# REVIEW ARTICLE

## RELEASE AND METABOLISM OF THE NEUROHYPOPHYSIAL HORMONES

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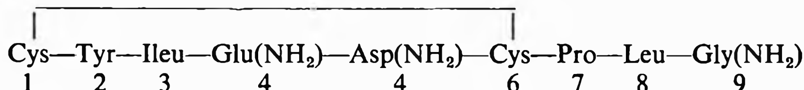
This article attempts to review a subject which is in a state of rapid expansion. The literature has not been covered completely, and the choice of works mentioned has been intentionally selective. Furthermore, points which are in process of elucidation have been omitted as their inclusion, at this stage, might have introduced controversial arguments.

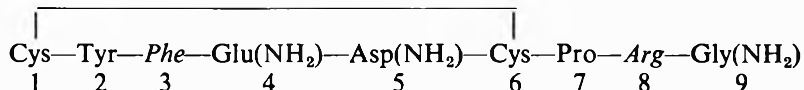
*Definitions*

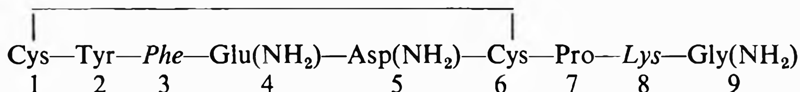
A substantial amount of confusion exists in the nomenclature of the neurophysical hormones, due mainly to the fact that commercial preparations have been used without any reference to their composition or even standardisation. For instance, preparations labelled as "vasopressin, B.P." suggest to the British research worker that they conform to the recommendations laid down by the British Pharmacopoeia, whereas in fact some of them are made according to the U.S. Pharmacopeia. The British Pharmacopoeia, in agreement with the Permanent Commission on Biological Standardisation of the League of Nations Health Organisation (Bangham and Musset, 1958), requires that the "Standard Preparation is a quantity of acetone-dried powder obtained from the posterior lobes of fresh pituitary bodies of *oxen*"; but the U.S. Pharmacopeia stipulates that the "Posterior Pituitary is a powder prepared from the clean, dried, posterior lobe of the pituitary body of *domestic animals* used for food by man," thus allowing the use of pigs' pituitaries as a source of vasopressin solution. With regard to the standardisation, it must be remembered that according to the British Pharmacopoeia the only suggested methods for the extract of the posterior pituitary lobe powder are assays of its oxytocic activity, with the result that it is not necessary to state its antidiuretic or pressor activity.

Oxytocin is the name given to the oxytoxic hormone extracted from the posterior lobe of the pituitary gland. Its formula is:



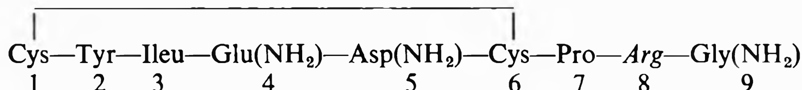

and, as far as it is known, its chemical structure is the same in all vertebrates. The name of the antidiuretic hormone of higher vertebrates is vasopressin. There are, however, at least two vasopressins, the arginine$^8$-vasopressin and the lysine$^8$-vasopressin, with the following formulae:





Arginine-vasopressin has been found in man, ox, horse, sheep, rat, dog, monkey, camel and marsupial (Acher and Chauvet, 1953; Acher, Chauvet and Lenci, 1959; Light and du Vigneaud, 1958; Sachs and Barrett, 1959; Sawyer, Munsick and van Dyke, 1960) whereas lysine vasopressin exists in hog and hippopotamus (Heller and Lederis, 1960; Popenoe, Lawler and du Vigneaud, 1952). Physiologically the two vasopressins differ markedly: the pressor activity of arginine-vasopressin is 600 u./mg. and that of lysine-vasopressin 300; when lysine-vasopressin and arginine vasopressin are injected intravenously in equipressor amounts in dog, rat or man, the latter has a much greater antidiuretic activity than the former (Dicker and Eggleton, 1961; Nielsen, 1958; Thorn. 1959; van Dyke, Engel and Adamson, 1956). It is therefore important that the antidiuretic hormone should be specifically defined as arginine-, or lysine-vasopressin. Works in which the secretion of antidiuretic hormone has been estimated using as standards commercial preparations of posterior pituitary extract or vasopressin solutions without reference to their composition or origin, must be considered with the utmost misgiving.

Finally, it must be remembered that the antidiuretic hormone of fish, amphibian and bird is arginine$^8$-vasotocin and different from that of other vertebrates (Heller and Lederis, 1958; Pickering and Heller, 1959; Sawyer, Munsick and van Dyke, 1959). Its formula is:



As for the term "neurohypophysis" it will be used in this text with the meaning of a morphological unit comprising the hypothalamic neurones of certain nuclei, their axones which run down the infundibular stem to end in the posterior lobe and the posterior lobe of the pituitary gland, also known as "pars neuralis." The hypothalamic nuclei are the supra-optic and paraventricular nuclei, which in the dog contain together some 100,000 neurones.

