REVIEW ARTICLE

5-HYDROXYTRYPTAMINE

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Until recently acetylcholine, noradrenaline and histamine have been the only three well-defined substances of natural origin which play an important role by regulating the activity of tissues locally. The role of acetylcholine and noradrenaline is principally recognised as that of chemical transmitter or mediator, although these two substances as well as histamine have additional physiological functions independent of nervous activity. The action of many drugs is beginning to find an explanation in their relation to acetylcholine, noradrenaline and histamine. Now there is a fourth substance, 5-hydroxytryptamine, which can be regarded as a local tissue hormone.

The early developments in our knowledge of 5-hydroxytryptamine (5-HT) are due principally to three groups of workers. Erspamer and his colleagues in Italy, described a substance which they called "enteramine" and which imparted to the argentaffin cells of the intestinal tract their histochemical characteristics. Enteramine was regarded as a specific secretion product of this enterochromaffin cell system. The other two groups, Page and his colleagues at Cleveland and Reid and Rand in Australia, arrived at this substance from a different approach. They were interested in the vasoconstrictor substance which appeared in blood under certain conditions and which they called serotonin. Serotonin was isolated by Page and Rapport and their colleagues, and identified as 5-hydroxytryptamine. It was later agreed that enteramine was the same substance. This interesting work, together with many other valuable contributions, has been the subject for review in 1954[1,2]; and, more recently, by Page, 1958[3]. Recently, the British Pharmacological Society organised a symposium on 5-hydroxytryptamine[4] and at this meeting many research workers in the field discussed their latest findings and views. The main outcome from the symposium was that the study of 5-HT has developed from its distribution, localisation and characterisation to the study of its biosynthesis, its fate (how it is stored, transported and released), and its physiological role in peripheral tissues as well as in the central nervous system.

Biochemical Studies of 5-HT

According to the work of Udenfriend and his colleagues[5–7] the essential amino acid, tryptophan, which is present as a dietary constituent, is the origin of the body's 5-hydroxytryptamine. These workers have shown that in toads tryptophan is converted to 5-hydroxytryptophan (5-HTP)[5]

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although this conversion has not yet been demonstrated in mammals. The decarboxylation of 5-HTP to 5-HT has been demonstrated in a number of tissues by Gaddum and Giarman[8]. Clark, Weissbach and Udenfriend[9] proposed pyridoxal phosphate (Vitamin B_6) as the coenzyme in the conversion of 5-HTP to 5-HT, but it remained for Buxton and Sinclair[10] to provide experimental proof. They showed that the activity of kidney 5-hydroxytryptophan decarboxylase was much lower in rats deficient in vitamin B_6 than in normal rats, and further, that the activity was restored by addition of pyridoxal phosphate.

Amino acid decarboxylases are not the only enzymes affected by pyridoxine deficiency. In animals[11] and man[12] suffering from this nutritional disorder, tryptophan metabolism is deranged and this derangement is increased by a high tryptophan intake.

In rats[13] and mice[14], a vitamin B_6 deficiency causes dermatitis of the peripheral parts of the body particularly in the feet, paws, ears and areas around the mouth. It may be significant that these are the sites in these species where large amounts of 5-HT are normally present in the mast cells of the skin[15].

Another site at which the derangement of tryptophan metabolism leads to symptoms which might involve 5-HT is in the brain. There is evidence[14] that pyridoxine deficiency gives rise to a convulsive disorder which appears to be more prevalent in young infants than in older children or adults. It has been found[16] that the pyridoxine deficient rats showed increased brain excitability measured by the decrease in electroshock threshold, while administration of pyridoxine increased the threshold. The possibility that a low concentration of 5-HT in the brain may lead to excitation will be discussed later.

Phenylketonuria is another condition in which there is a defect in tryptophan metabolism. Sandler,[4] following up the finding of Armstrong and Robinson[17] that abnormal indole metabolites were present in the urine of phenylketonurics, has concluded that in these patients phenylalanine competes with tryptophan for the same limited hydroxylating mechanism, eventually causing a diminished 5-HT production.

In man 5-hydroxyindole formation is not a major pathway of tryptophan metabolism. However, in patients suffering with carcinoid tumour the metabolism of dietary tryptophan is upset and in the tumour 5-HT formation is greatly increased[18]. Sometimes this increase is so great as to interfere with other pathways of tryptophan metabolism with the consequent symptoms of pyridoxine deficiency.

Recently an interesting observation was made on tryptophan metabolism[19] in which 5-HTP as well as 5-HT was found in the urine of a patient with argentiffinoma of kidney origin. It was concluded that large amounts of 5-HTP were formed in the argentaffin cells and that some of it was excreted before it could be metabolised to 5-HT. This is the most direct evidence available that the cells of the enterochromaffin system actually produce 5-HT as well as secrete it internally and, in fact, supports the view that the argentaffin cells constitute a diffuse but true endocrine system.

In man and some other mammals 5-HT conversion to 5-hydroxyindoleacetic acid (HIAA) is the final metabolic step. In some species this is a minor metabolic pathway and the main inactivation of 5-HT awaits elucidation. Monoamine oxidase is probably mainly responsible for the oxidative deamination of 5-HT and its importance in man is evident from the urinary excretion of HIAA[20]. Blaschko[21] has suggested that the primary physiological function of this enzyme may be the breakdown of 5-HT. In fact, monoamine oxidase may be for 5-HT what cholinesterase is for acetylcholine. The analogy may even go further. There may be more than one monoamine oxidase just as there are several cholinesterases. For instance, the monoamine oxidase inhibitor, iproniazid, is a poor inhibitor of amine oxidase in peripheral tissues although a potent inhibitor in the brain[22].

The rate of turnover of a substances is often an indication of its participation in the functional activity of a tissue. Some interesting findings have been made[4] on the rate of turnover of 5-HT. The most rapid turnover of 5-HT occurs in the brain where its half life is 1–2 hours. In the intestine the half life of 5-HT is 1 day and in the platelets 1–2 days, a value which is consistent with the survival time for platelets themselves and suggests that the liberation of 5-HT from platelets normally occurs only on their disintegration. There is a slow turnover rate of 5-HT in carcinoid tumours, where its half life is 5–6 days and on this basis it has been estimated that a carcinoid tumour of 2·8 kg. might contain as much as 3 mg. of 5-HT.

The Carcinoid Syndrome as an Indication of the Functional Significance of 5-HT

If we take the view of Masson[23] and Erspamer[2] that the enterochromaffin cells constitute a diffuse endocrine system designed for the production and storage of 5-HT then an indication of the physiological function of its secretion may be gained from observations on carcinoids, because in this condition there is hyperfunction of this diffuse endocrine organ.

Lembeck[4] has suggested that the two cardinal signs of carcinoid patients, vascular changes and diarrhoea, give an indication of the physiological role of 5-HT. The vascular changes consist of a flush sometimes accompanied by oedema and occasional petechial haemorrhages. 5-HT infused intra-arterially in man produces the same effects[24]. In addition, reserpine, which releases 5-HT (see page 537) causes flushing accompanied by nasal congestion in man[25]. The local vascular effects observed in carcinoid patients are usually not associated with systemic blood pressure effects.

Diarrhoea and increased intestinal motility are seen in most carcinoid patients. 5-HT is known to stimulate isolated smooth muscle and in cats close arterial injections into the coeliac artery of small amounts of 5-HT increase intestinal motility[4]. Injected subcutaneously into rats, 5-HT increases faecal excretion[26]. Lembeck discusses the possibility that the enterochromaffin cells secrete 5-HT to act on the neurons of Meissner's

plexus so that this 5-HT constitutes a physiological stimulus to intestinal motility.

The possibility that 5-HT is involved in the peristaltic reflex has also been considered by Ginzel[27], Kosterlitz and Robinson[28] and by Bülbring and Lin[29]. Ginzel as well as Kosterlitz and Robinson observed that 5-HT blocks the peristaltic reflex of the isolated guinea pig intestine. Bülbring and Lin however, introduced the 5-HT into the lumen of the isolated intestine and found the opposite effect. They observed a lowering of the threshold of the pressure required to elicit peristalsis and showed that the contractions so elicited were more frequent and expelled a larger volume of fluid. Further small amounts of 5-HT appeared in the fluid passing through the lumen of the isolated loop of intestine at rest, but increased two-fold when the intestine became active during peristalsis. When 5-HTP, the precursor of 5-HT, was added to the perfusing fluid a greater conversion of 5-HT occurred during activity. Bülbring and Lin concluded that there is an increased production of 5-HT in the intestinal wall during peristaltic activity, but they did not exclude the possibility that its release of 5-HT occurs as a result of the motor activity, that is by mechanical extrusion rather than by an increased formation. According to their view the increase in the intralumenal pressure releases 5-HT which sensitizes the sensory receptors in the mucosa which trigger the peristaltic reflex.

Action of 5-HT on Peripheral Nerve and Muscle Structures

The conclusion that 5-HT acts on ganglia or nerve endings in the intestine is in accord with a number of recent observations about an action of 5-HT on nerves and ganglia.

Trendelenburg found that it excited the cells of the superior cervical ganglion and that very small amounts of 5-HT potentiate the effects of preganglionic stimulation.[30] No 5-HT has yet been detected in the superior cervical ganglion, but 5-hydroxytryptophan decarboxylase, which is the enzyme that forms 5-HT, is present. The absence of 5-HT may only mean, as suggested by Dalgliesh, that in the ganglion 5-HT exists, in an active form, such as 5:6-dihydroxytryptamine, a substance which is extremely labile. In that case the very fact that it is so labile could explain the difficulty of detecting 5-HT as "active" 5-HT in the superior cervical ganglion. To settle this problem specific metabolic inhibitors are required which prevent its rapid destruction. If 5-HT were to play a role in ganglionic transmission we would have to assume that it is rapidly destroyed near its site of action. By analogy with acetylcholine the "active" form of 5-HT might elude detection without the aid of a potent and specific inhibitor of amine oxidase. However, the only experiments of this type carried out by Gertner, Paasonen and Giarman[31] with the aid of metabolic inhibitors do not support the idea that 5-HT is a ganglionic transmitter. They perfused the cat's superior cervical ganglion with a solution containing monoamine oxidase inhibitor iproniazid and found no 5-HT in the effluent after preganglionic stimulation. However, 5-HT appeared in the effluent after the ganglion had been perfused for 2–3

hours, but the significance of this finding must await further experimental results.

Gaddum and Picarelli[32] in studying the action of 5-HT and its antagonists on smooth muscle and peripheral nervous structures have found that the antagonists lysergic acid diethylamide (LSD), ergot alkaloids and dibenzyline were active 5-HT antagonists on the guinea pig ileum, whereas the drugs like atropine, morphine, and cocaine which did not antagonise the 5-HT response on rat uterus or had only an unspecific action of this kind, were effective antagonists on the guinea pig ileum. These differences may be associated with the fact that the rat uterus is composed only of smooth muscle whereas the guinea pig ileum contains, in addition, a complex nervous system.

It was shown by Gaddum and Picarelli that the response of the guinea pig ileum to 5-HT was reduced to nearly 50 per cent by low concentrations of dibenzyline, but increasing its concentration even 100 times caused no further reduction. The same result was observed when morphine was used as inhibitor for 5-HT. But when morphine and dibenzyline were used together, the response to 5-HT was abolished. These results suggest that each of these two inhibitors acted on a specific type of receptor both of which were stimulated by 5-HT. They have been called D and M receptors. The D receptors are antagonised by large doses of tryptamine, LSD, dibenzyline and gramine, while the M receptors are blocked by morphine, atropine, or cocaine. It seems likely that the D receptors are located in the smooth muscle fibres and are present in both the rat uterus and in the guinea pig ileum while the M receptors form part of a nervous structure, possibly the ganglion cells, and are present in the guinea pig ileum but not in the rat uterus. On this assumption the action of atropine may be due to antagonism of acetylcholine liberated at the endings of nerves stimulated by 5-HT, cocaine may act on the nerve fibre, while morphine probably exerts its antagonistic action by preventing the release of acetylcholine from the excited nerve endings. As morphine was shown by Trendelenburg to block the stimulating action of 5-HT on the superior cervical ganglion it seems likely that in the intestine the same mechanism may hold and that part of the effect is due to stimulation of ganglion cells. Thus if 5-HT plays a part in the physiological regulation of intestinal motility the constipating action of morphine may perhaps be explained in this way. Vane[33] has provided evidence that sympathomimetic amines which do not possess a phenolic hydroxyl group, like mescaline and amphetamine, act upon tryptamine or D receptors in certain smooth muscle preparations. He found that these amines contract the isolated rat stomach like tryptamine and 5-HT whereas sympathomimetic amines having phenolic hydroxyl groups, like, noradrenaline and adrenaline, relax this smooth muscle. Further, the contractions produced by mescaline and amphetamine are abolished by the antagonists of 5-HT, bromo-LSD and dibenamine whereas the relaxations caused by adrenaline and noradrenaline are unaffected by 5-HT antagonists. Another smooth muscle preparation, the guinea pig ileum responds by contracting to mescaline and amphetamine and also to large

doses of adrenaline, but only the contractions caused by mescaline and amphetamine are inhibited by bromo-LSD. Vane has pointed out that the sympathomimetic amines which act upon tryptamine (D) receptors are those which cause stimulation in the central nervous system. The possibility therefore exists of the presence in the central nervous system of tryptamine receptors on which these analeptics act.

Gaddum has drawn attention to the resemblance of the peripheral actions of 5-HT to those of acetylcholine. Both drugs have two receptors. Atropine antagonises the action of acetylcholine on smooth muscle revealing ganglionic effects and LSD antagonises the action of 5-HT on smooth muscle revealing effects which might well be ganglionic. Hexamethonium specifically blocks the ganglionic effects of acetylcholine while morphine is less specific and blocks also the effects of 5-HT.

In addition to effects on autonomic ganglia, 5-HT stimulates another type of nerve structure, the endings of sensory nerves. Armstrong, Dry, Keele and Markham[34] have shown in man that it causes pain when applied to the base of an exposed blister. Although human skin contains only minute amounts of 5-HT which could be liberated and stimulate nocireceptors it is possible that a high concentration in blood may occur in the vicinity of these receptors after the damage of platelets in an injured tissue.

Another type of sensory nerve ending stimulated by 5-HT is found in the chemo- and baroreceptors. Douglas and Toh[35] showed that the hyperpnoeic response to 5-HT was abolished by sinus nerve section. Since then many investigators have found evidence that it stimulates both the chemoreceptors and the baroreceptors. Ginzel and Kottegoda[36] showed in cats that 5-HT injected into the carotid sinus region caused a hyperpnoeic response accompanied by a fall in arterial blood pressure, the latter out-lasting the respiratory stimulation. Both responses were the direct outcome of stimulation of the receptors in the carotid sinus since the depressor response was unchanged when the animal was artificially respired and the respiratory response persisted when a constant pressure apparatus was used to neutralise the fall in blood pressure. Direct evidence of chemoreceptor stimulation was provided by McCubbin, Green, Salmoiraghi and Page[37] who demonstrated that there was an enormous increase in the number of impulses in the carotid sinus nerve when 5-HT was introduced into the carotid artery.

The response of the chemo- and baroreceptors to 5-HT disappeared when small doses were repeatedly injected in quick succession or after administration of a single large dose. Such tachyphylaxis is also encountered with the vascular responses of 5-HT[36] and the question arises, is its action on the sensory endings due to local vascular effects? This question has not yet been settled but it is interesting to note in this connection that Malcolm[4] found under certain experimental conditions that 5-HT depresses cortical responses evoked by stimulation of a sensory nerve and this effect ran parallel to oedema formation which occurred in the brain after the 5-HT. He pointed out that depression of the cortical responses may be a result of the vascular changes which follow intra-arterial injection of 5-HT.

Cardiovascular Effects

As 5-HT can produce both hypertension and hypotension in the same animal, Page and McCubbin[38] have introduced the term "amphibaric" to describe this blood pressure response. Page has given a comprehensive review[3] of the work carried out largely in his own laboratory on the cardiovascular effects of 5-HT. Some of the factors determining its effect on the arterial blood pressure are stimulation of baro- and chemoreceptors, an action on the vessels causing either vasoconstriction or dilatation, or both, as well as inhibition of neurogenic vasoconstriction. There is considerable species difference in the blood pressure response to 5-HT depending on which of the above factors plays the greater role.

The blood pressure response to 5-HT appears to depend largely on the vasomotor tone. When this is low, as for example after ganglionic blockade or destruction of spinal cord, the response is pressor, whereas when the vasomotor tone is high, for example, after section of buffer nerves, the response is depressor. Page has conceived the idea of 5-HT as a humoral antagonist to neurogenic vasomotor tone and speaks of a "chemical buffering system".

Erspamer[39] concluded that the antidiuresis caused by 5-HT was due to a preferential vasoconstriction of the afferent glomerular arterioles and that it was a hormone designed for the physiological regulation of renal function. But Abrahams and Pickford[40] studying its effect in the dog concluded that the antidiuresis was due to its action on the vascular system in general and not on the kidney vessels in particular. They found that antidiuresis occurred only when 5-HT raised the systemic blood pressure and that injected arterially into the kidneys a much larger dose was required to cause antidiuresis than on intravenous injection. 5-HT was even more effective in causing antidiuresis in rats when given by the subcutaneous route than when injected intravenously. Since in rats it produces a local oedema on subcutaneous injection, retention of fluid produced in this way may account for at least part of the strong antidiuretic effect seen under these conditions.

The Role of 5-HT in Anaphylaxis

The local oedema formation in rats at the site of a subcutaneous injection of 5-HT was first observed by Rowley and Benditt[41] and has been confirmed by Parratt and West[42]. It is known that in rat skin, oedema is a characteristic feature of the effects of histamine liberators, substances which produce their effects by disrupting mast cells and releasing the histamine contained in them. However, Benditt, Wong, Arose and Roeper[43] showed that the mast cells of rat contain not only histamine but 5-HT as well, and Bhattacharya and Lewis[44] have shown that 5-HT is released by histamine liberators. The oedema produced in rats by histamine liberators is in fact mainly the effect of the released 5-HT. This view is supported by the finding that after reserpine which depletes the mast cells of their 5-HT leaving their histamine intact[45], histamine releasers or, better, mast cell depleters, cause little or no oedema.

It seems likely that 5-HT plays a role in anaphylactic shock in rats because Lewis and Mota[46] found that 5-HT as well as histamine was released into the blood stream of rats sensitised with horse serum, after injection of antigen. However, no positive evidence has so far been obtained in perfusion experiments. When the antigen was injected into the hind-quarters of sensitised rats perfused with Locke's solution, neither 5-HT nor histamine appeared in the venous effluent.

5-HT is present in the mast cells of the skin of only mice and rats[47]. It seems possible, as suggested by Feldberg, that in the rat in particular, it takes over some of the functions which histamine has in other species. Certain human allergic states resemble the reaction in the rat and although normal skin does not contain appreciable amounts of 5-HT there is a possibility that the 5-HT content is increased in such conditions. There is no experimental evidence in favour of this view; in fact, West has observed that in a case of urticaria pigmentosa which is due to an increase of mast cells in the skin, the 5-HT content of the skin was not increased although the histamine content was raised considerably.

It is also not possible to explain the anaphylactic contractions of the histamine insensitive rat uterus by 5-HT because Brocklehurst[4] showed that 5-HT antagonists do not affect these contractions. Further, he showed that 5-HT does not contract but relaxes the medium sized bronchioles which are mainly responsible for the bronchospasm in human asthma. It therefore seems unlikely that it plays a role in these conditions.

In the guinea pig 5-HT causes a shock syndrome characterised by vascular collapse and bronchospasm. There is a rapid tachyphylaxis and it is interesting that during the period when the guinea pigs are insensitive to 5-HT, antigen does not produce an anaphylactic shock[48]. However, there is no evidence that 5-HT plays a role in anaphylaxis in the guinea pig, and 5-HT antagonists have no effect on the course of anaphylactic shock.

Humphrey and Jacques[49] found that 5-HT is released from platelets when antigen is added to blood from sensitised rabbits and this has been confirmed *in vivo*[50]. In spite of the fact that rabbit mast cells do not contain 5-HT[44] it is still possible that it plays some role in anaphylaxis in this species.

