at 28-29°C. After being set up the uterus generally relaxed slowly for about half-an-hour. The lever was then adjusted to bring the/

+ Modified - Gaddum, Peart and Vogt,(1948).

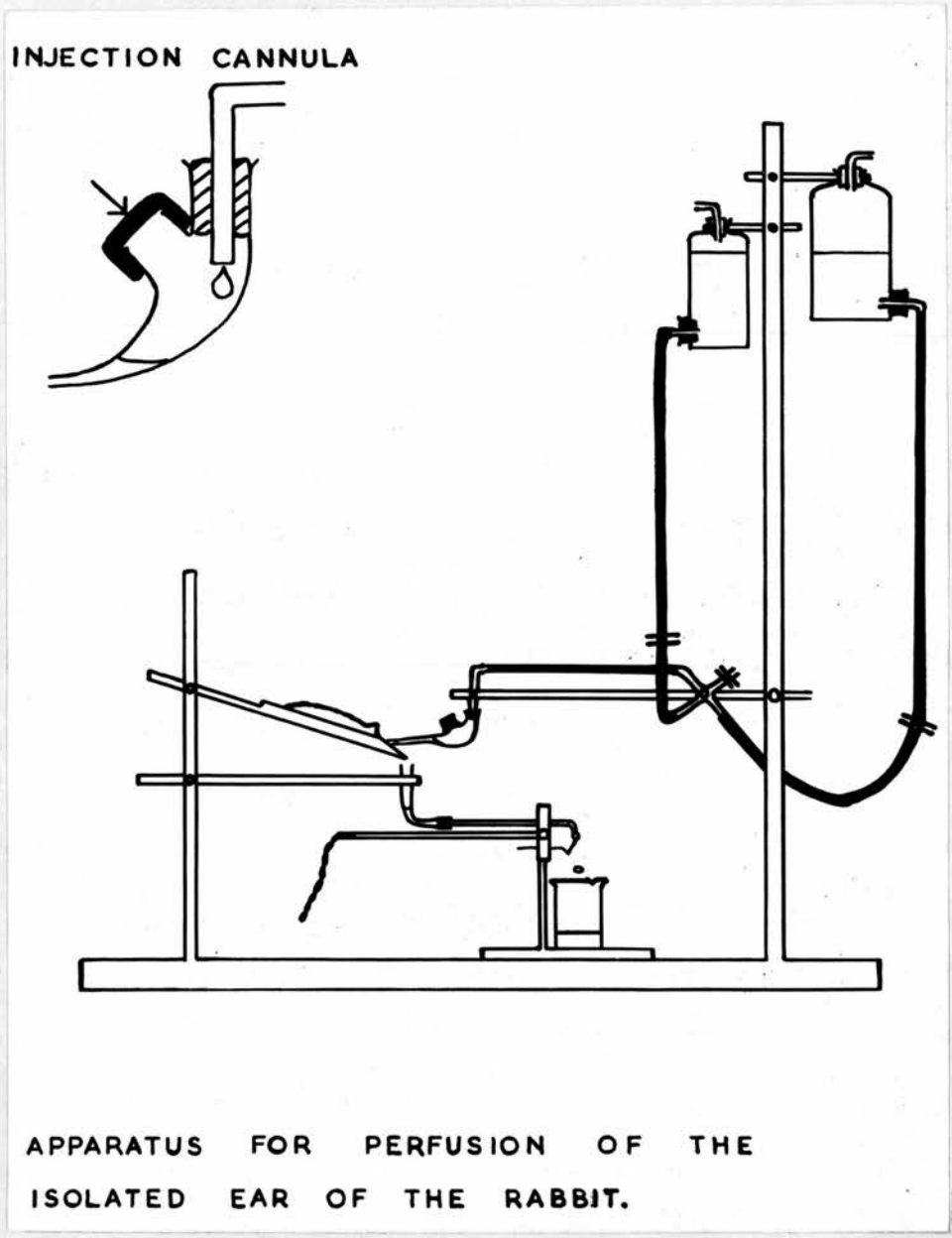
the arm bearing the writing point to an angle of about 10° below the horizontal. Dosing with 5-hydroxytryptamine was begun, and with a magnification of 10-12 x and a tension of 0.5-1.0 g. the range was usually between 2-5 ng (minimum response), and 20 ng (maximum response). Doses were repeated regularly at a 4 or 5 minute interval; and assay was made between 5 and 20 ng 5-hydroxy-tryptamine where the discrimination was ± 1-2 ng.

2) <u>The isolated perfused ear of the rabbit.</u>

The isolated ear of the rabbit was perfused in a manner similar to that used by Gaddum and Kwiatkowski (1938), and by Gaddum and Hameed (1954).

The rabbit was killed by a blow on the back of the head and its throat was cut. The ears were removed, as close to the skull as possible, and placed in physiological saline. As quickly as possible, the central artery was identified and dissected free from adipose tissue and fascia for about an inch of its length. A small polythene cannula was filled with saline, inserted into the artery and ligatured in position. The ear was washed/

Figure 1



APPARATUS FOR PERFUSION OF THE ISOLATED EAR OF THE RABBIT.

Apparatus for the perfusion

of the isolated ear of the rabbit.

Note the two reservoirs, which enable the supply of alternative perfusion fluid to be easily made; the injection cannula, which is described in the text; and the drop recorder, which measures the rate of fluid-outflow from the ear.

washed free from blood in the perfusing system while the second ear was similarly prepared. As soon as the perfusate was colourless, the second ear was washed free from blood. After this the ears were stored in a beaker of saline at 5°C for 3-4 days. Normally one ear was used on the day after dissection and the other on the following day.

The perfusion system comprised two reservoir bottles which could be raised to almost 1 m. above the platform on which the ear was mounted. Both these bottles were connected by an X-junction to the injection cannula and through it to the ear.

The injection cannula was designed so that the injected dose would reach the ear with as little dilution as possible, and also, that it would all be washed quickly into the ear. The flow from the reservoir passed dropwise into the cannula and thus the injection rate could easily be of controlled pressure, to be equivalent to the reservoir pressure. If the injection was made too slowly, drops of saline would also pass into the cannula; if too fast the saline would be pushed back up the tube by the excess pressure. Thus injections were made at such

a/

a rate that a hanging drop was held in position.

The venous outflow from the ear was collected on the supporting plate and allowed to run into a small funnel from which it was led to a drop-timer. This was arranged to record, on a smoked drum, the interval between successive drops. The higher the stroke, the longer was the interval; thus vaso-constriction was recorded as a peak.

The perfusion fluid was that recommended by Page and Green (1948), for use in the study of serum vasoconstrictor.

Composition of Page solution.

Sodium chloride	0.82%
Potassium chloride	0.084%
Calcium chloride, $2H_2O$	0.004%
Magnesium chloride, $6H_2O$	0.006%
Sodium bicarbonate	0.04%
Glucose	0.1%

To each litre of this solution is added 10 ml phosphate buffer prepared by mixing M. Potassium phosphate (4 vols.) with M. Potassium dihydrogen phosphate (1 vol.)

When perfused with Page solution, the sensitivity of the ear vessels to 5-hydroxytrypta-mine was 1-20 ng. in a volume of $\nearrow$ 0.2 ml saline.

3/

3) The heart of Mactra Solida - Method for assay.

Twarog and Page (1953) have used the heart of the quahog, Venus mercenaria, for the estimation of 5-hydroxytryptamine in tissue extracts. This mollusc is not found in British waters, but a survey of some of the molluscs available here has been made by Paasonen (1954). He has shown the heart of Mactra solida to be a suitable preparation, at least in the late summer and autumn months. This cockle lives below the low tide level and has been obtained from the sea-bed near St. Andrews, Fife. The molluscs were best stored in sea-water at about 10°C.

One shell of the bivalve was removed and the heart could be clearly seen. A thick walled ventricle is flanked on either side by very thin walled auricles. Ligatures were placed round each of these auriculo-ventricular junctions. By means of these ligatures, after the ventricle was dissected free from the auricles and aorta, the heart was suspended in a 2 ml organ bath, attached to a hook and a very light recording lever.

The heart is very sensitive to stimulation by 5-hydroxytryptamine and inhibition by acetylcholine; but/

but the acetylcholine effects can be abolished by the use of 10^{-6} - 10^{-5} mytolon in the bathing fluid. (This bathing fluid is a physiological saline having the ionic concentrations of sea-water). The heart is relatively insensitive to adrenaline, 1 µg being required to produce a stimulation whereas 1-2 ng of 5-hydroxytryptamine always caused at least a threshold stimulation. The discrimination was 0.5 - 1.0 ng 5-hydroxytryptamine/2 ml fluid.

Some extracts of dog brain samples were kindly assayed by Dr. Paasonen on this preparation in parallel with assays on the rat oestrous uterus.

4) <u>The isolated guinea-pig ileum</u>.

A young guinea-pig, weighing 150 g was fasted overnight. It was killed by a blow on the head and its throat was cut. The portion of ileum nearest the caecum was dissected out and washed through with Tyrode solution. A piece, about 1.5 cm, was suspended in a 2 ml organ-bath in Tyrode solution containing atropine (10^{-7}) and mepyramine (10^{-6}), at 37°C. The responses were recorded by a light frontal writing lever giving a magnification of about ten times.

<u>Preparation</u>/

Preparation of mushroom juice.

The acetone extracts of the tissues contained some proportion of adrenaline, which caused interference with the assay of their 5-hydroxytryptamine content, for while the latter caused a stimulation of the isolated rat oestrous uterus the former caused an inhibition. Adrenaline is an ortho-dihydroxy compound and 5-hydroxytryptamine is a monohydroxy structure. Thus it was thought that an enzyme which would specifically oxidise di- or polyhydroxy compounds might be used for the routine treatment of the extracts before their assay.

Keilin and Mann (1938) detailed the preparation of a polyphenoloxidase from edible mushrooms[+] - its purification, nature and properties. It had a comparatively high specificity, speedily oxidising ortho-dihydroxyphenols, but few monophenols.

Ten years later Mallette, Lewis, Ames, Nelson and Dawson (1948) described the purification of a similar enzyme preparation, also from mushrooms.

This/

[+] Psalliota (or, Agaricus) campestris - somewhat modified by continuous and intense cultivation.

This polyphenoloxidase, which is also known as tyrosinase (Sumner and Myrbäck, 1952; Sumner and Somers, 1953) occurs in bacteria, potatoes and mushrooms as well as in human skin and melanomata. It is an aerobic oxidase, containing copper, which converts catechol to o-quinone as the first step in the formation of melanin.

Preparations of this polyphenoloxidase possess, also, an action on monophenols. This latter activity is apparently linked to the other active entity but is less stable. For example, heating at 60°C reduces the activity of both, but of the mono-oxidase more than of the polyoxidase (Adams and Nelson, 1938). Various other treatments can alter the polyoxidase/mono-oxidase activity ratio. Both the purification methods referred to above were designed to give a high polyoxidase-active product.

Keilin and Mann (1938) stated that even a crude extract of mushrooms oxidised with great velocity, mainly ortho-dihydroxyphenols, and but few monophenols. This difference in oxidation rate of mono- and di- phenols was largely that of a more, or less, protracted lag period.

In the present work, edible mushrooms were chosen/

chosen as a convenient source of the enzyme for they are easily available and are free from the large quantities of starch which occur in the cheaper source, potatoes.

120 g of fresh mushrooms were sliced and then ground in a household mincer. The pulp was rubbed in a mortar with about 10 g silver sand for 20 minutes. The juice was squeezed out through linen, by hand. (No water was added). This yielded 80 ml of a dark brown turbid fluid, which was slightly frothy. It was stored at 5°C.

A one-in-ten dilution of this crude juice was used in the investigation of its activity.

Later, a larger quantity of juice was prepared in an exactly similar way. After storage at 5°C overnight - reported by Keilin and Mann (1938) often to cause an increase in activity, - a portion of it was distributed as 0.5 ml samples into a number of small test-tubes. These samples were freeze-dried by reduced pressure, in a centrifuge over phosphorus pentoxide.

When the juice was completely dry, the tubes were sealed and stored at 5°C. The residue was like a solid foam and was brown in colour. It redissolved readily to give a slightly turbid solution./

solution. One tube provided sufficient enzyme activity for the treatment of about eight samples of tissue extract.

This dried preparation of mushroom juice will be referred to later as the standard preparation of crude mushroom juice.

The remaining crude juice was divided into two parts and each was subjected to a simple purification, for the crude juice contained a considerable amount of coloured material. Some of the juice was dialysed in a cellophane tube (thickness, 0.0025mm), against slow flowing deionised water - 10 ℓ. in 24 hours. A little coloured matter passed out and the volume inside the tube was increased by 25%. The rest of the juice was filtered through Whatman No.41 paper; a small residue was held back.

Samples (0.5 ml) of the dialysed, and of the filtered juice were dried and sealed as before. With allowance for the dilution of the dialysed portion, neither of these portions showed any increase in activity or greater specificity than the standard preparation of crude mushroom juice.

RESULTS

A) Chromatographic separation

Paper chromatography.

The earlier part of this study was done on the assumption that extracts, prepared from tissues with acetone (see p.46) would be subjected to paper chromatography as the final stage of their purification. This was to be done with the aid of a solvent which would give such a separation of adrenaline from 5-hydroxytryptamine that the portion of the developed chromatogram carrying the 5-hydroxytryptamine could be eluted to give a sample free from adrenaline.

All the assays in this section of the work were done on the isolated oestrous uterus of the rat, prepared and set up as described on p. 52.

Samples of 5-hydroxytryptamine were placed on 6 x 12 cm. portions of Whatman No.1 paper, previously washed with 0.01N hydrochloric acid, in imitation of a chromatogram and various means and solvents were tried to achieve their satisfactory quantitative removal. It was found that a slow, flowing elution, from/

from a small trough down the paper, yielded, in about 22 hours, 6-8 ml of eluate which contained 70-100% of the added 5-hydroxytryptamine. To determine the progress of the 5-hydroxytryptamine down the paper, a spot of concentrated (4 µg/0.04 ml), 5-hydroxytryptamine solution was placed at the top of a number of portions (6 x 12 cm) of paper. These were eluted with 0.01N hydrochloric acid for various intervals, 2 hours, 16 hours and 22 hours; then the papers were removed, and dried. They were sprayed with Ehrlich's reagent, and the presence of a blue-purple spot on the '2 hour' and '16 hour' paper and its absence from the '22 hour' paper showed that in the latter case the 5-hydroxytryptamine was completely eluted. In a 'contact' elution where the whole strip was shaken in a stoppered tube with 14-15 ml eluting fluid, 60-80% recovery was obtained with 1 hour contact. The solvent in these cases (i.e. eluting fluid) was saline or 0.01N hydrochloric acid, in which the 5-hydroxytryptamine was found to be stable at room temperature for at least 24 hours. Samples of 1 µg and 2 µg 5-hydroxytryptamine were used/

[+]Ehrlich's reagent - (blue-purple colour with indoles). 1 g p-dimethylaminobenzaldehyde in 15 ml. ethanol and 15 ml conc. hydrochloric acid.

used and the activity estimated without dilution, but should the extracts contain less 5-hydroxytryptamine activity, the eluates would require to be concentrated.

Evaporation of the saline eluates under reduced pressure at 37°C yielded less than 10% recovery. No improvement was found by lowering the temperature to 20°C. Simple neutralisation of the acid eluates with sodium bicarbonate, before evaporation was not satisfactory. The recoveries were never greater than 25%.

Since the recovery is satisfactory when 5-hydroxytryptamine is added to tissue extracts and these are evaporated, 5-hydroxytryptamine was added to a tissue residue containing negligible 5-hydroxytryptamine; the mixture was acidified, neutralised with bicarbonate and evaporated under reduced pressure. The recovery was 60-100%; similar results were obtained by the addition of 1 µg or 2 µg lecithin to acid eluates.

It seemed at this time that this problem was more complicated than it had at first appeared. So, for a time, investigations were continued on the behaviour of simple dilutions of 5-hydroxytryptamine in/

in 0.01N hydrochloric acid, (i.e. no contact with chromatography paper). Since the recovery of 5-hydroxytryptamine fell as soon as evaporations were made, it was thought that heavy metal ions, possibly present in the water, were promoting oxidative destruction of the 5-hydroxytryptamine.

BAL - dimercaprol, which was shown by Bacq, Fischer and Lecomte (1948) to protect adrenaline from air oxidation and dithiocarbamate, another anti-oxidant, were each added to samples, but the recoveries after evaporation were inconsistent.

Various buffers were used to stabilize the pH at different levels, during evaporation. It was found that a few drops of 0.2 M. sodium phosphate buffer, used to give pH 6.5 gave the most satisfactory recoveries. The recovery estimates were made on the oestrous uterus of the rat. It was noted at this time that the pH of the sample at assay was partially responsible for the height of the uterine response. If the test sample was more acid than pH 4.5 the contraction elicited is not so great as the concentration of 5-hydroxytryptamine warrants. This can be shown by adjustment of the

pH/

pH to 6.5 and re-assay. (If the pH is more alkaline than pH 8, 5-hydroxytryptamine activity is lost, for it is unstable in alkaline media).

The addition of lecithin, and the use of phosphate buffers were then tried with the eluates of 5-hydroxytryptamine from the paper. It became clear that something else was washed out of the paper - this compound speeded the destruction of 5-hydroxytryptamine during evaporation, or formed a complex with it to reduce its activity on the uterus. Other types of paper, Whatman Nos. 3 and 4, were tried and various methods of pre-treating the paper were employed. These included:-

a) washing the paper with 0.01 N hydrochloric acid for 48 hours before use (instead of the usual 12 hours), or
b) washing the paper with ascorbic acid solution (50 mgm/100 ml.) or
c) washing the paper with Versene solution (ethylenediamine tetra-acetic acid, used by Eggleston and Hems (1952) in chromatography and believed to remove traces of metal impurities); or
d) washing the paper with 0.01N sodium hydroxide, before the usual acid washing.

No/

No significant improvement in the recovery of 5-hydroxytryptamine from paper treated in any of the above ways was found.

Vialli and Erspamer (1942) recognised a substance in their 5-hydroxytryptamine extracts of tissues, which gave the colour reactions of 5-hydroxytryptamine but had not the pharmacological activities of the latter. They found, however, that heating to 100°C at pH 7 for 10 minutes resulted in the appearance of pharmacological activites typical of 5-hydroxytryptamine. The treatment had therefore changed the 'enteramine I', or 'inactive enteramine', into 5-hydroxytryptamine, or enteramine. It was suggested that during these chromatographic and evaporation procedures, some of the added 5-hydroxytryptamine was being changed into an inactive form. So certain samples were placed at 100°C at pH7 for 10 minutes but increased recoveries were not found.

For a time, evaporation was abandoned and concentration of the samples was attempted with ion exchange resins.

Amberlite/

Amberlite resin 1RC 50

It had been found by Fingl (1953) that 5-hydroxytryptamine could be adsorbed on the cation exchange resin 1RC50, from a solution of pH 6-7 and could then be eluted by 0.1N hydrochloric acid. Here it was proposed that a similar method should be used for the concentration of eluates from paper chromatography, as an alternative to evaporation.

Preliminary experiment

50 mgm of fine 1RC 50 was packed into a tube, as described on p.44. Sodium phosphate buffer, 0.2 M and pH 7, was passed until the effluent was also pH 7. Then 100 µg 5-hydroxytryptamine base, in the same phosphate buffer was passed through the column at 0.2 ml/minute. The drops of the percolate, wash fluid and eluate, as they issued from the chromatogram tube were applied in succession as individual spots along a strip of Whatman No. 1 filter paper. The column was washed with 1 ml water and eluted with 2 ml 0.1N hydrochloric acid. The strip of paper was dried and then sprayed with Ehrlich's reagent (see p. 64). This showed that only the first part of the 5-hydroxytryptamine/

5-hydroxytrptamine had been adsorbed, for the first portion of the percolate gave a negative reaction, while the later part was positive. That is, after a certain time the column was saturated and could adsorb no more 5-hydroxytryptamine. However, the wash-water gave a negative reaction and only the first ml of acid eluate was positive. Thus the water-wash did not remove the adsorbed 5-hydroxy-tryptamine and what 5-hydroxytryptamine had been adsorbed was eluted by the first ml of 0.1N hydro-chloric acid.

In subsequent work, owing to the lower con-centration of base and salts in the samples, after the columns had been buffered with sodium phsophate, 0.2 M, buffer, they were washed through with 0.02 M buffer of the same pH. Then about 0.5 ml 0.2 M buffer was used to bring the samples to the same pH as the columns - these samples were now about 0.02 M.

In a few cases, the resin was not used in a column, but weighed amounts of resin were stirred with the samples, previously adjusted to the desired pH. The fluid was then decanted off, the resin was washed with water and then the resin was extracted twice/

twice with 0.1N hydrochloric acid.

Comparison of the two methods showed the column method to be more satisfactory.

The recovery results from these experiments are given below. Here the samples of 5-hydroxytryptamine were dried onto portions of chromatography paper. These were eluted by contact and shaking with 14 ml 0.01N hydrochloric acid for an hour. A 12 ml aliquot of each eluate was taken and adjusted to pH 6.5. One of these eluates was reserved at 5°C as a control while the rest were passed through 100 mgm columns of IRC 50 (prepared at pH 6.5), and the columns were eluted with 1 ml 0.25 N and 0.5 ml 0.01 N hydrochloric acid.

TABLE I

Recoveries from concentration of eluates from paper by passage through columns of IRC 50.

Expt No.	Paper eluate, control - Recovery of 1 µg 5-hydroxytryptamine	Eluates from paper and columns. Recovery of 5-hydroxytryptamine		
		2µg	1µg	0.5µg
I	60%	83%	35%	46%
II	-	75%	43%	70%
III	77%	50%	60%	30%

These results were considered to be not sufficiently consistent for routine work.

Freeze-drying

A method of freeze-drying was examined as an alternative to evaporation at $<40^{\circ}C$ under reduced pressure, or passage through IRC 50 columns, for concentration of chromatogram eluates.

A model experiment was done with various volumes of a solution of sodium chloride, of about the same molarity as the neutralised eluates. After freeze-drying, the residues were titrated with silver nitrate solution and no significant loss was found. It had been thought that perhaps there would be some blowing out of the fine residue as the apparatus was being opened, or perhaps some melting of the samples and spurting of the solution, should the intensity of the vacuum vary during the course of the evaporation.