## Sites of Formation of the Neurohypophysial Hormones

A controversy has been going on for many years concerning the relative importance of the hypothalamus and the posterior lobe. According to Fisher, Ingram and Ranson (1938), both the oxytocic and the antidiuretic hormones are elaborated in and liberated from the posterior lobe, their secretion and liberation from the pars neuralis being controlled by the hypothalamic neurones. According to modern views, however, the sites of formation of the hormones are the neurones of the hypothalamic nuclei. According to this theory, the hormone-containing secretion of the neurones migrates in or along the axones and is finally

deposited in the posterior lobe. Both groups of workers, however, hold that liberation of the hormones from the posterior lobe is controlled by hypothalamic neurones. As happens so often in science a reinterpretation of the facts may lead to a conception which, though a compromise, may be more nearly correct.

The first question is to examine the distribution of the hormones in the posterior lobe and the hypothalamus and to see what light such studies can throw on the problem under consideration. In adult animals the amounts of antidiuretic and oxytocic activities found in the posterior lobe vary according to the species and do not bear any obvious relation either to their size or to their needs for water conservation. Expressed in terms of weight, the posterior pituitary gland of the dog contains an average of 4,500, that of man 760, that of rat 300 m.u./mg. of each hormone. But for a few exceptions such as the guinea-pig, the wallaby and the camel, in which the amount of oxytocin is smaller than that of vasopressin, in all adult animals and man the vasopressin: oxytocin ratio (= V/O) is unity. The posterior pituitary gland of the camel, an animal which is known to be able to withstand a much more severe degree of dehydration than any other mammal, contains 2,000 m.u. antidiuretic hormone per mg. of tissue, whereas that of the dog contains as much as 4,500 m.u./mg. gland; on the other hand, the concentration of the anti-diuretic hormone in the posterior lobe of the kangaroo rat (*Dipodomys merriami*) is more than three-fold that of the ordinary rat.

In infant animals and babies the situation is different. Their posterior lobe contains a much larger proportion of antidiuretic than oxytocic activity. The disproportion is even more marked in foetuses. For instance, the V/O ratio was found to be 15 in a human foetus of 160 days and 6 in a foetus of 195 days; though at term the V/O ratio was near unity (Dicker and Tyler, 1953a). Both activities increase in amounts with age, but the rate at which the oxytocic activity increases is greater than that of vasopressin. Whether in foetuses, newly born or adult animals, there is however no known instance of a V/O ratio smaller than unity; that is to say, there is never more oxytocic than antidiuretic activity in the posterior lobe of the pituitary gland.

The presence of both activities in the hypothalamus was first reported by Abel (1924) and has been confirmed by several workers (Chamorro and Minz, 1957; Dicker and Tyler, 1953b; Kovacs and Bachrach, 1951; Sato, 1928; Trendelenburg, 1928; Vogt, 1953), the oxytocic activity being confined in the posterior part of the hypothalamus, whereas the anti-diuretic activity was located in the anterior part of it. A careful study of this region of the brain in the dog has shown that the paraventricular nuclei contain on the average 465 m.u. antidiuretic activity and 30 m.u. oxytocic activity, the supraoptic nuclei 1,700 m.u. antidiuretic activity and 60 m.u. oxytocic activity and the tuber cinereum 2,000 and 145 m.u. of both activities respectively. Thus, while the pars neuralis of the adult dog has equal amounts of both hormones (16,000 m.u./gland) the whole of the hypothalamus contains some 4,000 m.u. of antidiuretic activity but only 230 m.u. of oxytocic activity; that is, the oxytocic

activity of the hypothalamus represents about 5 per cent of its antidiuretic activity. The question then is whether the small amount of oxytocic activity in the hypothalamus represents the first appearance of the hormone and if not, what is implied? As a result of the observation that arginine-vasporessin exhibits some 5 per cent of intrinsic oxytocic activity, it has been suggested that the small amount of oxytocic activity found in the hypothalamus belongs intrinsically to the antidiuretic hormone. As the hog's antidiuretic hormone exhibits a much smaller intrinsic oxytocic activity than arginine-vasopressin, it would be interesting to see whether the amount of oxytocic activity in the hog's hypothalamus is similarly reduced in relation to its antidiuretic content. Such an investigation is long overdue. It might provide the answer to the question whether the oxytocic activity of the hypothalamus is that of a separate hormone or whether it belongs to the antidiuretic hormone. Indirect evidence supporting the latter hypothesis comes from the observation that the posterior pituitary gland of nursing animals contains less oxytocic than antidiuretic activity (Acher, Chauvet and Olivri, 1946; Acher and Fromageot, 1957; Dicker and Tyler, 1953a,b; van Dyke, Adamson and Engel, 1955) whereas no decrease of oxytocic or antidiuretic activity in the different parts of the hypothalamus has been observed. The implication of these findings is that most probably vasopressin is a precursor of oxytocin. The question of the origin of oxytocin remains, however, unanswered. Two possibilities have been suggested: it is either formed during the movement of the hypothalamic neurosecretion from the neurones to the posterior lobe or its site of formation is not in the hypothalamus, but possibly in the posterior lobe itself (Vogt, 1953).