Transport and Release of 5-HT

Page has stressed the importance of the chronic effects exerted by the continuous presence of minute amounts of 5-HT in the blood although there is no evidence of free 5-HT in plasma, all that in plasma probably being bound to platelets[51]. It seems possible that there is a continual release of 5-HT into the circulation and that this excess is taken up at once by the platelets which seem to control the amount available in plasma and its transport in the blood stream. The degree of absorption in plasma varies considerably in individual subjects and although patients with blood diseases often show a low 5-HT content in the platelets, there is no clear relationship between any pathological condition and degree of absorption

of 5-HT by the platelets. Born, Ingram and Stacey[52] found a proportionality between the concentrations of adenosine triphosphate (ATP) and 5-HT in platelets and discussed the possibility that ATP plays a role in the binding of 5-HT. Further, blood clotting leads to the disappearance of ATP as well as 5-HT from platelets[53]. But there are at least two conditions where there is no relation between the release of 5-HT and of ATP from platelets. Firstly, Hardisty and Stacey[54] found that in a case of myeloid leukaemia, a deficiency in platelet 5-HT was not accompanied by a corresponding deficiency in ATP. Secondly, reserpine greatly reduces the platelet 5-HT but does not alter the platelet ATP.

There is another more likely mechanism by which ATP may be involved with 5-HT in platelets. ATP may provide the energy for an active transport mechanism for concentrating 5-HT in platelets. Brodie and Shore[55] explained the action of reserpine in releasing 5-HT from platelets by this mechanism. Reserpine is known not only to release the 5-HT from platelets but also to prevent its accumulation in platelets. It is possible that it acts by upsetting this active transport system allowing the 5-HT present in the platelets to diffuse out and prevent further concentration until the transport mechanism is replenished.

Reserpine and several other rauwolfia alkaloids release the 5-HT not only from platelets and rat mast cells, but they also deplete the stores in the intestinal tract and brain[56–58]. At all these sites 5-HT is released, probably destroyed by monoamine oxidase as soon as it is freed, and the tissue remains depleted of it for many hours after reserpine has disappeared from the body. As in the case of the platelets, reserpine prevents the binding of 5-HT at its normal sites in the brain and intestine.

Brodie has developed the thesis that reserpine alkaloids exert their sedative effect by releasing 5-HT in the brain. Part of this evidence is derived from experiments with the reserpine alkaloid, rescinnamine, which provides a good example of the relationship between the intensity of the pharmacological effects and 5-HT release. This alkaloid induces slight sedation in the mouse where it causes only a small release of 5-HT, but in the rabbit where it causes marked sedation it causes a large release of 5-HT.

The rauwolfia alkaloids do not specifically release 5-HT but other substances as well. From the platelets, reserpine releases histamine, although it does not do so from the mast cells of rats[45]. On the other hand, reserpine releases catechol amines in most species[59–62], for instance, it releases the noradrenaline and adrenaline from the brain, the suprarenal glands and from sympathetic adrenergic neurones. It is possible that the effect on the suprarenals is central in origin since the depletion of the amines does not occur when reserpine is given after the glands have been denervated.

Role of 5-HT in the Brain

There are a number of papers which deal with the problem of explaining the central pharmacological actions of reserpine by release of 5-HT. The problem, however, becomes even more complex by the knowledge that the catechol amines are also released in the brain.

One method of studying the sedative action of reserpine in animals developed from the finding that it lengthened the barbiturate sleeping time. It is interesting that large amounts of 5-HT also increase the barbiturate sleeping time, since with these injections some of the 5-HT gets into the brain. These results are in favour of the view that the sedation following reserpine is due to the released 5-HT, although there are many drugs which lengthen barbiturate sleeping time[63].

Marazzi and Hart[64] reported that minute doses of 5-HT inhibited central synaptic transmission. They evoked activity in the cat cortex by transcallosal stimulation of the optical cortex and found that after intracarotid injection of 5-HT the response was greatly reduced. However, this appears to be a non-specific effect as they found the same effect with small doses of LSD, mescaline, bufotenine, adrenaline, noradrenaline and adrenchrome. Malcolm[4] also used intracarotid injections of 5-HT in cats and found that these injections inhibited the responses evoked by somatic sensory nerve stimulation only if large doses (200–300 μg. as a 1/1000 solution) were given.

Although 5-HT does not readily pass the blood brain barrier its precursor 5-HTP does so, and in this way the 5-HT content of the brain increases up to tenfold after administration of large doses of the precursor[6]. A further increase occurs when iproniazid, a potent inhibitor of amine oxidase is first administered. These facts have been used in a recent investigation by Lewis and Malcolm[65] in an attempt to throw some light on the role played by 5-HT in the central nervous system. They found that when the 5-HT level is raised by giving a large dose of 5-HTP (30 mg./kg.) the electrical responses evoked by cutaneous and muscle afferents in all areas of the brain explored are depressed. The responses were restored to normal by injection of reserpine. After previous iproniazid treatment 5-HTP caused a much greater depression which usually resulted in complete depression of the whole central nervous system and death. The depression in the latter case could not be reversed with reserpine. 5-HTP, iproniazid and reserpine produced a marked fall in blood pressure. They ruled out the possibility that hypotension was responsible for the central depression, since the effect also occurred when the blood pressure was kept constant by an automatic compensator.

These results showed that an increase in the level of "bound" 5-HT in the brain results in depression of central activity, but when this "bound" 5-HT is lowered by reserpine the activity returns to normal. This suggests that it is necessary for 5-HT to be present in a "bound" form to exert its central effects.

The sedation caused by reserpine in the anaesthetised animal therefore may not be due to its action in releasing 5-HT but to some other action, for instance, the release of catechol amines. Bogdanski, Weissbach and Udenfriend[66] reported that when the brain 5-HT is raised by administration of 5-HTP to unanesthetised cats, there was marked central excitation. This has not been confirmed in the experiments of Lewis and Malcolm, the only sign of excitation was some respiratory stimulation. The usual effects were slight depression after 5-HTP and after iproniazid the cats

showed a tendency to lie down until disturbed. The effects were similar to those reported by Feldberg and Sherwood[67] after injection of 5-HT into the cerebral ventricles of cats. Lewis and Malcolm found that there was never any sign of sham rage in cats after 5-HTP (30 mg./kg.) as reported by Udenfriend and his colleagues[66] or after iproniazid 100 mg./kg. followed by 5-HTP 30 mg./kg.

As the changes in electrical activity occurred in many areas of the brain and not particularly in those areas with a high 5-HT content, it was possible that the distribution of brain 5-HT was related to a cell type rather than to general anatomical areas. An attempt to determine its distribution at a cellular level by injecting ^{14}C labelled 5-HTP (0·1 mc./kg.) and examining the distribution on autoradiographs did not give very clear autoradiographs as the dose employed was insufficient. However, it indicated that 5-HT is distributed fairly evenly throughout the brain and is not related to nervous elements. The possibility exists that 5-HT is contained in the connective tissue cells—the glial cells—and its function is an action on the blood vessels around which glial cells are concentrated.

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RESEARCH PAPERS

A STEREOTYPED RESPONSE INDUCED BY MESCALINE IN MICE AS A MEANS OF INVESTIGATING THE PROPERTIES OF DRUGS ACTING ON THE CNS

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The effects of mescaline on spontaneous activity of mice placed in special cages have been studied. The typical response (stereotyped response) has been recorded and assessed in terms of percentage of animals showing the effect. Among drugs affecting the response chloropromazine shows the highest specificity. Promazine, hexobarbitone and pentobarbitone also show a definite but minor effect in doses that do not impair motor function. The response is little affected by phenobarbitone. Meprobamate, mephenesin and a thiadiazole derivative (L 1458) at non-paralyzing doses, and also azacyclonol, do not produce specific antagonistic effects. Nor is the effect significantly influenced by atropine, but it is enhanced by morphine.

MESCALINE has been intensively studied because of its impressive effect on the central nervous system of man and the laboratory animal. Mescaline induced hallucinations have been described in man[1] and experimental catatonia in animals[2]. Thus it may be possible to make use of the behavioural changes induced by mescaline to test the activity of drugs with reported beneficial effects in mentally-disturbed patients. The interest in studying the effect of mescaline and its antagonists has been intensified by the discovery and development of the so called "tranquillizing drugs". In the mouse, mescaline causes certain characteristic changes in behaviour, such as enhanced excitability and paroxysm of ear scratching[3]. Fellows and Cook[4] tested drugs for their ability to prevent or reduce the number of scratching episodes. Tripod[5] has described the antagonism of many sedative agents to mescaline-induced psychomotor stimulation in mice. But the "actographic" method of Hanschild[6] employed by Tripod[7] does not record movements[5].

It has been observed by us and others[8] that the scratching response may be induced by drugs other than mescaline, and it is known that psychomotor stimulation is a feature of the action of many CNS stimulants that produce hypermotility and hyperexcitability as, for instance, amphetamine does.

The present study has been undertaken to determine whether a quantitative characteristic behaviour response could be obtained in mice after mescaline.

Our observations were directed towards a quantitative evaluation of the integrated response of mice to mescaline treatment, using a simple apparatus,

and to assessing the ability of certain CNS depressants to prevent the effects of the drug.

Materials and Methods

Mice of both sexes of CFI strain and of our own breeding were used. Spontaneous activity was kymographically recorded by means of an apparatus first constructed some years ago by Mr. G. Ciuffi*. It consists of a cylindrical metal cage with a mobile floor fixed to the top of a spiral spring. A small rigid bar from the top of the spring is connected to a writing lever. This apparatus proved suitable for graphical recording of typical changes of the motility induced by mescaline in mice.

Aqueous solutions of the drugs used were injected intraperitoneally or intramuscularly. The intraperitoneal route was employed only for insoluble products, and these were suspended in an aqueous 10 per cent acacia gum solution.

Results

Effects of Mescaline

After administration of mescaline, 10 mg./kg. i.p. mice showed an increased sensitivity to touching and handling. Motor activity was slightly reduced. Movements were small and rapid and periods of intense scratching were observed. When animals were transferred from the cage to a new environment their usual curiosity and exploring activity was decreased.

After 30 mg./kg., mice showed more intense paroxysms of scratching of muzzle, ears and neck, with a further increase in sensitivity to touch. Spontaneous activity was abnormal. The movements of cleaning and periods of slight tremors and of tremors at rest were interrupted by short unidirectional movements. When stimulated to walk, some animals showed a slight motor incoordination. Righting reflex and muscular tone were normal. When higher doses were administered the symptoms appeared more marked and signs of autonomic involvement were noted (changes in rate and depth of respiration, lacrimation). A slight motor incoordination was present as well as a slight impairment of righting reflex. The maximum dose at which behavioural changes were still distinguishable from general toxic effects was commonly 100 mg./kg. The effects seen after i.p. or sub-cutaneous injection are similar.

When individual animals were placed in cages, observations and recordings of oscillations gave us a more detailed view of the phenomena.

The effect of 15 mg./kg. i.p. of mescaline depended upon the initial condition of the mouse. Mice with a high initial spontaneous activity usually quietened and gave the typical scratching response. Animals at rest before administration, showed an increased motor activity. In some mice, after 15 mg./kg. of mescaline we observed a rhythm in motor behaviour when periods of fine movements were followed by small and rapid displacements inside the cage. After 20 mg./kg. this rhythm took

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on a more definite character, and became distinguishable on the kymographic tracings (Fig. 1).

The observed changes consist of slow rhythmic waves, caused by unidirectional and regular movements of the animal, around the circumference of the cage by short stages. These movements were alternated with intense characteristic scratching or scratching-like movements. The frequency of these waves depends upon the duration of the scratching episodes and the amplitude of each stage of circular movement. We assigned to this behaviour the term *stereotyped response*. The behaviour of a single animal was found to be constant during the effect of mescaline.

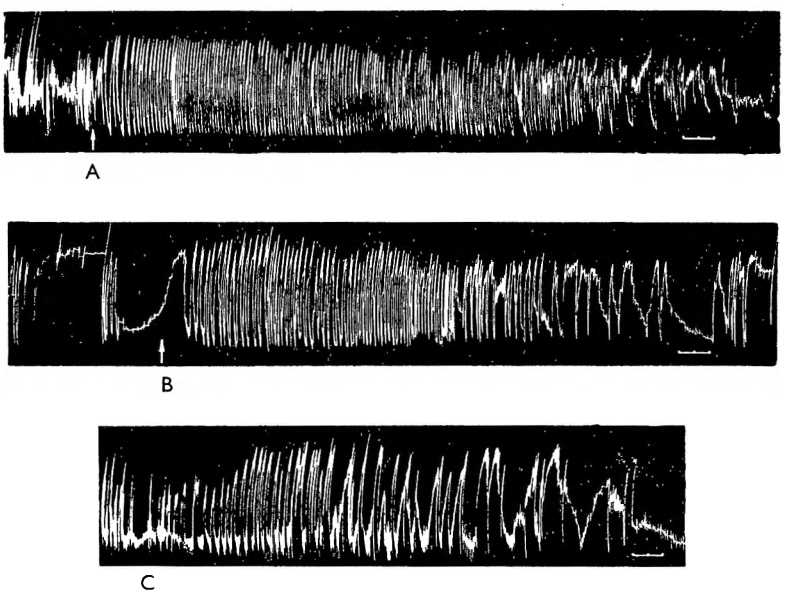


FIG. 1. Stereotyped response in mice following mescaline: A, B (at arrow), mescaline sulphate 50 mg./kg. i.p.; C mescaline sulphate 30 mg./kg. i.p.
Time = 5 minutes.

We have systematically investigated this phenomenon and concluded that it is one of the most prominent features of the action of mescaline that could be instrumentally recorded. By considering it as a quantal effect, experiments with a large number of animals have shown a linear relation between the log of the dose and probits of mice showing a "stereotyped response".

Figure 2 shows the straight line that reveals this dose effect function (according to Litchfield and Wilcoxon[9]). The resultant ED50 was 24·7 mg./kg. (19/20 confidence limits: 19·4–31·4).

An attempt to establish the average duration of the response for each dose used, failed because of individual variability and the difficulty of ascertaining the end of the response which always disappeared gradually.

However we observed that the duration of effect increased with increasing doses. At the level of the LD50 it lasted 90–150 minutes.

In Table I original data and the more important steps of our calculation are given.

TABLE I

CHANGES IN MOTOR BEHAVIOUR OF MICE FOLLOWING MESCALINE: EXPERIMENTAL DATA OF DOSE-ACTIVITY CURVE ACCORDING TO LITCHFIELD AND WILCOXON[9]

Doses of mescaline mg./kg.	Animals/ dose	Effective doses (ED) and confidence limits (C.L.)			Slope and C.L.	Heterogenity test χ^2 for P = 0·05	
		ED16	ED50	ED84		χ^2 found	χ^2 tabular
15–20 25–30 40–50 60	57	15·9 (21·9–11·5)	24·7 (31·4–19·4)	38·3 (52·8–27·7)	1·55 (1·95–1·23)	10·7	11·1

Effects of Other Drugs acting on CNS

We have compared under similar experimental conditions the effect of mescaline with that of drugs well known for their exciting effect on CNS and motor activity in mice.

Amphetamine sulphate, 3–10 mg./kg. s.c. showed the well known constant exciting effect with sudden enhancement of motor activity characterised by fast and continuous multidirectional movements. Swift scratching and cleaning movements were observed but only for short periods and were soon interrupted by the nearly paroxysmal hypermotility. The tracings (Fig. 3) show no similarity with those obtained for mescaline-treated mice.

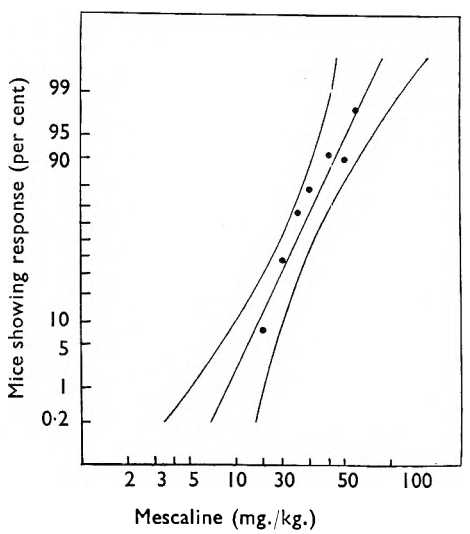


FIG. 2. Effects of increasing doses of mescaline on the frequency of stereotyped response in groups of mice (19/20 C.L. according to Litchfield and Wilcoxon).

After *caffeine* (10–20 mg./kg. i.p.) all mice showed an increased motor activity irrespective of the conditions before injection. Paroxysmal scratching movements were present in all mice but a regular succession of displacements and scratching—as seen with mescaline was never observed (Fig. 3).

Morphine induces a moderate hyperactivity at low doses (1 mg./kg.) and initially also with higher ones (3–5 mg./kg.) as well as the typical and well known rigidity and extension of the tail. However, it appears that the

reactions to handling and manipulating are decreased. Some morphine-treated mice developed a stereotyped motor behaviour, with circling movements. However, when the animals were placed in the cages and their activity recorded the tracings were unlike those obtained with mescaline because of the high frequency of the circling movements, the absence of rhythm and the great variability of the response (Fig. 3).

Nicotine tartrate-induced changes in motor activity also differed from those given by mescaline. After nicotine tartrate 5 mg./kg., spontaneous activity of mice was commonly reduced. The animals occasionally showed tremors and muscular twitches; with higher doses (7·5 mg./kg.) clonic convulsions appeared. Such observations are fully in accord with the effects of nicotine in the rat at the same dose level as reported by Blum[10]. But we have never seen patterns of motility similar to those seen with mescaline.

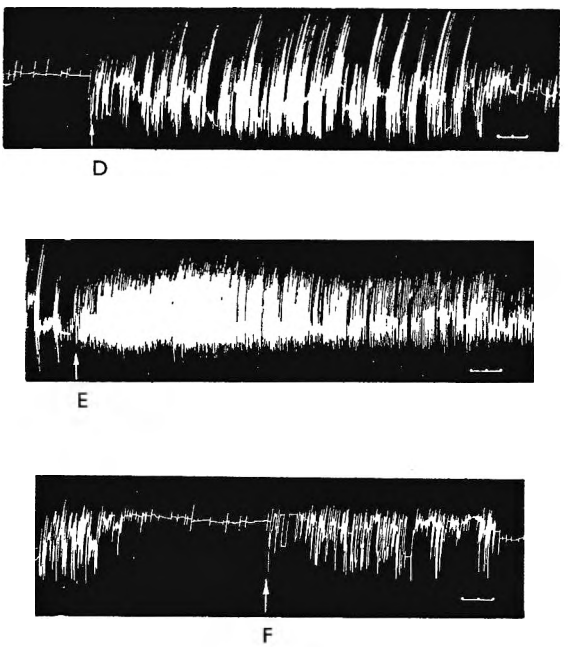


FIG. 3. Changes of motor activity in mice following: D (at arrow), caffeine 20 mg./kg.; E (at arrow), amphetamine sulphate 10 mg./kg.; F (at arrow), morphine hydrochloride 3 mg./kg.
Time = 5 minutes.

Changes in Mescaline-induced Stereotyped Response by different Drugs

The results convinced us that the method of recording this specific stereotyped activity induced by mescaline was suitable for studying interactions between mescaline and other drugs.

We therefore investigated the influence of drugs, given to mice half an hour after mescaline, upon the stereotyped response. To express the activity of the various substances, we inferred the expected effect of known doses of mescaline alone, from the fitted dose/activity curve.

The effect of a single dose of each of the drugs was then compared with the expected response after mescaline and the inhibition calculated. A per cent inhibition was plotted on log-dose probability paper and the ED50 calculated as well as its 19/20 confidence limits. Throughout our experiments, we constantly checked the effect of mescaline alone.

There were three types of response of the mice under the new conditions.

First a normal response to mescaline with a proportion of the mice presenting the stereotyped response for all doses within the limits of variability of expected effects of mescaline alone as seen with inactive drugs.

Secondly, non appearance of the stereotyped response in a significant proportion of mice treated with mescaline together with the drug being tested. This is evidence for specific block.

Thirdly, absence or disappearance of the stereotyped response due to failure of motor function either directly observed or demonstrated by a period of immobility in the tracings of the activity of mice. This is evidence of unspecific block.

We also observed, as a result of the effect of a drug, a potentiating action of the mescaline-induced responses. This potentiation was demonstrated by the increase either in the number of mice responding to the ED25 and ED50 of mescaline or in the duration of its effect. The effects of drugs on the stereotyped response induced by mescaline are shown in Tables II and III.

The response is little or not modified by atropine sulphate 2·5 and 5 mg./kg.

Morphine hydrochloride produces a potentiating effect that is easily demonstrable at 3 mg./kg. The response is increased by about 40 per cent, and its duration is much increased. The response to 20 mg. of mescaline lasting normally about 1–2 hours, is prolonged with morphine to 3 and even 4 hours (Fig. 4). With 5 mg./kg. of morphine some inhibition was seen as well as a significant suppression of spontaneous activity.

Meprobamate, 20 to 200 mg./kg., caused a block of the response only in doses that markedly affect motor function and caused immobility (Fig. 5).