After several experiments, it was found that if the tubes were very cold, (i.e. less than $-60^{\circ}C$) when placed in the apparatus, the drying process was needlessly prolonged. Initial temperatures of $-20^{\circ}C$ to $-40^{\circ}C$ were quite satisfactory. These were obtained by mixing ice with the solid carbon dioxide and acetone, when the samples were being frozen./

frozen.

It was found, too, that the pH of the sample at drying was very important to the recovery of the 5-hydroxytryptamine activity. Therefore samples were individually neutralised and buffered at a pH meter (glass electrode) instead of, as formerly, a control titration being done at the meter and appropriate volumes of sodium hydroxide and buffer added to the samples. The presence of a buffer system seemed quite essential and sodium phosphate, (to give a pH of 6.5, as was found most satisfactory during evaporation (see p.66) was employed.) The recovery estimates were made on the isolated oestrous uterus of the rat, and a number of uteri were very sensitive to inhibition of 5-hydroxytryptamine contractions by phosphate. Where this was the case, an equivalent concentration of phosphate was added to the 5-hydroxytryptamine standard solution and assays of samples were made with reference to this.

The results of an experiment in which eluates were freeze-dried after being adjusted to various pH, showed buffering at pH 6.5 to be most satisfactory.

Experimental results

A number of samples of 5-hydroxytryptamine were taken - some were simply diluted with 0.01 N hydrochloric acid, others were dried onto Whatman No. 1 paper (previously washed with 0.01 N hydrochloric acid) and eluted.

In the second experiment, a control sample was stored at 5°C during the time that the rest were being freeze-dried, subsequent to pH adjustment of all the samples in the manner described above.

Assays were made on the isolated oestrous uterus of the rat.

TABLE II/

TABLE II

Recovery on freeze-drying.

		Recovery of 5-hydroxytryptamine		
		2 µg	1 µg	0.5µg
Expt. I	Diluted samples - adjusted to pH 6.5 and freeze-dried.	58%	47%	47%
	Paper eluate samples - adjusted to pH 6.5 and freeze-dried.	47%	45%	54%
Expt. II	Diluted samples - as above.	84%	87%	55%
	Paper eluate samples - as above.	66%	76%	35%
	Paper eluate sample - control. adjusted to pH 6.5 and stored at 5°C		80%	

Underlined results obtained in comparison with standard containing phosphate.

Next, samples of 2 µg and 5 µg 5-hydroxytryptamine were chromatogrammed before freeze-drying. In the first experiment, sec butanol, acetic acid and/

and water (4:1:5) was used as the chromatography solvent. The appropriate strips were eluted with 0.01 N hydrochloric acid and the eluates were neutralised and buffered at the pH meter as before and then they were freeze-dried. The recovery of 5-hydroxytryptamine activity was very low, 25-30%, and it was found that the pH of the samples, after the addition of saline was 8-9. This must be because of the loss through volatilisation of some of the acetic acid which must have been in the samples at neutralisation.

However, increased recoveries were again obtained when n-butanol saturated with N hydrochloric acid was used for the chromatography. Here there was no 'pH-shift'.

This method was then used for 2.5-5 ml samples of dog serum. Before extraction with acetone, 2 µg 5-hydroxytryptamine was added to some of them. Then acetone extracts were made and these were defatted (see p.50). The dry residues were extracted successively with 1 ml, 0.5 ml and 0.5 ml of 75% ethanol, and these extracts were placed along the base-line of the sample portions of the 0.01 N hydrochloric/

hydrochloric acid washed chromatography paper. Control spots of 5-hydroxytryptamine were also placed on the base-line of the paper. (see p. 42).

When quite dry, the sheets were chromatogrammed in n-butanol saturated with N hydrochloric acid. After 22 hours, the chromatograms were removed and dried in air. The control strips were sprayed with Ehrlich's reagent in order to determine the position of the 5-hydroxytryptamine. The corresponding areas of the sample strips were cut out and eluted. Each sample was neutralised and buffered to pH 6.5 at the pH meter and subsequently frozen and freeze-dried. Assay of the 5-hydroxytryptamine content of the residue was made on the isolated rat oestrous uterus preparation.

The recoveries were low, 23-33%; but the results for 5-hydroxytryptamine content of dog serum, when corrected for this loss, agreed satisfactorily with results obtained later with other methods.

Chromatography solvents tested.

In this work only the ascending method of paper chromatography was used. The results would possibly have been different had the/

the descending method, for example, been applied. (see Discussion, p.105). With the ascending method and a common solvent for adrenaline chromatogram, phenol and hydrochloric acid, 5-hydroxytryptamine moved but little higher than adrenaline. (Where the solvent travels 22-30 cm the distance between the proximate edges of spots of substances required to be separately eluted should be at least 2.5 cm - a difference of at least 0.1 R_F in the absence of 'tailing'.)

Various solvent systems were tested and the nearest approach to this ideal condition was given by the solvent mixture sec-butanol, acetic acid and water, in the proportion by volume of 4:1:5. The spots were well defined. In some other solvent systems the R_F difference was about the same but the spots showed considerable 'tailing', which is unsatisfactory.

Unfortunately, repeated chromatograms of adrenaline and 5-hydroxytryptamine have shown the separation, even in the sec-butanol, acetic acid and water system, unsuitable for separate elution.

A/

A table of the mean R_F values is given for adrenaline, noradrenaline, tryptamine, 5-hydroxy-tryptamine, a preparation containing Substance P, and tryptophan in the various solvent systems.

Solvent systems.

1. Phenol: glacial acetic acid: water::99:1:100.
2. n-butanol saturated with N hydrochloric acid.
3. sec-butanol saturated with N hydrochloric acid.
4. equal parts of n- and sec- butanol, saturated with N hydrochloric acid.
5. n-butanol: glacial acetic acid: water::77:6:17.
6. sec- butanol: glacial acetic acid: water::4:1:5.
7. n-butanol: sec- butanol:glacial acetic acid: water::2:2:1:5.
8. tert-butanol:glacial acetic acid: water::4:1:5.
9. methylethyl ketone:glacial acetic acid:water::4:1:5.
10. acetone: water::4:1.
11. 0.001 N hydrochloric acid.

TABLE III/

Notes on Table III - (see also Methods, p.41)

The R_F value is the distance of a particular point in the chromatogram from the starting line expressed as a proportion of the distance travelled by the solvent from the starting line.

The adrenaline and noradrenaline were placed together at the same point on the starting line. In some solvent systems these bases moved at the same rate, in others they moved at different rates and two coloured spots were apparent after the strip had been sprayed with potassium ferricyanide reagent. The tryptamine, 5-hydroxytryptamine and tryptophan strips were sprayed with Ehrlich's reagent. This gives a characteristic blue-purple spot with indoles. In the chromatogram with 80% acetone, the 5-hydroxytryptamine spot showed extensive 'tailing' with three areas of fairly intense blue-purple colour after having been sprayed with Ehrlich's reagent.

The strip bearing Preparation P was sprayed with Pauly reagent. A colour reaction is not, however, necessarily indicative of the position of pharmacologically active material: see Amin (1954).

TABLE III

R_F values

Solvent	Adrena-line	Nor-adrena-line	Trypta-mine	5-hydroxy-trypta-mine	Prepar-ation P	Trypto-phan
Phenol:acetic acid:water	0.69 +++	0.31 ++	0.92 ++	0.79 ++	-	0.77 +++
n-butanol: N HCl	0.07 +	0.07 +	0.43 ++	0.16 +	-	0.34 +++
sec-butanol: N HCl	0.45 ++	0.52 ++	0.76 ++	0.47 +	-	0.74 ++
n- & sec-butanol: N HCl	0.1 +	0.16 +	0.49 +++	0.18 ++	0.04	0.43 +++
n-butanol:acetic acid:water	0.03 +	0.03 +	0.32 ++++	0.12 +++	-	0.16 +++
sec-butanol: acetic acid:water	0.58 ++	0.58 ++	0.76 +	0.67	0.75	0.67 +
n- & sec-butanol: acetic acid:water	0.35	0.39	0.60 ++	0.40 +	-	0.54 +
tert-butanol: acetic acid:water	0.71 ++	0.71 ++	0.84 +	0.70 +	0.81 ++	0.65 ++
methylethylketone acetic acid:water	0.73 +++	0.73 +++	0.89 ++	0.82 +	0.83 +	0.78 ++
acetone 80%	0.67 ++	0.67 ++	0.85 +++	0.80 +++++	-	0.49 +
0.001 N HCl	0.65 +++++	0.65 +++++	0.48 +++++	0.34 ++++	0.67 +++	0.59 +++

Spread 1.5 - 2.5 cm +
2.5 - 3.5 cm ++ etc.

Effects of antagonists on the responses of the isolated perfused ear of the rabbit.

When it seemed that chromatography would not provide a suitable separation of 5-hydroxytryptamine from adrenaline, attention was directed to the isolated perfused ear of the rabbit. It was hoped that under conditions produced by selective antagonism, vasoconstrictor responses to adrenaline and noradrenaline would be abolished while those to 5-hydroxytryptamine would remain unaffected. Thus the isolated perfused ear could be used under these conditions as an assay preparation in place of the isolated rat uterus; and the need for separation of the adrenaline from the extract activity would not be necessary.

During the experiments in which tests were made of possible perfusion conditions suitable for assay methods, one of the two reservoirs supplying the ear was filled with a particular concentration of an antagonist in Page solution. The ear was set up (see p. 54) and perfused with Page solution for 2-3 hours. The perfusion rate was adjusted to be about/

about 2 ml/minute, under a pressure of 30-40 cm saline. Similar responses to adrenaline and/or noradrenaline and 5-hydroxytryptamine were obtained-dose interval = 6-12 minutes. Then the antagonist solution was perfused, usually for about 20 minutes before dosage was resumed. Responses were again matched. From these matching responses the sensitivity ratio adrenaline/5-hydroxytryptamine, and (or) noradrenaline/5-hydroxytryptamine, was found.

Under ideal conditions for this assay method, the ratio, dose of adrenaline/dose of 5-hydroxy-tryptamine for equal effects, is high, but the ear vessels remain very sensitive to 5-hydroxytryptamine. Ephedrine was the first drug studied. The results of Gaddum and Hameed (1954) (see p.31) were confirmed. Ephedrine (10^{-6}) caused a sensitisation of the ear vessels to both adrenaline and 5-hydroxy-tryptamine. The sensitivity ratio (see Table IV) was little changed from that found with Page solution perfusion. Ephedrine (10^{-5}) gave an inhibition of adrenaline responses and a further sensitisation to 5-hydroxytryptamine.

TABLE IV/

TABLE IV

Ratio of doses causing similar vasoconstrictor responses in the isolated perfused ear of the rabbit under various conditions.

Perfusion fluid	Ratio - Adrenaline/5-hydroxytryptamine								
	Range	Individual values							
Page Soln alone	0.25-6.2	1.25			0.25	0.8	0.6-1	6.2	0.5
Page Soln and Ephedrine 10^{-6}	0.4-2	1.25		0.4	2				
" " " 10^{-5}	2-100	100	42			30	20	10	2
" " " 10^{-4}	1-10		1-10						
Page Soln and Ephedrine 10^{-6} with Piperoxane 10^{-7}	2-5			2	4-5				
Page Soln and Ephedrine 10^{-5} with Piperoxane 10^{-7}	2-8			2	8				
Page Soln and Ephedrine 10^{-5} with Piperoxane 10^{-6}	1						1		

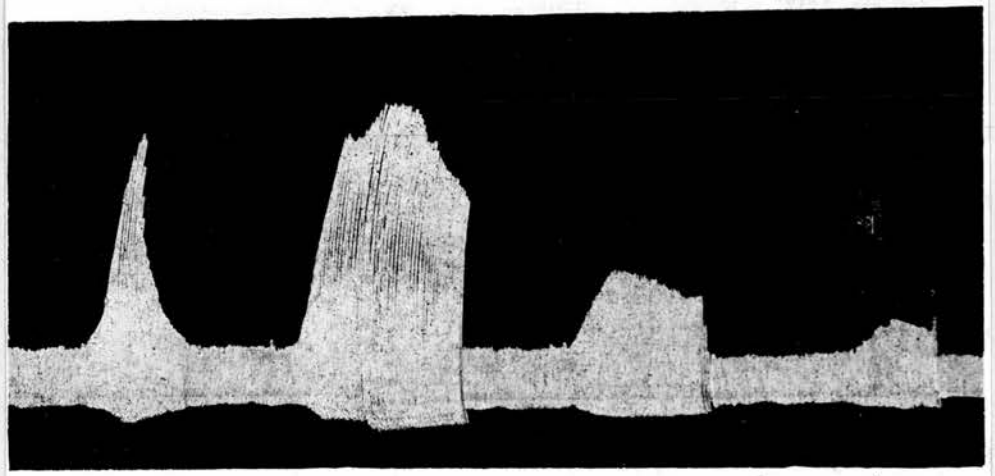
Table/

TABLE IV Contd:

Perfusion fluid	Ratio - Noradrenaline/5-hydroxytryptamine Range	Individual values							
Page Soln alone	0.15	0.1	0.2	0.2	0.33	0.1-.2	2	3	5
Page Soln and Regitine 10^{-9}	0.3-0.5	0.3-.5							
" " " " 10^{-8}	1	1							
" " " " 10^{-7}	8	8							
Page Soln and Ephedrine 10^{-5} with Regitine 10^{-7}	5		5						
Page Soln and Ephedrine 10^{-6}	10-15							15	10
Page Soln and Ephedrine 10^{-6} with Dibozane $5x10^{-8}$	1-10							1	10
Page Soln and Ephedrine 10^{-6} with Dibozane $5x10^{-7}$	1							1	
Page Soln and Dibenzyline	0.4-10			10	0.4	1-2.5			
Page Soln and Dibenzyline with Ephedrine 10^{-5}	0.6-50			50	0.6	5-10			
Page Soln and Ephedrine 10^{-5}	2-20						10	2	2	20
Page Soln and Ephedrine 10^{-5} with Dibenzyline	1-20						10	5	1	20

Results in each column are from a single experiment.

Figure 2



5 ng	5 ng	5 ng	5 ng
Adr	HT	HT	HT
12·26	12·34	12·49	1·04 pm

Perfusion of the isolated ear of the rabbit, with Page Solution containing ephedrine (10^{-5}).

Height of record shows interval between drops.

This tracing illustrates a tachyphylaxis of the ear vessels to 5-hydroxytryptamine, even with a dose interval of 15 minutes.

Adr. adrenaline.

HT. 5-hydroxytryptamine.

The ratio was increased. If the ephedrine concentration was raised, (e.g. 10^{-4}), responses to both drugs were reduced and the ratio returned to its previous low level. That is, the range over several experiments of the ratio of the dose of adrenaline to the dose of 5-hydroxytryptamine for equal effects was -

for Page solution alone, 0.25 - 6.2:

for Page solution containing ephedrine, 10^{-6}, 0.4 - 2:

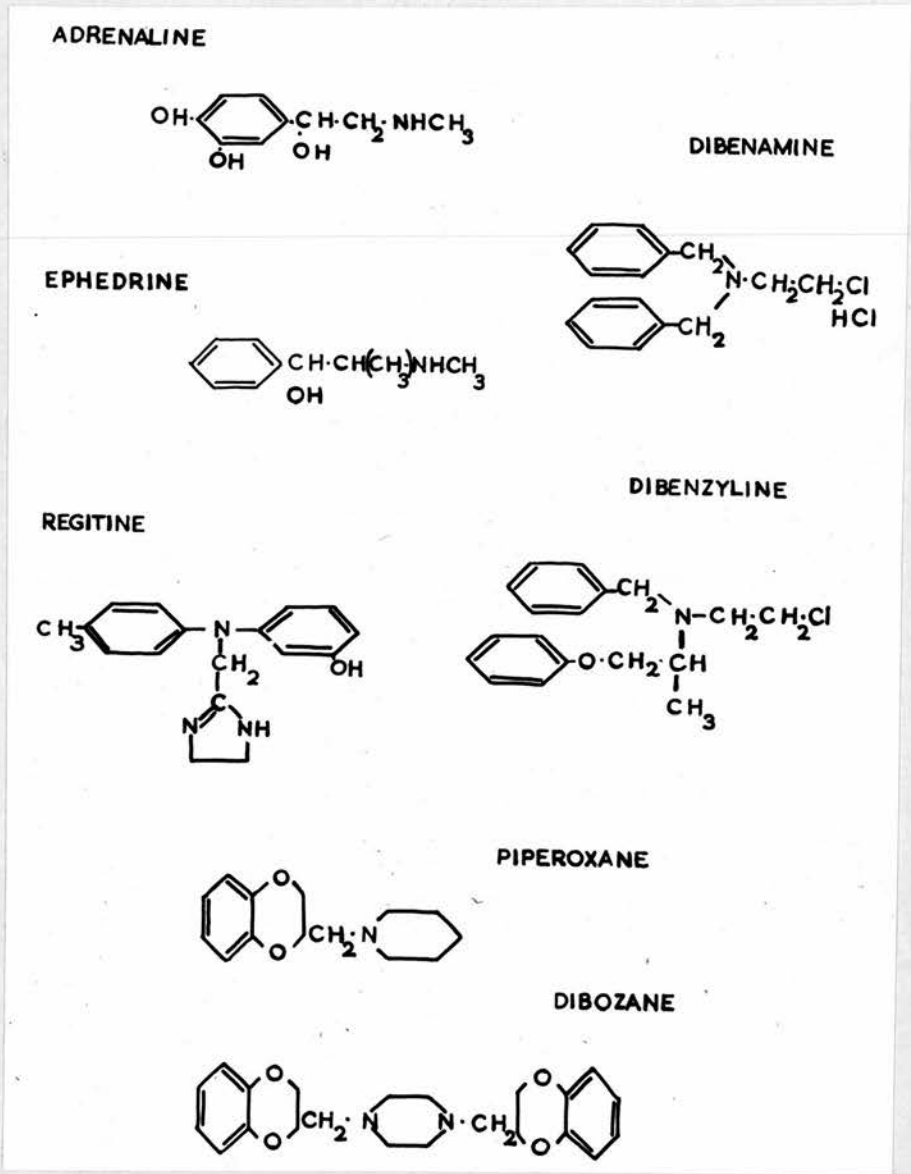
for Page solution containing ephedrine, 10^{-5}, 2 - 100 and

for Page solution containing ephedrine, 10^{-4}, 1 - 10.

Only on one occasion was tachyphylaxis to responses to 5-hydroxytryptamine recorded in the presence of ephedrine (10^{-5}). Part of this experiment is shown in Fig.2. Instead of the usual ratio of 10 - 100, the ratio here was about one, (before tachyphylaxis).

To the optimum concentration of ephedrine (i.e.10^{-5}) was added the adrenaline antagonist, piperoxane./

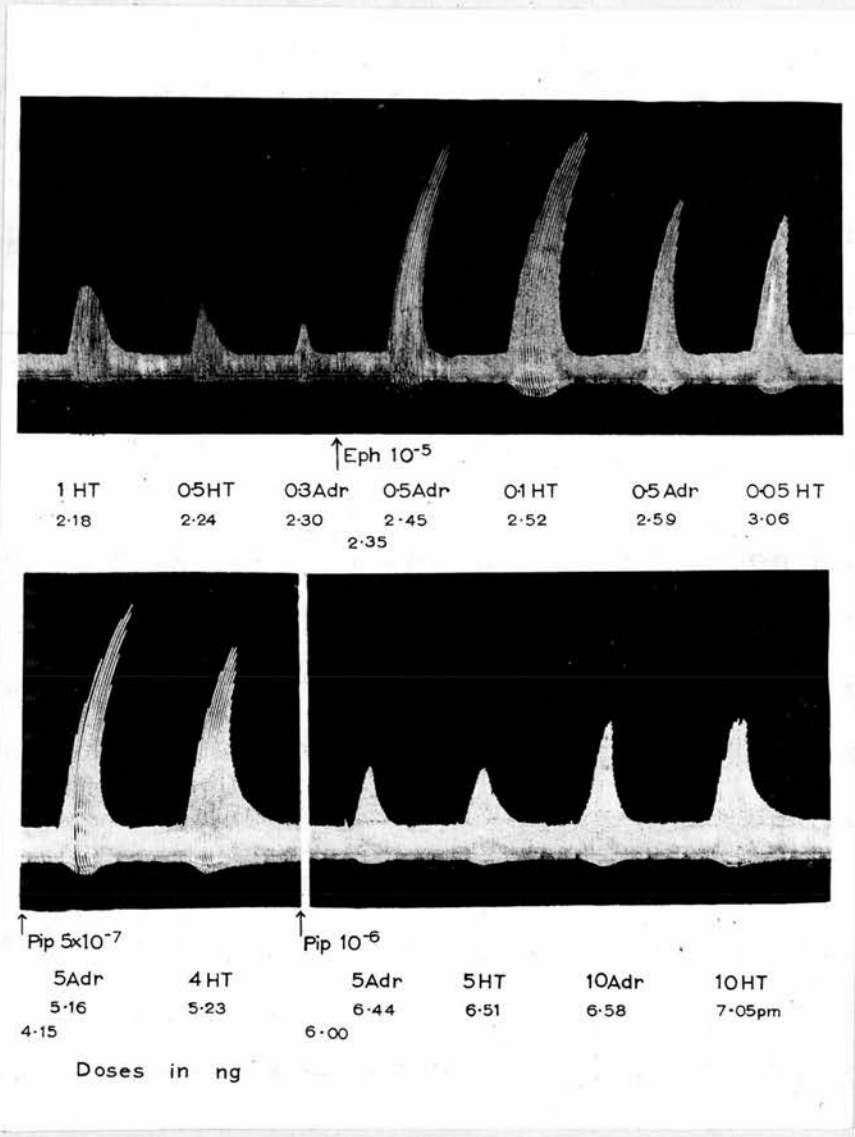
Figure 3



Chemical Formulae - Adrenaline
and certain adrenolytic drugs.

Figure 4

Perfusion of the isolated ear of the rabbit, with Page Solution.



Height of record shows interval between drops.

At 2.35 p.m. the Page Solution is replaced by Page Solution containing ephedrine, 10^{-5}:

At 4.15 p.m. ... Page Solution containing ephedrine 10^{-5} and piperoxane 5×10^{-7}:

At 6.00 p.m. ... Page Solution containing ephedrine 10^{-5} and piperoxane 10^{-6}.

Note the changes in sensitivity of the vessels of the ear to 5-hydroxytryptamine (HT) and adrenaline (Adr) during the perfusion with the various solutions.