The evidence so far reviewed based on physiological and pharmacological experiments is on the whole corroborated by results from anatomical and histochemical investigations. The nervous path along which it is assumed that the hormones from the hypothalamic nuclei travel consists of two parts, the supraopticohypophysial tract running in the ventral wall of the stalk and the tuberohypophysial tract which lies dorsally to it. Little is known about the latter. As to the former, it appears to have its origin in the supraoptic and paraventricular nuclei. According to histologists, the ganglionic cells of these nuclei appear to have some secretory activity and produce a substance of protein nature (Divry, 1934; Scharrer and Gaupp, 1933). The amount of the secretory material increases with age. It can be stained with chrome-alum-haematoxylin, a stain which according to Gomori (1941) is specific for neurohypophysial secretions. Granular secretions, Gomori-positive, have been described in the ganglion cells of the hypothalamic nuclei and are distributed in bead-like fashion along the entire length of their axones. Tracts laden with this material have been identified in all vertebrates as going from the paraventricular to the supraoptic nuclei and from there to the pituitary stalk, and Gomori-staining substances have been identified over most of the posterior lobe of the pituitary gland in adults, though more rarely in newborn animals. These findings have led the histochemists to believe

that the Gomori-staining granular formation represents the hormones, either during their migration from the hypothalamic nuclei, or in a stored form in the posterior lobe.

In favour of the neurosecretory theory are, *inter alia* the following data. Gomori-stainable material has been found in greater abundance in the neurohypophysis of dogs than in that of man (Hild and Zetler, 1952); similarly, the concentration of antidiuretic activity is greater in the former than in the latter. After pituitary stalk section in the dog there is a decrease of both hormonal content and neurosecretory Gomori-positive formation in the neural lobe, whereas on the hypothalamic side of the section, there is an increase above the control values of both. A similar correlation has been described in newborn animals, whose degree of maturity at birth varies according to their species. The degree of maturity is usually determined by the eruption of teeth, the development of locomotion, the opening of the eyes, the tolerance to anoxia, and the development of renal functions. By these criteria, the newborn hamster (Auer, 1951) and rat (Heller and Lederis, 1959) are less mature than the seal (Amoroso, Harrison, Harrison-Matthews, Rowlands, Bourne and Sloper, 1958) or the guinea-pig (Dicker and Heller, 1951). The small amount of antidiuretic activity in the pituitary gland of the newborn hamster is about the same as that found in the newborn rat; whereas that of the seal and of the guinea-pig is markedly higher. Similarly, there is much more Gomori-stainable material in the posterior pituitary gland of the newborn seal and guinea-pig than in the infant hamster or rat (Palay, 1957).

The main arguments against the neurosecretory theory are: first, the chrome-alum-haematoxylin stain lacks specificity: it stains equally well the subcommissural organ and microglial cells which have no connections with the neurohypophysis (Wingstrand, 1953; Wislocki and Leduc, 1952). Second, a mixture of absolute ethanol and chloroform will extract the stainable material from the neurohypophysis without affecting the amount of hormonal activity present in the tissue. Third, neither the antidiuretic nor the oxytocic hormones can be stained by the Gomori technique (Acher and Fromageot, 1955). Fourth, the relation between the presence of neurohypophysial activities and the Gomori-stainable material does not always hold; for instance, no trace of Gomori-stainable material was found in the gland of human foetuses younger than 23 weeks (Benirschke and McKay, 1953), but antidiuretic activity has been estimated in pituitary glands of human foetuses from the 14th week of intra-uterine life onwards (Dicker and Tyler, 1953a). Similarly, whereas antidiuretic activity is present in chick embryos after 10 days of incubation, the Gomori-granules appear only 4 days later (Wingstrand, 1953). The posterior lobe of the newborn rat contains appreciable amounts of both antidiuretic and oxytocic activities (Acher, Chauvet and Olivry, 1956; Dicker and Tyler, 1953a; Heller and Lederis, 1959), but there is no evidence of Gomori-granules before the 6th day of postnatal life. Finally, according to Ortmann (1951) heavy hydration is accompanied by a marked increase of the Gomori-stainable material; however, no increase of the antidiuretic

activity in the neurohypophysis has ever been observed in those circumstances.

What then is the so-called secretory Gomori-positive material? Van Dyke, Chow, Greep and Rothen (1942) isolated from the posterior pituitary gland of ox a protein which had both antidiuretic and oxytocic activities; its molecular weight was estimated to be about 20,000 (Block and van Dyke, 1952) though the synthesis of both hormones has fixed their molecular weight at only 1,084 and 1,007, respectively. Van Dyke and others (1942) expressed the view that in the posterior lobe of ox, both hormones are stored in association with a homogenous protein and similar conclusions have been reached by Acher, Manoussos and Olivry (1955) who called the protein neurophysine. The biological activity of van Dyke's protein complex is destroyed by the same enzymes and chemical reagents that affect the active peptides and it could be further shown that the complex is formed by the association of one molecule of vasopressin and one molecule of oxytocin per molecule of protein, both the antidiuretic and the oxytocic hormones being attached to the inert protein either by simple adsorption or as a result of electrostatic force (Acher and Fromageot, 1955).