Mephenesin, and also those other centrally acting muscle relaxants chemically related to the thiadiazoles[11,12] such as L 1458 (5-thienyl-2-amino-1 : 3 : 4-thiadiazole), causes a similar unspecific block.

An unexpected confirmation of the lack of specific inhibition by meprobamate and mephenesin was given by the reappearance of the stereotyped response, after a duration of immobility of about 10 to 15 minutes with higher doses of mephenesin and after half an hour with meprobamate (Fig. 5).

No specific action on the stereotyped response was observed with azacyclonol in doses one third of the reported LD50[13].

Phenobarbitone sodium only partially blocked the response. With doses that do not produce marked improvement of motor mechanism resulting in immobility, block was never complete (Fig. 5). Plotting the per cent inhibition and per cent immobility on log-dose/probability paper, the two straight lines converge at the top near the 60 per cent effects (Fig. 6).

Pentobarbitone sodium produces some specific block. At dose of 10 mg./kg. no mouse showed immobility, but the response was inhibited in a high proportion of all animals.

TABLE II

EFFECTS OF DIFFERENT DRUGS ON MESCALINE INDUCED RESPONSE AND MOTILITY

Compounds and doses mg./kg.		No. of animals	Per cent inhibition of response	Per cent immobility	LD50 mg./kg. mouse
Meprobamate	20	14	3	0	i.p. 719 [745–694][12]
	40	3	0	0	
	50	7	0	0	
	80	15	0	0	
	100	23	10	15	
	200	13	100	96	
Mephenesin	25	6	42	35	i.p. 518 [559–430][12]
	50	5	44	50	
	100	3	83	83	
L 1458	12·5	3	0	0	i.p. 374 [408–343][12]
	25	3	0	0	
	35	18	0	11	
	60	18	32	28	
	80	12	100	100	
Pentobarbitone	5	8	0	0	i.p. 128·76 ± 2·75[20]
	10	25	79	0	
	20	12	100	62	
Azacyclonol	40	9	0	0	i.p. 220 ± 31[13]
	80	15	11	7	
Atropine sulphate	2·5	6	23	0	i.p. 250 [190–330][21]
	5	12	19	0	
Morphine hydrochloride	1	3	−2*	0	s.c. 531[22]
	3	6	−42*	0	
	5	21	7	14	

* Potentiation of response.

TABLE III

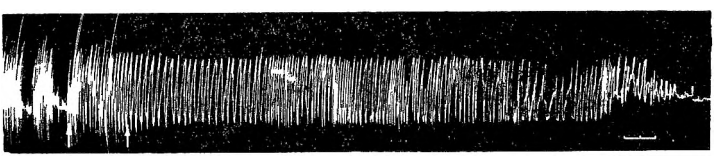
EFFECTS OF DIFFERENT DRUGS ON MESCALINE INDUCED RESPONSE AND MOTILITY. EXPERIMENTAL DATA OF DOSE-ACTIVITY CURVES ACCORDING TO LITCHFIELD AND WILCOXON[9]

Compound	Number of doses	Animals/ dose	ED50 and C.L.	Slope and C.L.	Heterogenity test χ^2 for P = 0·05		LD50 mg./kg. mouse
					χ^2 found	χ^2 tabular	
Chlorpromazine							
Block of response	4	12	0·16 (0·10–0·25)	2·33 (1·12–4·84)	4·70	5·99	i.p. 225–250[23]
Immobility	4	16	1·07 (0·82–1·38)	1·97 (1·31–2·95)	4·35	5·99	
Promazine							
Block of response	8	15	1·70 (1·36–2·12)	1·93 (1·62–2·30)	10·75	12·60	i.p. 200[23]
Immobility	5	14	4·00 (3·20–5·00)	1·50 (1·17–1·93)	2·78	7·82	
Hexobarbitone							
Block of response	4	14	11·40 (8·90–14·59)	2·21 (1·64–2·99)	1·25	5·99	i.p. 280 ± 20·4[24]
Immobility	3	16	53·00 (39·25–71·55)	2·02 (1·48–2·74)	0·72	3·84	
Phenobarbitone							
Block of response	5	15	26·3	3·06	8·14	7·82	i.p. 235 ± 12[25]
Immobility	5	15	53·2 (43·25–65·43)	1·30 (1·06–1·58)	0·30	3·84	

The activity of hexobarbitone was still more marked. As shown by Figure 6, the distance, on the dosage scale, between immobility and blocking is great and highly significant.

Chlorpromazine has shown high specificity in counteracting the response. As shown in Table III and in Figure 6, the abolition of the response has been observed within a range of doses almost completely independent of those that produce immobility. The scratching response and the alternating rhythmic activity were seen to be greatly reduced.

G



H

FIG. 4. Stereotype response in mice following mescaline: G (at arrow), mescaline 20 mg./kg.; H (at first arrow), mescaline 20 mg./kg., (at second arrow), morphine hydrochloride 3 mg./kg.
Time = 5 minutes.

Promazine also causes a specific block but it is much less effective than chlorpromazine (ED50 is about ten times that of chlorpromazine) and the specificity is less marked.

The median effective doses (ED50) abolishing the response and motor activity of the most extensively investigated compounds are given in Table III.

By analysis of the dose: activity relation, it was found that slopes of the linear functions representing respectively the block and the immobility are not statistically different with chlorpromazine, promazine, hexobarbitone and phenobarbitone. However, it has been noted that the slope of dose stereotyped response block curves was always greater than the slope of dose: immobility curves.

DISCUSSION

In our opinion the stereotyped response represents an aspect of total motor patterns concerned with the activity of mescaline. Only mescaline produces in mice a typical motor behaviour characterised by small movements—always or nearly in the same direction and scratching periods —in definite succession. Under suitable conditions the alternation is well defined and gives a characteristic kymographic record. The doses of mescaline used by us (within a range from 15 to 60 mg./kg. s.c. or i.p.) to demonstrate the response were apparently free from toxic effect.

Speck[14] studying toxicity and physiological effects of mescaline reported that in fasting rats, i.p. doses of 58 mg./kg. did not consistently reduce the blood glucose content and only slightly affected the heart rate. Moreover we found the LD50 by i.p. administration in mice to be 230 mg./kg. (19/20 confidence limits: 213–248).

Chlorpromazine was the most effective drug specifically blocking the response and its activity has been demonstrated in a range of doses close to those commonly used in man.

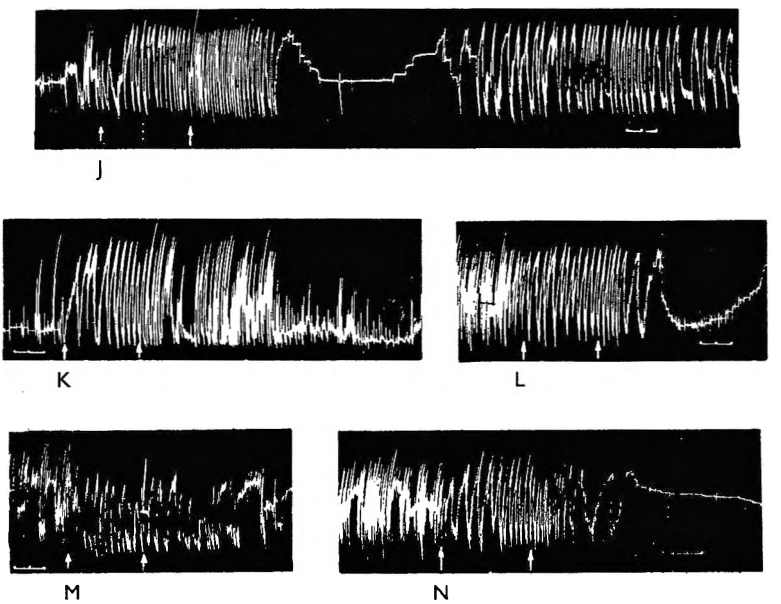


FIG. 5. Stereotyped response in mice following mescaline: J (at first arrow), mescaline 40 mg./kg., (at second arrow), meprobamate 100 mg./kg.; K (at first arrow), mescaline 40 mg./kg., (at second arrow), chlorpromazine 0·20 mg./kg.; L (at first arrow), mescaline 40 mg./kg., (at second arrow), chlorpromazine 1·20 mg./kg.; M (at first arrow), mescaline 40 mg./kg., (at second arrow), phenobarbitone 25 mg./kg.; N (at first arrow), mescaline 40 mg./kg., (at second arrow), phenobarbitone 50 mg./kg.
Time = 5 minutes.

On the other hand significant impairment of motor activity has been observed after doses as great as six times those that block the response.

According to Courvoisier[15] chlorpromazine in the rat also antagonizes all the symptoms that follow the administration of mescaline and Denber[16] reported that chlorpromazine was the most active agent in counteracting the psychic effects of mescaline in man.

Fellows and Cook[4] studying the scratching response in mice treated with mescaline reported it to be antagonized by chlorpromazine, reserpine, serotonin and morphine, but not by barbiturates or meprobamate.

According to Tripod[5] the motor stimulating activity of mescaline in

mice is antagonized by chlorpromazine, promazine, reserpine and serotonin, but also by meprobamate, 450 mg./kg. orally, and by azacyclonol, 200 mg./kg. orally. Also in the same paper the author reports that "les stimulations psychomotrices" induced by mescaline are enhanced by phenobarbitone and atropine.

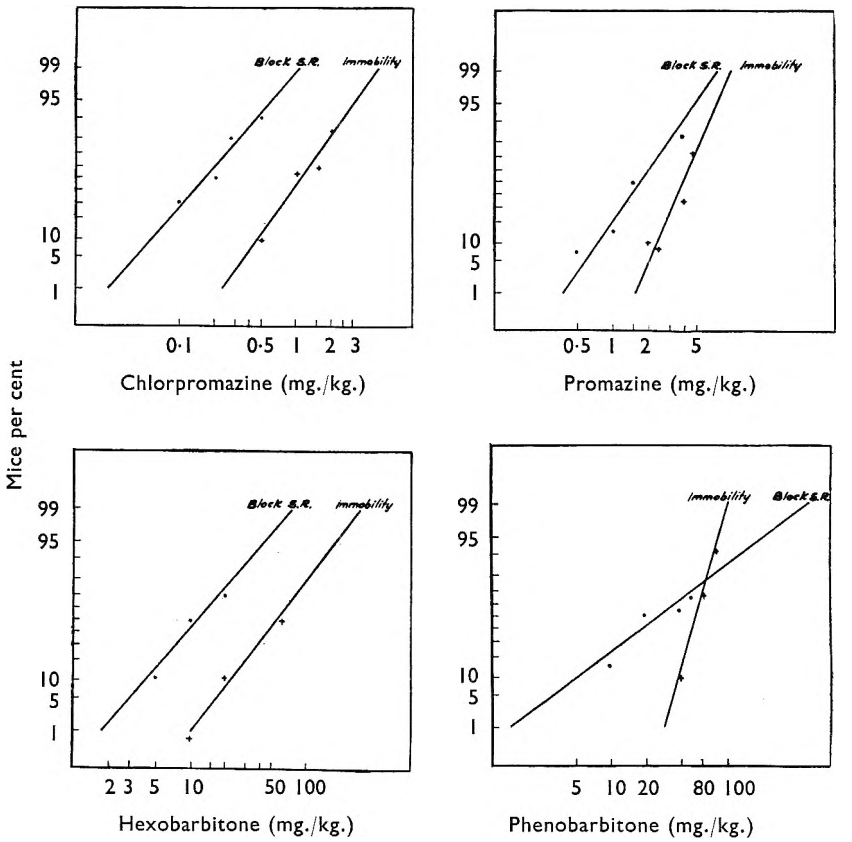


FIG. 6. Dose-activity curves of chlorpromazine, promazine, hexobarbitone and phenobarbitone. ●, block of stereotyped response; +, immobility.

Our results are not in full agreement with those of the authors quoted.

The most effective drug is chlorpromazine followed by promazine. The failure of meprobamate in specifically abolishing the stereotyped response, as we observed, and the scratching response, as found by Fellows and Cook[4]—contrasts with the antagonism of mescaline-induced "stimulations psychomotrices" reported by Tripod[5]. In our opinion the ED50 of meprobamate reported by this author is such to affect motor function, and can hardly be assumed to block specifically the effect of mescaline.

The three barbiturates studied, always inhibited the response and no

enhancement has been seen. Speck[14] also observed that phenobarbitone-induced anesthesia in rats may be easily and promptly counteracted by mescaline.

Among the drugs tested only morphine clearly enhanced the mescaline stereotyped response.

Fellows and Cook[4] found morphine to antagonise the scratching response and we also observed this effect in some animals. However the complex patterns of mescaline-induced motor behaviour as shown by the stereotyped response are, according to our observations, clearly potentiated by morphine. Also morphine alone is capable of inducing a stereotyped behaviour in mice, usually characterised by circling movements around the cage.

It may be interesting to remember that Cook and Weidley[17], studying the effect of drugs on conditioned avoidance response of rats, found that mescaline failed to produce any significant block of the conditioned response, but "in most instances" slightly enhanced the blocking activity on the conditioned response of promazine, reserpine, serotonin and morphine. On the other hand according to Wikler[18] mescaline-induced anxiety in man is relieved by barbiturates and not by morphine.

A poor effect on the response is given by azacyclonol, which is reported by Sturtevant and Drill[19] to change the psychomotor response of cats injected with mescaline from a catatonic to an excitatory type.

The many different technical approaches to testing such antagonism in mice has caused other authors to make use of the scratching response or "les stimulations psychomotrices". The study of the ability of drugs to abolish the effects of mescaline shows that different techniques may give different results. Further work is necessary to assess the usefulness of the systemic study of the stereotyped response as a means of differentiating the mode of action of certain CNS depressants.

From the practical point of view it must be said that although the method used by us is simple, the present investigation has required a relatively large number of animals to get statistically significant data.

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COMPARATIVE STUDIES ON PERCUTANEOUS ABSORPTION

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The percutaneous absorption of salicylic acid, sulphanilamide, copper acetyl-acetonate, and copper sulphate through the intact skin of rabbits and the efficiency of lard, emulsifying ointment B.P., and water in the form of a five per cent carboxymethyl cellulose gel, as carriers, is compared. Blood levels were accepted as a measure of absorption. The physico-chemical properties of the drugs seemed to dictate the amount absorbed; the influence of the base was also significant, although less so. Best absorbed was salicylic acid, next sulphanilamide, then copper acetyl-acetonate, and finally copper sulphate, although the differences were slight between the last two; lard was the best base, then emulsifying ointment, and finally water.

The *in vivo* methods used to study percutaneous absorption include blood, urine, faeces and tissue analyses and pharmacological, histological and clinical methods[1]. We have used the blood levels attained after the topical application of sulphanilamide to compare the efficiency of vehicles or bases as "carriers"[2]. The present study examines the amount of percutaneous absorption of four different drugs, namely, salicylic acid, sulphanilamide, copper acetyl-acetonate and copper sulphate, from three vehicles or bases, namely, lard, Emulsifying Ointment B.P. and water, as a 5 per cent carboxymethylcellulose gel.

After their cutaneous application, the detection and estimation of the drugs in the bloodstream of the animal permitted a comparative study of the efficiencies of the vehicles and bases and the effect on absorption of the properties of the drugs incorporated in these bases. The series of experiments undertaken were designed to provide sufficient results to make a complete statistical examination.

Experimental

Choice of Drugs

The drugs were selected to demonstrate a wide range of pharmacological and chemical properties but the choice was limited to those readily and accurately determined in blood. They were: salicylic acid, a lipid soluble organic acid, soluble in alcohol, ether, glycerol and chloroform, and possessing keratolytic and irritant properties; sulphanilamide, a neutral organic compound, soluble in water, ethanol, acetone and glycerol; copper acetyl-acetonate, a non-ionic compound of copper chelated with acetyl-acetone, insoluble in water, but soluble in alcohol and chloroform, and copper sulphate, an ionic compound, readily soluble in water and glycerol but insoluble in alcohol.

Choice of Vehicles

Previously reported experiments[2] with sulphanilamide had shown lard to be the most efficient of the vehicles and bases tested and water, as a

5 per cent carboxymethylcellulose gel, to be the least efficient. Emulsifying Ointment B.P. gave an efficiency value intermediate between these two[2]. It was decided to incorporate the four drugs in these bases to determine whether the same order of efficiency of the vehicles and bases held for drugs other than sulphanilamide.

Concentration of incorporated drug. The concentration of drug first used in the vehicles and bases was 10 per cent weight-in-weight. But with salicylic acid the concentration was lowered to 5 per cent to reduce the local irritant and keratolytic effect. This decrease in concentration would be unlikely to invalidate comparative blood level studies since others had found that an increase to over 5 per cent in the concentration of the incorporated drug did not lead to an increase in absorption[3].

Design of test and experimental details. These have been described in a previous publication[2] where a suitable test was designed for the comparison of vehicles and bases as "carriers" of drugs through the intact skin of rabbits.

Estimation of Drugs in Blood Samples

Salicylic acid. A modification of Smith and Talbot's colorimetric method was used[4]. 0·2 ml. of blood was added to 3·2 ml. distilled water followed by 0·6 ml. of a 25 per cent aqueous solution of trichloroacetic acid. To 2 ml. of the filtrate, 1 ml. 1·5N sodium hydroxide was added. Folin-Ciocalteu's reagent, 0·5 ml., diluted one to four with distilled water, was then added and the volume adjusted to 5 ml. with 1·5N sodium hydroxide. After standing, the solutions were filtered and extinctions measured against a reagent blank at a wavelength of 690 mμ. To correct for non-specific plasma phenols which are included in this estimation, the average reading of 36 normal blood samples treated as above was subtracted from the observed values before referring to a calibration curve, constructed by similarly treating known dilutions of sodium salicylate in distilled water. The concentration of reductable material in blood estimated by this method gave a value of 10·41 mg./100 ml.

Sulphanilamide. Sulphanilamide in blood was estimated by King's micro-modification of the method of Bratton and Marshall[5].

Copper. Copper, in ammoniacal solution, was estimated colorimetrically with sodium diethyl dithiocarbamate which is capable of detecting one part of copper in fifty million parts of solution. The colour produced is stable for several hours[6].

0·2 ml. blood was added to 3·2 ml. distilled water followed by 0·6 ml. of a 25 per cent aqueous solution of trichloroacetic acid. To 2 ml. of the filtrate was added 0·5 ml. of a 10 per cent solution of ammonia. Three drops of a 0·1 per cent solution of sodium diethyl dithiocarbamate were then added and the final volume adjusted to 5 ml. with distilled water. The extinctions of the solutions were measured against a reagent blank at a wavelength of 455 mμ. Estimated by this method, the concentration of copper present in normal circulating rabbit blood, taking the average of 24 blood samples, gave a value of 0·18 mg./100 ml. Copper solutions of known concentration were added to freshly drawn rabbit blood and the

procedure repeated. The estimation of copper was accurate to within 1 per cent so that, under the conditions of the assay, any complexes formed by the metal and the blood proteins appear to have broken down, the copper remaining in solution as the stable trichloroacetate.

Results

The results are summarised and shown in Tables I, II, III, and IV; the statistical analysis is given in Table V.

TABLE I

COMPARISON OF PERCUTANEOUS ABSORPTION IN THE RABBIT OF SALICYLIC ACID, SULPHANILAMIDE, COPPER ACETYL-ACETONATE AND COPPER SULPHATE, FROM LARD, EMULSIFYING OINTMENT B.P. AND A 5 PER CENT CARBOXYMETHYLCELLULOSE GEL

Drug	Base	Mean blood level of drug observed in 6 individual rabbits over 8 hours, mg./100 ml.						Mean blood level of drug observed in 6 rabbits over 8 hours, mg./100 ml.
		Rabbit Number						
		1	2	3	4	5	6	
Salicylic acid	Lard	1·09	1·13	1·24	1·21	1·13	1·17	1·16
	Emulsifying Ointment B.P.	0·86	0·84	1·01	0·99	1·08	1·09	0·98
	Water as a 5 per cent carboxymethyl cellulose gel	0·90	0·85	0·81	1·03	0·92	0·74	0·88
Sulphanilamide	Lard	0·88	1·03	0·91	1·02	0·82	0·75	0·89
	Emulsifying Ointment B.P.	0·48	0·56	0·53	0·39	0·48	0·35	0·47
	Water as a 5 per cent carboxymethyl cellulose gel	0·28	0·28	0·29	0·48	0·44	0·37	0·36
Copper acetyl-acetonate	Lard	0·25	0·25	0·28	0·26	0·24	0·26	0·26
	Emulsifying Ointment B.P.	0·23	0·23	0·24	0·21	0·22	0·23	0·23
	Water as a 5 per cent carboxymethyl cellose gel	0·19	0·19	0·19	0·18	0·17	0·19	0·19
Copper sulphate	Lard	0·21	0·21	0·22	0·19	0·24	0·24	0·22
	Emulsifying Ointment B.P.	0·20	0·21	0·21	0·20	0·20	0·23	0·21
	Water as a 5 per cent carboxymethyl cellulose gel	0·19	0·17	0·17	0·18	0·18	0·20	0·18

Table I shows the mean blood level obtained for the individual rabbits throughout the test. These figures are calculated by summing all the 16 observed half-hourly levels for each rabbit and dividing by the number of blood samples taken, i.e., 16. They provide a useful and practical index of efficiency for a particular vehicle or base.