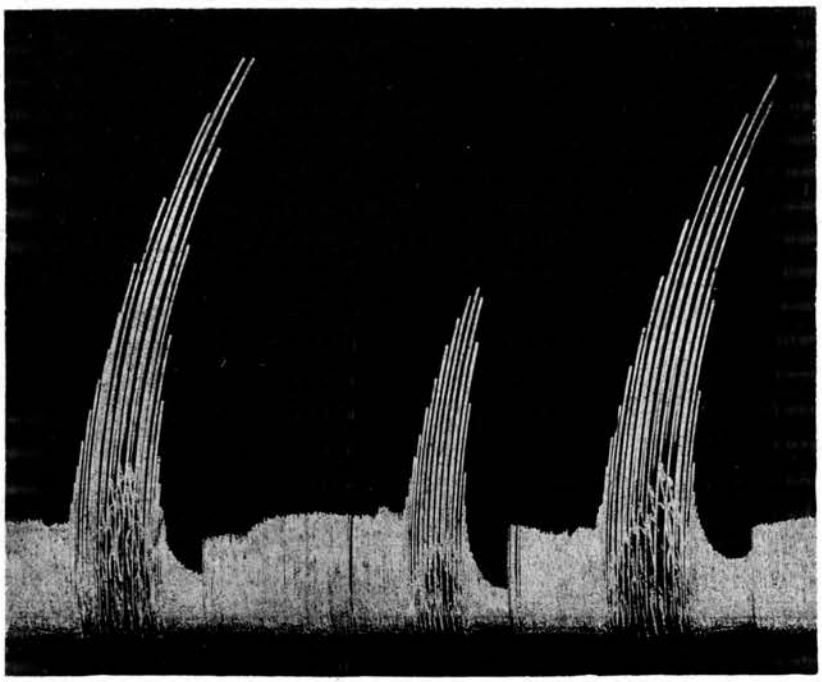
piperoxane. Their combined effect was to reduce the sensitivity of the vessels to both the constrictors. Even with less ephedrine, (e.g. 10^{-6}), piperoxane did not produce a purely anti-adrenaline effect. (The concentrations of piperoxane used, corresponded to those used by Gaddum and Hameed, 1954).

After a preliminary trial of regitine in various concentrations it was apparent that it had a definite anti-adrenaline effect though the ratio was not increased markedly. Regitine (10^{-7}) was combined with ephedrine (10^{-5}) and this gave a lower ratio than regitine (10^{-7}) alone had done.

Attention was then directed to the use of ephedrine alone. In a few cases, this drug caused a severe vasoconstriction and the experiment was abandoned unless excessively high pressure for a short time could cause a relaxation of the vessels.

When ephedrine 10^{-5} was perfusing satisfactorily the sensitivity of the preparation to adrenaline was less than it was to 5-hydroxytryptamine. In one experiment, the ear vessels were unusually sensitive to 5-hydroxytryptamine and the constriction, caused by an injection of this drug, was followed/

Figure 5



Perfusion of the isolated ear of the rabbit, with Page Solution containing ephedrine (10^{-5}).

Height of record shows interval between drops.

Note the high sensitivity of this preparation and the dilatation following the vasoconstriction caused by 5-hydroxytryptamine (HT), and by 5-hydroxytryptamine together with adrenaline (Adr).

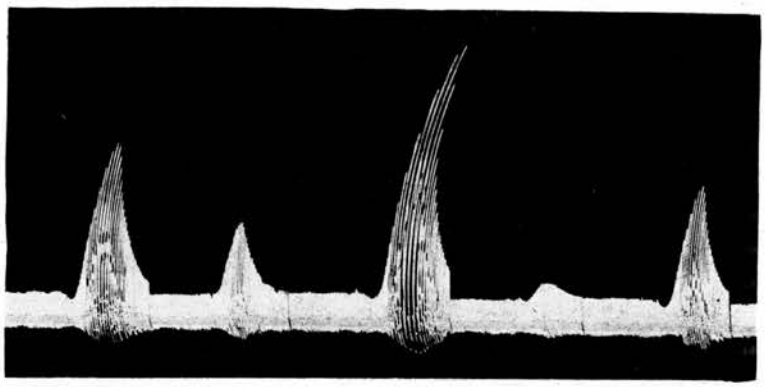
followed by a marked dilatation. The same occurred when a dose containing a mixture of adrenaline and 5-hydroxytryptamine was given ,(see Fig.5). In a few cases, small doses of noradrenaline caused a vasodilatation.

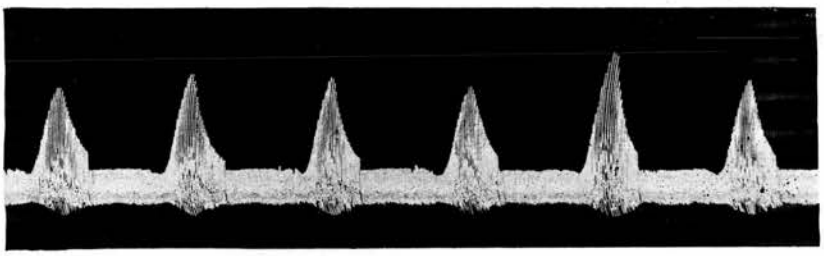
Again, in the presence of ephedrine, (10^{-5}), combined doses of adrenaline and 5-hydroxytryptamine were injected and the responses compared with those produced by the 5-hydroxytryptamine component alone. Where the amount of added adrenaline was the same (ng for ng) as the 5-hydroxytryptamine dose there was only a very slight increase in the response over that to the 5-hydroxytryptamine alone, whereas if there was 2-4 times as much adrenaline as 5-hydroxytryptamine in the dose, the response was much increased. This was in spite of the fact that the adrenaline dose, itself, caused only a small or threshold response.

A similar effect was recorded with noradrenaline and 5-hydroxytryptamine in combined doses, where the ear vessels were perfused with ephedrine, 10^{-5}. Here also, where the dose of noradrenaline was equal to one third of the dose of 5-hydroxytryptamine (ng for ng) there was a slight reduction/

Figure 6

Perfusion of the isolated ear of the rabbit with Page solution containing ephedrine (10^{-5}).





Doses in ng

Height of record shows interval between drops.

Note that although the sensitivity of the vessels of the ear is less to noradrenaline than it is to 5-hydroxytryptamine (sensitivity about 1/10), the response to a mixture of these drugs is greater than that to the 5-hydroxytryptamine component alone where the proportion of noradrenaline to 5-hydroxytryptamine is greater than 1 to 1.

Nor. noradrenaline. HT. 5-hydroxytryptamine.

reduction of the response compared with that to the 5-hydroxytryptamine dose alone. Where the proportion of noradrenaline to 5-hydroxytryptamine was greater than one, the response was increased.

In all these instances, the sensitivity of the preparation was greater to 5-hydroxytryptamine than to adrenaline or noradrenaline. In one case, the noradrenaline sensitivity was further reduced by the perfusion of noradrenaline (2.5×10^{-10}) itself, along with the ephedrine. Here, four times as much noradrenaline as 5-hydroxytryptamine in a combined dose gave a response equivalent to that elicited by the 5-hydroxytryptamine component alone. If the noradrenaline was increased to eight times as much as the 5-hydroxytryptamine the mixture gave an increased response. (see Discussion, p.111).

A search was therefore made for a more potent anti-adrenaline. Dibozane is reported to be ten times as active as piperoxane, in a test concerned with adrenaline responses in the isolated rat seminal vesicle, (Leitch, Liebig and Haley, 1954), but it did not satisfactorily improve the sensitivity/

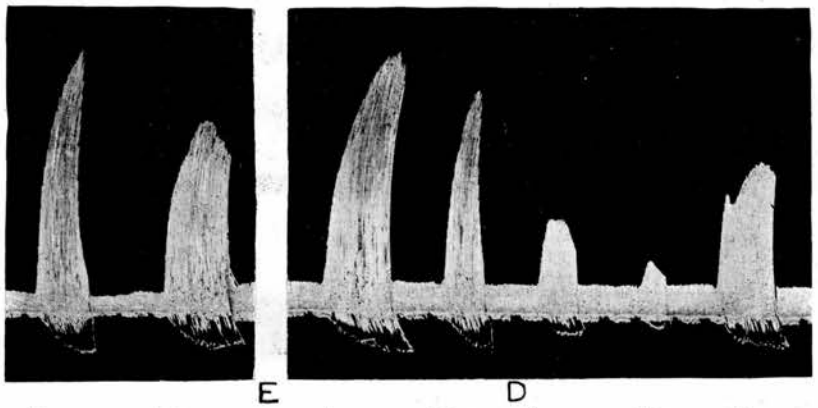
sensitivity ratio, adrenaline/5-hydroxytryptamine in the perfused rabbit ear. However, dibenzyline which was more active than dibozane in the seminal vesicle test, proved useful. It has a very powerful and long-lasting effect and was therefore given by injection in small volumes and not by continuous perfusion. The sensitivity of the ear vessels to dibenzyline varied from one preparation to another. The initial dose of dibenzyline was usually 75 µg in 0.5 ml/Page solution, followed by 25 µg doses until the desired effect was obtained. The full extent of the antagonism caused by any one dose was reached almost at once and the sensitivity of the vessels to noradrenaline and 5-hydroxytryptamine could be determined in a short time. Larger doses of dibenzyline eventually led to a decrease in the sensitivity and in the discrimination of the preparation towards 5-hydroxytryptamine.

After/

In these later experiments, the sensitivity ratio noradrenaline/5-hydroxytryptamine is quoted, for it was appreciated that this was of more value, (see Table IV), since the extracts were likely to contain more noradrenaline than adrenaline, sympathin being about 90% noradrenaline.

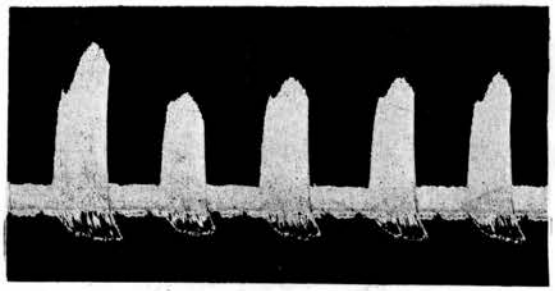
Figure 7

Perfusion of the isolated ear of the rabbit.



E D

5ng	50ng		1ng	10ng	1ng	10ng	10ng
Nor	HT		HT	Nor	HT	Nor	HT
12·20	12·30		2·30	2·45	3·05	3·15	3·25pm



0·10ml	5ng	0·10ml	10ng	0·10ml
A	HT	A	HT	A
4·55	5·10	5·25	5·40	5·55pm

Height of record shows interval between drops.

Nor. - noradrenaline. HT.- 5-hydroxytryptamine.

After E. - 12.40 p.m. - ephedrine (10^{-5}).

At D. - 2.55 p.m. - 150 ng dibenzyline in 0.5 ml.

Upper tracing shows sensitization by ephedrine before and after injection of dibenzyline.

Lower tracing shows part of the assay of a sample, A (acetone extract) of dog serum.

0.10 ml A contains 10 ng HT.

After it was found that ephedrine, 10^{-5}, and dibenzyline (doses varied with the individual ears), gave the most satisfactory conditions assay of several samples of dog serum was made.

Routine procedure:

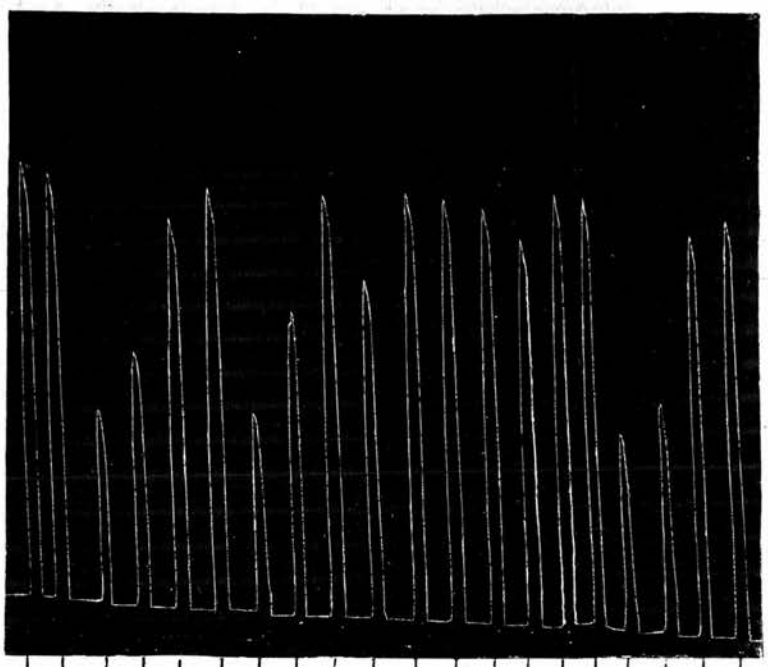
An ear was set up and perfused with Page solution; its sensitivity to noradrenaline and 5-hydroxytryptamine was found, then ephedrine (10^{-5}) was introduced. After redetermining the sensitivity of the preparation, to show that 5-hydroxytryptamine responses were increased, doses of dibenzyline were cautiously added until no improvement of the ratio was found.

The samples were then assayed in terms of 5-hydroxytryptamine. The sensitivity of the preparation to noradrenaline was checked again at the end of the assay, it was generally very little changed (2-4 hours).

Tracings of the preparation of an ear and its use during an assay are shown, (Fig.7). In this case, after the dibenzyline (given in one large dose) the responses of the ear vessels were blocked for up to 5 ng noradrenaline, 2.5 ng adrenaline, 1 µg acetylcholine and 100 ng histamine.

Evaluation/

Figure 8



s. 10ng. HT

M. 10ng. HT incubated with 10ng. Adrenaline and Mushroom juice

C. Control as M without Mushroom juice

Rat Oestrous uterus 2ml. bath

Effect of incubation with mushroom juice at room temperature on the inhibition of responses to 5-hydroxytryptamine by adrenaline.

Evaluation of crude mushroom juice activity.

Tests of enzyme activity were always based on the time taken for a given amount of mushroom juice to eliminate the inhibitory effect of a certain amount of adrenaline on the spasm of rat oestrous uterus caused by 5-hydroxytryptamine.

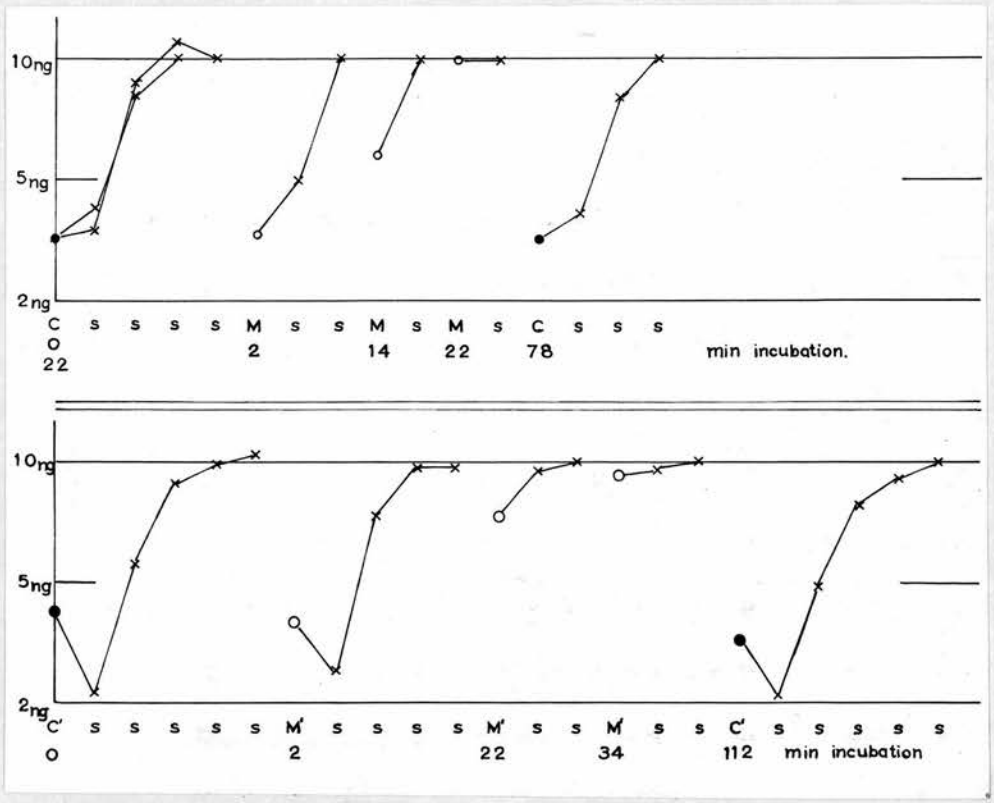
The portion of rat oestrous uterus was set up as usual in the 2 ml organ-bath (p.52), and regular contractions were obtained to, say, 10 ng 5-hydroxytryptamine. Then at C, 10 ng of 5-hydroxytryptamine was again given but within the same dose volume was also 10 ng adrenaline. (Each dose was left in for 45 seconds and washed out carefully - 4 minutes interval between doses). The presence of the adrenaline caused inhibition of the response and the inhibitory effect gradually disappeared as the following responses to 10 ng 5-hydroxytryptamine (alone) were recorded. This gives a measure of the inhibitory effect of a mixture containing equal quantities of the two drugs, and/

and the time required for full recovery from inhibition.

The dose at M contained 10 ng of each drug as before and 0.25 ml of a 1 in 10 dilution of the crude mushroom juice per ml (i.e. one fifth of each dose volume is diluted mushroom juice; or one fiftieth is crude mushroom juice.) If this dose is tested, 1-4 minutes from addition of the mushroom juice, the inhibition and recovery is very similar to that for a dose of C. After full recovery of the 10 ng 5-hydroxytryptamine responses, another dose of M, now 14 minutes from preparation, was given. This caused a reduced response but did not inhibit responses to subsequent doses of 5-hydroxytryptamine. At 22 minutes from preparation, M showed no inhibitory effect.

Thus by standing at room temperature with adrenaline at pH 4.5 - 6, the enzyme preparation was able to abolish in 22 minutes the inhibitory effect of 10 ng adrenaline on the uterine contraction caused by 10 ng 5-hydroxytryptamine. It was found too that the mushroom juice, in the same dose as was present in the above mixture, M, had no effect/

Figure 9



Upper graph shows the results of the experiment shown in Fig. 8.

Ordinate - Height of responses to 5-hydroxytryptamine.

s - Response to 10 ng 5-hydroxytryptamine.
M - Response to 10 ng 5-hydroxytryptamine incubated with 10 ng adrenaline and mushroom juice.
C - Response to control as M, <u>without</u> mushroom juice.

Lower graph -

s' - Response to 10 ng 5-hydroxytryptamine.
M' - Response to 10 ng 5-hydroxytryptamine incubated with 50 ng adrenaline and mushroom juice.
C' - Response to control as M', <u>without</u> mushroom juice.

effect itself on the 5-hydroxytryptamine response.

Where there was more adrenaline than 5-hydroxytryptamine in a mixture, e.g. 5:1, it was found that the response to the next dose of 5-hydroxytryptamine following the mixed dose, was smaller than that to the mixed dose. The adrenaline must take some time to reach its maximum inhibitory effect on this preparation. It was therefore convenient, for a number of investigations, to add the adrenaline, or the adrenaline-mushroom juice mixture to the organ-bath, one minute before a 5-hydroxytryptamine dose was due in order to give the adrenaline time to produce its maximum effect. In experiments carried out in this manner, the record showed a regular pattern of increasing responses to 5-hydroxytryptamine as the adrenaline effect wore off, until responses at the pre-adrenaline level were again obtained.

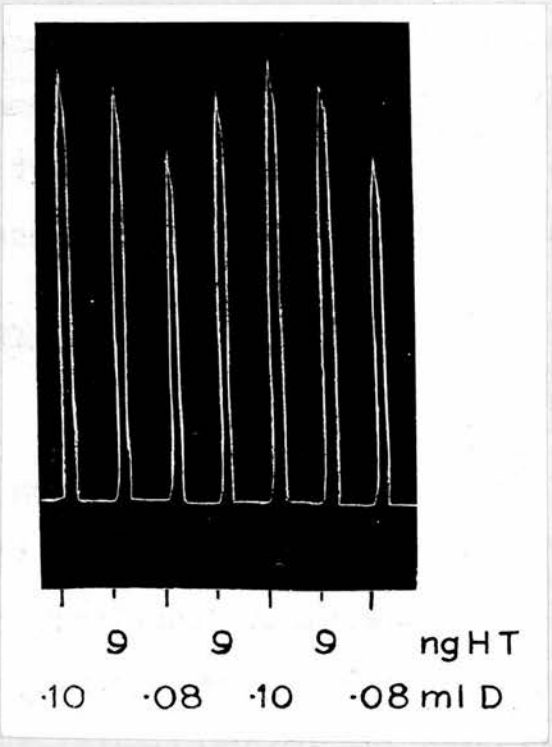
Later of course, further tests had to be made of mixtures of adrenaline and 5-hydroxytryptamine or of noradrenaline and 5-hydroxytryptamine.

The results of such experiments showed that the inhibitory action of adrenaline, when present in/

in concentrations up to 10 times that of the 5-hydroxytryptamine could always be abolished within 40 minutes by treatment of the mixture with mushroom juice. Under similar conditions, concentrations of noradrenaline up to 100 times that of the 5-hydroxytryptamine could be dealt with. 5-hydroxytryptamine itself proved resistant to the enzyme action for at least 80 minutes and only a slight decrease in its spasmogenic activity was noted after 2 hours treatment with the mushroom juice.

Based on these findings a standard method was developed, to eliminate interference due to the presence of adrenaline and/or noradrenaline in the assay of a tissue extract for its 5-hydroxytryptamine content. It comprised the addition of 0.25ml of a 1 in 10 dilution of the standard preparation of crude mushroom juice (p. 60) to each ml of the reconstituted acetone extract residue of the tissue samples, (or, of these diluted if necessary). Thus each dose volume of tissue extract placed in the organ-bath contained one fifth part of diluted mushroom juice. The mushroom treated sample was allowed to stand at room temperature and was not assayed until at least 40 minutes from preparation, certainly/

Figure 10



Assay of sample of dog serum (acetone extract).

Rat oestrous uterus, in 2 ml bath, with de Jalon's Solution (modified) containing atropine 10^{-6}.

D - 1/2 dilution of extract residue in saline.

certainly before 2 hours had elapsed. The pH was tested and found to lie between pH 4.5 and pH 6.