Recently, Chauvet, Lenci and Acher (1960) have shown that the complex could be dissociated in its three components oxytocin, vasopressin and neurophysine, from which they were able to reconstitute the original molecule; and they could even bring about associations between the hormones and neurophysine of different species. Acher and Fromageot (1955) had previously found that the neurophysine in the presence of the hormones take the Gomori-staining readily, whereas the hormones alone do not. Finally, according to Barrnett and Seligman (1954) the granules in the hypophysis and the hypothalamus which take Gomori's chrome-alum-haematoxylin staining can also be stained by reagents which are specific for the presence of disulphide bonds. As van Dyke's protein is known to be rich in cystine it is reasonable to conclude that van Dyke's protein, Acher's neurophysine and Gomori's granules are the same substance, and cannot therefore be identical with the hormones. It may be of interest to mention here that according to Pardoe and Weatherall (1955) the oxytocic and vasopressor material in the rat's pituitary gland is present in cytoplasmatic particles which behave like mitochondria, and that the two substances do not appear to be contained in the same particles. These observations, however, are at variance with those of Lederis and Heller (1960).

Little is known about the nature of the bonds by which the hormones are attached to the protein-carrier. There are, however, some observations which suggest that the bond that fixes oxytocin is much more labile than that which fixes vasopressin. Acher and Fromageot (1955) confirming an old observation by Dudley (1923) found that oxytocin is preferentially extracted by acetone from the gland of adult animals, and is more soluble in organic solvent than the antidiuretic hormone, a fact which led to the separation of the two peptides by the counter-current distribution method. Heller and Lederis (1959, 1960) also found that the "solubility"

of oxytocin in acetone was greater in newborn than in adult rats, in lactating females than in males. Since the variations in the amount of oxytocin extracted by acetone cannot be attributed to changes in actual solubility of the molecule of oxytocin, this can be explained only either by changes in the nature of the bond between the carrier and the hormone or by physicochemical changes of the protein carrier. The observation by Dawson (1953) that the neurosecretory material in the pituitary gland of infant rats stains differently from that of adult animals may be worth remembering. Though the reason for the greater lability of oxytocin is still not clear, the fact that it can be separated from the protein-carrier more easily than vasopressin is of interest and will have to be borne in mind when considering the mechanism which regulates the release of the hormones from the posterior pituitary gland.

It may then be concluded that in mammals the antidiuretic activity (hormone?) is manufactured somewhere in the hypothalamus and travels with an inert protein (neurophysine?) along some nervous path, until it is stored in the posterior pituitary gland. As for oxytocin, it is not known where it is formed; however, when in the neural lobe, it appears to be bound on the same protein as vasopressin. In the pars neuralis of adult animals, both hormones are stored in equal amounts, one molecule of oxytocin and one molecule of vasopressin being associated with one molecule of the inert neurophysine.

### *Estimation of the Hormones*

Though it may not be necessary to use highly sensitive methods to study the fate of large amounts of intravenously injected vasopressin or oxytocin, they are the limiting factor when it comes to measuring the minute amounts of the hormones normally present in the peripheral blood or in the urine. Since there are no suitable chemical methods for estimating the concentrations of these substances in the body fluids, they must be assayed biologically. An ideal method of assay would be completely specific, extremely sensitive and precise. Simplicity, though not absolutely necessary, is also an important consideration. It is safe to say that no known method fulfils all these requirements.

How specific are any of the methods? One mg. of pure oxytocin assayed in terms of the International Standard has 500 units of oxytocic (rat uterus), depressor (fowl's blood pressure), and milk ejecting (rabbit) activity, and exhibits at the same time 7 units pressor (rat) and 3 units of antidiuretic (dog) activity. One mg. of pure arginine-vasopressin has 600 units of antidiuretic (dog), and pressor (rat) activity, 120 units of milk ejecting (sow) activity and 30 units of oxytocin (rat uterus) activity. Thus there is no complete specificity of an assay method for either hormone; vasopressin is quantitatively the more versatile; however, as 1 mg. of pure oxytocin has 3 units of antidiuretic activity, whereas vasopressin has as much as 600 units, it can be said that the antidiuretic action of vasopressin approaches true specificity. The same thing can be said about the oxytocic activity of oxytocin, but not of its milk ejecting activity. The specificity is limited further by the choice of techniques

used. For example, if oxytocin is injected in a non-pregnant woman it will be less potent than vasopressin in stimulating the uterus muscle. Likewise, when small amounts of antidiuretic hormone such as those which exist in plasma are assayed by intraperitoneal or subcutaneous injections into hydrated rats, non-specific antidiuresis is likely to occur (Dicker and Ginsburg, 1950); for instance, the titre of endogenous antidiuretic hormone of plasma can be up to 25 times higher if assayed by intraperitoneal instead of by intravenous injections (Ames and van Dyke, 1952).

As to sensitivity, the rat under ethanol anaesthesia (Jeffers, Livesey and Austin, 1942) kept with a constant water load (Dicker, 1953; J. Heller, 1959; Thorn, 1957) appears to be the most sensitive animal preparation for antidiuretic assays; its sensitivity varies from 0·001 to 0·005 m.u. Other preparations using dogs, rabbits or mice have a range of sensitivity varying from 0·5 to 2·5, from 0·1 to 0·4 and from 0·01 to 0·05 m.u., respectively. For oxytocin, the isolated rat's uterus (Holton, 1948) is by far the most suitable preparation, though it has obvious limitations: not less than 1 to 2·0 m.u. of oxytocin can reliably be estimated; furthermore, body fluids with low concentrations of the hormone will modify the uterine response, producing non specific contractions.