The average response is found by summing the above totals over all six rabbits and dividing by the new total number of observations i.e., 96. The analysis was worked on totals rather than means since the former require fewer arithmetical operations and are less liable to error. These totals are given in Table II.

Table III shows the total amount observed in six rabbits for one drug from a particular vehicle or base and the mean rabbit response to the drug in that vehicle or base under the conditions of test.

Table IV is a table of comparative efficiencies and shows the order of efficiency of the three vehicles or bases for each drug tested. For each drug, lard is the most efficient vehicle or base; water, as a 5 per cent carboxymethylcellulose gel, is the least efficient. The extent of absorption

TABLE II

COMPARISON OF PERCUTANEOUS ABSORPTION IN THE RABBIT OF SALICYLIC ACID, SULPHANILAMIDE, COPPER ACETYL-ACETONATE AND COPPER SULPHATE, FROM LARD, EMULSIFYING OINTMENT B.P. AND WATER, AS A 5 PER CENT CARBOXYMETHYLCELLULOSE GEL

Drug	Base	Total amount observed in the blood of 6 individual rabbits over 8 hours (each value is the sum of 16 samples), mg./100 ml.						Total amount of drug observed in 6 rabbits over 8 hours, mg./100 ml.
		Rabbit Number						
		1	2	3	4	5	6	
Salicylic acid	Lard	17·56	18·04	19·80	19·42	18·15	18·72	111·69
	Emulsifying Ointment B.P.	13·71	13·47	16·18	15·86	17·33	17·27	93·82
	Water, as a 5 per cent carboxymethyl cellulose gel	14·45	13·65	13·02	16·42	14·78	11·78	84·10
Sulphanilamide	Lard	14·14	16·05	14·54	16·24	13·03	11·92	85·92
	Emulsifying Ointment B.P.	7·62	9·08	8·45	6·27	7·71	5·57	44·70
	Water, as a 5 per cent carboxymethyl cellulose gel	4·46	4·47	4·64	7·67	7·19	5·93	34·36
Copper acetyl-acetonate	Lard	3·96	4·05	4·54	4·19	3·85	4·24	24·83
	Emulsifying Ointment B.P.	3·71	3·67	3·78	3·42	3·48	3·74	21·80
	Water, as a 5 per cent carboxymethyl cellulose gel	3·03	2·97	2·99	2·94	2·74	3·00	17·67
Copper sulphate	Lard	3·40	3·37	3·47	3·18	3·78	3·83	21·03
	Emulsifying Ointment B.P.	3·27	3·41	3·35	3·21	3·17	3·65	20·06
	Water, as a 5 per cent carboxymethyl cellulose gel	2·99	2·75	2·77	2·94	2·89	3·21	17·55

of the incorporated drugs can also be seen. Salicylic acid was absorbed more readily than sulphanilamide while the acetyl-acetonate and the sulphate of copper were poorly absorbed by comparison.

Table V shows the statistical analysis of variance on the results. The wide variation in the level of reaction to the different drugs and the wide

TABLE III

COMPARISON OF PERCUTANEOUS ABSORPTION OF THE FOUR DRUGS IN THE RABBIT FROM THREE VEHICLES OR BASES

	Lard		Emulsifying Ointment B.P.		Water, as a 5 per cent gel	
	Total amount observed in 6 rabbits, mg.	Mean individual rabbit response, mg.	Total amount observed in 6 rabbits, mg.	Mean individual rabbit response, mg.	Total amount observed in 6 rabbits, mg.	Mean individual rabbit response, mg.
Salicylic acid	111·69	18·62	93·82	15·64	84·10	14·02
Sulphanilamide	85·92	14·32	44·70	7·45	34·36	5·73
Copper acetyl-acetonate	24·83	4·14	21·80	3·63	17·67	2·95
Copper sulphate	21·03	3·53	20·06	3·34	17·55	2·93

variation in the variances makes the result of an analysis of variance rather more difficult to interpret than in the orthodox case.

Because of the death of two of the test animals it was impossible to replicate the determinations for sulphanilamide. Accordingly, these results were excluded from the analysis of variance which thus became a two factor analysis with replication. The difference between drugs was highly significant although the difference between the two compounds of copper tested by the *t*-test was not significant. This implied that there was a very large difference between the vehicles and bases containing salicylic acid and those containing the two compounds of copper.

TABLE IV

TABLE OF COMPARATIVE EFFICIENCIES

THE BLOOD LEVEL FOR COPPER SULPHATE IN WATER, AS A 5 PER CENT CARBOXYMETHYLCELLULOSE GEL, TAKEN AS UNITY

	Lard	Emulsifying Ointment B.P.	Water, as a 5 per cent carboxy-methylcellulose gel
Salicylic acid	6·32	5·32	4·75
Sulphanilamide ..	4·85	2·54	1·95
Copper acetyl-acetonate	1·42	1·23	1·01
Copper sulphate ..	1·19	1·14	1·00

The drug-base interaction was significant at a level between $P = 0{\cdot}01$ and $P = 0{\cdot}05$. Examination showed that this was almost entirely due to the unexpectedly high efficiency of the salicylic acid-lard interaction. The apparently large differences between the bases was found to be due almost entirely to this interaction and when allowance was made for this, the differences between the bases were not significant.

TABLE V

ANALYSIS OF VARIANCE

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F	Remarks
Between Drugs ..	2	21351	11576	116	$F = 61{\cdot}3$ for $P = 0{\cdot}001$
Between Vehicles or bases	2	489·8	244·9	2·72	$F = 6{\cdot}9$ for $P = 0{\cdot}05$
Drug x Vehicle or base interaction ..	4	359·6	89·89	4·42	$F = 3{\cdot}6$ for $P = 0{\cdot}05$
Residual	9	182·8	20·31		$F = 6{\cdot}4$ for $P = 0{\cdot}01$

Incorporation of the sulphanilamide experiments modified these conclusions as follows:

(1) There were significant differences between salicylic acid, sulphanilamide, and the two compounds of copper, although these did not differ significantly from each other.

(2) Lard is particularly effective as a "carrier" for both salicylic acid and sulphanilamide.

(3) The experiments have not been sufficiently sensitive to give differences between vehicles or bases large enough to produce significant results in the analysis.

Nevertheless, when account is taken of the manner in which differences between the bases occur (lard was always shown to be more efficient than Emulsifying Ointment B.P. which was always more efficient than water, as a five per cent carboxymethylcellulose gel) it is almost certain that more extensive and refined experiments would show such differences between bases to be significant.

Discussion

Salicylic acid, sulphanilamide, copper acetyl-acetonate and copper sulphate, when applied in lard, Emulsifying Ointment B.P. and water, as a 5 per cent carboxymethylcellulose gel, to the intact skin of rabbits enter the circulation in measurable quantities. The order of efficiency of vehicles or bases as "carriers" remained as found previously[2]; lard proved to be the most efficient and water, as a 5 per cent carboxymethylcellulose

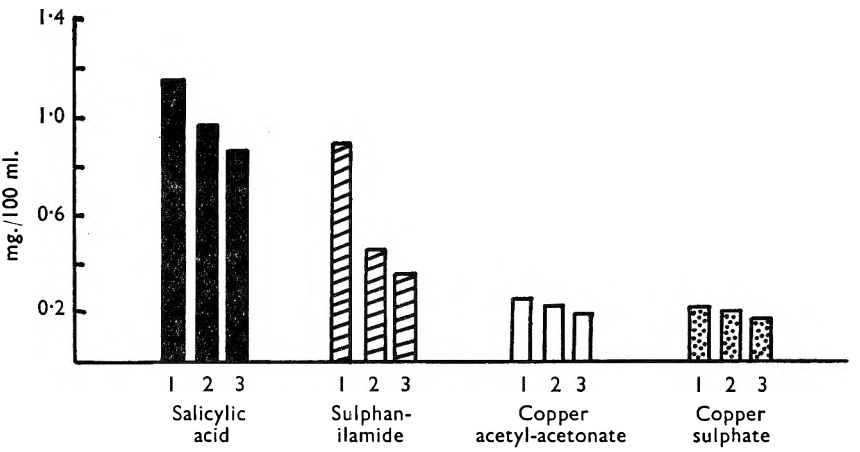

Fig. 1. The mean blood levels of salicylic acid, sulphanilamide, copper acetyl-acetonate and copper sulphate seen in rabbits after their application in lard (1), Emulsifying Ointment B.P. (2) and water (3) as a 5 per cent carboxymethylcellulose gel.

gel, to be the least efficient. It seems that the physico-chemical nature of the applied drugs is of the greatest importance in dictating absorption through the skin. This is clearly illustrated in Figure 1. That these properties outweigh the influence of the vehicle is well shown by a consideration of the data shown in Figure 2. Indeed, it is surprising that the differences in absorption of each drug from water, emulsifying ointment and lard are so small.

The differences in the amounts of drugs absorbed from the three bases may be explained in terms of their local actions and lipid solubilities. Whether other pharmacological properties play a part in the absorption of drugs is a question for further enquiry. Others have suggested that the known irritant and keratolytic action of salicylic acid[7,8] may influence absorption. Certainly in our experiments localised vasodilatation was observed after removal of the salicylic acid preparations

from the backs of the animals. Sulphanilamide is neutral and appears to exert neither an irritant nor an astringent effect when applied topically and probably produces no reactions which would be expected to increase or decrease percutaneous absorption. In contrast, copper is astringent and is used locally for its precipitant action on proteins. Whether this action influences the absorption of copper is still unknown. The slightly higher concentrations of copper acetyl-acetonate may be due to the non-ionic character of the copper which is chelated with acetyl-acetone molecules increasing its lipid solubility in contrast to the lipid insolubility of the

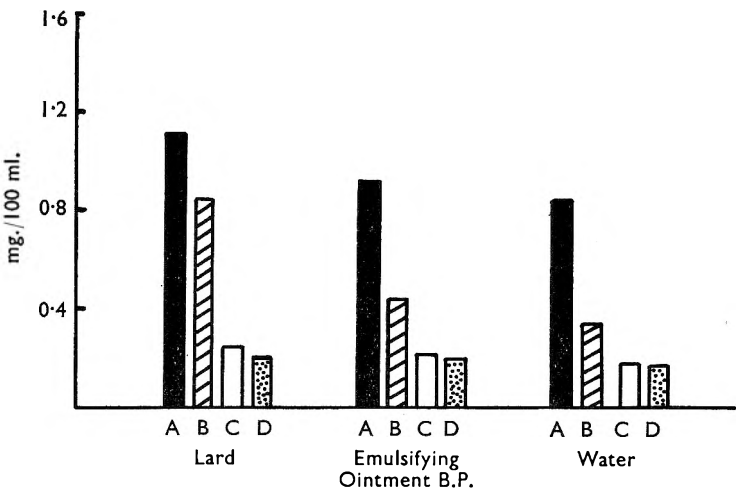

FIG. 2. The mean blood levels of salicylic acid (A), sulphanilamide (B), copper acetyl-acetonate (C) and copper sulphate (D) seen in rabbits after their application in lard, Emulsifying Ointment B.P. and water, as a 5 per cent carboxymethylcellulose gel.

sulphate and so facilitating penetration to the dermal blood supply since passage through the sebum, a mixture of triglycerides, free fatty acids, unsaponifiable matter and cholesterol, at the bases of the follicles may be simplified.

In the present experiments maximum absorption occurred when the drug was presented to the intact skin in an oil phase. Accepting the view that penetration takes place by way of the appendages and that the final barrier to the dermal blood supply is lipoid in nature[9,10], greater absorption would be expected from an oil phase miscible with the skin glycerides and fatty acids and not repelled by this barrier. It may be further postulated that if the mechanism of absorption through the skin depends on the partitioning of the drug across this barrier[11], drugs which are lipid soluble will be absorbed more rapidly than those which are lipid insoluble[12]. The low levels of absorption from water, as a 5 per cent carboxymethylcellulose gel, support the view that absorption through intact rabbit skin is enhanced when the drug is presented to the skin in an oil phase.

These results reinforce the conclusions drawn previously that the intact healthy skin of the rabbit presents a very effective barrier to the passage of drugs applied percutaneously.

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ON THE ASSAY OF THROMBIN PREPARATIONS

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Different techniques of assaying thrombic activity have been compared. The relative strength of three commercial thrombin preparations has been compared with that of an arbitrary standard. Heparinized plasma as used by Burstein and Guinand and TAME, toluene-sulphonyl-arginine methyl ester, as used by Sherry and Troll are both unsuitable as clotting substrates. Only purified fibrinogen can be used. Fibrinogen prepared according to M. and B. Blombäck proved to be suitable, giving only 3–7 per cent error in the thrombin determinations. It is suggested that the strength of the thrombin standard should be expressed by its capacity to split *N*-terminal glutamic acid from fibrinogen with the appearance of *N*-terminal glycine.

Since 1949 a committee has been working on the preparation of a Scandinavian Pharmacopoeia for the three Scandinavian countries and Finland. It is hoped that the first edition will be published within a few years. Simultaneously a subcommittee, Nordisk Biologisk Standardiseringskommitté, NBS, has been engaged in selecting or working out methods for the assay of the activity of biological products, vitamins, hormones, antibiotics, and cardiac drugs, as well as toxicity tests. Also included are assays of heparin and thrombin preparations.

A critical analysis was made in this laboratory of the methods in use for the assay of heparin preparations[1–4] and the results were summarised by one of the present authors[5]. Only whole blood methods using fresh whole blood or blood preserved with sodium sulphate according to the B.P. 1953 method could be recommended, since they gave the same result as *in vivo* tests in sheep. An extended use of the B.P. 1953 method in the routine assay of heparin has since proved it to be comparable to the whole blood method using fresh ox blood. It has therefore been recommended by the subcommittee for the new pharmacopoeia.

This paper is concerned with the determination of thrombin activity. The disagreement in the results obtained with different methods is still more pronounced than with the assay of heparin. The very sensitive enzyme reactions involved in blood coagulation are strongly influenced by the milieu. Hence endeavours should be made to eliminate all external factors and to work with a thrombin-fibrinogen system which is as pure as possible.

Plasma Methods

In the earlier techniques oxalated bovine plasma was commonly used as a clotting medium. Astrup and Darling[6,7], defined as a thrombin unit the amount of thrombin which will clot 1 ml. of oxalated ox plasma in

30 seconds at 37°, the concentration of thrombin in the test being proportional to the reciprocal of the clotting time. Subsequently they found that plasma was not suitable for the purpose. The individual variations were too large and the relation between thrombin concentration and the reciprocal of the clotting time was not always linear. Plasma is, however, still recommended for clinical analyses[8].

For the assay of thrombin preparations of differing strengths and purities, plasma methods cannot be used because of the presence of varying amounts of different less well defined antithrombins in plasma.

Fibrinogen Methods

At an early date Warner, Brinkhous and Smith[9] introduced the use of purified fibrinogen as a clotting medium. The fibrinogen was precipitated from oxalated bovine plasma with 1/3 volume of saturated ammonium sulphate solution, reprecipitated twice and dialysed for 90 minutes against an oxalated saline solution, the final volume being 1/3 of the original plasma volume. They also suggested the use of a clotting interval of 15 seconds as a standard for determining the thrombin unit. The extent to which the unknown solution was to be diluted to give a 15 second clotting time gave a measure of the amount of thrombin present. An interpretation curve was used to correct for deviations of one or two seconds from the 15 second end point. A 15 second coagulation time with a plasma diluted for 1 : 223 corresponded to a concentration of 223 thrombin units/ml. of undiluted plasma.

The technique for the use of purified fibrinogen for the bioassay of thrombin was elaborated by Seegers, Brinkhous, Smith and Warner[10]. The clotting medium contained a stabilising substance, 2 per cent acacia, dissolved in 0·9 per cent physiological saline solution. The pH of the reagent was 7·1 to 7·3 and the temperature 26–29°. Dried thrombin was used as a standard. In studying the details of the method Seegers and Smith[11] demonstrated the difference between plasma and fibrinogen in this respect. Thrombin prepared from bovine plasma did not clot pig plasma as well as it clotted bovine plasma, whereas purified fibrinogen of the two species clotted with equal ease. Some kinds of animal plasmas needed 4 to 5 times more of the bovine thrombin than did the same volume of bovine plasma for coagulation in 15 seconds at 28°.

In 1942 Astrup and Darling[12] used fibrinogen prepared in a number of different ways for the thrombin assay. They found the ammonium sulphate-precipitated bovine fibrinogen to be superior to the Mellanby fibrinogen recovered from the euglobulin fraction. On that occasion they made an extensive study of the different procedures then available for the preparation of fibrinogen. Their technique of assaying thrombin activity was described as follows. To 0·1 ml. of a thrombin solution, 1·0 ml. of ice-cold fibrinogen solution was added and the mixture placed in a water bath at 37°. With the Mellanby fibrinogen no linear relation but a curved line was obtained, whereas the ammonium sulphate-precipitated fibrinogen gave a straight line passing through zero; the thrombin concentration being proportional to the reciprocal of the clotting time.

The Present Work

Before studying the fibrinogen methods we decided to apply one of the plasma methods, that of Burstein and Guinand[13], and the method of Sherry and Troll[14] using toluene-sulphonyl-arginine methyl ester (TAME) as substrate. As expected, neither the plasma method of Burstein and Guinand nor the ester splitting method of Sherry and Troll gave satisfactory responses with the thrombin enzyme. Therefore only fibrinogen methods remained to be tried. For this purpose we selected as substrate the highly purified bovine fibrinogen prepared by Blombäck and Blombäck[15] with their glycine extraction technique. When the clottability with thrombin is 94 to 97 per cent, this fibrinogen may be regarded as free from prothrombin, plasmin and proactivators of plasminogen.

We compared three commercial brands of thrombin: Thrombin Topical (Parke, Davis & Co., Detroit, Mich., U.S.A.) No. 030164–B, Topostasin "Roche" (F. Hoffman-La Roche, Basel) No. B 501087 and Thrombin Upjohn (The Upjohn Company, Kalamazoo, Mich., U.S.A.) No. FA. 620, against a thrombin standard of our own, T 49.

The same number of each brand was used throughout the experiments. In order to prevent inactivation by adsorption to the glass surfaces, all thrombin solutions were stored in siliconised vessels at 0°.

Determination of Thrombin by the Method of Burstein and Guinand

Principle. Thrombin is allowed to act in the presence of heparin upon bovine oxalated plasma whereby the antithrombic activity of the heparin is utilised. To 2 ml. of bovine oxalated plasma is added 1 ml. of a heparinised thrombin solution of varying thrombin concentration. The time necessary for the mixture to reach a certain optical density is noted. A curve is plotted showing the relation between this time and the thrombin concentration of the solution.

Standard preparation. Thrombin T 49. *Material.* Bovine oxalated plasma (1 vol. 0·1M sodium oxalate + 9 vol. blood), Heparin Vitrum 1000 IU/ml., NaCl (0·15M) for dilution of the thrombin and heparin solutions.

Procedure. A Coleman Jr. spectrophotometer is adjusted to zero against a blank consisting of 2 ml. plasma and 1 ml. of 0·15M NaCl at a wavelength of 600 mμ. The optical density of clotted plasma 60 seconds after addition of 1 ml. of T 49 thrombin solution with 60 NIH units/ml. to 2 ml. of oxalated plasma is then determined. The standard preparation, T 49, is then diluted to 60, 57, 54, 51, 48, 45 and 42 NIH units/ml. with 0·15M NaCl, each thrombin dilution containing 1·5 IU heparin/ml.

Determinations are then made of the time necessary for each of the thrombin dilutions to give an optical density of one-third and one-half, respectively, of the known optical density of the plasma clot determined earlier. Each time 1 ml. of thrombin solution is added to 2 ml. of plasma. Similar determination were made with Thrombin Topical, Topostasin Roche, and Thrombin Upjohn, which, on the basis of data on the

ampoules, were diluted with 0·15M NaCl to concentrations of 60, 54, 48 and 42 NIH units/ml., each dilution containing 1·5 IU heparin/ml. All determinations were made at room temperature (20°).