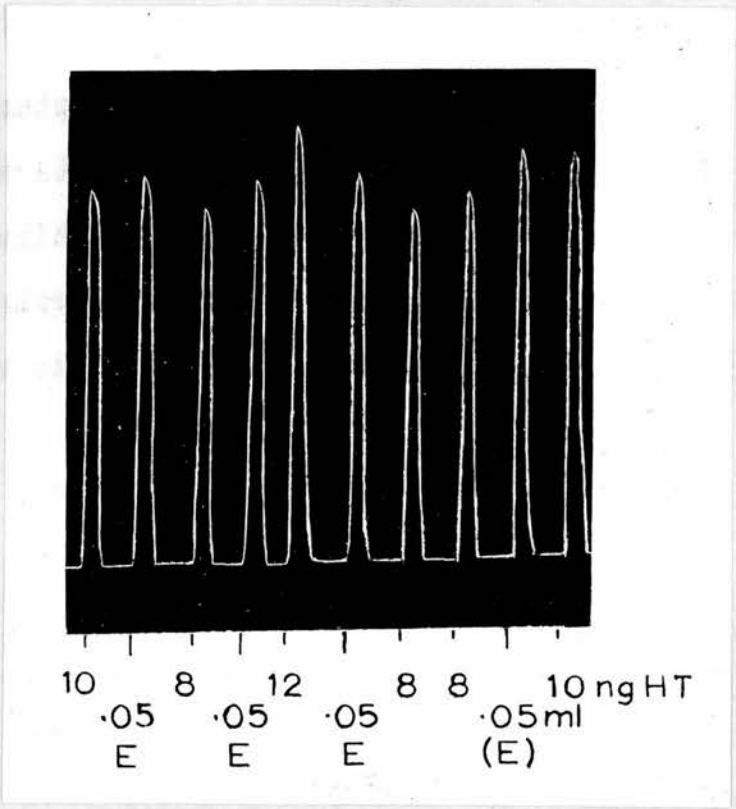
In view of the relatively high concentration of adrenaline and noradrenaline in the adrenal glands, the extract residues of these were treated with twice the standard volume of mushroom juice for twice the standard time, in an effort to destroy all the adrenaline and noradrenaline present.

B. Estimates of 5-hydroxytryptamine in tissues.

Using the acetone extraction method for the samples of tissues and the mushroom treatment for removal of the adrenaline inhibitory effect, the estimates of 5-hydroxytryptamine activity were made by assay on the isolated atropinised oestrous uterus of the rat. A few assays were done on the rabbit ear, and for the dog brain samples parallel assays were made on the heart of Mactra solida. In most cases three samples of each tissue, from different animals, were estimated.

The results are given in the following tables - /

Figure 11



Assay of sample of rabbit gut mucosa (acetone extract).

Rat oestrous uterus, in 2 ml bath, with de Jalon's Solution (modified) containing atropine 10^{-6}.

E - 1/20 dilution of extract residue in saline containing 0.20 ml diluted mushroom juice in each ml.

(E) - 1/20 dilution of extract residue in saline <u>without</u> mushroom juice.

Note no change in the responses of the uterus.

tables -

Table V - Dog serum 5-hydroxytryptamine estimates.

Tables V - VII Summary of 5-hydroxytryptamine equivalents of various tissues of the rabbit, (mean estimates).

Table VIII- 5-hydroxytryptamine eqivalents in various tissues of the rabbit.

TABLE V/

TABLE V

Dog serum - 5-hydroxytryptamine estimates.

	Results - corrected for recovery.
1. Unanaesthetised dog - serum - acetone extract - chromatogrammed eluates freeze-dried. Uterus assay[+] - duplicate samples	0.48µg/ml (0.4-0.6) 0.40µg/ml (0.3-0.53) 23-33% recovery
2. Unanaesthetised dog - serum - acetone extract Ear assay[+] - (ephedrine 10^{-5}, with 75µg dibenzyline)	0.52µg/ml (0.32-0.84) 50-95%
Uterus assay	0.62µg/ml (0.46-0.80) 50%
3. Unanaesthetised dog - serum - acetone extract Ear assay - (ephedrine 10^{-5}, with 150µg dibenzyline)	0.39µg/ml (0.30-0.59) 76%
4. Chloroform anaesthetised dog - serum - acetone extract Ear assay - (ephedrine 10^{-5}, with 150µg dibenzyline)	0.30µg/ml (0.20-0.40) 15-20%
	Mean = 0.45µg/ml (6)
	Excluding No.4. - Mean = 0.48µg/ml (5)

[+]Uterus assay = estimation on isolated oestrous uterus of the rat.

Ear assay = estimation on perfused isolated ear of the rabbit.

TABLE VI

Summary of 5-hydroxytryptamine equivalents of various tissues of the rabbit.

(mean estimates)

Spleen	11.72 µg/g (6)	stimulated	11.19 µg/g (2)

(see p. 49 and 119)

Mucosae -

Stomach	6.15 µg/g (3)
Small intestine upper	2.20 µg/g (3)
Small intestine lower	1.64 µg/g (3)
Large intestine	3.82 µg/g (3)

Blood -

Whole	2.0 - 2.5 µg/ml
Serum	4.4 µg/ml

These estimations were made on the isolated oestrous uterus of the rat, and the stimulation caused by the extract in each case was abolished by lysergic acid diethylamide, (i.e. L.S.D. +).

TABLE VII

Summary of 5-hydroxytryptamine equivalents of various tissues of the rabbit.

(mean estimates)

Brain -		
Hypothalamus	0.395 µg/g	(+)
Mid-brain	0.370 µg/g	(-)
Olfactory bulbs	0.128 µg/g	(-)
Cerebellum	0.040 µg/g	(-)
Liver -		
with portal perfusion	0.348 µg/g	(-)
without portal perfusion	0.943 µg/g	(+)
Bone marrow -	0.327 µg/g	(+)
Lung -	0.177 µg/g	(+)
with direct pulmonary perfusion	0.136 µg/g	(+)
Thyroid	0.164 µg/g	(+)
Pancreas	0.116 µg/g	(+)
Diaphragm	0.091 µg/g	(+)
Salivary glands	0.089 µg/g	(+)
Thymus	0.049 µg/g	(+)
Heart	0.035 µg/g	(-)
Bladder	0.030 µg/g	(-)
Kidney	0.025 µg/g	(-)
Skeletal muscle	<0.018 µg/g	
Sciatic nerve	<0.067 µg/g	
Bile	<0.030 µg/g	(-)
Adrenal	not detectable	

Where extraction activity was abolished by lysergic acid diethylamide estimates are marked (+), where activity not abolished (-).

TABLE VIII

5-hydroxytryptamine-equivalent in µg/g rabbit tissue.

Spleen	13.8 (9.4 - 18.7)	11.1 (8.0-12.5)	7.6 (6.4 - 9.0)
	21.0 (18 - 26)	9.1 (8.0-10.0)	7.71 (6.4 -10.0)
Stimulated	13.3 (8 - 16)	9.07 (7.3-10.8)	
Mucosae			
Stomach	6.4 (5.8 - 8.0)	7.49 (3.75-14.9)	4.56 (3.7 - 6.06)
Small intestine, upper	1.4 (1.14- 1.7)	4.05 (3.37-5.14)	1.17 (0.93 - 1.56)
Small intestine, lower	0.67 (0.5 - 1.0)	3.74 (2.49-7.48)	0.52 (0.4 - 0.8)
Large intestine	3.5 (2.78- 4.17)	3.97 (3.2-6.4)	2.98 (2.27 - 3.64)
Blood	Siliconed-unclotted	Glass-rapid clot	Siliconed slow-clo
I Ether	2.0µg/ml (1.6-2.4)	2.0µg/ml (1.6-2.4)	2.0µg/ml (1.6-2.4)
	2.0µg/ml (1.6-2.4)	2.0µg/ml (1.6-2.4)	2.0µg/ml (1.6-2.4)
II Urethane	2.5µg/ml (2-3)	2.5µg/ml (2-3)	
	2.5µg/ml (2-3)	2.5µg/ml (2-3)	
	2.0µg/ml (1-2.2)		
Serum	Direct estimation	After mushroom treatment	
	4.4µg/ml (4.0-4.8)	4.4µg/ml (4.0-4.8)	
Brain			
Hypothalamus	0.42 (0.31-0.50)	0.406 (0.31-0.50)	0.36 (0.27-0.43)
Mid-brain	0.126(0.09-0.19)	0.488(-) (0.43-0.57)	0.496(0.37-0.74)
Olfactory bulbs	0.21 (0.17-0.28)	0.038(-)	0.137(0.09-0.28)
Cerebellum	0.037	0.054(-)	0.028
Liver/			

Figures in brackets indicate the range of each estimate.

Where extract activity was not abolished by lysergic acid diethylamide, estimates are marked (-)

TABLE VIII Contd:

Liver	0.48 (0.32-0.64)	0.90 (0.72-1.08)	1.45 (1.0 - 2.0)
with portal perfusion	0.25(-) (0.13-0.38)	0.126(-) (0.10-0.16)	0.445(0.32 - 0.64)
Bone marrow	0.29 (0.25-0.33)	0.30 (0.20-0.40)	0.39 (0.34 - 0.43)
Lung	0.30 (0.20-0.40)	0.075(0.06-0.09)	0.157(0.11 - 0.21)
direct pulmonary perfusion	0.03 0.32(-) (0.28-0.38)	0.234(0.16-0.31) 0.125(0.09-0.15)	0.156(0.09 - 0.25)
Thyroid	no perfusion 0.53	0.232	0.095
Pancreas	0.055(0.05-0.06)	0.213(0.17-0.26)	0.08 (0.06 - 0.10)
Diaphragm	<0.02 0.04(-)	0.12	0.18
Salivary glands			
Parotid	0.066	0.070	
Submaxillary	<0.03(-)	0.132	
Thymus	no perfusion 0.137(0.10-0.20)	0.050	0.048
Heart	<0.028	0.042	0.030(-)
Bladder	0.030	0.135(-)	0.110(-)
Kidney	0.100	0.025	0.150(-)
Skeletal muscle	<0.02	<0.01	<0.025
Sciatic nerve	<0.055	<0.07	<0.076
Bile	0.02	<0.02(-)	<0.05(-)
Adrenals	not detectable because of inhibition of standard		

Figures in brackets indicate the range of each estimate.

Where extract activity was not abolished by lysergic acid diethylamide, estimates are marked(-)

One series of rabbit tissue extracts were assayed both before and after the standard mushroom treatment. The increases in the 5-hydroxytryptamine-equivalent are shown in the table below. No increase in the equivalent was found with serum which is unlikely to contain significant amounts of adrenaline or noradrenaline. In this experiment, none of the extracts gave any response after the uterus was treated with lysergic acid diethylamide, so the increase is probably due to an unmasking of more 5-hydroxytryptamine activity.

TABLE IX

Acetone extracts of rabbit's tissues.

	5-HT-equivalent µg/g.		
	Direct estimation	After mushroom treatment	RATIO
Kidney	0.020	0.025	1.25
Lung	0.060	0.075	1.25
Pancreas	0.147	0.213	1.45
Liver	0.560	0.900	1.80
Serum	4.400	4.400	1.00

Parallel/

Parallel assays - Dog brain samples.

Extracts of certain areas of dog brain were prepared with acetone (see p.46). The following day, the dry residues were taken up in 1 ml distilled water and diluted with saline if necessary. This solution was assayed directly on the Mactra heart, while an aliquot was treated with standard crude mushroom juice and assayed on the isolated rat uterus in oestrus.

TABLE X

Acetone extracts of dog's brain.

	5-HT-equivalent ng/g.		
	Rat uterus	Mactra heart	RATIO
Olfactory bulbs	40	62	0.65
	33	31	1.06
	43	25	1.72
Cortex of carebellum	0(< 12)	12	< 1.0
	0(< 20)	7	< 2.85
	0(< 30)	8	< 3.75
Sympathetic ganglia	260	0(< 59)	> 4.4
	162	0(< 19)	> 8.5
	240	0(< 29)	> 8.3
Caudate nucleus	45	88	0.51
	117	200	0.58
Hypothalamus	1010	555	1.82
	415	280	1.48
	930	224	4.15

$1 \text{ ng} = 10^{-3} \mu g$

TABLE XI

Acetone extracts of dog hypothalamus and caudate nucleus, (mushroom treated), see p. 125.

	Hypothalamus	Caudate nucleus
5-HT-equivalent ng/g		
Rat uterus	770	130
Guinea-pig ileum	<400	<60
Substance P equivalent units/g.		
Rat uterus	215	1.38
Guinea-pig ileum	<35.9	<0.20

TABLE XII

Acetone extract of dog hypothalamus, see p.125.

5-HT-equivalent ng/g

Rat uterus	Rabbit ear
900	1000
(670-1000)	(500-1500)

DISCUSSION

Chromatography, including the use of IRC 50 and freeze-drying.

In the chromatography experiments which set out to find a separation of the position reached by adrenaline from that reached by 5-hydroxytryptamine, only one method, the ascending method on paper, was used. This limited, necessarily the time for development, for once the solvent had reached the top of the sheet of paper there was no further increase in the distance travelled by the amines. If there is a real, though slight difference in the rate of progress of adrenaline from that of 5-hydroxytryptamine, it might have been better exploited by a descending method, where the development time can be so much increased.

Pure solutions of 5-hydroxytryptamine are rather unstable to heat and to evaporation under reduced pressure at 37°C. If such solutions are acid or alkaline (pH <4 or >8) the loss of 5-hydroxytryptamine activity on evaporation is almost complete. Similar losses were noted by Erspamer (1954).

There/

There is however, a fairly satisfactory recovery of 5-hydroxytryptamine added to acetone extracts of tissues, or in the presence of adequate buffering at pH 6-7. Yet when the solution of 5-hydroxytryptamine had had previous contact with chromatography paper (Whatman No.1, washed with 0.01 N hydrochloric acid) it was again less stable to evaporation despite buffering at pH 6-7. Something seemed to come out of the paper and either aid in the oxidative destruction of 5-hydroxytryptamine or complex with this in such a way that its ability to stimulate the isolated oestrous uterus was impaired. For, all the assays of the samples were made on the isolated oestrous uterus of the rat. Yet, how something could still be present on the paper, and coming off in sufficient quantity to have such an effect on the 5-hydroxytryptamine is strange - after the paper was washed even for 48 hours with 0.01 N hydrochloric acid, or washed with 0.01 N sodium hydroxide and then 0.01 N hydrochloric acid. In similar work, Fingl (1953) was unable to make quantitative recovery of small amounts ($< 2\mu g$) of 5-hydroxytryptamine.

The/

The results from the concentration of solutions of 5-hydroxytryptamine by passage through the IRC 50 resin columns are puzzling. On some occasions, recoveries were high and on others, low - and even where the columns were being treated strictly in parallel the result from one might be high and from another, low. Fingl (1953) did some work on this problem also, but he was not concerned to remove the adsorbed 5-hydroxytryptamine in a very small volume. He was interested in the separation of 5-hydroxytryptamine from histamine and acetylhistamine, and he did show quantitative recovery of 10 µg samples of 5-hydroxytryptamine at pH 6.9.

The columns used here, were however, smaller. The concentration of phosphate, as buffer, in the percolate, made the testing of whether all the 5-hydroxytryptamine had in fact been adsorbed, rather difficult. The response of the isolated uterus is inhibited by phosphate. Where tests were made it was found that a good part at least of the 5-hydroxytryptamine was taken up. But the elution from the columns was not quantitative.

Then, when freeze-drying was used for the concentration of the samples it was found just how important/

important is the control of pH during drying. Phosphate buffer was the most satisfactory at the required pH (i.e. pH 6.5). Yet the sensitivity of the uterus to inhibition by phosphate prevented the excessive use of this buffer; the isolated rat oestrous uterus preparation was used for all assays at this time. In a few cases, where the uterus was particularly sensitive to the phosphate, a similar concentration of phosphate was added to the standard 5-hydroxytryptamine solution and the buffered freeze-dried samples compared with this. The recovery of 5-hydroxytryptamine from chromatograms, the eluates of which were freeze-dried, was 50% whereas the recovery of 5-hydroxytryptamine added to serum and similarly treated was only 23-33%. This loss remains unexplained.

Isolated perfused ear of the rabbit

The experiments with the perfusion of ephedrine through the vessels of the rabbit ear, gave the expected changes in sensitivity to adrenaline and 5-hydroxytryptamine responses. Low concentrations of ephedrine (10^{-6}) caused an increase in the responses to both drugs; higher concentrations (10^{-5}) caused some inhibition of adrenaline responses/

responses with further sensitisation of the ear vessels to 5-hydroxytryptamine; ephedrine (10^{-4}) blocked responses to both drugs. These changes are discussed by Gaddum and Kwiatkowski (1938) and by Gaddum and Hameed (1954) on the following theory. Ephedrine causes inhibition of amine oxidase, therefore, since this enzyme destroys both drugs, it potentiates the response to both adrenaline and 5-hydroxytryptamine. But at a concentration of 10^{-5}, ephedrine tends to block the adrenaline receptors, though still higher concentrations are required before it will block the tryptamine receptors.

Ephedrine 10^{-5} proved to be the optimum concentration for partial blockade of adrenaline responses, and stimulation of 5-hydroxytryptamine responses; thus giving a high equivactive dose-ratio adrenaline/5-hydroxytryptamine. This ratio was not improved by the addition of a fairly specific anti-adrenaline drug, e.g. piperoxane, (Gaddum and Hameed, 1954) or regitine, and in fact, the sensitivity of the ear vessels to 5-hydroxytryptamine was much more reduced than that to adrenaline so that the ratio became less. Was there a displacement of the ephedrine from the adrenaline receptors by the piperoxane, so that the ephedrine was now free/

free to block the tryptamine receptors? Or, was the piperoxane concentration effect simply additive, the molecules filling available receptors of either kind?

In the presence of ephedrine 10^{-5}, a dose containing equal quantities of adrenaline and 5-hydroxytryptamine causes a vaso-constriction equal to that caused by the 5-hydroxytryptamine component alone. Where the combined dose contained more adrenaline, the response was increased despite the fact that the adrenaline component alone was considerably inhibited by the ephedrine. Similar effects were recorded with noradrenaline and 5-hydroxytryptamine given together, during ephedrine perfusion, even when the noradrenaline dose, alone, gave a threshold vaso-constriction or sometimes even a vasodilatation.

Also, while noradrenaline (2.5×10^{-10}) was perfused along with ephedrine, noradrenaline responses were further inhibited and when four times as much noradrenaline as 5-hydroxytryptamine were given together, the response was not increased over that to 5-hydroxytryptamine alone. But where more noradrenaline was added to the 5-hydroxytryptamine dose/

dose, the response was again increased.

This increased response effect was important because though a test with adrenaline or noradrenaline alone would give no response and the ear be considered insensitive to these drugs, yet the presence of either or both of these in the tissue extract, would have an influence on the 5-hydroxytryptamine estimate.

So more powerful adrenolytic drugs were tested. Dibenzyline was found satisfactory as an inhibitor of adrenaline vasoconstriction, though the dose required in the various ear preparations varied. The required effect had to be built up with added doses. Unfortunately, dibenzyline, in the presence of ephedrine, was not entirely specific. In a few cases, together they further inhibited the noradrenaline response and also flattened the 5-hydroxytryptamine dose-response curve. In other cases, dibenzyline caused marked inhibition of the 5-hydroxytryptamine responses, as the ephedrine and piperoxane together had done.

This preparation was considered unsuitable for routine assays, because of the time which it took to prepare and the poor discrimination between doses of 5-hydroxytryptamine after dibenzyline.

Mushroom/

Mushroom polyphenoloxidase.

The enzymes in crude mushroom juice were very suitable for the destruction of adrenaline activity, in mixtures of adrenaline and 5-hydroxytryptamine. The preparation was easy to use, since it is active at pH 4-6, which was the reaction of the extracts, and since it is active at room temperature.

The activity of the juice is not purely polyphenoloxidase; it has a monophenoloxidase property. Fortunately for our purpose the lag time before the monophenoloxidase reached its maximum activity was such that the concentrations of adrenaline used were oxidised by the polyoxidase before the mono-oxidase attacked the 5-hydroxytryptamine. By measurement of oxygen uptake and loss of biological activity, Philpot (1940) showed a conversion of over 95% of adrenaline to adrenochrome by a preparation of 20% pure polyphenol oxidase from mushrooms.

Erspamer and Boretti (1951) sprayed chromatograms with diluted mushroom juice and showed a coloration of spots identified as enteramine (5-hydroxytryptamine), octopamine (p-hydroxyphenylethanol amine), tyrosine and tyramine. These reactions were probably due to the monophenoloxidase, giving oxidation products which polymerised to form coloured compounds.

B. Dog serum - 5-hydroxytryptamine estimates.

The mean serum 5-HT-equivalent for a normal dog was 0.48 µg/ml. This estimate includes five values obtained from serum of the same dog, some as duplicate determinations and all on samples of blood taken within a three month period. They range from 0.39 µg/ml - 0.62 µg/ml. (A single estimate was made in blood from a dog under chloroform anaesthesia. This value was 0.30 µg 5-hydroxytryptamine per ml serum).

All the assays were made on extracts which had been prepared from the serum with 20 volumes of acetone. The acetone was removed under reduced pressure and lipids extracted from the aqueous residue with petroleum ether. The resulting extract was subjected in one case to paper chromatography.

Each estimate was governed by a recovery determination, i.e. pure 5-hydroxytryptamine was added to a corresponding sample of serum and extraction completed. For the acetone extraction, the recovery is 50 - 95%. In the chromatography experiment the recovery was 23 - 33%.

Because/

Because these estimates were made on a single dog they cannot be compared with the mean figure obtained by Erspamer and Faustini (1953) for normal dogs, i.e. 0.21 µg 5-hydroxytryptamine per ml serum. However, the mean estimate obtained in the present work lies within the range of their figures which was 0.08 µg/ml - 0.57 µg/ml and covered ten estimates. Erspamer's extraction method was more prolonged and employed a lower concentration of acetone than that used here. The blood was allowed to stand at room temperature for 3 - 5 hours and then for 16 - 20 hours in the refrigerator, until the clot was contracted and the serum had separated; whereas, here, the blood was not standing at room temperature for more than 20 minutes before the serum was separated by centrifugation. Then Erspamer and Faustini used just four volumes of acetone, a concentration of 80%, whereas here a concentration of 95% was used.