In view of the lack of sensitivity of any of these methods, attempts at concentrating the hormones have been made either by extraction (Bisset and Lee, 1957; Ginsburg and Smith, 1958) or chromatographic methods (Arimura and Dingman, 1960), combined with one of the methods of assay already mentioned; for example, the rat under ethanol anaesthesia with constant water load for the antidiuretic hormone, or the isolated superfused rat uterus (Gaddum, 1953) for the oxytocic hormone.

The use of unsuitable methods of assay (Birnie, Eversole, Boss, Osborn and Gaunt, 1950) or the lack of a suitable standard of comparison (Mirsky, Stein and Paulisch, 1954) has led to unrealistic claims for the concentration of the neurohypophysial hormones in the peripheral blood. For instance, reports of 140 to 530 m.u. of antidiuretic activity for 100 ml. plasma have repeatedly been made (Hawker, 1953). To assess such results critically it must be borne in mind that an intravenous injection of as little as 1·0 m.u. of arginine-vasopressin ($= 1{\cdot}7 \times 10^{-6}$ mg.) causes a clear cut antidiuresis in a hydrated human subject. Assuming that none of the antidiuretic hormone injected is either inactivated, destroyed or excreted, its concentration in the plasma would be about 0·03 m.u. per 100 ml. plasma (see also p. 458). As a matter of fact, very few methods, if any, have a sufficient sensitivity to estimate such small amounts.

It is because the methods of assaying the hormones in biological fluids are so unsatisfactory that research workers have tried to equate the reactions which follow a stimulation of the neurohypophysis with that produced by a suitable intravenous infusion of the hormone under consideration. This method of matching the response to a stimulation with that produced by the administration of the hormone has proved very useful in animals (Harris, 1947) and in man (Burn and Singh Grewal, 1951; Lauson, 1951). It has, however, obvious limitations and lacks the

precision of a biological assay. Furthermore, some experimental procedures may produce the secretion of hormones in much greater concentration than normally required, and it is a well known observation that very large doses of posterior pituitary extracts may have effects other than those ascribed to vasopressin and oxytocin.

Lastly, when some oxytocic or antidiuretic activity has been found in body fluids or their extracts, its identification with one or the other neurohypophysical hormone must be achieved. The extract will need to possess all the pharmacological characteristics of oxytocin or vasopressin; thus, in the instance of oxytocin it will not only have to contract an isolated rat's uterus but produce milk ejection and exhibit some antidiuretic activity in the required proportion; likewise for vasopressin, it will have to have 5–7 per cent of oxytocic activity besides its antidiuretic activity. When assaying either substance, the dose-response curve will have to be parallel with that of the hormones.

*Release of the Hormones*

It is common knowledge that the release of the antidiuretic hormone is controlled, *inter alia*, by suitably adjusted osmotic stimuli. In theory, the stimulus could be produced either by a lowering or by an increase of the osmotic pressure. In the former hypothesis, an increased water load would alter the degree of stimulation of osmoreceptors and so produce a reflex inhibition of the secretion of the antidiuretic hormone. According to the other theory, the osmoreceptors are stimulated by an increased osmotic pressure, after water restriction, and so produce an enhanced secretion of the hormone. Whichever point of view is adopted, dehydration is accompanied by a release of the antidiuretic hormone.

The release of neurohypophysial hormones can also be achieved by other means such as electrical stimulations of the supraopticohypophysial tract and of the supraoptic and paraventricular nuclei, by haemorrhage and administration of anaesthetics, by emotional stimuli of various kinds, and more specifically in the female by stimulation of the mammilla, dilatation of the cervix and of the body of the uterus as well as by coitus. At first sight it is difficult to see how such different stimuli can produce the same effect. All these stimuli can, however, be divided into two main groups, one concerned with the regulation of body water (for example, dehydration, intravascular injections of hypertonic saline solutions, haemorrhages); the other related to physical or emotional stress. As it would appear that the neurohypophysis is fully dependent on its nerve supply represented by the supraopticohypophysial tract, the broad outline of the mechanism regulating its activity can be summarised in the following way. First, its activity depends on its connections with the hypothalamus. Second, it is influenced, directly or indirectly, by changes in the internal environment. Third, superimposed on the latter, comes the influence of the central nervous system in response to changes in the external environment, which are closely associated with states of stress and act by nervous reflex paths involving the hypothalamus. The supraoptic nuclei are assumed to function as minute osmometers (Jewell, 1953) more specifically