The results are summarised in Figure 1. Since the times were noted for two optical densities, each preparation is represented by two curves (I and II). Figure 1 shows that the method is very sensitive to small differences in thrombin concentration. Evidently the method cannot be used for comparing the activity of different thrombin preparations. One of the preparations, Topostasin "Roche" according to this method was found to be five to ten times weaker than the other two preparations.

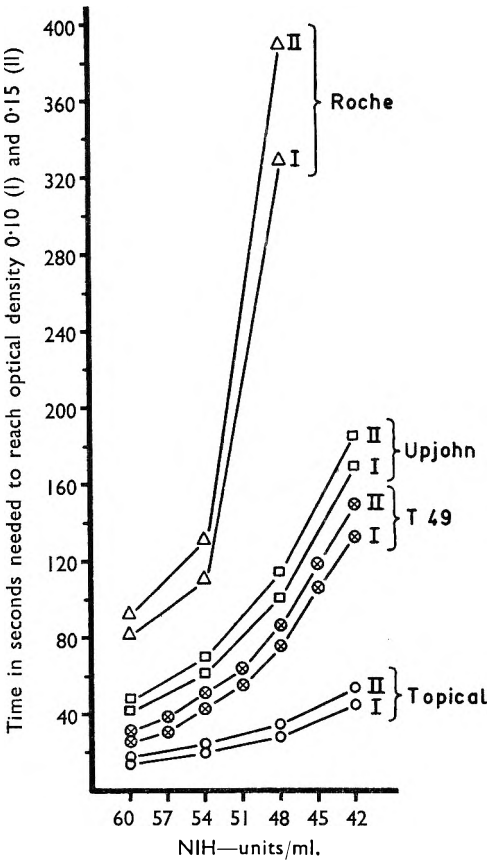

FIG. 1. The assay of the four thrombin preparations with the plasma method of Burstein and Guinand.

Determination of Thrombin According to Sherry and Troll

Principle. Thrombin is allowed to act under definite conditions for a certain time upon TAME (toluene-sulphonyl-arginine-methyl ester). The thrombin activity is directly proportional to the number of released μmols of carboxyl groups. One TAME unit of thrombin is the amount of thrombin which will release one μmol. carboxyl from TAME in 10 minutes under standardised conditions.

Standard preparation. Thrombin T 49. *Material.* TAME (0·2M 6·8 per cent pH 7·0 with NaOH); NaCl (0·15M, 0·88 per cent).

Tris buffer. Tris (hydroxymethyl)aminomethane $(CH_2OH)_3{\cdot}CNH_2$, Sigma 121, Sigma chem. Comp., St. Louis, U.S.A. 1. Tris base (0·3M). 3·64 g. base is dissolved in 100 ml. of 1·75 per cent NaCl. 2. Tris salt (0·3M). 3·64 g. base is dissolved in 30 ml. of N HCl and diluted with water to 100 ml. Tris buffer pH 9 = 90 ml. base + 10 ml. salt.

Procedure

Control of substrate. Eight samples each containing 0·2 ml. of 0·2M TAME + 0·6 ml. of tris buffer (pH 9·0) and 0·2 ml. of Thrombin T 49, diluted to 50 NIH units/ml. with 0·15M NaCl, are incubated at 37°. The reaction is terminated by addition of 1·0 ml. of formalin after 5, 10,

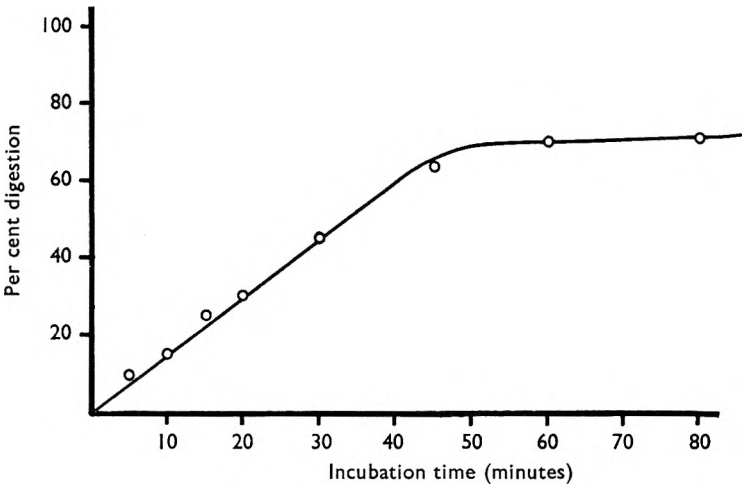

FIG. 2. The relation between the time of incubation and the degree of hydrolysis of the substrate in the TAME method.

15, 20, 30, 45, 60 and 80 minutes, respectively. The number of released μmols carboxyl groups was determined by titration with 0·05N NaOH with 0·01 per cent phenol red as indicator. As a blank, use was made of 0·2 ml. of 0·2M TAME + 0·6 ml. tris buffer + 1·0 ml. of formalin + 0·2 ml. of Thrombin T 49 to 50 NIH units/ml. and titration at time 0.

The digestion of TAME at each interval was calculated in per cent of the amount added (see Fig. 2).

Results. The rate of the reaction is constant up to 70 per cent digestion of the substrate within which region the tests were carried out.

Determination of the Activity of the Different Thrombin Preparations

On the basis of data given on the ampoules the various

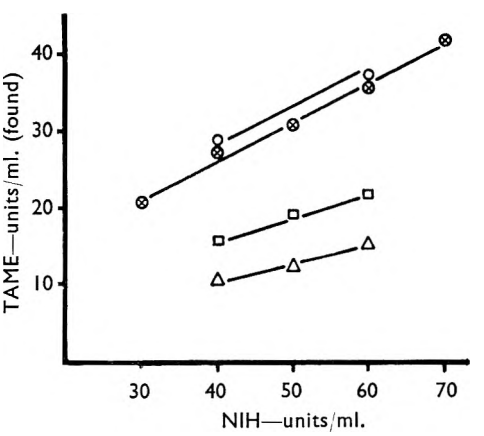

FIG. 3. Assay of the four thrombin preparations with the TAME method of Sherry and Troll.

○ Thrombin Topical ⊗ T 49
□ Upjohn △ Roche

thrombin preparations were diluted to certain definite concentrations in NIH units/ml. A sample of each concentration was incubated for 30 minutes under the same conditions as those described above, the number of released μmols of carboxyl groups was assessed by titration and the number of TAME units/ml. was calculated.

TABLE I

THE TAME METHOD
Incubation time 30 minutes

Date	Preparation	NIH units/ml.	0·05N NaOH consumed ml.		Acid produced μmol.	Digestion per cent	TAME units/ml. found
			Sample	Sample-blank			
15·7	T 49	30	0·650 0·620	0·255	12·75	31·88	21·25
	"	40	0·720 0·710	0·335	16·75	41·87	27·92
	"	50	0·750 0·750	0·370	18·50	46·25	30·83
	"	60	0·825 0·815	0·440	22·00	55·00	36·67
	"	70	0·860 0·910	0·505	25·25	63·12	42·08
	Topostasin "Roche" B 501087	40	0·520 0·530	0·125	6·25	15·63	10·42
	"	50	0·560 0·540	0·150	7·50	18·75	12·50
	"	60	0·600 0·580	0·190	9·50	23·75	15·83
	Thrombin Upjohn FA 620	40	0·600 0·600	0·200	10·00	25·00	16·67
	"	50	0·635 0·635	0·235	11·75	29·38	19·58
	"	60	0·650 0·660	0·255	12·75	31·88	21·25
	Thrombin Topical Parke, Davis & Co. 030174-B	40	0·740 0·740	0·340	17·00	42·50	28·33
	"	60	0·845 0·855	0·450	22·50	56·25	37·50

It is thus apparent that, as judged by this method with T 49 as reference, only Thrombin Topical complies with data given on the ampoules. Topostasin Roche and Thrombin Upjohn give only about 30 per cent and 50 per cent, respectively, of the values given on the ampoules (see Table I and Fig. 3).

Determination of Thrombin using Highly Purified Fibrinogen

Principle. Thrombin is tested for its power to clot a well-defined fibrinogen solution. *Standard preparation.* Thrombin T 49.

Material. Fibrinogen (bovine, prepared according to Blombäck and Blombäck[15]), fraction I–2 with a purity of 94 to 97 per cent with thrombin coagulable fibrinogen.

Method of preparation. Bovine blood was allowed to run into a solution of trisodium citrate (one part 3·8 per cent trisodium citrate ($\times$ $2H_2O$) to 9 parts of blood). The blood cells were separated by centrifugation at 2000 *g* for about 50 minutes.

Fraction I was precipitated by the method of Cohn and others[16]. The plasma is stirred gently and cooled to 0°. The stirring is continued while sufficient sodium acetate-acetic acid buffer in a 53·5 vol. per cent (at 25°) ethanol-water mixture is added through a capillary jet to bring the pH to 7·2 $\pm$ 0·2 and the final ethanol concentration of the system to 8 per cent. During the addition, which takes about 15–25 minutes per litre plasma, the temperature is allowed to fall from 0° to $-$ 3°. This step requires 0·177 l. of 53·3 vol. per cent ethanol (measured at $-$ 5°) per litre of plasma (measured at 0°), and about 1 ml. of 0·8M sodium acetate, adjusted with acetic acid to pH 4·0, is usually sufficient for the pH adjustment. Fraction I consists of between 40 and 50 per cent of thrombin clottable protein. The precipitate is removed by centrifugation at $-$ 3 to $-$ 5° during about 15–20 minutes at 2000 *g*. The precipitate is extracted with an aqueous glycine-citrate-ethanol mixture as described by Blombäck and Blombäck[15].

A 1·6 per cent solution of the fibrinogen in 1·62 per cent (0·055M) trisodium citrate solution (M.V. 294) is freeze-dried. Of the dry powder, which contains 50 per cent trisodium citrate, a 3·2 per cent solution is prepared. This solution has a fibrinogen content of 1·6 per cent and an ionic strength of 0·3.

NaCl (0·30 and 0·15M) 1·75 per cent and 0·88 per cent, respectively.

Tris buffer (0·3M, pH 7·2 = 11 ml. tris base + 89 ml. tris salt).

Siliconised glass vessels, non-siliconised test tubes 60 mm., 9 mm. inner diameter, 10 mm. outer diameter; platinum loops, water bath, stop watches.

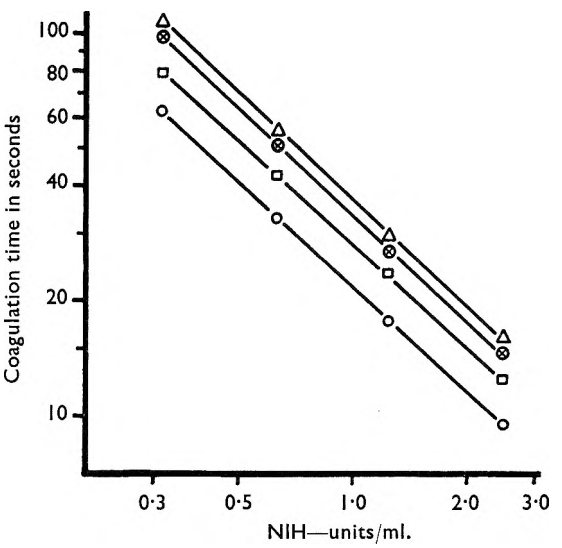

FIG. 4. Assay of the four thrombin preparations using pure fibrinogen.

○ Thrombin Topical ⊗ T 49
□ Upjohn △ Roche

The fibrinogen solution. To 1 part (25 ml.) of the 1·6 per cent fibrinogen solution with the ionic strength of 0·3 is added a mixture of 2 parts (50 ml.) of distilled water and 1 part (25 ml.) of 0·3M tris buffer of pH 7·2. The fibrinogen solution will then contain fibrinogen in a concentration of

0·4 per cent, it will have an ionic strength of 0·15 and a pH of 7·2. The volume is sufficient for testing of 2–4 thrombin preparations against a standard sample.

The thrombin solution. Of the standard preparation, T 49, four dilutions are prepared in siliconised vessels with 0·15M NaCl to concentrations of 25, 12·5, 6·25 and 3·125 NIH units/ml. Corresponding dilutions are made with commercial preparations. Theoretically the clotting time of the strongest solution should not be less than 15 seconds and the corresponding time for the weakest solution should not exceed 110 seconds. In order to avoid inactivation of the thrombin, the dilutions were prepared just before the test and were kept in siliconised vessels at 0°.

Procedure

The determinations were made in a water bath at 37°. The fibrinogen solution and the non-siliconised test tubes were warmed beforehand in the water bath. The various thrombin dilutions were studied separately in series and at least 3 determinations were made of each thrombin dilution.

Exactly 0·1 ml. of diluted thrombin solution was blown into a pre-warmed test tube, 60 × 9 (10) mm., after which 1·0 ml. of pre-warmed fibrinogen solution was added. A platinum loop was slowly moved up and down in the test tube. The interval between the addition of the fibrinogen solution and the visible appearance of fibrin threads caught by the platinum loop was measured with a stopwatch.

Results. The results are summarised in Figure 4. The curves represent the relation between logarithms of the clotting times and the logarithms of the concentrations of the thrombin. In contrast to what was found with the use of the plasma and TAME methods in their relation to T 49 none of the preparations are seen to differ appreciably from what might be expected from the data given on the ampoules.

Discussion

The wide differences in the effect of the different thrombin preparations on heparinised oxalated plasma in Burstein and Guinand's method prohibit the use of the latter.

A comparison between the fibrinogen and TAME methods clearly showed that the activity of the different thrombin preparations in a TAME system is not proportional to their activity in a fibrinogen system. This is due to the presence in varying amounts of esterases in the different thrombin preparations, as pointed out by Landabury and Seegers[17] in 1957. Two preparations, Roche and Upjohn, which in Blombäck and Blombäck's fibrinogen method agree fairly well with the standard preparation T 49, show only 30 per cent and 50 per cent respectively of the activity of T 49, as measured by the TAME method. Consequently, the TAME method is not suitable for the biological standardisation of thrombin preparations.

The only technique that remains is that using fibrinogen. The fibrinogen method works with pure fibrinogen and a system with the ionic

strength, pH and temperature well defined. A statistical analysis of the results shows a quite insignificant margin of error.

An analysis of the data given in Figure 4 showed that the thrombin activity of Topostasin Roche (No. B 501087) corresponded to 60 per cent, that of our standard preparation T 49 to 64 per cent and that of Thrombin

TABLE II

THE FIBRINOGEN METHOD OF BLOMBÄCK AND BLOMBÄCK

Sample	NIH units/ml.	Coagulation time seconds		
T 49	20	16·1	17·2	17·0
	10	27·2	30·0	29·5
	5	52·0	53·0	51·0
"Roche" B 512107	20	15·8	18·0	16·7
	10	29·6	26·5	30·5
	5	50·2	50·6	52·0
Topical Parke Davis 030566 B	20	11·5	11·7	12·6
	10	20·0	18·5	20·1
	5	36·4	36·0	36·8

Upjohn (Fa 620) to 78 per cent of the activity of Thrombin Topical, Parke Davis & Co. (No. 030164 B) all of them within the limits of error of 97 to 103 per cent.

On another occasion, one year later a comparison was made between T 49 and new batches of Topostasin Roche (No. B 512107) and of

TABLE III

STANDARD: THROMBIN TOPICAL (030566 B). TEST: TOPOSTASIN ROCHE (B 512107)

Groups	S_1 5 U/ml.	S_2 10 U/ml.	S_3 20 U/ml.	T_1 5 U/ml.	T_2 10 U/ml.	T_3 20 U/ml.	Sums
1	1·5611	1·3010	1·0607	1·7007	1·4713	1·1987	8·2935
2	1·5563	1·2672	1·0682	1·7042	1·4232	1·2553	8·2744
3	1·5658	1·3032	1·1004	1·7160	1·4843	1·2227	8·3924

Adjustment for mean $= \frac{623{\cdot}01658}{18} = 34{\cdot}612032$

Analysis of variance:

Variation due to	d.f.	Sum of squares	Mean square	Variance ratio
Between doses ..	5	0·80933 (1)	0·161866	381·8
Between groups ..	2	0·00133 (2)	0·000665	1·57
Error	10	0·00424	0·000424 = s^2	
Total ..	17	0·8149011 (3)	s = 0·02059	

(1) $\frac{106{\cdot}26410783}{3} - 34{\cdot}612032 = 35{\cdot}42136 - 34{\cdot}612032 = 0{\cdot}80933$

(2) $\frac{207{\cdot}68021537}{6} - 34{\cdot}612032 = 34{\cdot}61336 - 34{\cdot}612032 = 0{\cdot}00133$

(3) $35{\cdot}42693313 - 34{\cdot}612032 = 0{\cdot}8149011$

Thrombin Topical, Parke Davis & Co. (No. 030566 B). This time the figures found were for Topostasin Roche 64 per cent and for T 49, 64 per cent of that of Thrombin Topical, Parke Davis & Co. The limits of error were 94–107 per cent (P = 0·05). (See Tables II, III and IV.)

TABLE IV

FACTORIAL ANALYSIS (3 × 3)

Variation due to	Factorial coefficients for dose						(1) Divisor $n \cdot S(x^2)$	(2) Sum of products $S(x \cdot T_d)$	(3) Mean square $\frac{2}{1}$ (fg = 1)	(4) Variance ratio
	S_1	S_2	S_3	T_1	T_2	T_3				
1. Differences between preparations ..	−1	−1	−1	+1	+1	+1	18	1·3925 = T_a	0·107725 = D^2	254·1
2. Slope	−1	0	+1	−1	0	+1	12	−2·8981 = T_b	0·699915 = B^2	1651
3. Parallellism	+1	0	−1	−1	0	+1	12	0·0097	0·0000078	0·018
4. Curvature	+1	−2	+1	+1	−2	+1	36	0·2097	0·0012215	2·881
5. Departure from curvature	−1	+2	−1	+1	−2	+1	36	−0·1297	0·000467	1·1014
Total effect per dose = T_d	4·6832	3·8714	3·2293	5·1209	4·3788	3·6767				

$n = 3$
$i = 0{\cdot}30103$
$N = 18$
$t = 2{\cdot}228$ $\quad (P = 0{\cdot}05;\ d.f. = 10)$ $\quad t^2 = 4{\cdot}9640$

$$M = \frac{4.i.T_a}{3 \cdot T_b} = \frac{4.0{\cdot}30103.1{\cdot}3925}{3\,(-2{\cdot}8981)} = \frac{1{\cdot}676737100}{-8{\cdot}6943} = -0{\cdot}1928547 = 0{\cdot}8071453 - 1$$

Antilog: 0·64141 or 64 per cent

$$b = \frac{T_b}{4.i.n} = \frac{-2{\cdot}8981}{4.0{\cdot}30103.3} = \frac{-2{\cdot}8981}{3{\cdot}61236} = -0{\cdot}8022733$$

$$s^2_M = \frac{s^2}{b^2}\left[\frac{4}{N}\left(1 + \frac{D^2}{b^2 - s^2.t^2}\right)\right] = \frac{0{\cdot}000424}{0{\cdot}643641966529}\left[\frac{4}{18}\left(1 + \frac{0{\cdot}107725}{0{\cdot}699915 - 0{\cdot}002104736}\right)\right]$$

$$= 0{\cdot}0006588\ [0{\cdot}2222(1{\cdot}15438)] = 0{\cdot}0001464.1{\cdot}15438 = 0{\cdot}0001690$$

$$t.s_M = 2{\cdot}228\ \sqrt{0{\cdot}000169} = \pm \underset{(0{\cdot}971036-1)}{0{\cdot}028964}$$

Antilog: 1·0690 resp. 0·9355 or 94 per cent—107 per cent

A New Principle for the Determination of the Strength of the Standard Thrombin

Since we now have good knowledge about the chemical processes taking place in the transformation of fibrinogen to fibrin, there seems to be every reason for measuring the thrombin activity by means of a chemical titration. The proteolytic liberation of fibrinopeptides from fibrinogen is accompanied by the appearance in the solution of *N*-terminal glycine. As shown by Bettelheim and Bailey[18], two peptides A and B are liberated from ox fibrinogen, the first one containing the *N*-terminal glutamic acid of fibrinogen. During the initial phase of thrombin action *before any clotting of fibrin takes place*, there is a linear relation between the incubation time and the amount of *N*-terminal glycine appearing in the solution[19] (see Fig. 5).

As shown later by Blombäck and Vestermark[20], only peptide A is liberated during this phase. It could furthermore be demonstrated[21] that the appearance of *N*-terminal glycine in the "soluble fibrin" was followed by a stoichiometric disappearance of glutamyl residues from the fibrinogen (see Fig. 6).