Twarog and Page (1953) made similar acetone extracts of serum to those of Erspamer and their estimates for two samples of dog serum were 0.12 µg 5-hydroxytryptamine/ml and 0.2 - 0.3 µg/ml.

A higher 5-HT-equivalent for dog blood was found by Udenfriend and Weissbach (1954). They made a butanol extract of the platelets only and estimated/

estimated the 5-hydroxytryptamine in it by a fluorescence method. Their value is 0.25 µg 5-hydroxytryptamine/ml whole blood.

5-hydroxytryptamine content of rabbit tissues.

The 5-HT-equivalent for rabbit blood is high, compared with that of other animals, (Erspamer and Faustini, 1953; Erspamer, 1954a or b), thus the blood had to be removed as far as possible from the tissues before sampling. This was done with an isotonic saline and was complete in about 15 - 20 minutes. It may also have washed some 5-hydroxytryptamine out of the tissues themselves, though perhaps this is not of any real significance except in a loosely packed tissue containing a high 5-HT-equivalent, e.g. spleen. Another danger, possibly incurred by this perfusion treatment is that of causing oedema of the tissues in which case the 5-HT-equivalent, determined per gram of sample would be low. There was no evidence of oedema, to the naked eye inspection, except in one case of thyroid and submaxillary glands which were rejected. The lung tissue invariably floated readily in the acetone.

When/

When the perfusate was almost colourless, it contained probably less than 1% of blood. Thus if a 2 gm sample of tissue were to contain 1 ml of this saline perfusate, the "blood-5-hydroxytryptamine" content of this sample would not be more than 0.025 µg: (rabbit blood contains 2.5 µg 5-hydroxytryptamine/ml). Thus tissue 5-HT-equivalents were considered to be quite significant when they were more than 0.100 µg/g.

The estimate of 5-hydroxytryptamine in rabbit blood was 2.0 - 2.5 µg/ml whole blood and 4.4 µg/ml serum, from acetone extracts in each case. The 5-HT-equivalent for rabbit serum given by Erspamer and Faustini (1953) is 3.53 µg/ml. Another result is given by Erspamer and Sala (1954): it is 4.3 µg/ml serum from ten samples. (No explanation is given of this difference). Udenfriend and Weissbach (1954) quote a value of 4.0 µg/ml of whole blood, which is high compared with these other estimates. Their method is a chemical one, involving the fluorimetric estimation of 5-hydroxytryptamine in an extract of the platelets.

From the work of Reid and Rand (1951) and Zucker (1951) it seems quite clearly indicated that 5-hydroxytryptamine occurred within the platelets and/

and was only released into the blood with their normal breakdown in the body or with clotting. The metabolic studies of Udenfriend and Weissbach (1954) using C_{14} labelled tryptophan, confirmed this view. These authors suggested that 5-hydroxytryptamine synthesis occurs at the site of platelet formation, and, having been incorporated in the platelets remains inert within them until they are ruptured and disintegrate. Yet, Humphrey and Toh (1954) showed that the platelets were able to adsorb 5-hydroxytryptamine against a concentration gradient, from the medium in which they were suspended.

Two experiments here showed that there was no synthesis of 5-hydroxytryptamine during in vitro clotting, (p. 51/100). The estimate of 5-hydroxytryptamine per ml whole blood was the same, whether the blood was prevented from clotting by being passed immediately from siliconed vessels into acetone, or was allowed to clot either rapidly or slowly.

Free-flowing rabbit blood was collected through a polythene cannula into a siliconed tube. One ml samples were measured with a siliconed pipette,/

pipette, directly into 20 ml acetone (duplicates A and B), into dry siliconed glass flasks (C and D), and into dry non-siliconed glass flasks (E and F). Extraction of the samples A and B was proceeded with at once, while C, D, E and F were left to clot at room temperature. For some time the samples C and D were quite fluid but after 5 hours they had clotted, whereas E and F clotted within a few minutes of pipetting. These samples were then extracted. Assay of the extracts was made on the atropinised oestrous uterus of the rat and for each sample the 5-HT-equivalent was within the range 1.6-2.4 µg/ml serum.

In the second experiment, one ml samples of blood were measured and some placed directly into 20 ml acetone while others were placed in dry flasks and left to clot. Extraction of the samples in acetone was proceeded with at once, and after one hour the clotted samples were similarly extracted. The 5-HT-equivalents were again in good agreement, (see Table VIII).

A high 5-HT-equivalent was found in the spleen, though the mean figure was lower than that found by Erspamer (see Table XIII). This discrepancy is/

is possibly due to the blood content of the spleens sampled by Erspamer (1954a and b). The contraction of the spleens caused by electrical stimulation and leading to expulsion of perfusion fluid from the sinuses, gave no difference in their 5-hydroxytryptamine content per gram.

TABLE XIII

Comparison of 5-hydroxytryptamine content of rabbit spleen and mucosae.

	Erspamer's estimates (Erspamer 1954a & b)	Author's estimates.
Spleen	19.6 μg/g$_{(5)}$ (16.4 - 22.5)	11.72 μg/g$_{(6)}$ (7.6 - 21)
Mucosae:-		
Stomach	Fundus 4.90 μg/g Pylorus 0.85 μg/g	6.15 μg/g$_{(3)}$
Small intestine, upper.	3.3 μg/g	2.20 μg/g$_{(3)}$
lower.	3.7 μg/g	1.64 μg/g$_{(3)}$
Large intestine	2.7 μg/g	3.82 μg/g$_{(3)}$

The values for 5-hydroxytryptamine in the mucosae of the various areas of the intestinal tract correspond to those of Erspamer (1954a & b). However, his estimates show a decreasing concentration (excluding the pylorus mucosa) from the stomach fundus to the colon, whereas the figures found in the present study represent a high 5-hydroxytryptamine concentration in both the stomach and large intestine/

intestine mucosae, with lesser concentrations in the small intestine mucosa.

Using the atropinised rat colon for assays, Feldberg and Toh (1953) obtained higher 5-HT-equivalents for acetone extracts of rabbit gastric mucosa, viz. Fundus mucosa 7.5 - 10 µg 5-hydroxytryptamine per gram and pylorus mucosa 1.25 µg 5-hydroxytryptamine per gram.

Yet the relatively high 5-hydroxytryptamine content throughout these mucosae samples is consistent with their high content of enterochromaffin (argentaffin) cells.

The bone marrow had a fairly consistent, significant content of 5-hydroxytryptamine but it would be difficult to say whether this was due entirely to that in the platelets of the blood circulating through the vascular sinuses, or whether part of it was due to 5-hydroxytryptamine in the megakaryocytes (already synthesised for the time when these break down to form platelets).

When the liver was perfused with saline via the portal vein as well as via the aorta, the 5-HT-equivalent of the samples was lower than it was if only aortic perfusion was made. The most likely reason for this difference is that in the second

case blood was still trapped in the portal sinuses. A volume of 0.25 ml blood per g in the tissue would contribute 0.5 µg 5-hydroxytryptamine per g tissue. This proportion, 1 in 4, of portal blood to tissue is perhaps rather high, but it may be that though less blood is present, excess of platelets are trapped prior to their destruction by the liver cells, and that these platelets as well as the blood be washed out by the saline perfusion.

The 5-HT-equivalents for the samples of lung tissue varied rather widely. The lungs contain amine-oxidase which destroys 5-hydroxytryptamine and some workers believe that megakaryocytes from which platelets are formed occur there, also. Should these cells contain 5-hydroxytryptamine it may be that the differing balance between these activities of production and destruction caused the various estimates.

Other tissues which contained small but significant amounts of 5-hydroxytryptamine, more than 0.1 µg/g, were the thyroid and the pancreas. It is interesting to note that Erspamer (1937) described enterochromaffin cells in the pancreas of certain mammals, including the hare. The pancreas of the rabbit/

rabbit was not examined by him.

All these 5-HT-equivalents were found from assay of mushroom treated acetone extracts of the tissues on the atropinised oestrous uterus of the rat. On this preparation, the spasm caused by 5-hydroxytryptamine is specifically blocked by lysergic acid diethylamide. After each assay, sufficient lysergic acid diethylamide was added to the organ-bath to abolish the standard response to 5-hydroxytryptamine. In the case of all the tissues mentioned above, blood and serum, spleen, mucosae, bone marrow, liver, lung, thyroid and pancreas, except the 'portal perfused' liver, the extract activity was abolished by lysergic acid diethylamide. Thus in these extracts, at least a very large proportion of the response was probably due to 5-hydroxytryptamine. The tests of the liver samples, positive when the 5-HT-equivalent was high and negative when it was low, suggested the presence of some less active substance in the extracts.

In the extracts of each of the other tissues sampled the activity was neither consistently abolished by lysergic acid diethylamide nor was it consistently resistant to this antagonist. The mean estimate for each of these tissues was never more/

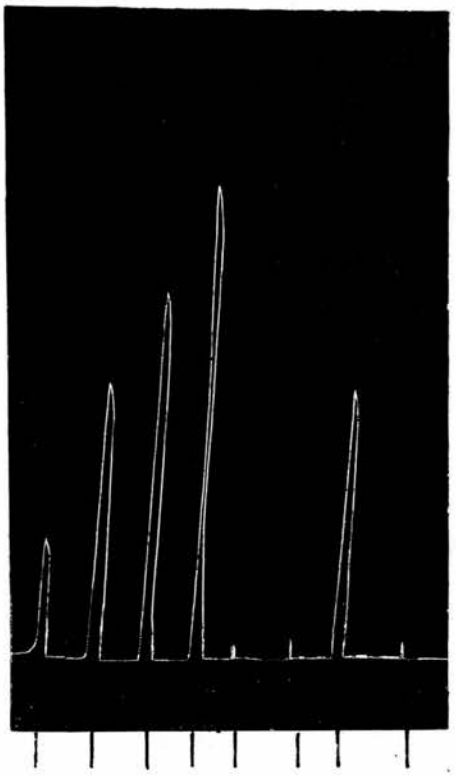
more than 0.1 µg 5-hydroxytryptamine per gram. It may be said that there was a trace of 5-hydroxy-tryptamine in the diaphragm, salivary glands and thymus, but in the heart, bladder, kidney, skeletal muscle, sciatic nerve, and bile, no definite evidence for the presence of any 5-hydroxytryptamine was obtained.

No uterus stimulating activity was recorded from the adrenal extracts, but only an inhibitory effect on subsequent 5-hydroxytryptamine doses. This observation results presumably from an incomplete destruction of the adrenaline content of these glands, even with twice the usual amount of mushroom juice and twice the standard incubation time. The extracts became faintly red in colour probably owing to the relatively high concentration of adrenochrome.

Parallel assays of dog brain samples.

The 5-HT-equivalents for the olfactory bulbs and for the cerebellar cortex from assay on the rat oestrous uterus and on the heart of Mactra solida agreed fairly well. There was a small amount of activity in the olfactory bulbs, (mean of estimates on rat oestrous uterus = 38.7 ng/g., and on/

Figure 12



D. Dog hypothalamus extract
15 mg. tissue
Rat Oestrous Uterus
2 ml. bath

Blockade of 5-hydroxytryptamine responses by lysergic acid diethylamide.

At L.S.D. 2 µg of lysergic acid diethylamide were in contact with the uterus for 10 minutes.

on Mactra heart = 39.3 mg/g) and the activity in the cerebellar cortex was negligible. The other results suggest either varying proportions of 5-hydroxytryptamine and some other principle in the areas sampled, or 5-hydroxytryptamine plus another principle peculiar to each area. These samples were all treated with mushroom juice before assay on the rat oestrous uterus, but they had not been treated with mushroom juice when assayed on the Mactra heart. (This latter preparation is very insensitive to stimulation by adrenaline). The estimates made on the rat oestrous uterus were higher than those found by Amin, Crawford and Gaddum (1954) for similar extracts untreated with mushroom juice.

After treatment of the rat uterus preparation with the specific 5-hydroxytryptamine antagonist, lysergic acid diethylamide, the mushroom treated samples of sympathetic ganglia, hypothalamus and caudate nucleus caused responses, whereas Amin et al (1954) had found that the response to similar samples not treated with mushroom had been abolished.

No specific antagonist for 5-hydroxytryptamine stimulation in the Mactra heart has yet been found, so/

so it is not possible to say whether the stimulation of it caused by the acetone extracts was indeed due entirely to 5-hydroxytryptamine.

Though it was thought unlikely that the samples might contain substance P, parallel assays were made of mushroom treated extracts on the rat oestrous uterus and guinea-pig ileum preparations. From this experiment (p. 104, Table XI), the active principle, other than 5-hydroxytryptamine is shown not to be substance P which, though present in the tissues, is —— as designed in the method of extraction —— left in the insoluble residue during the treatment with 20 volumes of acetone.

A parallel assay of one sample of dog hypothalamus was made on the isolated oestrous uterus of the rat, and on the perfused ear of the rabbit. The extract was prepared as usual with acetone and the lipids were removed, but it was not subjected to mushroom juice treatment. The estimates were in agreement but were much higher than was usual for non-mushroom treated samples. The reason for this it not known.

It might be inferred from the results of the parallel assays on the rat uterus and Mactra heart preparations that a new pharmacologically active principle had been discovered. However, in view of the small number of experiments done and the possibility of there being an artefact caused by this particular mushroom juice preparation, that conclusion cannot yet be drawn.

SUMMARY

Recently Amin, Crawford and Gaddum (1954a and b) developed a method for the extraction and estimation of 5-hydroxytryptamine in samples of brain tissue, which depended for assay on responses evoked in the isolated oestrous uterus of the rat. This preparation is sensitive to inhibition by adrenaline and it may be that there was sufficient adrenaline in certain of the extracts to make the 5-hydroxytryptamine estimate too low. This study aimed to eliminate the adrenaline interference in estimation of various tissues for 5-hydroxytryptamine.

An attempt was made to separate 5-hydroxytryptamine from adrenaline by paper chromatography, elute/

elute the 5-hydroxytryptamine from the appropriate strip, and estimate the activity - now free from any adrenaline - on the oestrous uterus of the rat. However, a solvent giving more than a small R_F difference was not found among those tested. In addition, it was found that in the eluates from chromatography paper 5-hydroxytryptamine was rather unstable, particularly during the evaporation necessary for the concentration of eluates before estimation. If careful control of pH was made at pH 6-7, eluate activities could be stabilised during freeze-drying and samples were thus prepared for estimation - always in parallel with 'recovery' samples to which standard 5-hydroxytryptamine had been added before chromatography.

As a result of this failure with physical methods of separating 5-hydroxytryptamine from adrenaline, attention was turned to an examination of pharmacological methods.

For a time, the assay method was changed to calibration of vasoconstriction in the isolated ear of the rabbit. This is a sensitive preparation to 5-hydroxytryptamine, under certain conditions, provided/

provided the vasoconstrictor effects of adrenaline and noradrenaline can be minimised. This was done by the use of ephedrine and dibenzyline together. The treated preparation was fairly satisfactory, and was used on a number of occasions, but the dibenzyline tended to lower its discrimination to varied doses of 5-hydroxytryptamine.

It was found that by the activity of the polyphenoloxidase enzyme present in mushroom juice, the inhibitory effect of adrenaline on the rat uterus could be eliminated. It thus became possible to return to the more regular and sensitive rat oestrous uterus preparation for assays. All the tissue extracts were treated with a standard preparation of crude mushroom juice previous to 5-hydroxytryptamine estimation.

A survey of the 5-hydroxytryptamine content of acetone extracts of rabbit tissues was made. Since rabbit blood has a relatively high 5-hydroxy-tryptamine equivalent, efforts were made to wash the blood from the animal by saline perfusion before the tissues were sampled.

Significant/

Significant amounts of 5-hydroxytryptamine were found in the spleen (11.7 μg/g); various areas of gut mucosa (1.6-6.15 μg/g); blood (2.0-2.5 μg/ml) and serum (4.4μg/ml) as previously determined by Erspamer (1954a and b). The hypothalamus, the liver and the bone marrow showed smaller (0.3-0.9 μg/g) but still significant amounts of 5-hydroxytryptamine. In the liver, possibly part of the estimate is due to 5-hydroxytryptamine from the blood content of the tissue at the time of extraction; marrow, too, contains circulating blood which it would be almost impossible to wash out.

Other tissues contained little 5-hydroxytryptamine (0.05-0.2 μg/g), for example, lung, thyroid, pancreas and diaphragm; while in yet others 5-hydroxytryptamine was not detectable, skeletal muscle, bile and adrenals (< 0.03 μg/g).

The only test of specificity of the extract activity was by its influence on the lysergic acid diethylamide treated uterus. Lysergic acid diethylamide is a specific antagonist of 5-hydroxytryptamine contractions in the oestrous uterus of the rat.

The/

The test was positive (i.e. lysergic acid diethylamide abolished the extract response) for extracts of spleen, gut mucosa, blood, serum, bone marrow, hypothalamus, lung, thyroid, pancreas, diaphragm, salivary glands and thymus. It was negative for midbrain, olfactory bulbs, cerebellum, and the tissues containing the equivalent of less than 0.04 µg 5-hydroxytryptamine per gram.

The 5-hydroxytryptamine equivalent of the serum of a dog was estimated by various methods including paper chromatography with freeze-drying of the eluates, and parallel assays on the rat uterus and rabbit ear preparations. Estimates ranged between 0.39 µg and 0.62 µg. 5-hydroxy-tryptamine with the mean at 0.48 µg/ml serum.

Certain areas of dog brain and dog sympathetic ganglia were extracted with acetone, and the extracts estimated directly on the Mactra heart, while aliquots were treated with mushroom juice and estimated on the isolated oestrous uterus of the rat. The assay results for the sympathetic ganglia, the hypothalamus and the caudate nucleus did not agree and the discrepancies suggested that the extracts contained some pharmacologically active principle other/

other than 5-hydroxytryptamine. It was shown too that these particular extracts were still stimulatory to the uterus after 5-hydroxytryptamine responses had been abolished by lysergic acid diethylamide. However, sufficient experiments have not been done for there to be confidence that another pharmacologically active principle is here revealed, and not just some artefact arising perhaps from the procedure or mushroom treatment.

PART II

5-hydroxytryptamine antagonism measured on the isolated guinea-pig ileum.

These studies on the effects of antagonists on the responses induced by 5-hydroxytryptamine in the isolated guinea-pig ileum, were undertaken as an investigation of the possible role of 5-hydroxytryptamine in the animal body.

5-hydroxytryptamine itself, or a compound very closely related to it, is present in a wide variety of animal tissues. Also, the recent work of Udenfriend and Weissbach (1954), and of Erspamer, (1955) on the metabolism of 5-hydroxytryptamine showed that the whole body content of this substance was completely replaced in 2 - 5 days. Erspamer, (1954) believes that the 5-hydroxytryptamine is synthesised by the enterochromaffin cells, that it is a hormone concerned with the control of diuresis, and that its presence in the platelets is merely as a/

a convenient mode of transport. He emphasises the 'physiological' nature of the action of 5-hydroxytryptamine on renal haemodynamics and function. Udenfriend and Weissbach (1954), however, believe that 5-hydroxytryptamine is incorporated in the platelets at the site of their formation, and though inert within them is liberated wherever platelets are destroyed.

The presence of 5-hydroxytryptamine in nervous tissue (Amin, Crawford and Gaddum, 1954) and its various spasmogenic, vasoconstrictive and cardiac activities, suggest that it may have some other function in the control of the normal working of the animal body. Thus these studies were done with certain antagonists in a search for effects peculiar to 5-hydroxytryptamine-induced spasm of the isolated guinea-pig ileum. This is a smooth muscle organ containing a plexus of parasympathetic ganglion cells.

Certain antagonists were given for a short period only - 1-10 minutes - and their immediate inhibitory effect on 5-hydroxytryptamine and a 'control' drug, either nicotine, acetylcholine or histamine, was recorded. The recovery of the responses/

responses from the inhibition was also recorded.

A quantitative study of atropine and certain atropine-like compounds was made. The pA_{10} index was used as a measure of inhibitory effect on spasm of the isolated guinea-pig ileum. This index was determined by the method of Schild and is defined as the negative logarithm of the molar concentration of the antagonist in the presence of which a 10-fold dose of spasmogenic drug causes a response equal to that elicited by a single dose of the drug, in the absence of the antagonist (Schild, 1947).

A. Experimental Method. Isolated guinea-pig ileum.

Small guinea-pigs (~150 g) were fasted overnight and killed by a blow on the head. Portions of the ileum (1.5 - 2 cm long) were taken from nearest to the caecum and were suspended in Tyrode solution at 36 - 37°C. These were found to be sensitive to 5 - 40 ng. 5-hydroxytryptamine (2 ml bath), and showed satisfactory, steady responses, which were recorded with a light frontal writing lever having a magnification of 10 - 15X.

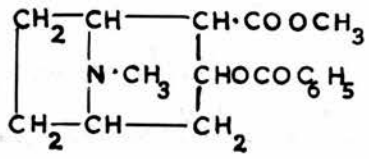
Some/

Some antagonists were tested for their immediate short-term effects on 5-hydroxytryptamine responses of the ileum, compared with their effects on acetylcholine, nicotine or histamine responses. Once steady responses were obtained to a suitable dose of 5-hydroxytryptamine and one (or more) of the control drugs, an antagonist dose was given immediately after a drug dose had been washed out. It was left in either a) until the next drug dose had been given and both drug and antagonist were washed out together, (e.g. cocaine and 6-amino-tetrahydrocarbazole) or, b) for 2 - 10 minutes after which it was removed and the dosage regimen resumed in the presence of Tyrode solution, (e.g. dihydro-ergotamine). Yohimbine itself caused a contraction and was washed out after 60 - 90 seconds contact with the tissue.

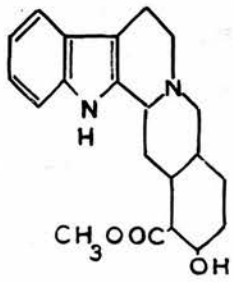
By adding the antagonist dose on one occasion immediately before a 5-hydroxytryptamine dose, and after full recovery of the responses, repeating that antagonist dose before a control dose, the pattern of inhibition and recovery of 5-hydroxytryptamine and control responses could be clearly/

Figure 13

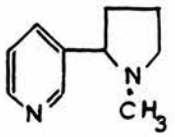
COCAINE

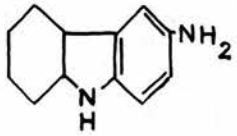


YOHIMBINE

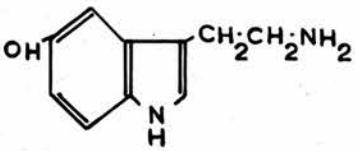


NICOTINE

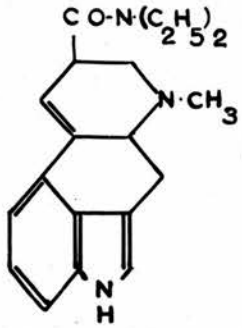




6-AMINO TETRAHYDRO-CARBAZOLE



5-HYDROXYTRYPTAMINE



LYSERGIC ACID DIETHYLAMIDE

Chemical formulae.

clearly seen. The antagonist dose was then increased and the experiment repeated.