adapted to regulate the secretion of the antidiuretic hormone, when stimulated by changes in the osmotic pressure. For instance, an increase of 1 per cent in the osmotic pressure of the aortic blood lasting over a period of 10 to 40 min., will reduce a water diuresis to about 10 per cent of its initial value, corresponding to a release of about 50 $\mu$u. of vasopressin ($= 2 \times 10^{-9}$ mg. of arginine-vasopressin) per min. Such a change in the osmotic pressure of arterial blood is within physiological ranges. As for all other stimuli (physical, traumatic, emotional stress or excitement) it is likely that they activate nervous pathways to the supraoptic nuclei. Though the anatomy of these afferent nerves is still unknown, there is evidence that certain fibres end on and modify the activity of the supraoptic nuclei and that at least some of them are cholinergic in nature. Intravenous injections of acetylcholine produce antidiuresis. As this can be prevented by removal of the neural lobe, but not by atropine, it can be concluded that the mechanism of antidiuresis is similar to that produced by the nicotinic action of acetylcholine (Burn, Truelove and Burn, 1945; Pickford, 1939). Pickford (1947) localised the site of action of acetylcholine by showing that the injection of 2–40 $\mu$g. of acetylcholine directly into the supraoptic nuclei of dogs produces a release of the antidiuretic hormone. Further evidence for a central action of acetylcholine comes from the observation that much smaller amounts are effective when injected into the carotid artery rather than intravenously. All these results suggest that it is the cells of the supraoptic nuclei which are sensitive to acetylcholine and not the cells upon which the supraopticohypophysial tract terminates, an interpretation which is entirely consistent with the findings that very low values for choline acetylase (an enzyme concerned with the formation of acetylcholine) are found in the neurohypophysis, but substantially higher amounts of it in the supraoptic nuclei (Feldberg and Vogt, 1948). It is interesting to note that small injections of adrenaline, in amounts corresponding to those likely to be liberated from the adrenals during physical or emotional stress, can prevent the liberation of the antidiuretic hormone. Though the inhibitory action of adrenaline has not yet been explained satisfactorily, it is likely that it acts through some specific interference in the chain of chemical reactions initiated in the nervous system by emotional stimuli, and there are grounds to believe that the adrenaline may act on the supraoptic nuclei.

While the sites from which a stimulus produces a release of the antidiuretic hormone from the pars neuralis appear to be located in the supraoptic nuclei, those responsible for the liberation of oxytocin appear to be situated in the paraventricular nuclei (Olivecrona, 1957). Bilateral destruction of these nuclei produces a loss of extractable oxytocic activity from the neurohypophysis, though the antidiuretic activity remains unaffected; conversely, electrical stimulation of the paraventricular nuclei produces a release of oxytocic activity, as estimated by milk ejection, without any decrease of the antidiuretic activity. Whether acetylcholine is the chemo-transmitter for the release of oxytocin (Walker, 1957) is still under discussion (Chaudhury, 1960).

As in the neurohypophysis both oxytocin and vasopressin are bound in the proportion of 1 : 1 to one molecule of inert protein, the possibility of a separate release of one hormone without the other is difficult to conceive, unless it can be shown that the so-called neurosecretory material which contains the hormones breaks up into its constituents before the entry of the hormones into the blood stream. According to Hanström (1952), Rothballer (1953) and Scharrer and Scharrer (1954) stainable granules of the same kind as those in the nerve endings of the supra-opticohypophysial tract have been seen within the blood vessels of the posterior lobe, suggesting that the neurosecretory substance is discharged as such into the circulation. In the blood stream, however, vasopressin is adsorbed on a protein (Heller, 1957; Hipsley and McKellar, 1960) which has been identified as a beta globulin (Thorn and Silver, 1957) and cannot therefore be identical with the protein carrier in the neurohypophysis, which has a molecular weight of about 20,000 only (Block and van Dyke, 1952). If this is so, one has to accept the possibility of the hormones being released from the gland with an inert protein (Scharrer and Scharrer, 1954) eluted from the latter (where and when is not known) and readsorbed in the blood on another protein.

Whatever the final explanation, one thing is certain: any stimulus that releases one hormone releases the other simultaneously. For instance, intracarotid or intravenous injections of hypertonic NaCl or sucrose solutions into a bitch inhibits a water diuresis by liberating the antidiuretic hormone and produces increased uterine activity as a result of the release of oxytocin (Abrahams and Pickford, 1954). Similar results have been observed after electrical stimulation of the hypothalamus in the conscious rabbit (Harris, 1947). Likewise the release of oxytocin which follows suckling or coitus is accompanied by that of vasopressin, in animals and man (Cross, 1951; Friberg, 1953; Kalliala and Karvonen, 1951; Kalliala, Karvonen and Leppänen, 1952; Peeters and Coussens, 1950; Peeters, Coussens, Bouckaert and Oyaert, 1949).

In an attempt to estimate the amount of hormones released after the stimulation of the neurohypophysis, its effects (antidiuresis, uterine activity) have been matched with those obtained after adequate intravenous injections of the hormones. All the results agree and show that whatever the stimulus, both hormones are released together, but that the amount of oxytocin released always exceeds that of vasopressin. Electrical stimulation of the neurohypophysis of conscious rabbits causes antidiuresis and uterine contraction, which could be reproduced by the intravenous injections of a mixture of the hormones in which the oxytocin concentration was more than twenty times that of vasopressin (Harris, 1947). In other experiments specifically designed for estimating the amount of oxytocin released in cows and rabbits, it was found that suitable stimuli produced a simultaneous secretion of both hormones, in a ratio of vasopressin : oxytocin = 1/100 (Cross, 1956; Peeters and Coussens, 1950). In bitches an intravenous injection of hypertonic saline solution causes the release of 15 to 20 times as much oxytocin as vasopressin (Abrahams and Pickford, 1954).