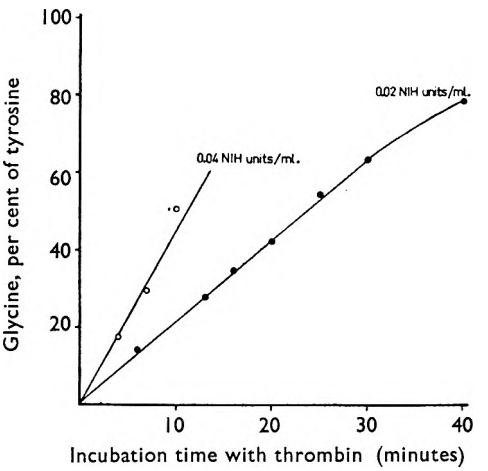

Fig. 5. Appearance of *N*-terminal glycine in the fibrinogen solution at different enzyme concentrations. ●, 0·02 NIH units/ml.; ○, 0·04 NIH units/ml.

There is consequently a possibility of expressing the proteolytic phase of the thrombin action in chemical terms, for example, as μmols of *N*-terminal glycine deriving from pure ox fibrinogen under well defined experimental conditions. Blombäck and Yamashina[19] and Blombäck[21] found that one unit of our thrombin sample T 49 on two different occasions released 0·1 and 0·09 μmols of *N*-terminal glycine from 75 mg. of fibrinogen in 10 minutes as measured with Edman's PTH method.

The fibrinogen used for the assay must be free from prothrombin. In these analyses it should be dissolved in a sodium chloride solution, not in the citrate solution used by Blombäck and Blombäck[15]. The citric acid dissolves in ethyl acetate together with the phenylthiohydantoins of the *N*-terminal amino acids. The experimental conditions governing the substrate and the enzyme concentration, the ionic strength, the pH and the temperature were studied in detail by Blombäck[21].

The Rate of Appearance of N-terminal Glycine in a Fibrinogen-thrombin System

Fibrinogen. The preparation of fibrinogen coagulable with thrombin in excess of 98 per cent has been described by Blombäck and Blombäck[15].

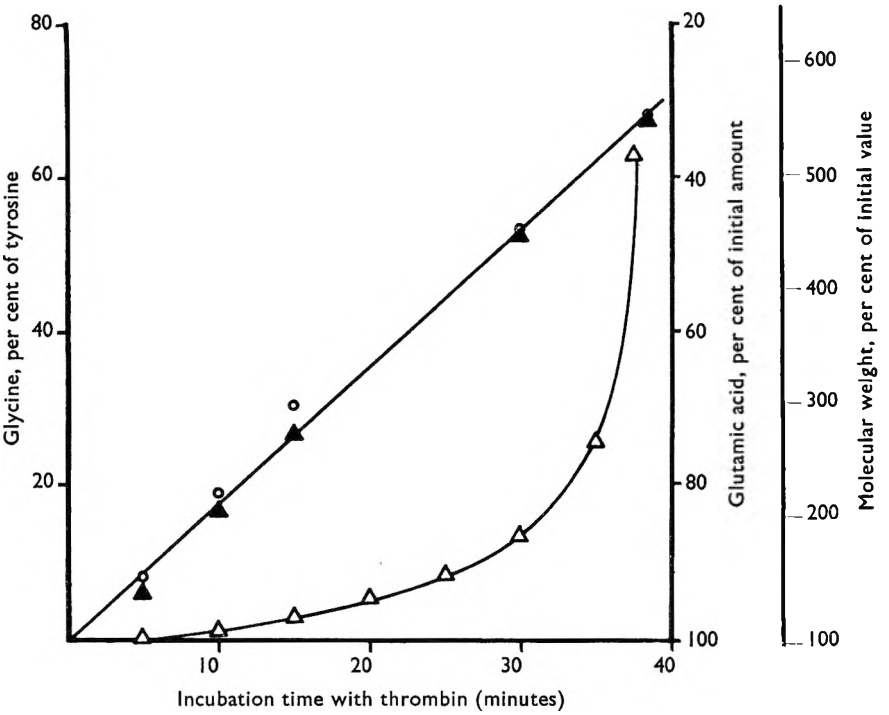

FIG. 6. Changes in *N*-terminal amino acids in the fibrinogen and the increase in light-scattering after addition of thrombin. ○, gly; ▲, glu; △, molecular weight in per cent of initial value.

The fibrinogen made free of glycine through dialysis against three changes of 0·3M sodium chloride at + 5° during 24 hours is diluted with a 0·3M NaCl solution to a protein concentration of 0·5 per cent.

Thrombin. A solution of thrombin containing 1·8 NIH units/ml. is made up in a siliconized glass vessel. The solution is to be used within one hour.

Buffer. A tris (tris(hydroxymethyl)aminomethane)-imidazol buffer is used. The following stock solutions were prepared:

I. A mixture of equal volumes of 0·1M imidazol hydrochloride and 0·1M tris-hydrochloride.

II. A mixture of equal volumes of 0·1M imidazol and 0·1M tris base, both in 0·1M sodium chloride.

These two stock solutions were then mixed in such proportions that a pH of 9·0 is obtained. After dilution of this final buffer with an equal volume of 0·2M sodium chloride, the ionic strength was 0·15.

Procedure. Into several beakers of the same size was put 15 ml. fibrinogen solution (0·5 per cent). Thirty ml. of trisimidazol buffer of pH 9·0 and ionic strength 0·15 were then added. Temperature 20°. The reaction was started by adding thrombin to a concentration between 0·02 and 0·04 NIH units/ml. The reaction was stopped at different intervals (e.g. 5, 10, 15 and 20 minutes) by coupling with phenylisothiocyanate in pyridine (45 ml. pyridine and 2·25 ml. phenylisothiocyanate). The coupling was complete within two hours. The precipitation of the PTC protein as

well as the further treatments including the chromatographic analyses were described in detail by Blombäck and Yamashina[19].

The *N*-terminal glycine is expressed in μmoles. The blank value obtained from the zero time determination is substracted.

Irrespective of the fact that the fibrinogen method described here for the determination of thrombin activity is easy to perform and works with a very small margin of error (3–7 per cent), it may be of value to have a reference standard of thrombin whose activity is checked by chemical means and expressed in chemical terms. A suitable entity would be the equivalent of one or one and a half μmols of *N*-terminal glycine released from pure ox fibrinogen under strictly defined experimental conditions. Of course the same accuracy is not to be expected with this technique as with the coagulation method. The relation between Topostasin Roche, T 49 and Thrombin Topical was namely 68·5 ; 70 : 100, whereas the coagulation method gave a relation of 64 : 64 : 100. For routine work therefore the coagulation technique is still to be recommended, but the chemical titration may have some value for the expression of the strength of the international thrombin standard.

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A NOTE ON THE ASSAY OF SOME SULPHYDRYL COMPOUNDS

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An assay of thioglycollic acid has been developed using potassium iodate solution which is considered to be as accurate as the Pharmacopoeial method. The proposed method has been applied to the assay of cysteine hydrochloride, dimercaprol and glutathione.

During an investigation of the antagonism of mercurial bacteriostats by sulphydryl-containing compounds a suitable rapid and simple method of assay of these compounds was required. The requirements were that its accuracy should be of the same order as that of other methods of assay, and it should be applicable to aqueous solutions of the compounds and their sodium salts.

Thioglycollic Acid

Fresh commercial samples of thioglycollic acid are liable to contain small quantities of dithiodiglycollic acid and dithioglycollide.

Older, or improperly stored, samples may contain appreciable amounts of dithiodiglycollic acid and possibly some 2:2:5:5-tetracarboxymethylmercapto-1:4-dithiane[2].

The assay of thioglycollic acid, described in Appendix I of the British Pharmacopoeia 1958 (p. 800), is in two stages, determining both functional groups; the acidity being determined by titration with sodium hydroxide solution and, after neutralisation, the sulphydryl content by titration with iodine solution in the presence of excess sodium bicarbonate. The colour of the indicator (cresol red) remaining after neutralisation and addition of the sodium bicarbonate makes the use of starch mucilage imperative in the second part of the assay. The titration with sodium hydroxide solution will determine all material having titratable –COOH groups, i.e., thioglycollic, thiodiglycollic and dithiodiglycollic acids and the tetracarboxy acid; whereas the titration with iodine solution will determine only sulphydryl-containing substances, viz. thioglycollic acid.

In a sample of thioglycollic acid which has deteriorated the apparent $HS{\cdot}CH_2{\cdot}COOH$ content, by alkali titration, will be high whilst that indicated by iodimetric titration will be lower[2]. Since the Pharmacopoeia gives no upper limit for the $HS{\cdot}CH_2{\cdot}COOH$ content of samples, the object of the first stage of the assay is not apparent, other than as a test for the absence of salts of thioglycollic acid. A further factor not taken into account by the official method is temperature, which is reported[1] to markedly affect the iodine consumption of thiol acids, the theoretical iodine consumption occurring only at or near 0°.

To determine the $HS{\cdot}CH_2{\cdot}COOH$ content of thioglycollic acid directly and rapidly, the following method is suggested:

Dissolve about 0·3 g., accurately weighed, in 20 ml. of water, add 2 ml.

of glacial acetic acid and 0·2 g. of potassium iodide. Shake gently until solution is complete and titrate with M/60 potassium iodate solution until a faint permanent yellow colour is obtained. The addition of starch mucilage is unnecessary. Each ml. of M/60 potassium iodate is equivalent to 0·009212 g. of thioglycollic acid.

Three different samples of thioglycollic acid were assayed by both the Pharmacopoeial and the above methods, with the results shown in Table I.

TABLE I

PERCENTAGE (W/W) THIOGLYCOLLIC ACID WITH (IN PARENTHESES) STANDARD DEVIATION

Sample	B.P. method		Potassium iodate method
	(i) Alkimetric	(ii) Iodimetric	
A	90·92 (0·06)	86·46 (0·19)	86·18 (0·02)
B	90·43 (0·12)	84·77 (0·36)	84·33 (0·01)
C	93·93 (0·03)	90·40 (0·13)	89·55 (0·06)

RESULTS AND DISCUSSION

From the results in Table I it can be seen that the iodine titrations give results slightly higher than those obtained by potassium iodate titration. This is believed to be due to the formation of a small amount of thioglycollic acid or *S*-thioglycollylthioglycollic acid or both, by hydrolysis of dithioglycollide, reactions which can occur at room temperature in the presence of alkali[2].

Hydrolysis of the dithioglycollide may be expected to occur during the official assay where alkali is present, and since the extent of hydrolysis occurring will vary with the time taken to complete the assay, replicate titrations will possibly give inconsistent results.

The larger standard deviations obtained with the iodimetric titrations are taken as indicative of this hydrolysis.

With the potassium iodate method, an acid reaction prevails and no hydrolysis of dithioglycollide occurs, resulting in a greater reproducibility in replicate determinations, as shown by the smaller standard deviations.

A further advantage of the proposed method lies in the greater stability of potassium iodate solution compared with iodine solution, and in the case of assay of preparations such as ammonium or sodium thioglycollate.

The iodate titration can, if required, be used in place of iodine in the second stage of the Pharmacopoeial assay, similar results being obtained by either method. In this case the use of starch mucilage is necessary. Using the equation

$$pH = 1/2\ pKw + 1/2\ pKa + 1/2 \log C$$

it may be calculated that the neutralised thioglycollic acid at the completion of the first stage of the official assay has a pH of ca. 8·3.

No suitable indicator has been found for the neutralisation which has an exponent of this order and is colourless in acid solution. Such an

indicator would obviate the need for starch mucilage in the second part of the assay. Phenolphthalein has been shown[3] to be unsuitable for the alkimetric titration of thioglycollic acid, since ionisation of the sulphydryl group occurs about the pH value at which the indicator becomes coloured.

Thymolphthalein may be expected to behave similarly since its titration exponent is of the same order of that of phenolphthalein.

It is considered, however, that if both the alkimetric and the potassium iodate titrations are to be carried out, separate samples should be used for each determination.

Application to other Sulphydryl Compounds

Cysteine hydrochloride. This has been introduced into the B.P. 1958 (p. 956) as an inactivator for streptomycin and dihydrostreptomycin in Tests for Sterility of preparations containing these substances. The recommended assay (B.P. 1958, p. 751) for this material is by a Kjeldahl determination of nitrogen, a lengthy and complex procedure. Further, any cystine formed by oxidation will also be determined and hence the true cysteine content may be lower than indicated by the assay results.

By the use of potassium iodate titration, this is obviated and the time required for assay reduced to a minimum.

The weight of cysteine hydrochloride suggested for assay by the method described is about 0·4 g. Each ml. of M/60 potassium iodate is equivalent to 0·01576 g. of cysteine hydrochloride.

Both pH and temperature are reported[1] to affect the amounts of iodine combining with cysteine and it is believed that the presence of potassium iodide in the cysteine solution can reduce this error. Lucas and King[1] reported the quantitative determination by indirect iodine titration (excess iodine and back titration with sodium thiosulphate) at 0° and normal acidity.

Glutathione. Lucas and King[1] observed that glutathione could be determined quantitatively by iodimetric titration only at temperatures below 25° and reactions below pH 5. The potassium iodate method has however been satisfactorily applied to the determination of this material. The method may be applied to the determination of small quantities of glutathione on a semi-micro scale, using M/600 potassium iodate solution. The weight of glutathione suggested for assay is 20 to 50 mg. Each ml. of M/600 potassium iodate is equivalent to 0·003073 g. of glutathione.

Dimercaprol. Potassium iodate titration has been satisfactorily applied to dimercaprol which is at present officially assayed (B.P. 1958, p. 227) by titration with N/10 iodine solution. About 0·2 g. of sample is taken and each ml. of M/60 potassium iodate is equivalent to 0·006211 g. of dimercaprol. The insoluble disulphide of dimercaprol produced during the titration does not interfere with the visibility of the end point.

References

1. Lucas and King, *Biochem. J.*, 1932, **26**, 2076.
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3. Larsson, *Z. anal. Chem.*, 1929, **79**, 170.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Cinchophen, Determination of, in the Presence of Aspirin, Sodium Bicarbonate, Caffeine, Hexamine and Lactose. W. Kamp and J. W. Kampman. (*Pharm. Weekbl.*, 1958, **93**, 545.) Since the official methods for the assay of cinchophen in the British Pharmaceutical Codex and Danish Pharmacopoeia are not applicable in the presence of the above-named substances, the following method is proposed:—The sample is extracted with an aqueous sodium carbonate solution and the filtrate evaporated to dryness on a water bath. The residue is then dissolved in a little glacial acetic acid, and after warming to almost boiling point, a known amount of 0·1N iodine solution is added with constant shaking. After cooling the liquid in the flask is made up to a known volume, and the excess iodine is determined in an aliquot portion of the filtrate by titration with 0·1N sodium thiosulphate solution. Cinchopen forms a black precipitate of formula $(C_{16}H_{11}NO_2)_2 \cdot HI \cdot I_3$ so that 1 g. atom of iodine is equivalent to 2/3 mol. phenylcinchoninic acid. Results are still on the high side (up to 2·8 per cent high), but the method seems the most satisfactory. D. B. C.

Digitalis Glycoside Mixtures, Quantitative Determination of, by Paper Chromatography. L. Fuchs, M. Wichtl and H. Jachs. (*Arch. Pharm. Berl.*, 1958 **291**, 193.) The authors report conditions suitable for a quantitative estimation by paper chromatography of the most important glycosides in digitalis leaves or extracts thereof using the Baljet reaction (i.e., using as the reagent a solution containing 95 volumes of 1 per cent picric acid in water and 5 volumes of 10 per cent NaOH solution in water). The molar extinction coefficients of different cardio-active glycosides and aglycones were determined using the pure substances for comparison, measurements being taken 20 minutes after the addition of the reagent to obtain the maximum colour intensity. For the separation by paper chromatography a solvent mixture of chloroform, tetrahydrofuran and formamide in the ratio 50 : 50 : 6·5 was used for the determination of the primary glycosides and mixture of xylene and methylethyl ketone, ratio 1 : 1, saturated with formamide was used for the determination of secondary glycosides. It was found necessary to add formamide to the glycoside solutions which were transferred to the paper to avoid certain disturbances in the separation. Examples are reported for the quantitative determination of lanatoside A, B and C, strospeside, acetyldigitoxin, acetylgitoxin, acetyldigoxin and digoxin besides small quantities of digitoxigenin and gitoxigenin and the sum of water-soluble glycosides (digitalinum verum, gitorin and lanatoside D, etc.) in the leaves of *Digitalis lanata* and purpurea glycosides A and B, glycogitaloxin, strospeside, verodoxin, gitaloxin, digitoxin, gitoxin besides a small amount of digitoxigenin and the sum of water-soluble glycosides in the leaves of *Digitalis purpurea*. The small amount of aglycone is attributed to the unfavourable drying conditions employed for both drugs. Two other examples are given. A control determination of the total cardiac glycosides, calculated as digitoxin, was performed on the starting solution before chromatographic separation, and this corresponded well with the result calculated from the sum of the extinction coefficients of the separated substances. D. B. C.

Polyoxypropylene Glycols, Analysis of. E. H. Vogelenzang and D. J. Stöver. (*Pharm. Weekbl.*, 1958, **93**, 550.) Determination of the hydroxyl content by acetylation with acetic anhydride and pyridine (used for polyoxyethylene glycols) gives very high results when applied to polyoxypropylene glycols due to hydrolysis of ether bonds adjacent to methyl groups. The following method using propionic anhydride and *p*-toluene sulphonic acid as catalyst was found to give more satisfactory results:—About ½ millimol of polyoxypropylene glycol, accurately weighed, is transferred to a dry conical flask with a ground glass stopper together with a known amount of a reagent containing propionic anhydride and *p*-toluene sulphonic acid. After standing 30 minutes at 20°, an accurately measured excess of a standard solution of aniline in dry benzene is added. After 15 minutes the excess aniline is titrated with standard perchloric acid in glacial acetic acid using a solution of crystal violet as indicator which changes from blue-green to green. The whole operation is repeated without the sample. It is also necessary to determine the water content of the material by the Karl-Fischer method and to apply a correction.

D. B. C.

Reserpine, Deserpidine and Rescinnamine, Separation and Determination of, by Partition Column Chromatography. A. L. Hayden, L. A. Ford and A. E. H. Houk. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 157.) The suggested method is suitable for determination of the purity of crystalline specimens of the alkaloids, and for the analysis of commercial mixed alkaloids. Separation is accomplished by chromatography on a column of diatomaceous silica (Celite 545), using formamide as immobile solvent, and eluting with a mixture of heptane 715, chloroform 110, morpholine 1. Deserpidine is eluted first, followed by reserpine and finally rescinnamine. The quantity of each alkaloid in solution can be estimated by measurement of the ultra-violet absorption of the appropriate fraction against a solvent blank, at the maxima at 272 mμ for deserpidine, 267 and 295 mμ for reserpine and 304 mμ for rescinnamine. The result is calculated by comparison with the absorption of standard solutions. The method is sufficiently sensitive to detect 1–5 per cent of one of the alkaloids occurring as an impurity in another. G. B.

Strychnine and Brucine, Separation of, by Paper Chromatography. G. Dušinský and M. Tyllová. (*Nature, Lond.*, 1958, **181**, 1335.) A new paper chromatographic procedure for the separation of strychnine and brucine is described, based on the finding that small amounts of brucine (but not of strychine) are quantitatively oxidised in 4N nitric acid to *o*-brucichinone. An aqueous or ethanolic solution (1 ml.) containing not less than 1 mg. of strychnine and brucine is mixed with concentrated nitric acid (d = 1·41; 0·3 ml.), the acid neutralised after 1 minute by addition of sodium hydroxide solution (2 ml., 15 per cent), and the pH finally adjusted to 4–5 with glacial acetic acid. The solution (equivalent to 30–100 μg.) is spotted on to strips of Whatman No. 1 filter paper, which have been impregnated with formaldehyde-methanol (1 : 1) and chromatographed (descending technique) with butanol-acetic acid-water (4 : 1 : 5) at 22° for about 6 hours. After drying the chromatogram, strychnine (R_F 0·76) is detected with Dragendorff's reagent, brucichinone (R_F 0·1) appearing as a red spot near the starting line. The method can be applied quantitatively as an assay for strychnine. J. B. S.