The effect of a large dose of 5-hydroxytryptamine and of nicotine was also investigated. In these experiments the 'antagonist' was given either as one large dose or by continuous perfusion.

The antagonistic drugs studied, and their effects as recorded here.

Cocaine.

On the isolated guinea-pig ileum, cocaine causes a reversible inhibition of the spasm induced by 5-hydroxytryptamine, without having much effect on histamine or acetylcholine responses, (Sinha and West, 1953; Robertson, 1953; Rocha e Silva, Valli and Picarelli, 1953; Gaddum and Hameed, 1954). This was explained by the various workers on the theory that 5-hydroxytryptamine acts through the nerves of the intestinal preparation and that cocaine prevents its action by paralysing these nerves.

In the present work, low doses of cocaine were/

were shown to potentiate the 5-hydroxytryptamine induced contractions of the isolated guinea-pig ileum. In higher doses (5×10^{-5}), cocaine caused some slight inhibition of the responses, while with a cocaine concentration of 10^{-4}, the effects of both 5-hydroxytryptamine and nicotine were reduced to a similar extent, but the responses to histamine were unaffected.

Yohimbine.

The responses of the isolated rat uterus to 5-hydroxytryptamine and to tryptamine were depressed by yohimbine (5×10^{-6}). This antagonism was not very specific for the response to acetylcholine was also depressed, though to a lesser degree (Gaddum and Hameed, 1954). Erspamer (1952) reported that yohimbine (2×10^{-6}) caused very little change in the response of the rat uterus to 5-hydroxytryptamine.

The antagonistic action of yohimbine on 5-hydroxytryptamine stimulation of isolated strips of carotid artery is marked, reversible and surmountable, (Reid and Rand, 1952). No indication is given of the specificity of this action.

Page and McCubbin (1953b) recorded a reversal of the 5-hydroxytryptamine pressor action in dogs. This was particularly well displayed when the pressor effect had been potentiated by a ganglion blockade caused by hexamethonium.

The guinea-pig ileum is stimulated directly by yohimbine. In the experiments reported here, when the contraction was complete and relaxation had begun, the yohimbine was washed out of the organ-bath (60 - 90 seconds contact). The maximum inhibitory effect of a dose of yohimbine, on other responses in the ileum occurred about 12 minutes from the beginning of the yohimbine contraction. This inhibition was possibly slightly more effective against nicotine or acetylcholine. Yohimbine was tested in concentrations of 10^{-6} and 10^{-5}.

6-amino 1:2:3:4-tetrahydrocarbazole.

In 1953, the studies of Woolley and Shaw on antimetabolites of 5-hydroxytryptamine, included the investigation of the action of 6 amino-tetrahydrocarbazole. It was among the more active members of a series of structural intermediates between 5-hydroxytryptamine and yohimbine. It/

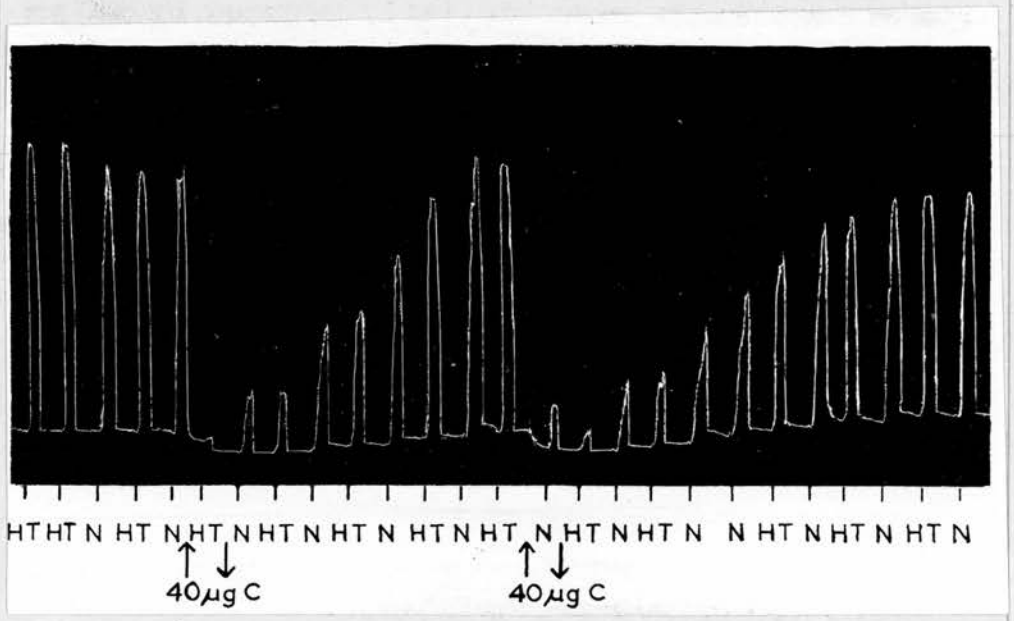
It prevented the 5-hydroxytryptamine induced contraction in a concentration similar to that required for 2:3 dimethyl 5:amino indole. The inhibitory effect was surmounted by higher doses of 5-hydroxytryptamine. (Woolley and Shaw, 1953; Shaw and Woolley, 1953).

On the isolated oestrous uterus of the rat, 6-amino tetrahydrocarbazole exerted a feeble, though specific, antagonistic effect on spasm caused by 5-hydroxytryptamine. This antagonist reached its maximum effect in 10 minutes and its inhibitory action could easily be removed by washing, (Hameed, 1954). It was noted that the '6' position in the carbazole compound corresponds to the '5' position in the tryptamines.

A sample of 6-amino tetrahydrocarbazole was supplied through the courtesy of Glaxo Laboratories. It was reported by Mr. Tomish, working at Glaxo, to be more active in the inhibition of 5-hydroxy-tryptamine-induced spasm, than of acetylcholine spasm in the isolated guinea-pig ileum.

This effect was shown here but the discrimination of the antagonist between the two drugs seems/

Figure 14



Guinea-pig ileum in Tyrode at 37°C. 2 ml bath

HT - Response to 20 ng hydroxytryptamine.

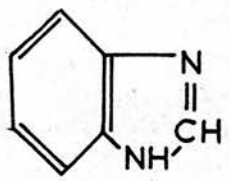
N - Response to 1000 ng nicotine.

Dose interval - 3 minutes.

40 µg C - 40 µg 6-amino tetrahydrocarbazole added at ↑ and washed out at ↓.

seems very small. Also, the activity of 6-amino-tetrahydrocarbazole was only one tenth that reported by Tomish. Matching responses to nicotine and 5-hydroxytryptamine were similarly reduced by this antagonist (2×10^{-5}) and recovered in parallel after its removal.

Benzimidazole.



o-phenylene-formamidine.

Benzimidazole was first studied pharmacologically by Goodman, Gilman and Hart (1943), who showed that it produced a reversible flaccid paralysis. This effect was obtained orally and by injection and was evident in decreased muscle tone, decreased voluntary movement and a loss of postural reflexes. There was no alteration in the spontaneous electrical activity of the cortex so the conclusion of these workers was that benzimidazole has a highly selective/

selective depressant action on the cerebro-spinal axis. Other experimentors showed that this paralysis was not due to a curare-like action but to a selective depressive effect on spinal interneurones. (Domino, Unna and Kerwin, 1952; Funderburk, King, Domino and Unna, 1953).

A slight effect on 5-hydroxytryptamine responses of the isolated guinea-pig ileum was recorded here with a high concentration (1.6×10^{-4}) of benzimidazole, though there was no effect on histamine-induced contractions.

Dihydroergotamine.

On the guinea-pig ileum, the antagonism between dihydroergotamine and 5-hydroxytryptamine is weak and not clearly specific. When concentrations of 2.5×10^{-6} — 2.5×10^{-5} were used, the response to 5-hydroxytryptamine was depressed, but the response to histamine was also depressed, sometimes more than that to 5-hydroxytryptamine. (Gaddum and Hameed, 1954).

Similar effects were recorded here. A single dose of 5 µg dihydroergotamine (bath concentration = 2.5×10^{-6}) was left in contact with the isolated guinea-/

guinea-pig ileum preparation for 5 - 10 minutes. The responses to 5-hydroxytryptamine were depressed and recovered slowly; while those to histamine were less depressed and recovered rapidly.

Nicotine.

Rocha e Silva et al (1953) have studied the antagonistic effect of high doses of nicotine on the responses of the guinea-pig ileum to nicotine, 5-hydroxytryptamine, acetylcholine, histamine and bradykinin. They showed that "moderate paralysing doses of nicotine", (i.e. 3 - 13 x 10^{-6} nicotine base in the bathing fluid) abolished the responses to 5-hydroxytryptamine and depressed those to the other drugs studied. If larger doses of nicotine (26 x 10^{-6}) were given, the muscle "escaped" from the inhibitory effect and responded again to 5-hydroxytryptamine as well as to the other drugs. In both these cases, responses to the routine dose of nicotine (0.6 x 10^{-6}) were completely abolished.

It was shown here that, in a concentration of 10^{-4}, nicotine caused a large contraction of the isolated guinea-pig ileum with spontaneous relaxation;/

relaxation; after this, responses to small doses of nicotine were blocked while responses to 5-hydroxytryptamine, acetylcholine and histamine were all reduced. A single dose of nicotine, which gave a bath-concentration of 10^{-5} caused a block of nicotine responses and a marked reduction of the 5-hydroxytryptamine responses. Increased single doses, from 2.5×10^{-5} — 10^{-7} caused a complete block of the effect of 5-hydroxytryptamine. The tissue was still responsive to these large single doses of nicotine (see discussion, p. 165).

5-hydroxytryptamine.

It is possible to induce a specific desensitisation of the guinea-pig ileum to 5-hydroxytryptamine, by exposure of the tissue to high concentrations of this drug. Gaddum, (1953), showed that this desensitisation prevented also, responses to tryptamine, and that, following a large dose of tryptamine, responses to both it and 5-hydroxytryptamine were abolished, but not those to substance P.

After doses of up to 6×10^{-6} 5-hydroxytryptamine, Rocha e Silva et al (1953) showed that responses/

responses to this drug were blocked whereas those to nicotine, acetylcholine, histamine and pilocarpine were unaffected. This block developed quickly and was easily reversible.

In the present experiments, a block of 5-hydroxytryptamine responses in the guinea-pig ileum was obtained with a continuous concentration of 10^{-6} 5-hydroxytryptamine in the bathing fluid. Responses to nicotine, acetylcholine and histamine were only slightly reduced. This confirms some of the results of Rocha et al. (1953).

B. Atropine and the atropine-like compounds.

Atropine has an anti-5-hydroxytryptamine action on the spasm caused by this drug in the isolated guinea-pig ileum. It was thought that the nature of this inhibitory action might be better understood if quantitative studies were made of the antagonism between atropine and 5-hydroxytryptamine, and/

and also between atropine and acetylcholine, and atropine and histamine. These comparisons were made, together with similar experiments where an atropine-like compound was used in place of atropine. In each case, the pA_{10} at equilibrium of each drug-antagonist pair was found.

The atropine-like compounds were four synthetic drugs supplied through the courtesy of Dr. A.C. White of the Wellcome Research Laboratories. They had been shown to be similar to atropine by various spasmolysis and mydriasis tests. Some notes are given later of the activities of each, (see p.148-149).

Atropine is known to antagonise 5-hydroxytryptamine responses on the guinea-pig ileum when it is used in larger doses than those which antagonise acetylcholine. This has been explained on the theory that 5-hydroxytryptamine acts on nerve cells and that atropine antagonises its effect by blocking the acetylcholine liberated by these cells. An alternative theory is that atropine acts on both acetylcholine and 5-hydroxytryptamine-receptors. If this were so, some other drug might have a more specific/

specific action on acetylcholine-receptors than atropine itself, and it was in the hope of finding such a drug that these experiments were done.

Atropine.

In the isolated guinea-pig ileum, concentrations of atropine which are just sufficient to abolish responses caused by acetylcholine and carbachol, do not abolish responses due to 5-hydroxytryptamine. But these responses to 5-hydroxytryptamine may be reduced; and they are abolished by higher concentrations of atropine. Responses induced by histamine are less affected. Various workers have described these effects, (Rapport and Koelle, 1953; Rocha e Silva et al. 1953; Gaddum and Hameed, 1954).

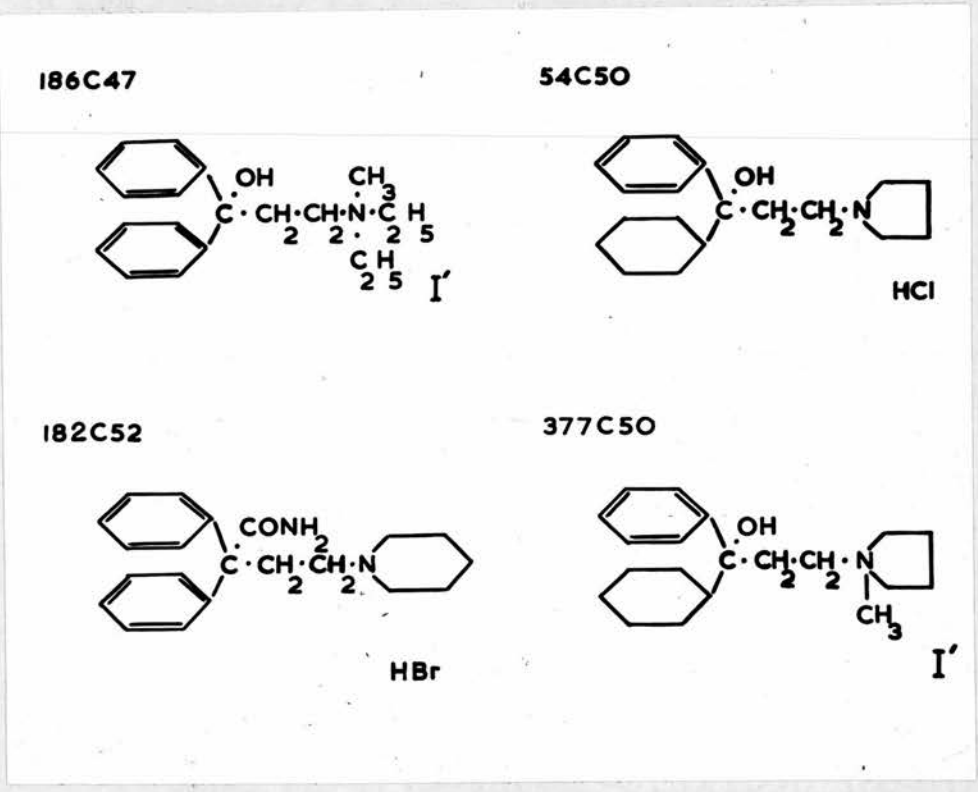
The effect of atropine on responses of this tissue is thus to inhibit those due to 5-hydroxytryptamine less than those due to choline esters and more than those due to histamine. Nicotine-induced spasm is inhibited by atropine to about the same extent as that of 5-hydroxytryptamine, (Hameed, 1954).

Cambridge/

Cambridge and Holgate (1955) estimated the effect of atropine on the responses to these three drugs. They used the technique of superfusion, (Gaddum, 1953) for a portion of guinea-pig ileum which was stimulated regularly by the same concentration of 5-hydroxytryptamine. As the concentration of atropine in the superfusion fluid was increased from 10^{-9} - 10^{-8} the response of the ileum was progressively reduced to 50 - 70% of its original height. Increase of atropine to 10^{-6} did not reduce this response further. Beyond a concentration of 10^{-6}, increase in atropine concentration again caused decrease in the 5-hydroxytryptamine response. The first portion of this atropine concentration-effect curve followed that of atropine/acetylcholine, then after the plateau the curve lay beside that of atropine/ histamine. These authors suggested that 5-hydroxytryptamine has two types of action, one on the nerve net and one more directly on the muscle. The former would be more easily blocked by atropine and the latter would only be blocked by much higher concentrations of atropine.

Atropine-like/

Figure 15



Chemical formulae of certain compounds having atropine-like activities.

Atropine-like compounds.

186C47 3.3 diphenyl propan-3-ol diethylamine methiodide. This is Compound 22, of White, Green and Hudson, (1951).

Comparisons of 186C47 with atropine showed that both had very similar activities with reference to carbachol, pilocarpine and histamine. 186C47 was 0.7 - 1.0 times as active as atropine in tests on the same strip of isolated rabbit ileum, stimulated by carbachol or pilocarpine. The two compounds were almost equivactive against the spasm of isolated guinea-pig ileum produced by histamine. In various other tests, including one on mydriasis and another on histamine-induced asthma the activity of 186C47 was found to be not less than half the activity of atropine, (White, 1953).

54C50 3-pyrrolidino-1-phenyl-1-cyclohexyl propan-1-ol hydrochloride. Kemadrin (Green, 1952).

54C50 was about 0.14 times as active as atropine in the abolition of acetylcholine spasm in isolated guinea-pig ileum. It was active also against/

against carbachol in the guinea-pig ileum and against both these spasmogenic drugs on rabbit ileum. In higher concentrations, 54C50 could abolish contractions due to histamine and to barium. In mydriasis and salivation tests the activity of 54C50 was about 0.04 that of atropine and the effect was of shorter duration. (White, 1953).

377C50 3-pyrrolidino-1-phenyl-1-cyclohexyl propan-1-ol methiodide. Methiodide of Kemadrin.

The methiodide, 377C50, had a somewhat greater atropine-like activity than 54C50. It had about 0.5 the potency of atropine on acetylcholine spasm of isolated guinea-pig ileum. It was about 0.5 as active as atropine also, on a mydriasis test in mice. (White, 1953).

182C52 1,1 diphenyl 3-piperidino propane-1-carbonamide hydrobromide.

The atropine-like activity of 182C52 and of the base from which it is derived, were equal, on a molar basis in a mouse mydriasis test. The base itself was more fully investigated by White and he stated/

stated that it was about 1.5 times as active as atropine in the antagonism of acetylcholine-induced spasm of isolated guinea-pig ileum. It was, however, about 0.7 times as active as atropine in the mouse mydriasis test (White, 1953).

Other investigations on the base were made by Schaumann (1951); it is his compound 9980. He stated that it was about 0.16 times as effective as atropine in the reduction of histamine responses of the isolated guinea-pig colon. But in a salivation test in kittens it was twice as potent as atropine.

Experimental method.

The organ-bath was closely connected by a two-way tap, to one reservoir containing Tyrode solution and to another containing Tyrode solution plus a particular concentration of antagonist. A change to permit contact of the tissue and the antagonist solution could thus be made without interruption of the dosage regimen. It was usually done during the washing out of a drug dose.

In the estimation of the effects of atropine and the atropine-like compounds, regular responses of/

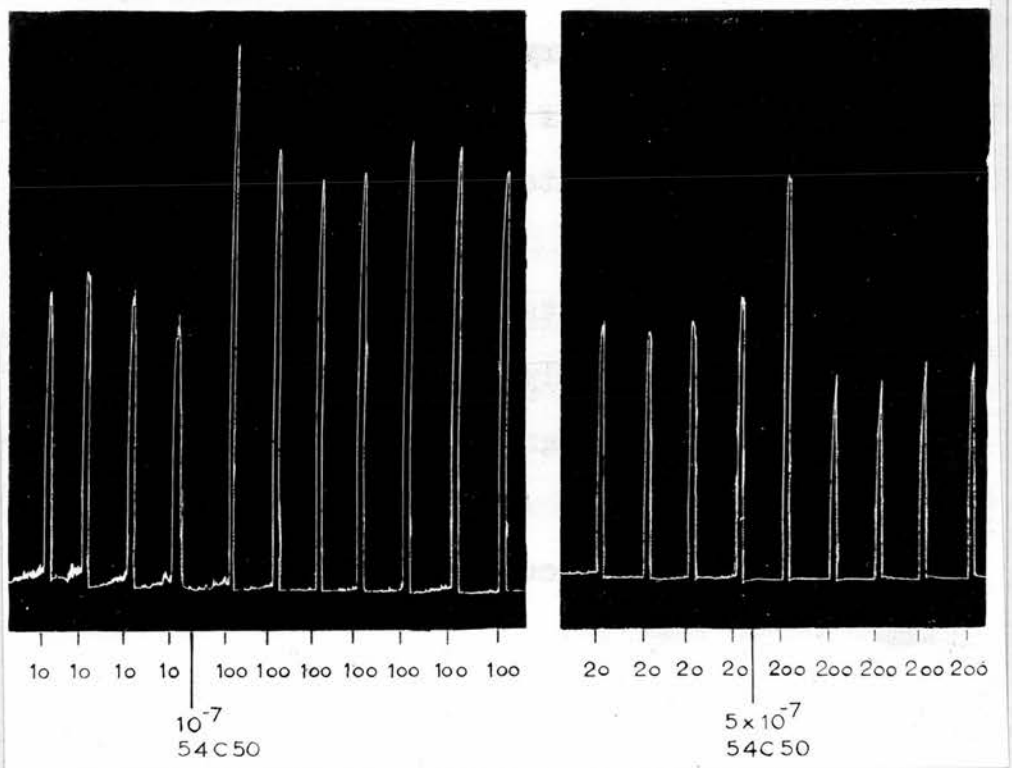
of the isolated guinea-pig ileum to 5-hydroxy-tryptamine (or acetylcholine or histamine) were first obtained in the presence of Tyrode solution. Then this solution was replaced by Tyrode containing antagonist. The drug dose was increased by ten times and given until the response was again steady, (16 - 20 minutes). Sometimes the antagonist - Tyrode solution was removed and the recovery of the responses to the single dose of 5-hydroxy-tryptamine in Tyrode solution was recorded.

The index, pA_{10}, is different from that of Schild (1947), only in that Schild expressed all responses in terms of the maximum response obtained in the absence of antagonist, whereas here responses are measured in terms of the steady submaximal response to the single dose of drug.(Reuse, 1947). Also, Schild's index was always qualified by the length of contact of the antagonist and the tissue, but here dosage was continued until the response reached a steady level (equilibrium response).