These results raise a series of problems. From the evidence reviewed above, according to which both hormones are bound together on one molecule of inert protein in the pituitary gland, it is difficult to see why any stimulus releases more oxytocin than vasopressin. It may be that the "bonds" which fix oxytocin to the protein carrier are looser than those of vasopressin: evidence to that effect has already been mentioned. Alternatively, it may be that, in agreement with the results of experiments during which the glands of nursing animals have been depleted of their hormones, vasopressin is a precursor of oxytocin, the latter being released as soon as manufactured. As for the discrepancy between the ratio of oxytocic and antidiuretic activities released, it must be remembered that all the above experiments were made before pure hormones were available, and thus the authors used as standards of comparison commercial preparations of unspecified composition. For instance, it is possible that if arginine-vasopressin had been used instead of Pitressin solutions (which in all likelihood contained lysine-vasopressin) the ratio of oxytocin to vasopressin observed in dogs (Abrahams and Pickford, 1954) would be different from that stated. Finally it must be borne in mind that no satisfactory quantitative estimation of plasma hormonal content has yet been achieved. Also, the comparisons of the actions of two different hormones on different effector organs assumes, among other things, that the two hormones under investigation are metabolised, inactivated or excreted at a similar rate. That this is unlikely to be so will be shown later on. It is clear, however, that whatever the interpretation both oxytocin and vasopressin are released simultaneously and that whenever they are released the amount of oxytocin secreted exceeds by far that of vasopressin.

*Role of Age on the Mechanism of Release of Hormones*

It is well known that the kidneys of newborn animals and babies concentrate their urine less well than those of adults and that in old age there is a decrease in the concentrating power of the kidneys. The lack of urinary concentration immediately after birth may be due to an inadequate amount of antidiuretic hormone available, a deficiency in the mechanism of its release, an inability of the kidneys to respond to the hormone, or a combination of these factors. There is some difficulty in assessing the adequacy of available antidiuretic activity present in the neural lobe of newborn infants and animals, due mainly to the difficulty of choosing the right parameter in which to express it: hormonal contents have been expressed in terms of glandular weight, wet or dry, in terms of kidney weight or body surface. However, irrespective of the parameter adopted, the amounts of antidiuretic and oxytocic activities in the neurohypophysis of newborn babies or animals is smaller than that of adults (Dicker and Tyler, 1953a,b; Heller and Lederis, 1959; Heller and Zaimis, 1949): it is about 1/8th of that in adults. This is more than twice the minimum amount needed by adults to prevent the occurrence of diabetes insipidus. It is therefore unlikely that the lack of urinary concentration can be attributed to an inadequate amount of available hormone. Is the

mechanism of release deficient? The most powerful physiological stimulus producing the liberation of the antidiuretic hormone in adults is dehydration, after prolonged water deprivation. According to J. Heller and Stulc (1960) whereas there is a tenfold rise in the plasma antidiuretic activity after 24 hours of dehydration in adult rats, in newborn animals no similar rise has been noted and no plasma antidiuretic activity could be estimated. However, as antidiuretic activity, presumably of neurohypophysial origin, has been repeatedly found in the urine of newborn animals and babies (Ames, 1953; Dicker and Eggleton, 1960) its absence in the blood can be explained only by a lack of sensitivity of the method used. It is possible, however, that the antidiuretic hormone is synthetised at a slower rate in newborn animals than in adults, a hypothesis which would agree with the results obtained by comparing the neurohypophysial content during dehydration in newborn and adult animals: water deprivation for 24 hours leads to a decrease of the antidiuretic activity of the neurohypophysis of new born rats (Heller and Lederis, 1959) whereas in adults it produces a marked increase of it (Ames and van Dyke, 1950; Dicker and Nunn, 1957). Thus, there is no clear evidence of a faulty mechanism in the release of the antidiuretic hormone in newborn animals or babies, and thus the inability of infants to concentrate their urine may be attributed more plausibly to the immaturity of their kidneys (Dicker and Eggleton, 1960; Heller, 1944; McCance, Naylor and Widdowson, 1954; McCance and Widdowson, 1954).

In ageing animals, the pattern of salt and water excretion resembles that of animals with diabetes insipidus. This has been attributed to a decreased ability of the neurohypophysis to respond to osmotic stimuli (Friedman, Hincke and Friedman, 1956). Dicker and Nunn (1958), however, were unable to find any change in the responsiveness of the neurohypophysis to osmotic stimuli and concluded that the decreased ability to concentrate the urine arose from changes in the kidney functions.

*Fate of the Neurohypophysial Hormones*

Gilman and Goodman (1937) found that after dehydration some antidiuretic material appeared in the urine. This was confirmed by Boylston and Ivy (1938) and then refuted by Arnold (1938), Walker (1939) and Krieger and Kilvington (1951). The apparent conflict between these opinions was due to unsatisfactory methods of assay. It is now accepted that antidiuretic activity is present in the urine of dehydrated animals, or in that of animals which have been injected with the antidiuretic hormone (O'Connor, 1951; Dicker, 1954; Dicker and Eggleton, 1960; Dicker and Nunn, 1958; Ginsburg and Heller, 1953; Thorn, 1959). When a commercial preparation of the antidiuretic hormone is injected intravenously into a conscious rat, about 10 per cent of its activity can be recovered from the urine (Dicker, 1954; Ginsburg, 1954; Heller, 1952). The antidiuretic material excreted in the urine of dog or rat after stimulation of the osmoreceptors or after an intravenous injection of vasopressin would appear to be a large molecule (Ames, Moore and van Dyke, 1950; Thorn, 1959). Whether this large molecule is the same as that described



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by Thorn and Silver (1957) in the blood or whether it represents the pituitary octapeptide adsorbed on to a urinary protein is still unknown. Since the antidiuretic activity in the urine can be inactivated by trypsin, indicating the presence of a basic peptide, and can be destroyed after treatment with sodium thioglycollate in the same way as purified vasopressin (Thorn, 1959) it is likely that the kidneys do excrete the hormone.