ORGANIC CHEMISTRY

Dihydro-6-desoxy Morphine, a New Preparation of. R. Bognár and S. Makleit. (*Arzneimitt.-Forsch.*, 1958, **8**, 323.) 3-Acetyldihydromorphine was first produced either by the hydrogenation of morphine in the presence of palladium to give dihydromorphine followed by acetylation of the phenolic hydroxy group with actic anhydride and sodium bicarbonate, or by acetylation as described to give 3-acetylmorphine and subsequent hydrogenation with a palladium catalyst. Both processes gave excellent yields. The 6-tosyl derivative was then formed by the reaction of 3-acetyldihydromorphine in pyridine at room temperature with *p*-toluene sulphonyl chloride. This, on reacting with lithium aluminium hydride in tetrahydrofuran solution, underwent deacetylation and removal of the tosyl group which was replaced by hydrogen giving the desired dihydro-6-desoxy morphine. In addition to ultra-violet data, this was characterised by methylation with diazomethane to give dihydro-6-desoxy codeine which was also produced by hydrogenation of the known compound α-chlorocodid. D. B. C.

***iso*Nicotinic Acid Hydrazide, New Method of Preparation.** H. B. Thomasen. (*Acta chem. scand.*, 1957, **11**, 1787.) A new synthesis of *iso*nicotinic acid hydrazide in 90 per cent yield is described in which *iso*nicotinic acid hydrazonium salt is submitted to azeotropic distillation with a suitable water entrainer, such as amyl alcohol or xylene. J. B. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Adrenal Cortical Hormones and Formation of Histamine and 5-Hydroxytryptamine. R. Hicks and G. B. West. (*Nature, Lond.*, 1958, **181**, 1342.) The effects of cortisone and certain of its analogues on the tissue levels of histamine and 5-hydroxytryptamine have been studied in rats. Histamine-levels in the skin areas of rats treated for 14 days with cortisone or hydrocortisone (50 mg./kg./day) fell to about half those of animals in control groups. Degranulation of skin mast cells was noticeable, but no disruption of the cell membrane. In the jejunum and spleen only histamine depletion was detected. Deoxycortone acetate failed to alter the tissue levels of either of the amines, or the morphology of the mast cells. The recovery rate of tissues depleted of histamine by treatment with Polymixin B was greatly retarded by administration of cortisone, whilst the effect on tissue 5-hydroxytryptamine was even more pronounced. Concentrations of the latter in the skin areas (only) fell to about 20 per cent of that in controls—a fall comparable with that found after treatment with reserpine. Deoxycortone acetate showed no such effect. It is suggested that cortisone and other glucocorticoids lower the activity of histidine decarboxylase and 5-hydroxytryptophan decarboxylase, the mineralocorticoids being devoid of such action. J. B. S.

Antigen-antibody Complexes, Soluble, Production of Anaphylaxis in the White Mouse with. S. Tokuda and R. S. Weiser. (*Science*, 1958, **128**, 1237.) A soluble antigen-antibody complex was prepared by adding heat inactivated rabbit anti-serum to excess of antigen (bovine serum albumin), the mixture then

being incubated and centrifuged. When injected intravenously into mice this complex produced death in 90 per cent of the animals with all the symptoms of anaphylaxis. One-fifth of this toxic dose of complex killed only about 28 per cent of the mice but was 100 per cent lethal to mice which had received *Haemophilus pertussis* vaccine 5 days previously. It is well known that such pertussis treatment renders mice highly susceptible to active and passive anaphylaxis induced by the usual methods. The fact that anaphylaxis produced with the soluble antigen-antibody complex was typical in all respects, suggests that the site of antigen-antibody action and the ensuing events leading to anaphylaxis are similar regardless of whether shock is induced in the usual manner by injection of antigen or by injection of the soluble preformed antigen-antibody complex. The speed and severity of the shock is probably determined by the rate at which the preformed complex reaches the site where antigen-antibody injury is produced. This work represents a fresh approach for investigating the mechanism of anaphylaxis in the mouse but care is necessary before applying these results to anaphylaxis in other species. W. C. B.

m-Methoxynoradrenaline, Presence, Formation and Metabolism of, in the Brain. J. Axelrod. (*Science*, 1958, **127**, 754.) Chromatographic studies on extracts of the brains of rats indicated that noradrenaline is metabolised in the rat brain to *m*-methoxynoradrenaline (normetanephrine) and that this reaction requires *S*-adenosylmethionine as the methyl donor. The *m*-methoxynoradrenaline is then deaminated by the brain mitochondria and oxidised to form 3-methoxy-4-hydroxymandelic acid. W. C. B.

BIOCHEMICAL ANALYSIS

Adrenaline and Noradrenaline in Plasma, Fluorimetric Determination of. R. Robinson and F. D. Stott. (*Biochem. J.*, 1958, **68**, 28P.) The authors have modified the method of Euler and Floding, and using a fluorimeter constructed for high sensitivity were able to determine the two amines at concentrations as low as 0·2 μg./l. of adrenaline and 0·3 μg./l. of noradrenaline. By use of a differential oxidising procedure both adrenaline and noradrenaline in mixtures can be determined. The amines are isolated from plasma on micro columns of alumina. Aliquots of the eluate buffered to pH 3·5 and 6·0 are treated with potassium ferricyanide solution, when at pH 3·5 all the adrenaline but only about 4 per cent of noradrenaline is oxidised, while at pH 6·0 both amines are quantitatively oxidised to the corresponding chromes. Alkali and ascorbic acid convert the chromes to trihydroxyindoles and stabilise them, when the fluorescence can be measured. Prevention of the disintegration of platelets was found to lower the adrenaline and noradrenaline concentration, which is in harmony with the view that the platelets may act as vehicles for transport of the amines. J. R. F.

3-Hydroxytyramine, Presence of, in Brain. A. Carlsson, M. Lindqvist, T. Magnusson and B. Waldeck. (*Science*, 1958, **127**, 471.) The study of 3-hydroxytyramine has been hampered by the lack of sensitive and specific assay methods. The authors have observed that if the pH of samples prepared essentially according to the fluorimetric method of Euler and Floding was adjusted to about 5 with acetic acid the fluorescence for 3-hydroxytyramine, weak with the original method, is enhanced. Further, the activation and fluorescent peaks were at much shorter wavelengths that those from adrenaline and noradrenaline, which therefore do not interfere. Using this method, together with ion exchange chromatography, 3-hydroxytyramine has been

found in the rabbit brain at a concentration of 0·4 μg./g., which is roughly equal to the noradrenaline content. Intravenous injection of reserpine (5 mg./kg.) caused almost complete disappearance of 3-hydroxytyramine from brain, and an intravenous injection of the precursor 3:4-dihydroxyphenylalanine (5 mg./kg.) caused a marked increase, accompanied by central excitation. Both phenomena were enhanced by iproniazid. J. R. F.

PHARMACOLOGY AND THERAPEUTICS

Aluminium Glycinate in Peptic Ulcer. T. Clark and J. N. Hunt. (*Practitioner*, 1958, **180**, 334.) A comparison *in vitro* of the antacid action of magnesium oxide and an aluminium glycinate preparation showed that the aluminium compound, even in gross excess, did not, in contrast to magnesium oxide, raise the pH of the gastric contents above that of the plasma. Tests in four patients with duodenal ulcers showed that the continuous sucking of four tablets of an aluminium glycinate preparation an hour raised the pH of the gastric contents to 4 but did not carry it beyond pH 7. On the other hand, intermittent doses of aluminium glycinate were less effective in maintaining the pH of the gastric contents above 4 continuously. In these experiments aluminium glycinate produced an acid deficit in the urine corresponding to not more than one-quarter of its antacid power measured to pH 4. This would allow a dose corresponding to 6 hours' effective antacid control per day without danger of alkalosis. On this basis patients with normal renal function have the power to excrete alkali corresponding to an oral intake of about 300 aluminium glycinate tablets daily. S. L. W.

Barbiturate and Tranquillizer Drugs, Comparative Sedative Effects of. T. A. Loomis and T. C. West. (*J. Pharmacol.*, 1958, **122**, 525.) In a series of controlled experiments, the comparative sedative effects of orally administered quinalbarbitone sodium (100 mg.), chlorpromazine hydrochloride (50 mg.), meprobamate (400 mg.), phenaglycodol (300 mg.) and a placebo consisting of 200 mg. of corn starch were evaluated on normal human male subjects. The subjects, all of whom had received previous training, were required to operate an automobile driving test apparatus which automatically recorded driving faults. Quinalbarbitone, chlorpromazine and meprobamate caused significant impairment of performance on the test apparatus. Phenaglycodol and the placebo were without effect. Quinalbarbitone produced the most intense impairment of function. Chlorpromazine produced impairment after a delayed onset of action. Meprobamate produced delayed impairment after the first dose and immediate impairment after the second. W. C. B.

Benactyzine in Patients with Violent Tempers and with Parkinsonism. P. W. Nathan. (*Brit. med. J.*, 1958, **1**, 926.) Twelve patients (10 of them epileptics) with violent tempers formed the subject of this investigation. Benactyzine was given orally in tablets containing 1 mg., and these were alternated with control tablets. In half the patients the first supply of tablets consisted of benactyzine, and in the other half it consisted of the control tablets; on subsequent occasions the tablets and the patients were reversed. The dosage of benactyzine varied between 3 and 12 mg. a day. In most of the patients the investigation lasted between one and two years. The results showed that benactyzine was of no greater value than the control tablets in reducing outbursts of temper. Benactyzine was also tested on 18 patients with Parkinsonism, in a dose varying from 3 to 10 mg. a day; control tablets were also employed in

this group. No patients were improved by the benactyzine who were not also improved by the control tablets, or who, on other occasions, received no benefit from benactyzine. S. L. W.

Benzchlorpropamide in Epilepsy. D. S. Sharpe, G. Dutton and J. R. Mirrey. (*Brit. med. J.*, 1958, **1**, 1044.) Twenty-five cases of epilepsy in mental defectives were treated over a period of six months with the anticonvulsant benzchlorpropamide (Nydrane). Irrespective of what other drugs were being used, 0·5 g. of benzchlorpropamide daily was added to the patients' treatment for the first 7 days. Following this, other anticonvulsants were gradually withdrawn and substituted by 0·5 g. of benzchlorpropamide weekly until the maximum dose was reached—usually, from 3 to 4 g. a day given in three equal parts. With grand mal convulsions, 68 per cent improved, 20 per cent showed no change, and 12 per cent were worse. General improvement in behaviour was shown in 32 per cent. It was not possible to control the epilepsy with benzchlorpropamide alone; in all except 3 cases a maintenance dose of barbiturates had to be given in addition. There was a notable freedom from signs and symptoms of toxicity, except that transient renal disturbances were observed in some cases. S. L. W.

Captodiame, Pharmacology and Toxicology of. R. Kopf and I. Møller Nielsen. (*Arzneimitt.-Forsch.*, 1958, **8**, 154.) It has already been reported that captodiame (*p*-butylmercaptobenzhydryl-β-dimethylaminoethylsulphide) has marked sedative and spasmolytic actions. In rats and mice it has been found to potentiate the anaesthetic effect of *n*-methylated barbiturates and thiobarbiturates as well as the analgesic effect of ketobemidone, pethidine, l-*iso*-methadone and morphine. Whereas captodiame has no effect on leptazol- or strychnine-induced convulsions it does have a certain protective effect against electric shock treatment. In rats it causes a moderate fall in body temperature. It has no neuromuscular blocking action in the rat and no ganglion blocking effect in the cat. Oedema of the rat's foot was inhibited by 50 mg./kg. of captodiame. The thyroid gland, adrenals and ovaries of rats were not morphologically or functionally affected by moderate doses of captodiame. Subtoxic doses caused ascorbic acid depletion of the adrenal gland which appeared to be mediated via the pituitary gland. Administration of up to 100 mg./kg. orally, daily for 3 months did not have adverse effects as judged by the general condition, body weight, blood picture and histology of the essential organs in rats and cats. M. M.

Chlorophenols, Biological Action of. M. E. Farquaharson, J. C. Gage and J. Northover. (*Brit. J. Pharmacol.*, 1958, **13**, 20.) Chlorination of phenol produces a series of nineteen compounds comprising mono-, di-, tri- and tetrachloro-isomers and one pentachloro-form. The lower compounds are used as antiseptics, the high as herbicides, fungicides and insecticides. A study of the effects of progressive chlorination of phenol on toxicity has shown that changes in toxicity occur in correlation with the dissociation constants. The higher chlorinated phenols interfere with oxidative phosphorylation and this property may be attributed to the chlorophenate ion. The lower compounds have a convulsant action probably due to the undissociated molecule. The higher chlorinated phenols produce a contracture of the isolated phrenic nerve diaphragm and a stimulation of *in vitro* oxygen uptake in rat brain homogenate. G. F. S.

Ciba 10870 in Parkinsonism. W. Hughes, J. H. Keevil and I. E. Gibbs. (*Brit. med. J.*, 1958, **1**, 928.) A controlled trial of a parasympatholytic drug, 3 - phenyl - 3(β-diethylaminoethyl) - 2:6 - dioxopiperidine hydrochloride (Ciba 10870) was made in 16 cases of Parkinsonism. Patients were given six 2·5 mg. tablets daily, the drug being alternated with a placebo every 4 days. The preparation relieved some of the most troublesome symptoms in every case, and in some cases produced very good results. The general impression was that it was equal, if not superior, to any therapy in current use. The best effect was on salivation in post-encephalitics; on rigidity the results on the whole were good; its effect on tremor was no better than that achieved by current therapy. Mental confusion occurred in one case and disappeared when the drug was stopped. No effects on bowel function were observed and no pupillary changes were seen. No blood dyscrasias were observed. S. L. W.

Cinnamic Acid, Action of Esters of, on Capillary Permeability. J. J. Pocidalo and M. Chaslot. (*Thérapie*, 1958, **13**, 72.) A 20 per cent preparation of the ester under examination in a sodium alginate emulsion base was applied to a rectangular patch of the skin of a rabbit's abdomen, the hair having been removed 24 to 48 hours previously. Similar rectangles were treated with the base alone, and with soft paraffin. Application was followed by gentle massage for 3 minutes, and after an interval of 15 minutes, a 1 per cent solution of trypan blue (1 ml./kg.) was injected. Five minutes later, chloroform was applied to the skin with the aid of cotton wool, and left in contact for 1 minute. The delay in appearance of a blue colour on the skin was used as a measure of the reduction of permeability of the capillaries. Neither the soft paraffin nor the sodium alginate emulsion base appeared to have any effect in these experiments. Methyl cinnamate showed a marked effect in reducing permeability, but the most powerful ester was ethyl cinnamate. Higher homologues showed progressively less effect, the hexyl and higher esters being ineffective. The allyl and phenylethyl esters were also ineffective. G. B.

Dieldrin Poisoning in Man. T. B. Patel and V. N. Rao. (*Brit. med. J.*, 1958, **1**, 919.) An account is given of 20 cases of poisoning by dieldrin among spray teams in filaria and malaria control work in India. A 50 per cent dieldrin wettable powder was used, the strength of the final diluted suspension being 1·25 per cent for malaria control and 2·5 per cent for filaria control work. The rate of spraying in both cases was the same, and the deposits were 28 and 56 mg./sq. ft. (290 and 580 mg./sq. metre) respectively. There were 10 cases of poisoning among 105 sprayers in the filaria unit, and 10 cases among 192 workers in the malaria unit. In the filaria unit poisoning was seen from 14 to 154 days (average 57 days) after the first exposure to dieldrin. In the malaria unit the cases occurred from 60 to 116 days (average 99 days) after exposure. No protective clothing or masks were used and washing with soap and water was not done. The symptoms started with giddiness, headache and twitching of muscles, going on to convulsive attacks occurring up to several times a day, with loss of consciousness for periods of up to 2 hours. There were no deaths. Most of the cases of poisoning occurred during a second period of exposure. The teams were in the habit of using their bare hands for transferring the powder from the containers to the water and for stirring the suspensions prepared from the powder. The incidence of poisoning will probably be reduced by the introduction of suitable precautions. S. L. W.

4-Diphenylmethyl-(±)-tropyltropinium Bromide, a new Ganglion-blocking Agent. K. Nádor and L. Gyermek. (*Arzneimitt.-Forsch.*, 1958, **8**, 336.) In an attempt to find a ganglion-blocking agent with little parasympatholytic effect the authors examined some simple alkyl quaternary derivatives of tropine esters which possessed both actions. Ganglion-blocking action was considerably increased and parasympatholytic action decreased by substituting aralkyl instead of alkyl groups, especially *para*-substituted aralkyl groups. Among the best of the compounds examined from a therapeutic point of view, the 4-diphenylmethyl quaternary derivative of atropine combined marked ganglion-blocking action with slight parasymptholytic action. A detailed description of the pharmacology of this compound is given. D. B. C.

Framycetin in Infantile Gastro-enteritis. R. Louwette and A. Lambrechts. (*Brit. med. J.*, 1958, **1**, 868.) Framycetin has a very favourable action on gastro-enteritis due to pathogenic *Escherichia coli* in premature babies and infants. Twenty-five infants, with ages ranging from 7 days to 7 months, were successfully treated; 14 were premature babies and 8 others were undernourished. Framycetin is well tolerated and in a dosage of 50 mg./kg. daily by mouth for 5 days does not appear to be toxic. The framycetin was administered in the form of a flavoured water-soluble powder, the daily dose being divided into two portions. It acts with the same speed and potency as neomycin. Diarrhoea, dehydration, and vomiting disappear very rapidly, often within two days after establishing treatment; stool cultures usually become negative within three days. S. L. W.

Laminarin Sulphate, Antilipaemic Activity of. S. Mookerjea and W. W. Hawkins. (*Canad. J. Biochem. Physiol.*, 1958, **36**, 261.) The antilipaemic activity of heparin is reflected in its effects upon the concentrations of glyceride, phospholipid and cholesterol. In addition, there is a splitting off of lipid from β-lipoprotein, the protein particularly involved in the transport of cholesterol. There is also evidence that heparin, presumably because of this property, inhibits the development of atherosclerosis in hypercholesterolaemic rabbits. A number of compounds of relatively high molecular weight, most of them sulphated, have been shown to have antilipaemic activity. Among them, a preparation of laminarin sulphate, with a low sulphate content and a low anticoagulant activity, has been shown to reduce hyperlipaemia and correspondingly to affect the β-lipoprotein. In this paper a sample of laminarin sulphate is used which was obtained from *Laminaria digitata*. It contains 1·7 sulphate groups per glucose unit, somewhat less than does heparin. Its molecular weight is about 10,000, also less than that of heparin. It has about one-third of the anticoagulant activity of heparin and appears to act like it, principally as an antithrombin. It reduces alimentary lipaemia in dogs and rats. The experiments described in this paper concern the antilipaemic activity of this compound in comparison with that of heparin. Rabbits were chosen because of the high levels of blood lipids, particularly of cholesterol, which can be induced in them by dietary means. It was found that when the rabbits were fed on a diet containing 1 per cent cholesterol the blood serum levels of neutral fat, phospholipid, free and esterified cholesterol and β-lipoprotein were greatly increased. After the parental administration of laminarin sulphate or of heparin, all these were decreased and the electrophoretic mobility of the β-lipoprotein was increased. Laminarin sulphate showed the same effect as heparin on the hyperlipaemia. There was no definite relationship between the magnitude of dosage of either compound and the degree of its antilipaemic effect. M. M.

2-Methyl-3-*o*-tolylquinazol-4-one, a New Hypnotic Drug, Pharmacology of. J.-R. Boissier, C. Dumont and C. Malen. (*Thérapie*, 1958, **13**, 30.) The toxicity of 2-methyl-3-*o*-tolylquinazol-4-one was determined in mice, rats, rabbits and guinea pigs, using various routes of administration. The hypnotic action was determined by experiments using fish, mice, guinea pigs and rabbits. The compound was shown to have a suitable hypnotic action and a relatively low toxicity, the ratio of hypnotic to lethal doses being of the order of 4, compared with 2 to 2·5 for phenobarbitone. Onset of hypnotic action was rapid, and the drug antagonised the effect of leptazol, but not of strychnine or picrotoxin. It opposed the effects of amphetamine and other stimulants, and potentiated the action of pentobarbitone, methylpentynol, chlorpromazine and reserpine. It appeared to be free from objectionable side-effects. G. B.

Morphine; Treatment of Side-effects. G. Christie, S. Gershon, R. Gray, F. H. Shaw, I. McCance and D. W. Bruce. (*Brit. med. J.*, 1958, **1**, 675.) Provided the sensitivity of the patient is first ascertained to small doses, morphine is not a dangerous drug. All the side-effects can now be controlled. The most frequent side-effect is not respiratory depression but nausea and vomiting, of which the incidence may be as high as 30 per cent. Large doses of morphine (up to 200 mg. four times daily) may be required to control chronic pain. The patient should not be denied the benefit of complete analgesia on the score of side-effects or addiction. The results in over 400 cases show such fears to be groundless when the appropriate antagonists are employed. These are nalorphine, which is used for the control of respiratory depression; amiphenazole which combats central depression and is essential for the long-term treatment of intractable pain with large doses of morphine; and cyclizine which completely controls the nausea and vomiting. Both amiphenazole and cyclizine are completely harmless. Larger doses of amiphenazole (40 to 100 mg. four times daily) than hitherto used are now employed, and wide experience shows the drug to be one of the safest in medicine. S. L. W.