The/

Figure 16

Guinea-pig ileum in Tyrode at 37°C. 2 ml bath.



First tracing:

Responses to 10 ng hydroxytryptamine; from ↑, 10^{-7} 54C50 present in Tyrode until the end of the experiment and doses of hydroxytryptamine increased to 100 ng.

Second tracing:

Fresh portion of ileum in Tyrode. Responses to 20 ng hydroxytryptamine; from ↑, 5×10^{-7} 54C50 present in Tyrode and doses of hydroxytryptamine increased to 200 ng.

The effect of atropine and the atropine-like compounds on 5-hydroxytryptamine responses was compared in a short series of experiments with their effect on nicotine responses. Doses of nicotine and 5-hydroxytryptamine which caused similar steady responses (3 minute interval) were found and then given alternately throughout the rest of the experiment. A particular concentration of one of the antagonists was introduced, and allowed to remain continuously during dosing until steady responses were again obtained (8 - 20 minutes). The antagonist solution was then removed and doses were given until the recovery from inhibition appeared to be complete.

Results of estimations on atropine and the atropine-like compounds.

For each of the antagonists the pA_{10} was found for 5-hydroxytryptamine, as well as for acetylcholine and histamine.

The equilibrium response to 10 times the standard dose, in the presence of a given concentration of antagonist, was compared with the response/

response to a single dose, in the absence of antagonist. All the estimates were made on portions of the isolated guinea-pig ileum taken within 10 cm of the caecum.

The results are tabled for each drug-antagonist pair, showing the negative logarithm of the molar concentration of the antagonist and the equilibrium response to 10 times the dose of the drug. The best line fit was calculated from the results by the use of the formula for the simple regression line.

That is:-

$$y - \bar{y} = b\,(x - \bar{x})$$

$\bar{y}$ = mean of y values.
$\bar{x}$ = mean of x values.

$$\text{where } b = \frac{S\left[y\,(x - \bar{x})\right]}{S\,(x - \bar{x})^2}$$

$$= \frac{\text{Sum of the products of y and the differences of x from their mean}}{\text{Sum of the squares of the differences of x from their mean.}}$$

The point at which this line has y = 100 is the pA_{10}. Or, on the graph, the point at which this line crossed the line corresponding to 100% is the pA_{10}. This is the negative logarithm of the molar concentration of the antagonist, in the presence of which, 10 times the dose of drug is required, to cause/

Effect/

cause a response equal to that given by the single dose in the absence of antagonist.

For example, for 5-hydroxytryptamine and atropine, to find best line fit -

$y = \bar{y} + b(x - \bar{x})$

x = neg. log. molar concentration antagonist.

y = response.

x	$x - \bar{x}$	y
6.83	+0.767	142
6.53	+0.467	129
6.13	+0.067	109
5.83	-0.233	81
5.83	-0.233	60
5.23	-0.833	38
36.38		559

$\bar{x} = 6.063$ $\qquad \bar{y} = 93.17$

$S(x - \bar{x})^2 = 1.6133$ $\qquad S\left[y(x - \bar{x})\right] = 111.95$

$$b = \frac{S\left[y(x - \bar{x})\right]}{S(x - \bar{x})^2} = 69.4$$

$$y = 93.17 + 69.4x - (69.4 \times 6.063)$$

for pA_{10} $\quad y = 100 = 93.17 + 69.4x - 420.77$

$$x = 6.310 = pA_{10}$$

Two points on the best line were found by substitution of values of x and the line was placed on the graph.

Effect of atropine.

Under the experimental conditions which were used here, fairly high concentrations ($> 10^{-7}$) of atropine were necessary to cause inhibition of the responses of the isolated guinea-pig ileum to the 10-fold dose of 5-hydroxytryptamine. In fact, the atropine concentration corresponding to pA_{10} for 5-hydroxytryptamine was more than 100 times greater than that for acetylcholine. For histamine, the atropine concentration was more than 10 times greater than that for 5-hydroxytryptamine.

Where an attempt was made to measure the inhibitory effect of a concentration of atropine less than 10^{-7}, on the responses to 5-hydroxy-tryptamine, instead of the expected $> 100\%$ response to the 10-fold dose there was a rapid progressive decrease in response. In one experiment where dosage was continued for 40 minutes after the introduction of the antagonist, the responses recovered somewhat and became steady at 60%. This may indicate that the lower concentrations of atropine take some time to reach their full effect and that meanwhile, the uninhibited large doses of 5-hydroxytryptamine cause a degree of tachyphylaxis. (see Discussion).

In/

In a single experiment, where responses to 5-hydroxytryptamine and nicotine were matched, atropine (10^{-6}) was introduced into the Tyrode. The responses to both nicotine and 5-hydroxytryptamine were similarly reduced and recovered in parallel on removal of the atropine.

Atropine

Acetylcholine - equilibrium response to ten times standard dose, expressed as percentage of standard response.

Atropine concentration.	Neg. log. molar concentration of atropine.	Equilibrium response. %
10^{-9}	8.83	143
10^{-9}	8.83	120
2.5×10^{-9}	8.43	75
5×10^{-9}	8.13	106
5×10^{-9}	8.13	50

5-hydroxytryptamine - as above./

5-hydroxytryptamine - equilibrium response to ten times standard dose, expressed as percentage of standard response.

Atropine concentration.	Neg. log. molar concentration of atropine.	Equilibrium response %
10^{-7}	6.83	142
2×10^{-7}	6.53	129
5×10^{-7}	6.13	109
10^{-6}	5.83	81
10^{-6}	5.83	60
4×10^{-6}	5.23	38

Histamine - equilibrium response to ten times standard dose, expressed as percentage of standard response.

Atropine concentration	Neg. log. molar concentration of atropine.	Equilibrium response %
4×10^{-6}	5.23	130
4×10^{-5}	4.23	45

5-hydroxytryptamine - equilibrium response to 10 times standard dose, expressed as percentage of standard response.

186C47 concn	Neg. log. molar concn.	Result %	54C50 concn	Neg. log. molar concn.	Result %
2×10^{-9}	8.34	143	5×10^{-9}	7.81	160
5×10^{-9}	7.94	108	2×10^{-8}	7.21	130
5×10^{-9}	7.94	100	5×10^{-8}	6.81	115
10^{-8}	7.64	120	10^{-7}	6.51	152
2×10^{-8}	7.34	104	10^{-7}	6.51	125
2×10^{-8}	7.34	76	10^{-7}	6.51	94
10^{-7}	6.64	100	10^{-7}	6.51	80
10^{-7}	6.64	100	2×10^{-7}	6.21	86
10^{-7}	6.64	80	5×10^{-7}	5.81	136
10^{-7}	6.64	42	5×10^{-7}	5.81	90
			10^{-6}	5.51	100

377C50/

5-hydroxytryptamine - equilibrium response to 10 times standard dose, expressed as percentage of standard response.

377C50 conen.	Neg. log. molar conen.	Result %	182C52 conen	Neg. log. molar conen.	Result %
5×10^{-9}	7.93	178	5×10^{-9}	7.90	160
10^{-8}	7.63	143	10^{-8}	7.60	110
2×10^{-8}	7.33	133	2×10^{-8}	7.30	97
2×10^{-8}	7.33	70	2×10^{-8}	7.30	82
2.5×10^{-8}	7.23	117	5×10^{-8}	6.90	70
5×10^{-8}	6.93	84	10^{-7}	6.60	90
10^{-7}	6.63	10	10^{-7}	6.60	42
10^{-7}	6.63	44	2×10^{-7}	6.30	32

Acetylcholine - equilibrium response to 10 times standard dose, expressed as percentage of standard response.

186C47 concn.	Neg. log. molar concn.	Result %	54C50 concn.	Neg. log. molar concn.	Result %
2×10^{-9}	8.34	170	10^{-8}	7.51	150
6.6×10^{-9}	7.82	90	2×10^{-8}	7.21	150
2×10^{-8}	7.34	26	5×10^{-8}	6.81	95
			10^{-7}	6.51	75

377C50 concn.	Neg. log. molar concn.	Result %	182C52	Neg. log. molar concn.	Result %
10^{-9}	8.63	128	2×10^{-10}	9.30	200
5×10^{-9}	7.93	107	5×10^{-10}	8.90	126
2×10^{-8}	7.33	92	2×10^{-9}	8.30	46
10^{-7}	6.63	30	5×10^{-9}	7.90	32

Histamine/

Histamine - equilibrium response to 10 times standard dose, expressed as percentage of standard response.

186C47 conen.	Neg. log. molar conen.	Result %	54C50 conen.	Neg. log. molar conen.	Result %
10^{-6}	5.64	150	10^{-6}	5.51	125
5×10^{-6}	4.94	117	5×10^{-6}	4.81	62
10^{-5}	4.64	10	2×10^{-5}	4.21	10

377C50 conen.	Neg. log. molar conen.	Result %	182C52 conen.	Neg. log. molar conen.	Result %
			5×10^{-7}	5.90	200
10^{-6}	5.63	160	4×10^{-6}	5.00	200
10^{-5}	4.63	150	5×10^{-5}	3.90	Spontaneous contractions.

Results of calculations of pA_{10} values.

$y = \bar{y} + b(x - \bar{x})$; where $y = 100$, $x = pA_{10}$

Antagonist / Drug	$y = 100 = \bar{y} + bx - b\bar{x}$	pA_{10}
Atropine		
5-hydroxytryptamine	93.17 + 69.4x - 420.77	6.310
acetylcholine	98.4 + 90.57x - 767.13	8.590
histamine	87.5 + 85.0x - 402.0	4.876
186C47		
5-hydroxytryptamine	96.7 + 28.56x - 208.77	7.428
acetylcholine	95.33 + 144.40x - 1131.1	7.868
histamine	92.3 + 125.22x - 634.86	4.972
54C50		
5-hydroxytryptamine	115.3 + 23.16x - 149.85	5.809
acetylcholine	117.5 + 83.62x - 586.18	6.801
histamine	65.66 + 88.55x - 428.85	5.22
377C50		
5-hydroxytryptamine	97.0 + 110.92x - 798.5	7.25
acetylcholine	89.25 + 49.02x - 358.76	7.856
histamine		<4.63
182C52		
5-hydroxytryptamine	85.375+ 65.70x - 464.0	7.283
acetylcholine	101.0 + 122.07x - 1049.8	8.612
histamine		<5.0

Effects of the atropine-like compounds.

All the four atropine-like compounds were more active in the antagonism of acetylcholine responses than in the antagonism of 5-hydroxytryptamine responses. Their activity against histamine was much less.

Where the effect of these atropine-like compounds on responses to 5-hydroxytryptamine was compared with their effect on responses to nicotine, these were found to be very similar. Matching responses to nicotine and 5-hydroxytryptamine were similarly reduced by a given concentration of one of these compounds, and, on removal of the antagonist, the responses to both drugs recovered in parallel.

Suppose the "dose-ratio" is a measure of the increase in the dose of agonist which is required to produce, in the presence of the antagonist, a response equal to that produced by a certain dose of agonist in the absence of antagonist. Then in the presence of 186C47 (10^{-7}) the dose ratio for nicotine or 5-hydroxytryptamine was 10, while that for acetylcholine was 50 and that for histamine was 1.

The/

The pA_{10}'s of the two quaternary compounds, 186C47 and 377C50, for 5-hydroxytryptamine are almost the same. These values are only a little less than the pA_{10}'s of these compounds for acetylcholine. The other two antagonists, 54C50 and 182C52, both show a greater difference between the pA_{10} for acetylcholine and that for 5-hydroxytryptamine, - but whereas with 54C50 there is a very wide scatter of the estimates of effect, with 182C52 the estimates lie close to the calculated lines.

For 186C47 and 54C50 the concentration/effect curves for histamine are shown and the pA_{10}'s were calculated. No antagonism was recorded for histamine responses by 377C50 in concentrations less than $10^{-4.6}$ molar. 182C52 (10^{-4} molar), itself, caused spontaneous contractions of the guinea-pig ileum and this made estimation of the pA_{10} impossible.

In most cases, any small spontaneous contractions occurring while Tyrode solution was the bathing fluid, were reduced or eliminated by the antagonist solution.

Discussion.

A. The antagonistic effect of certain drugs on 5-hydroxytryptamine responses in the isolated guinea-pig ileum.

The striking similarity between the actions of 5-hydroxytryptamine and nicotine has been confirmed by this study of antagonistic effects. Nicotine and 5-hydroxytryptamine are not, however, interchangeable, for a large dose of either will not block the normal response to the other.

Cocaine, 6-aminotetrahydrocarbazole, atropine and the four atropine-like compounds, all inhibited matching responses of the isolated guinea-pig ileum to 5-hydroxytryptamine and nicotine to the same extent. Responses to both drugs recovered in parallel after removal of the antagonist. That atropine had this action was shown by Robertson, (1953) and by Rocha e Silva et al. (1953); and that cocaine acted similarly was shown also by Rocha e Silva et al (1953).

Yohimbine was the only antagonist which showed a slight discrimination between the two drugs. It caused (in a concentration of 10 μg/ml) a complete inhibition of the 5-hydroxytryptamine response,/

response, and about 60% inhibition of the nicotine response. The recovery of the responses to 5-hydroxytryptamine was slightly slower than that to nicotine. This experiment was done only twice and the discrimination shown between 5-hydroxy-tryptamine and nicotine may not have been real.

Benzimidazole and dihydroergotamine were both slightly more effective against 5-hydroxy-tryptamine than against histamine.

In the presence of 5-hydroxytryptamine (1 μg/ml) the isolated guinea-pig ileum will not respond to this drug nor to tryptamine (Gaddum, 1953). It will however, respond to nicotine, as shown by Rocha e Silva et al (1953) and confirmed here.

When the ileum was bathed with a solution containing 100 μg of nicotine per ml, the response to a small dose of nicotine was blocked while responses to 5-hydroxytryptamine, acetylcholine and histamine were all slightly reduced. In other experiments, single doses of 20 - 100 μg nicotine per ml of Tyrode solution themselves caused responses but small doses of 5-hydroxytryptamine caused no response.

These effects are similar to those described by Rocha e Silva et al (1953), but these workers reached/

reached the high level of nicotine block by repeated groups of doses of nicotine (60 to >100 µg/ml), and the second and following doses in each group generally gave very small responses. With the progressive increase in the nicotine block they showed a progressive "escape" from inhibition of the 5-hydroxytryptamine response. They explain this by postulating that at the lower level of nicotine block, when responses to other drugs are also inhibited, nicotine itself is stimulating a sympathetic inhibitory mechanism (Ambache, 1951; Ambache and Edwards, 1951), and that with higher levels of nicotine block, this inhibitory function is also paralysed. The difference in these effects is shown more clearly by these workers, possibly because they used a routine dose of 5-hydroxy-tryptamine more than 20 times larger than that employed here.

The results of all these experiments bear out the conception that 5-hydroxytryptamine acts through the nervous structures in this preparation, both because of the similarity of effect of the various antagonists on nicotine and 5-hydroxy-tryptamine responses and particularly because both responses are blocked by cocaine. It is believed that/

that nicotine acts on the nervous structures via the parasympathetic ganglia, and that cocaine inhibits conduction through post-ganglionic fibres. But since excessive nicotine block is specific and 5-hydroxytryptamine block is specific (to tryptamines) the receptors for the two drugs cannot be the same (Gaddum, 1955). From this, Rocha e Silva et al (1953) conclude that 5-hydroxytryptamine must act along the parasympathetic post-ganglionic fibres. But they leave unexplained the fact that nicotine is still active when 5-hydroxytryptamine has been blocked by itself. This must mean that conduction down the post-ganglionic fibres is unimpaired though their surface is unresponsive to stimulation.

Alternatively, nicotine and 5-hydroxytryptamine might have distinct receptors on the ganglion cells. Paton and Perry (1953) consider that the paralysing action of large doses of nicotine on the superior cervical ganglion of the cat is caused by a depolarisation of the whole ganglion. Should this be the case also in the parasympathetic ganglia of the isolated guinea-pig ileum, it is difficult to presume that 5-hydroxytryptamine could

cause/

cause a stimulation through such a nicotine-depolarised ganglion. Thus, Gaddum and Hameed (1953) suggest that 5-hydroxytryptamine and nicotine act on similar but distinct ganglion cells.

B. Atropine and the atropine-like compounds.

When the pilot experiments were done for the measurement of the pA_{10} of atropine and 5-hydroxytryptamine, the responses to the 10-fold dose in the presence of atropine were rapidly diminished. If the response at a particular time, (say 10 minutes) after the introduction of the atropine was measured in each experiment there were inconsistencies in the results. It appeared that certain portions of guinea-pig ileum were more sensitive to tachyphylaxis to stimulation by 5-hydroxytryptamine. Tachyphylaxis of this tissue to this drug has been noted elsewhere. (Gaddum, 1953; Rocha e Silva et al. 1953). If doses of 5-hydroxytryptamine were continued there was some recovery of the responses; this must be because the atropine had reached its full effect and tachyphylaxis was no longer having its influence. In later experiments with higher concentrations of atropine/

atropine and in all the experiments with the atropine-like compounds, the antagonist rapidly reached its full effect, and the responses to 5-hydroxytryptamine rapidly reached equilibrium without tachyphylaxis.

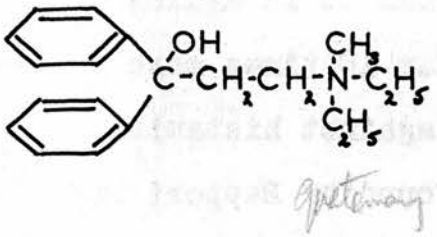
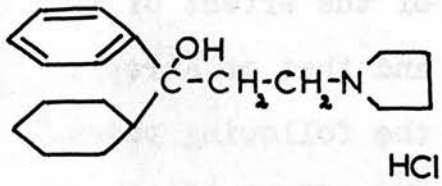
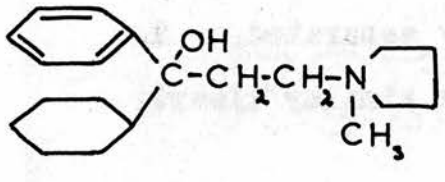
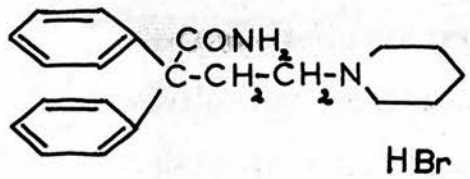
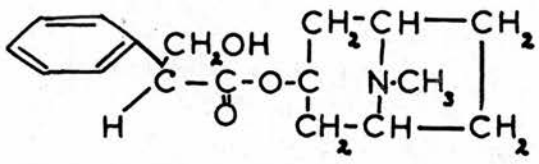
Thus the application of Schild's method, as used by Reuse (1948) was employed here. In this, the equilibrium response to the 10-fold dose is expressed as a percentage of the steady response to the single dose in the absence of antagonist. Equilibrium was reached in 15-20 minutes. Schild (1947) expressed the response to the 10-fold dose at a particular time after introduction of the antagonist in terms of the maximum response obtained in the absence of the antagonist.

Despite the difference in method, the effective concentrations for atropine/acetylcholine and atropine/histamine antagonism are of the same order as those obtained here.

	Schild pA_{10} at 14 min.	Author's results pA_{10} at equilibrium.
Atropine/acetylcholine	8.05	8.59
Atropine/histamine	4.60	4.88
Atropine/5-hydroxy-tryptamine	-	6.31

The/

pA_{10} Estimates for atropine and the atropine-like compounds.

	Acetyl-choline	5-hydroxy-tryptamine	Hist-amine
186 C 47  quaternary I′	7.87 (3)	7.43 (10)	4.97 (3)
54 C 50  HCl	6.80 (4)	5.81 (11)	5.22 (3)
377 C 50  quaternary I′	7.86 (4)	7.25 (8)	<4.63 (2)
182 C 52  HBr	8.61 (4)	7.28 (3)	<5.0 (2)
ATROPINE 	8.60 (5)	6.31 (6)	4.88 (2)

The pA_{10} for atropine/5-hydroxytryptamine showed that atropine is more than 100 times more effective against acetylcholine than it is against 5-hydroxytryptamine; and more than 10 times more effective against this drug than against histamine. This order of activity was that found by Rapport and Koelle (1953).

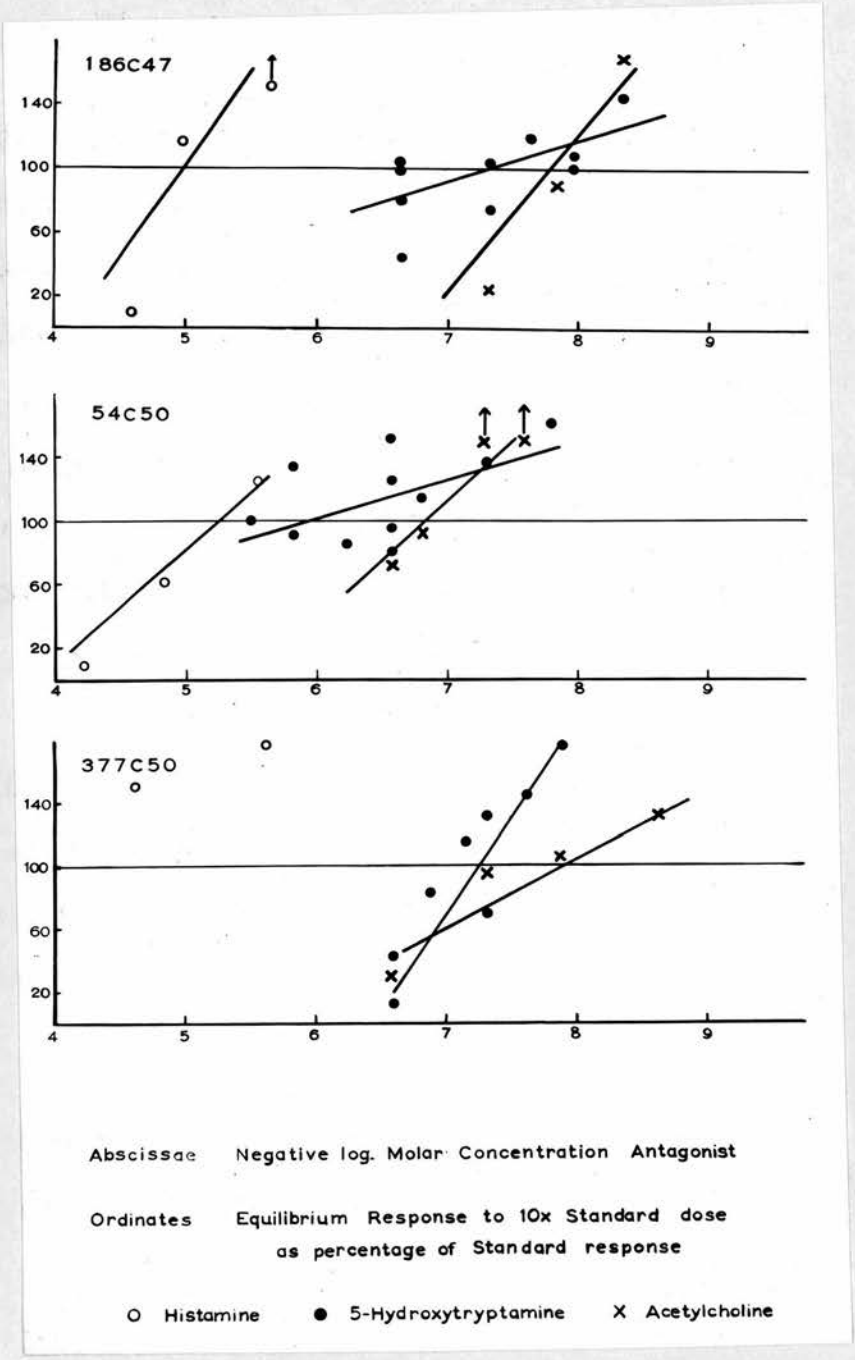
Comparison of the graphs of the effect of the various atropine-like compounds and that of atropine, on the spasmogenic drugs showed the following points.

a) The graphs of the effect of atropine on acetylcholine, 5-hydroxytryptamine and histamine were clearly separated. The graphs for 182C52 showed a similar clearly defined activity.

b) The pA_{10} values of 54C50 for acetylcholine and 5-hydroxytryptamine were themselves separated by one unit but there was a wide scatter of the effects on both drugs, which reduced the significance of this result.

c) The pA_{10} values of 186C47 and 377C50 for acetylcholine and 5-hydroxy-tryptamine were less separated ($\sim$ 0.5 unit) but the results of the individual estimations were/

Figure 17



pA_{10} Graphs - 186C47; 54C50; 377C50.

were less scattered.

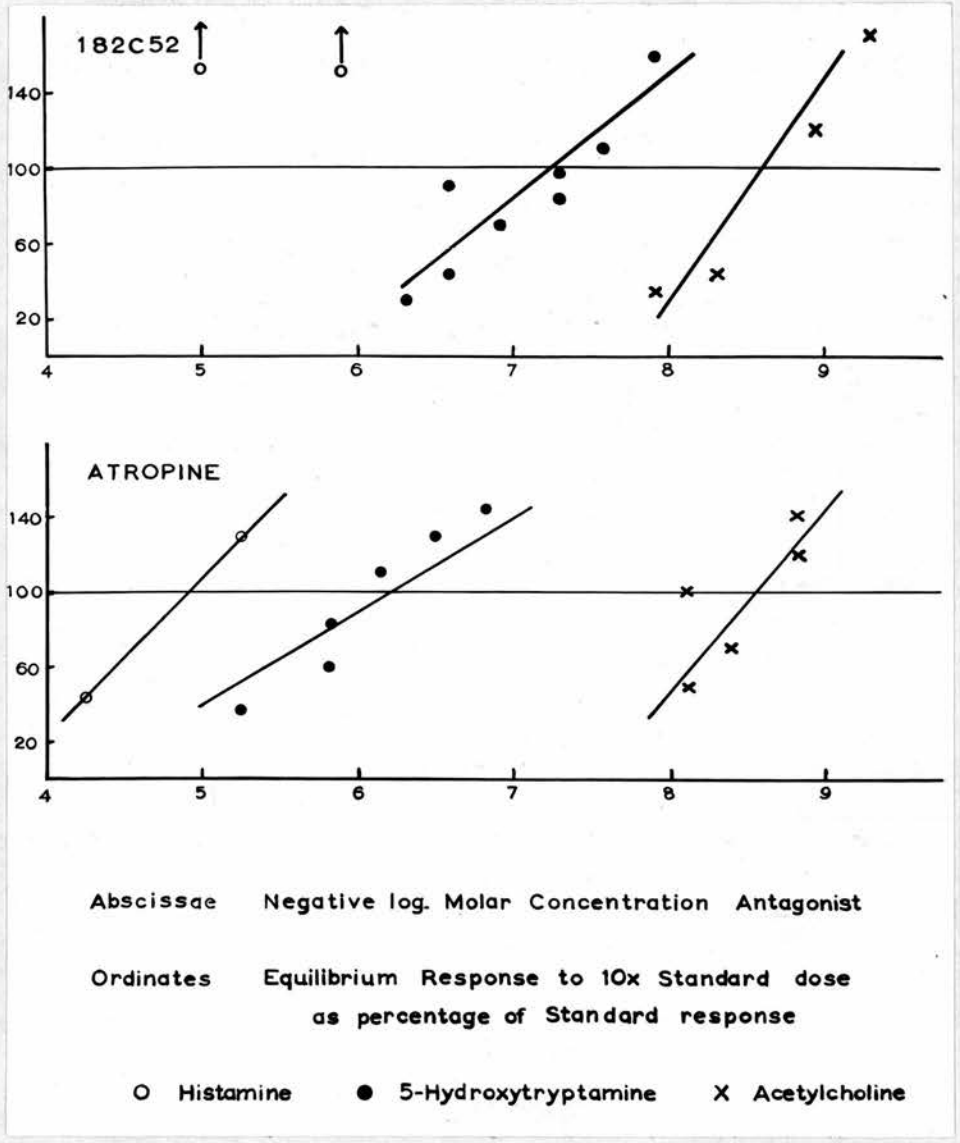
d) The activity of 182C52 on acetylcholine responses was very slightly greater than that of atropine against acetylcholine, (pA_{10} 8.61 cf. 8.59). The corresponding activity of the other compounds was less than that of atropine (7.87, 6.80, 7.86 cf. 8.59).

e) 186C47, 377C50 and 182C52 were all considerably more active against 5-hydroxytryptamine responses than was atropine, (pA_{10}, 7.43, 7.25 and 7.28 cf. 6.31); 54C50 was less active, (pA_{10} 5.81 cf. 6.31).

f) The activities of all the compounds, where measurement was made, were of the same order with reference to histamine responses. (pA_{10} all about 5).

From these results, it was interesting to note that though 186C47, 377C50 and 182C52 are not more active anti-acetylcholines than atropine they are all more active anti-hydroxytryptamines than atropine. This suggested that they are not preventing 5-hydroxytryptamine spasm by antagonism of the effect of acetylcholine, released by the 5-hydroxytryptamine stimulation. Or, if they were doing/

Figure 18



pA_{10} Graphs - 182C52; Atropine.

doing so, it must be that they penetrate more readily to the site of acetylcholine release than does atropine. Alternatively, they have a more specific anti-5-hydroxytryptamine effect on its receptors (possibly on the ganglion cells. See p. 167).

The difference between the activities of 54C50 and 377C50 must be due to the quaternary ammonium radicle in the latter. 186C47, whose activity closely follows that of 377C50, also bears a quaternary ammonium grouping. This radicle cannot however, be essential for activity since it is absent from the most active compound of the series, 182C52. The nitrogen in this compound is contained in a piperidine ring. This compound bears also an amide group in the position in which the other compounds carry a hydroxyl group.

Summary/

Summary

The study of the antagonism, of the responses of the isolated guinea-pig ileum to 5-hydroxytryptamine, by cocaine, 6-aminotetrahydrocarbazole, atropine and the atropine-like compounds, showed very clearly the similarity between nicotine and 5-hydroxytryptamine stimulation. These antagonists inhibited matching responses to these two drugs to the same extent, and the responses recovered in parallel on removal of the antagonist.

However, an excessive dose of either nicotine or 5-hydroxytryptamine did not block the normal response to the other.

Consideration of the various results lends support to the theory of Gaddum and Hameed (1954), that 5-hydroxytryptamine and nicotine both act on ganglion cells, but each has its distinct type of ganglion cell bearing its own receptors.

The quantitative comparison of anti-acetylcholine and anti-5-hydroxytryptamine activities of atropine and the atropine-like compounds supplied through the courtesy of Wellcome Research Laboratories, showed that three of them had a more specific anti-5-hydroxytryptamine effect than had atropine.

BIBLIOGRAPHY

Adams, M.H. and Nelson, J.M. (1938). J. Amer. Chem. Soc. 60, 2474.

Ambache, N. (1951). Brit. J. Pharmacol. 6, 51.

Ambache, N. and Edwards, J. (1951). Brit. J. Pharmacol. 6, 311.

Amin, A.H. (1953). Thesis for Ph.D. University of Edinburgh.

Amin, A.H., Crawford, T.B.B. and Gaddum, J.H. (1952). Communication to the British Pharmacological Society, July, 1952.

Amin, A.H., Crawford, T.B.B. and Gaddum, J.H. (1954). J. Physiol. 126, 596.

Armstrong, Desiree, Dry, R.M.L., Keele, C.A. and Markham, J.W. (1952). J. Physiol. 117, 70P.

Armstrong, Desiree, Dry, R.M.L., Keele, C.A. and Markham, J.W. (1953). J. Physiol. 120, 326.

Asero, B., Colo, V., Erspamer, V. and Vercellone, A. (1952). Annalen. 576, 69.

Bacq, Z.M., Fischer, P. and Ghiretti, F. (1952). Arch. int. Physiol. 60, 165.

Bacq/

Bacq, Z.M., Fischer, P. and Lecomte, J. (1948). Arch. int. Physiol. 56, 25.

Barac, G. (1953). Arch. int. Physiol. 61, 400.

Barter, R. and Everson Pearse, A.G. (1953) Nature, London, 172, 810.

Battelli, F. (1905). J. Physiol. Path. gen. 7, 625 and 651.

Bayliss, L.E. and Ogden, E. (1933). J. Physiol. 77, 34P.

Blaschko, H. (1952). Biochem. J. 52 X. Pharm. Rev. 4, 415.

Brodie, T.G. (1903). J. Physiol. 29, 266.

Cambridge, G.W. and Holgate, J.A. (1955). To be published - (Communicated to Pharmacol. Soc. Jan. 1955).

Comroe, J.H. jr., Van Lingen, B., Stroud, R.C. and Roncoroni, A. (1953). Amer. J. Physiol. 173, 379.

Consden, R., Gordon, A.H. and Martin, A.J.P. (1944). Biochem. J. 38, 224.

Correale, P. (1954). Arch. int. Pharmacodyn. 97, 106.

Correll, J.T., Lyth, L.F., Long, S. and Vanderpoel, J.C. (1952). Amer. J. Physiol. 169, 537.

Domino,/

Domino, E.F., Unna, K.and Kerwin, J. (1952). J. Pharm. Exp. Ther. 105, 486.

Duncan, D., Garven, Jean D. and Gibbons, J.L. (1955). To be published.

Eggleston, L.V. and Hems, R. (1952). Biochem. J. 52, 156.

Ek, A. and Witkop, B. (1953). J. Amer. Chem. Soc. 75, 500.

Erspamer, V. (1935). Biochimie e terapie sperimentali, 22, 390. Quoted by V. Erspamer (1954b).

Erspamer, V. (1937). Z. Anat. Entw. Gesch. 107, 574.

Erspamer, V. (1940). Arch. exp. Path. Pharmak. 196, 343, 366 and 391.

Erspamer, V. (1942a). Arch. exp. Path. Pharmak. 200, 43.

Erspamer, V. (1942b). Arch. exp. Path. Pharmak. 200, 60.

Erspamer, V. (1943). Virchow's Arch. 310, 59.

Erspamer, V. (1946a). Arch. Sci. biol., Napoli. 31, 86.

Erspamer, V. (1946b). Experentia 2, 369.

Erspamer, V. (1948a). Acta Pharmacol. 4, 213.

Erspamer/

Erspamer, V. (1948b). Arch. int. Pharmacodyn. 76, 308.

Erspamer, V. (1952a). Ricerca Sci. 22, 694.

Erspamer, V. (1952b). Ricerca Sci. 22, 1568.

Erspamer, V. (1952c). Arch. intern. Med. 90, 505.

Erspamer, V. (1953). Arch. int. Pharmacodyn. 93, 293.

Erspamer, V.(1954a). Ciba Foundation Symposium on Hypertension. London: Churchill. p.78.

Erspamer, V. (1954b). Rendiconti sci. Farmitalia, I, 1-193.

Erspamer, V. (1955). J. Physiol. 127, 118.

Erspamer, V. and Asero, B. (1951). Ric. Scient. 21, 2132.

Erspamer, V. and Asero, B. (1952). Nature, London. 169, 800.

Erspamer, V. and Boretti, G. (1951). Arch. int. Pharmacodyn. 88, 296.

Erspamer, V. and Faustini, R. (1953). Naturwiss. 40, 317.

Erspamer, V. and Ghiretti, F. (1951). J. Physiol. 115, 470.

Erspamer, V./

Erspamer, V. and Ottolenghi, A. (1950). Experentia, 6, 428.

Erspamer, V. and Ottolenghi, A. (1951). Experentia, 7, 191.

Erspamer, V. and Ottolenghi, A. (1952). Experentia, 8, 31.

Erspamer, V. and Sala, G. (1953). Brit. J. Pharmacol. 9, 31.

Erspamer, V. and Vialli, M. (1951). Nature, London. 167, 1033.

Erspamer, V. and Vialli, M. (1952). Ric. Scient. 22, 1420.

Euler, U.S. von and Gaddum, J.H. (1931). J. Physiol. 72, 74.

Feldberg, W. and Toh, C.C. (1953). J. Physiol. 119, 352.

Fingl, E. (1953). Personal Communication to Prof. J.H. Gaddum.

Fingl, E. and Gaddum, J.H. (1953). Fed. Proc. 12, 320.

Florey, E. and Florey, E. (1953). Naturwiss. 40, 413.

Freyburger, W.A., Graham, B.E., Rapport, M.M., Seay, P.H., Govier, W.M., Swoap, O.F. and Vander Brook, M.J. (1952). J. Pharmacol. 105, 80.

Funderburk,/

Funderburk, W.H., Domino, E.F. and Unna, K. J. Pharm. Exp. Ther. 107, 356.

Gaddum, J.H. (1953a). J. Physiol. 119, 363.

Gaddum, J.H. (1953b). J. Physiol. 121, 15P.

Gaddum, J.H. (1953c). Brit. J. Pharmacol. 8, 321.

Gaddum, J.H. (1954). Ciba Foundation Symposium on Hypertension. London, Churchill. p.75.

Gaddum, J.H. and Hameed, A. (1954). Brit. J. Pharmacol. 9, 240.

Gaddum, J.H., Hebb, C.O., Silver, Ann and Swan, A.A.B. (1953). Quart. J. Exp. Physiol. 38, 255.

Gaddum, J.H. and Kwiatkowski, H. (1938). J. Physiol. 94, 87.

Gaddum, J.H., Peart, W.S. and Vogt, Marthe (1949). J. Physiol. 108, 467.

Ginsburg, M. and Heller, H. (1951). J. Physiol. 115, 43P.

Ginsburg, M. and Heller, H. (1953). J. Endocrinol. 9, 274.

Goodman, L., Gilman, A. and Hart, Nancy (1943). Fed. Proc. 2, 80.

Green, A.F. (1952). Lancet. 1952 I, 583.

Hameed/

Hameed, A. (1954). Thesis for Ph.D. University of Edinburgh.

Hamlin, K.E. and Fischer, F.E. (1951). J. Amer. Chem. Soc. 73, 5007.

Herxheimer, H. (1953). J. Physiol. 120, 65P.

Heymans, C., Bouckaert, J.J. and Moraes, C. (1932). Arch. int. Pharmacodyn. 43, 468.

Holgate, J.A. (1953). J. Physiol. 122, 46P.

Humphrey, J.H. and Jaques, R. (1953). J. Physiol. 119, 43P.

Humphrey, J.H. and Jaques, R. (1954). J. Physiol. 124, 305.

Humphrey, J.H. and Toh, C.C. (1954). J. Physiol. 124, 300.

Janeway, T.C. and Park, E.A. (1912). J. Exp. Med. 16, 541.

Janeway, T.C., Richardson, H.B. and Park, E.A. (1918). Arch. intern. Med. 21, 565.

Jepson, J.B. and Stevens, B.J. (1953). Nature, London. 172, 772.

Keilin, D. and Mann, T. (1938). Proc. Roy. Soc. (London). Series B. 125, 187.

Landis, E.M., Wood, J.E. jr. and Guerrant, J.L. (1943). Amer. J. Physiol. 139, 26.

Leitch,/

Leitch, J., Liebig, C. and Haley, T. (1954). Atomic Energy Project. Report 285. University of California. Los Angeles.

Lembeck, F. (1953). Nature, London. 172, 910.

Lembeck, F. (1954). Arch. exp. Path. Pharm. 221, 50.

Mallette, M.F., Lewis, S., Ames, S.R., Nelson, J.M. and Dawson, C.R. (1948). Arch. Biochem. 16, 283.

Page, I.H. (1952). J. Pharm. Exp. Ther. 105, 58

Idem Fed. Proc. 11, 116.

Page, I.H. and Green, A.A. (1948). Methods in Medical Research, Chicago. Year Book Publishers. Vol.1, p.123. Editor - Van R. Potter.

Page, I.H. and McCubbin, J.W. (1953a). Circulation Research. I, 354.

Page, I.H. and McCubbin, J.W. (1953b). Amer. J. Physiol. 174, 436.

Paasonen, M. (1954). Personal Communication.

Paton, W.D.M. and Perry, W.L.M. (1953). J. Physiol. 119, 43.

Pernow, B. and Waldenström, J. (1954). Lancet, 1954 II, 951.

Philpot,/

Philpot, Flora J. (1940). J. Physiol. 97, 301.

Rand, M. and Reid, G. (1951). Nature, London. 168, 385.

Rapport, M.M. (1949). J. Biol. Chem. 180, 961.

Rapport, M.M., Green, A.A. and Page, I.H. (1947). Fed. Proc. 6, 184.

Rapport, M.M., Green, A.A. and Page, I.H. (1948a). J. Biol. Chem. 174, 735.

Rapport, M.M., Green, A.A. and Page, I.H. (1948b). Science. 108, 329.

Rapport, M.M., Green, A.A. and Page, I.H. (1948c). J. Biol. Chem. 176, 1237 and 1243.

Rapport, M.M. and Koelle, G.B. (1953). Arch. int. Pharmacodyn. 92, 464.

Reid, G. (1952). J. Physiol. 118, 435.

Reid, G. and Rand, M. (1951). Aust. J. Exp. Biol. 29, 401.

Reid, G. and Rand, M. (1952). Nature, London. 169, 801.

Reuse, J.J. (1948). Brit. J. Pharmacol. 3, 174.

Robertson, P.A. (1953). J. Physiol. 121, 54P.

Rocha/

Rocha e Silva, M., Valli, J.R. and Picarelli, Z.P. (1953). Brit. J. Pharmacol. 8, 378.

Schaumann, O. and Lindner, E. (1951). Arch. exp. Path. Pharm. 214, 93.

Schild, H.O. (1947). Brit. J. Pharmacol. 2, 189.

Schneider, J.A. and Yonkman, F.F. (1953). Fed. Proc. 12, 128.

Idem Amer. J. Physiol. 174, 127.

Shaw, E. and Woolley, D.W. (1953). J. Biol. Chem. 203, 979.

Shepherd, D.M., West, G.B. and Erspamer, V. (1953). Nature, London. 172, 357.

Sinha, Y.K. and West, G.B. (1953). J. Pharm. and Pharmacol. 5, 370.

Speeter, M.E. and Anthony, W.C. (1954). J. Amer. Chem. Soc. 76, 6208.

Speeter, M.E., Heinzelmann, R.V. and Weisblat, D.I. (1951). J. Amer. Chem. Soc. 73, 5514.

Stewart, G.N. and Zucker, T.F. (1913). J. Exp. Med. 17, 152.

Stoll, W.A. (1947). Schweiz. Arch. Neurol. Psychiat. 60, 279.

Sumner/

Sumner, J.B. and Myrbäck, K. (1951). The Enzymes. Vol.2, part 1, p.456. Academic Press Inc. New York.

Sumner, J.B. and Somers, G.F. (1953). Chemistry and Methods of Enzymes. (3rd Ed.) Academic Press Inc. New York. p.240.

Thorson, A., Biörck, G., Björkman, G. and Waldenström, J. (1954). Amer. Heart J. 47, 795.

Titus, E. and Udenfriend, S. (1954). Fed. Proc. 13, 411.

Twarog, Betty M. and Page, I.H. (1953). Amer. J. Physiol. 175, 157.

Udenfriend, S. (1954). Personal Communication, 13.8.54.

Udenfriend, S., Clark, C.T. and Titus, E. (1952). Experentia 8, 379.

Udenfriend, S., Clark, C.T. and Titus, E. (1953). Fed. Proc. 12, 282.

Idem J. Amer. Chem. Soc. 75, 501.

Undenfriend, S. and Weissbach, H. (1954). Fed. Proc. 13, 412.

Vialli, M. and Erspamer, V. (1933). Z. Zellforsch. 19, 743.

Vialli,/

Vialli, M. and Erspamer, V. (1940). Arch. Fisiol. 40, 239.

Vialli, M. and Erspamer, V. (1942). Arch. Sci. biol. Napoli, 28, 122.

Welsh, J.H. (1953). Arch. exp. Path. Pharm. 219, 23.

Welsh, J.H. (1954). Fed. Proc. 13, 162.

Welsh, J.H. and Taub, R. (1948). Biol. Bull. 95, 346.

White, A.C. (1953). Personal Communication (27.7.53)

White, A.C., Green, A.F. and Hudson, Audrey (1951). Brit. J. Pharmacol. 6, 560.

Woolley, D.W. and Shaw, E. (1953). Fed. Proc. 12, 293.

Zucker, Marjorie B. (1944). Amer. J. Phys. 142, 12.

Zucker, Marjorie B. (1951). Fed. Proc. 10, 151.

Reviews:

Page, I.H. (1954). Physiol. Rev. 34, p.536-583 - 157 References.

Erspamer, V. (1954). Pharmacol. Rev. 6, p.425-479. 307 References.

Woolley, D.W. and Shaw, E. (1954). Brit. Med. J. Pt.II, 1954. p.122-126. 41 References.

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