Noradrenaline in Artery Walls; Dispersal by Reserpine. J. H. Burn and M. J. Rand. (*Brit. med. J.*, 1958, **1**, 903.) It has been shown that noradrenaline is present in artery walls but hitherto it has not been thought to affect vascular tone. The vasoconstriction caused by certain substances, such as nicotine, in the vessels of the rabbit ear has now been shown to be due to the release of noradrenaline from the artery wall, since the vasoconstriction was absent in the ears from rabbits treated with reserpine. On extracting the skin of rabbit ears a noradrenaline-like substance was found which was not present in ears from reserpine-treated rabbits. Chromaffin cells, present in the ears of normal rabbits, are absent in the ears of reserpine-treated rabbits. Strips of aorta from normal rabbits, suspended in a bath, contracted on addition of nicotine; they were found to contain noradrenaline in a mean amount of 0·5 μg./g. Strips of aorta from rabbits treated with reserpine did not contract on addition of nicotine, contained only traces of noradrenaline and were highly sensitive to this amine, indicating that the store of noradrenaline in the vessel wall must normally reduce the effect of noradrenaline, and probably the effect of sympathetic impulses also. Conditions such as Raynaud's disease may be due to a release of noradrenaline from the store in the artery wall and might therefore benefit from treatment with reserpine. S. L. W.

Raunescine and *iso*Raunescine, Effects of, on Behaviour and on the 5-Hydroxytryptamine and Noradrenaline Contents of Brain. M. K. Paasonen and P. B. Dews. (*Brit. J. Pharmacol.*, 1958, **13**, 84.) Raunescine and *iso*raunescine are rauwolfia alkaloids which reduce the adrenaline and noradrenaline content of the heart of rats. A study of the behaviour effects of these alkaloids in trained pigeons has shown that both alkaloids in sufficient dosage have effects very similar to reserpine but both were much less potent. In rats both raunescine and *iso*raunescine, like reserpine, caused a reduction in the concentration of both 5-hydroxytryptamine and noradrenaline in the brain. The results of these studies are compatible with the postulated indirect effect of the rauwolfia alkaloids on the brain through the release of noradrenaline and 5-hydroxytryptamine. G. F. S.

Sulphamerazine: Toxicity in Children. G. C. Arneil. (*Lancet*, 1958, **1**, 826.) Over a period of 10 years, a total of 29 patients have been treated at the Royal Hospital for Sick Children, Glasgow, for haematuria associated with taking sulphonamides. In 27 of the 29 children the preparation administered contained sulphamerazine. Most of the children were aged from 1 to 6 years, and the sulphonamide was given for periods of from 1 to 6 days in daily doses of from 100–400 mg./kg. bodyweight. The haematuria lasted from one to six days, and albuminuria persisted a few days longer. Anuria, hypertension, encephalopathy, and uraemia were seen in some patients but all subsequently recovered under conservative treatment. To justify giving a drug potentially noxious in therapeutic doses it must be shown to have significant advantages over other preparations. Sulphamerazine seems to have no such outstanding virtue and it is doubtful whether its use is justified. S. L. W.

Tienmulilminine, Hypotensive Effect of. K. Kuo-Chang and H. Bin. (*Acta physiol. Sinica*, 1958, **22**, 71.) Tienmulilminine is a new alkaloid isolated from the root of Chinese *Veratrum schindleri Loes. f.* Being a tertiary amine, this alkaloid yields alkamine by hydrolysis in acidic or alkaline medium. In anaesthetised cats, intravenous injections of tienmulilminine at the dosage of 0·45–0·6 mg./kg. produced a prompt fall of artial pressure, which, after $1\frac{1}{2}$–3 minutes, decreased 50–130 mm. Hg below the original level (a reduction of 32–75 per cent). In the lapse of 15–60 minutes, the pressure gradually recovered. During the period of hypotension, the pressor reflex from temporary occlusion of common carotid artery was diminished. Repeated administrations showed no tachyphylaxis. The fall of blood pressure was, as a rule, accompanied by bradycardia, respiratory depression or sometimes apnoea. The hypotensive effect was very much reduced or even abolished in vagotomised cats, significantly reduced after previous injection of procaine-HCl mg./kg., but did not change remarkably in atropinised cats. A minute quantity of tienmulilminine (50–90 μg./kg.), ineffective by intravenous injection, produced a significant hypotension by injection into left ventricle. It appears, therefore, that the vagus is an important afferent pathway for the hypotension. The isolated carotid sinus was prepared according to Aviado's method. Injection of 30–50 μg./kg. into thyroid artery exhibited some hypotensive effect, but administrations of 80–400 μg./kg. raised the arterial pressure 40–140 mm. Hg. Thus, the carotid sinus was demonstrated as a secondary sensory region for the hypotensive effect of tienmulilminine. Its acute LD50 for intravenous and subcutaneous injections in mice was 3·2 and 26 mg./kg. respectively.

BOOK REVIEWS

CHROMATOGRAPHY. By Edgar Lederer and Michael Lederer. Second, revised and enlarged, edition. Pp. xx + 711 (including Index). Cleaver-Hume Press, Ltd., London, 1957. 72s.

This is the second edition of the most comprehensive book on the subject. The distinguished authors claim with unnecessary modesty that the unexpected success of the first edition and the ever-increasing development of chromatographic methods prompted them to prepare this second edition. It has been reported that this revision will be the last by these authors. Let us examine why this should be so. The book under review contains 700 pages, 139 figures, 175 tables and 3704 references, an enlargement of roughly 50, 55, 40 and 100 per cent respectively over the first edition, and this in the short space of four years. No significant reference can have escaped the painstaking eyes of the the authors. Nor is this all; comprehensive indices of authors and subjects, an extremely detailed table of contents, with tables of retention values interspersed throughout the text, enable the reader quickly to find his way about the book. This expansion could not continue indefinitely without the work losing its character as a book devoted to a technique, and becoming a catalogue. The situation has been remedied by the establishment of a journal, limited to this subject, with one of the authors as senior editor.

What has the book for readers of this journal? Firstly, the techniques of chromatography have proved admirably suitable for the detection, isolation and determination of substances of biological (including pharmacological) importance, both in the animal and plant, *post* and *ante mortem*. Secondly, applications are more frequently appearing in the field of pharmaceutical analysis, and here might be mentioned the chapter headings: natural pigments, vitamins, hormones, antibiotics. Inorganic chromatography receives its due place in the book. A newer application—gas chromatography—is very briefly discussed in the text with a supplementary list of references. This application, the results of which have already received mention in the research pages of this journal, is likely to achieve an important place in pharmaceutical and pharmacological analysis. While not out of place, this introduction will not carry the investigator very far, and he will need to consult the specialised books which are beginning to appear.

Preceding the chapters on the individual chemical classes is a useful account of the general theoretical side including such topics as the modes of operation of chromatographic systems, a review of the different chromatographic porous media and descriptions of apparatus and procedure. While not exactly an introduction to the subject, this section is valuable in that it discusses those aspects from which many a practical chromatographer will derive enlightenment. The presentation is excellent and the price not excessive for such a useful compilation.

TUDOR S. G. JONES.

TECHNIQUE OF ORGANIC CHEMISTRY, edited by A. Weissberger. Vol. X. *FUNDAMENTALS OF CHROMATOGRAPHY*, by H. G. Cassidy. xvii + 447 (including Indices). Interscience Publishers, London, 78s. New York, $9.75.

This book is apparently addressed to those interested in the theory of chromatography. It is at present the only work which has any pretention to detailed discussion of the background of a subject which is usually studied for its practical applications. In the reviewer's opinion, this neglect of the theoretical background is much to be deplored and has tended to reduce books on the subject to recitals of recipes for dealing with specific groups of substances. While the study required to master the rather difficult mathematical descriptions of the chromatographic process, which have yet to achieve unanimity, may not repay the effort, an understanding of the principles involved will do much to improve the application. The background is certainly given in this book, mathematics and all. In fact, much introductory chemistry is present, relevant enough to the subject, but which might be assumed as knowledge appropriate to the level of education of the presumed reader. At the same time, the author gives instruction on technique, exemplified by such chapter headings as "on recognising and evaluating zones", "on choosing mobile and stationary phases" and "on using chromatography". The book is full of "know-how", including mention of many proprietary substances available in America and even a list of suppliers, with addresses, some in this country. The book retains many traces of the interest of the author in non-chromatographic adsorption, being a successor to an earlier volume which dealt with both adsorption and chromatography.

The wide coverage, well-indexed, ensures that the book contains much of value for the pharmacologist. There is not, however, very much of specific interest. The book is well produced in the usual manner of the series.

TUDOR S. G. JONES.

LETTERS TO THE EDITOR

Tryptamines in Edible Fruits

SIR,—Earlier this year, Udenfriend and others[1] reported the rather surprising finding that bananas contain large amounts of two physiologically important agents, 5-hydroxytryptamine (5-HT) and noradrenaline. The presence of these amines in a food as widely used as the banana is of clinical interest since the ingestion of bananas might lead to the erroneous chemical diagnoses of carcinoid tumours and phaeochromocytomas by producing an increased urinary excretion of 5-HT and noradrenaline and their metabolites. The finding is also of biochemical interest as clues might be provided about the biosynthesis and function of these amines in nature.

We have now confirmed that the ripe yellow banana contains 5-HT, noradrenaline and dopamine which are concentrated in the peel rather than in the pulp. The fresh fruits were extracted with acetone (1 g./5 ml.) or with 0·1N HCl (1 g./2 ml.). After reducing the extracts to a small volume, they were subjected to chromatography using 5 different solvent-mixtures. Indoles were detected when Ehrlich's reagent was used as the spray reagent, and noradrenaline and dopamine when potassium iodate was sprayed on to the chromatograms. Duplicate spots were eluted and tested on the isolated rat uterus or on the cat blood pressure. Green under-ripe bananas were then secured and subjected to the same extraction and identification procedures, whilst other green bananas were allowed to ripen or over-ripen before extraction. Whereas large quantities of noradrenaline and dopamine (1–20 μg./g.) were found in all extracts, the peel from the green bananas contained only traces of 5-HT (Table I). The appearance of 5-HT in the peel coincided with the development of the yellow colour and therefore may be related to the maturity of the fruit. Although 5-hydroxyindoleamines have been found in plants previously, they have usually been associated with plants regarded as toxic to animals. The significance of 5-HT in the metabolism of the banana is not clear. No evidence of tryptamine, 5-hydroxytryptophan or 5-hydroxyindoleacetic acid was obtained. Further work on the formation and fate of 5-HT in the banana is now in progress.

TABLE I

THE 5-HT ACTIVITY (μG./G.) OF PARTS OF THE BANANA AT DIFFERENT STAGES OF MATURITY

Stage	Outer peel	Inner peel	Pulp
Under-ripe	0·1	0·2	25·0
Ripe	52·0	40·0	19·0
Over-ripe	39·0	30·0	22·0

During the systematic examination of other edible fruits, the tomato was found to contain indole derivatives but no noradrenaline or dopamine. Estimated as 5-HT, the activity of green unripe tomatoes was 0·18 μg./g., of red ripe tomatoes 3·75 μg./g., and of over-ripe tomatoes 2·90 μg./g. When the acetone extracts were subjected to paper chromatography, the activity was found to consist of 5 parts of tryptamine to 1 part of 5-HT. The significance of tryptamine in the metabolism of the tomato is also not clear at present. Traces of 5-HT in this fruit may simply represent a means of removing unwanted tryptamine, or the tomato may form its 5-HT through the intermediate substance tryptamine, and not through 5-hydroxytryptophan as in mammals.

These results are of interest since indole derivatives and noradrenaline-like substances have not been detected in extracts of strawberries, cherries, rhubarb, raspberries, blackcurrants, gooseberries, lemons, oranges, apples, figs, prunes or potatoes.

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July 25, 1958.

REFERENCE

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Penicillin-induced Round Bodies in Gram-negative Bacteria

SIR,—Lederburg[1,2] has recently shown that if penicillin is allowed to act upon growing cultures of *Escherichia coli* and *Salmonella typhimurium* in hypertonic medium in the presence of Mg^{++}, spherical forms or round bodies are generated, due it is postulated, to complete or partial inhibition of cell-wall synthesis during cell division. Similar results are reported for *Pr. vulgaris*[3] and *Alcaligenes faecalis*[4]. The term round body rather than protoplast has been retained in describing these forms in accordance with the suggestion of Brenner and others[5] that the term protoplast should be applied only when there is additional evidence to show that the round bodies contain no cell wall residues.

It is obviously of interest to apply this elegant but simple experiment to other Gram-negative organisms. In our experiments 0·15 ml. of a 17 hour culture of the organism containing about 10^7 viable cells was inoculated into 10 ml. of a medium containing in each litre: sucrose 114, $MgSO_4.7H_2O$ 2·5, NaCl 5, Lab Lemco 10 and peptone (Oxoid) 10 g., and varying quantities of the potassium salt of benzylpenicillin. Growth was allowed to proceed at 37° for 4–5 hours and the cultures examined by interference microscopy.

The results for 12 organisms are summarised in Table I.

TABLE I

Organism	Concentration of penicillin to induce round bodies (units/ml.)	Diameter of round body (μ)	Size of organism in hypertonic medium without penicillin (μ)
E. coli[1]	4000	3·5–6·0	0·52 × 2·6
E. coli[2]	25–100	4·0–5·0	0·52 × 1·7
Cloaca cloacae[3]	100–200	6·2–6·8	0·78 × 1·7
Citrobacter freundii[4]	3000	4·8–5·2	0·52 × 1·7
Klebsiella aerogenese[5]	1500–2000	4·3–5·2	0·78 × 2·1
Serratia marcescens[6]	1000–5000	3·5–5·5	0·52 × 0·78
Proteus vulgaris[7]	1000	3·4–4·3	0·78 × 2·6
Proteus morganii[8]	5000	5·2–6·0	0·52 × 1·7
Pseudomonas aeruginosa[9]	250–2000	6·0–6·5	0·52 × 2·1
Pseudomonas hydrophila[10]	4000–5000	5·5–6·5	0·52 × 1·7
Vibrio cyclosites[11]	100	2·6	0·78 × 2·6
Vibrio neocistes[12]	100	2·6	0·78 × 2·6

[1]*E. coli* NCTC 86. [2]*E. coli* originally NCTC 5934. [3]*Cloaca cloacae* NCTC 8155. [4]*Citrobacter freundii* NCTC 8165. [5]*Aerobacter aerogenese* NCTC 8197. [6]*Serratia marcescens* isolated in the laboratory. [7]*Proteus vulgaris*—Constantinople OX19 NCTC 7052. [8]*Proteus morganii*—692 NCTC 417. [9]*Pseudomonas pyocyanea* NCTC 7244. [10]*Pseudomonas hydrophila*—Kulp NCTC 7810. [11]*Vibrio cyclosites* NCIB 2581. [12]*Vibrio neocistes* NCIB 2582.

Although the majority of the organisms recorded are not human pathogens and the concentration of penicillin used is very much higher than blood-level concentrations achieved clinically it is interesting to find a further unification and extension of this fundamental biochemical action of penicillin. The above experiments have in the case of *E. coli* NCTC 5934, *Serratia marcescens* and *Ps. hydrophila* been repeated on the 250 ml. scale and by careful centrifugation followed by resuspension in 0·33M sucrose a washed suspension of protoplasts was obtained suitable for further study.

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July 10, 1958.

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4. Lark, *Canad. J. Microbiol.*, 1958, **4**, 165.
5. Brenner and others, *Nature, Lond.*, 1958, **181**, 1713.

On the Quantitative Estimation of Amino Acids by Paper Chromatography

SIR,—The quantitative estimation of amino acids as their dinitrophenyl (DNP) derivatives by Levy [1] was made by two dimensional paper chromatography using a toluene:chloroethanol:0·8N ammonium hydroxide system in the first direction followed by 1·5M phosphate buffer in the second direction. Chloroethanol is poisonous[2] and it is desirable to replace it. The method of Rockland and Dunn[3] was used for screening various organic liquids (hydrocarbons, ketones, alcohols, ethers, esters) as developers in the chromatography of DNP-amino acids. Low R_F values were obtained using the hydrocarbons, ethers and chloroform whilst higher values were obtained with the alcohols.

A system referred to as "Ethyl benzene" was devised and consists of ethyl benzene:*tert.*-amyl alcohol:1·6N ammonium hydroxide 1:3:2 (v/v/v), it was used in the first direction, followed, after drying the paper, by 1·5M phosphate buffer in the second direction. Figure 1 shows the separation obtained with a mixture of DNP-amino acids.

The factors given by Levy were found not to be applicable to the analysis of β-lactoglobulin under the conditions of this experiment. The reactions of amino acid with dinitrofluorobenzene (DNFB) by Sanger's[4] method in 66 per cent ethanol and in aqueous solution[1,5] was investigated. A mixture of amino acids containing 0·00002M of each was reacted with DNFB, the DNP-amino acids were subjected to quantitative paper chromatography using the ethyl benzene system in the first direction followed by 1·5M phosphate buffer. From the optical density reading of each DNP-amino acid and the concentration of the amino acid, a factor "F" was deduced which gives the concentration of the amino acid in moles for an optical density reading of 1. The factors obtained by reaction in 66 per cent ethanol for 1½ and 2 hours differed and when the factors for 1½ hours reaction was applied to a protein hydrolysate, the values for valine, leucines, lysine and phenylalanine were low. The results were consistent and reproducible $\pm$ 5 per cent when the reaction was carried out in 0·1N potassium chloride for 1½ and 2 hours. The factors "F" $\times$ 10^7 are, Asp and Glu 0·635; Gly 0·784; Ala 0·713; Val 0·65; Leu's 0·692; Ser 0·765; Thr 0·695; CyS 0·383;

CySH 0·766; Met 1·0; Arg 0·744; His 1·55; Lys 0·392; Tyr 0·9; Pro 0·56; Phe 0·785; Orn 0·421; Hypro 0·6.

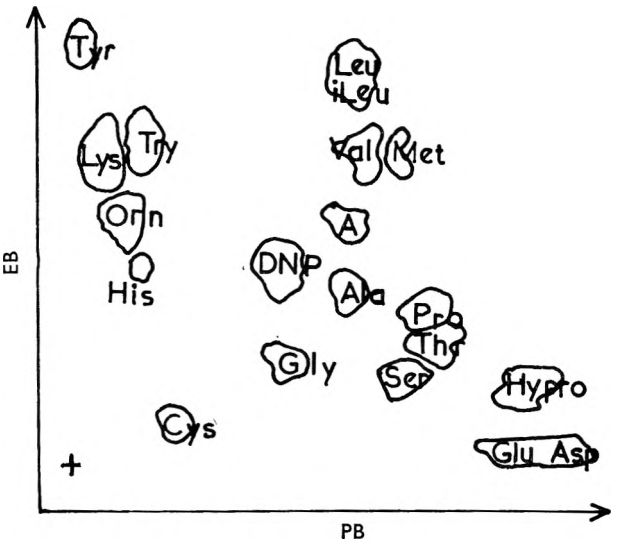

FIG. 1. Two-dimensional chromatogram of a known mixture of DNP-amino acids. EB, direction of flow of ethyl benzene system developer. PB, direction of flow of phosphate buffer developer. Spots (amino acids as DNP derivatives): A, α-aminobutyric acid; Ala, alanine; Asp, aspartic acid; Cys, cystine; DNP, dinitrophenol; Gly, glycine; Glu, glutamic acid; His, histidine; Hypro, hydroxyproline; Leu, leucine; iLeu, *iso*leucine; Lys, lysine; Met, methionine; Orn, ornithine; Pro, proline; Ser, serine; Tyr, tyrosine; Thr, threonine; Try, tryptophane; Val, valine.
+, Point of application of initial mixture.

Histidine gives rise to Di-DNP histidine in 66 per cent ethanol while in aqueous solution both the mono and di DNP-histidine are obtained.

Quantitative paper chromatography of DNP-amino acids has been applied to the analysis of β-lactoglobulin using the system and the factors reported above; the results obtained compared favourably with the reported data[6,7].

I wish to thank Professor H. G. Cassidy for his helpful advice and suggestions throughout the work which was supported by a research grant RG 3207 (C4) from the Division of Research Grants, The National Institute of Health, Public Health Service.

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