Since about 10 per cent only of the injected antidiuretic hormone or of the secreted endogenous polypeptide can be recovered from the urine, the question is what happens to the remainder. The likelihood is that it is used up, destroyed or inactivated by tissues. This problem has been studied in *in vitro* and *in vivo* experiments. From *in vitro* experiments it is known that vasopressin is inactivated after incubation with slices or homogenates of kidney (Dicker and Greenbaum, 1954), of liver (Birnie, 1953; Eser and Tuzunkam, 1950; Eversole, Birnie and Gaunt, 1949), of spleen and of duodenum (Christlieb, 1940) but not by that of muscle (Dicker and Greenbaum, 1956). Most of the renal enzyme responsible for the inactivation of vasopressin can be extracted from the particle free supernatant fluid of the tissue homogenate from which it can be precipitated at pH 8 with 40–50 per cent $(NH_4)_2SO_4$ saturation. The inactivation of vasopressin by this fraction can be partly reversed by oxygenation. It could be further shown that the inactivation of the antidiuretic activity of vasopressin is partly due to the reduction of the –S–S–bond of the polypeptide to –SH and that the renal enzyme is in all likelihood an –SH enzyme with a maximum activity in the –SH form (Dicker and Greenbaum, 1958; Fong, Silver, Christman and Schwartz, 1960). However, the inactivation of vasopressin by the renal enzymic preparation cannot be due solely to the reduction of the disulphide link because by treating the reduced vasopressin with agents such as oxidised glutathione or cysteine, only a partial restoration of the hormone's initial antidiuretic activity can be obtained. This suggests that the enzymic preparation has therefore other possible inactivation mechanisms. As it is known that the kidney is rich in amide splitting enzymes, such as glutaminase or asparaginase, it is reasonable to assume that a point for enzymes to attack the vasopressin molecule might be at the amide linkage of the three amides present: glycineamide, asparagine and glutamine. When vasopressin and the crude enzymic preparation are incubated in the presence of an excess of one, two or three amides, for example, under conditions of competitive inhibition, the addition of each of the amides —glutamine, asparagine or glycineamide—causes a reduction in the inactivation process and when all three amides are present there is an almost complete inhibition of the enzymic destruction. It follows that the amide groups of the vasopressin are necessary for its antidiuretic activity and that one of the mechanisms by which the kidney destroys it, besides the reduction of the –S–S–link, may be an attack on the amide groups of the molecule (Dicker, 1960).

These views on the mechanism of inactivation of vasopressin are in good agreement with results of *in vivo* experiments. Ginsburg (1953)

showed that the decreased concentration of intravenously injected vasopressin followed an exponential course, but that the tying up of the coeliac and mesenteric arteries or the removal of kidneys, or both, retarded the rate at which the hormone disappeared. It could be calculated that the kidneys account for 50 per cent of the vasopressin cleared, and the splanchnic area for about 40 per cent, the remaining 10 per cent being excreted in the urine. If tissues such as intestine, spleen, liver and kidneys all take part in the inactivation of vasopressin, how is it that 10 per cent of either exogenous or endogenous vasopressin escapes destruction and can be recovered from the urine, especially as it would appear that the kidneys extract in a single circulation all the vasopressin from the blood which passes through them? (Crawford and Pinkham, 1954). In animals killed 3 min. after an intravenous injection of vasopressin, no antidiuretic activity can be found in the renal tissues, though some of it can be found in the bladder, ureters and kidney dead space (Heller and Zaidi, 1957). As the process of inactivation appears to be confined to the tubular cells, it may be either that some vasopressin is filtered unaltered through the glomeruli, or alternatively, since vasopressin circulates bound to some proteins (Thorn and Silver, 1957), that some of it is secreted by the renal tubules (Ginsburg, 1957).

When oxytocin is injected intravenously into rabbits it disappears quickly from the blood stream but its half life is twice as long as that of vasopressin (Heller, 1960). This suggests that the mechanisms of inactivation of the two hormones are different in spite of the similarity of their molecules. The ligation of the splanchnic vascular area does not affect the rate of disappearance of injected oxytocin, though it retards the elimination of vasopressin considerably. When both kidneys and splanchnic vascular area are excluded, the plasma concentration of oxytocin falls exponentially during the first 7 min. after administration, but thereafter remains constant. Heller's (1960) interpretation of these experiments is that the initial fall in concentration is due to equilibration of the hormone in the extravascular space, but that once this process is completed no inactivation takes place. The volume of distribution of oxytocin at equilibrium is 43·5 ml./100 g. weight as compared with 11·8 ml./100 g. for inulin, suggesting that the hormone distributes itself in a volume markedly greater than the extracellular fluid space (Heller, 1960).

The differences between the rates of inactivation of vasopressin and oxytocin *in vivo* are corroborated by *in vitro* experiments. Whereas both cysteine and glutathione reduce the antidiuretic activity of commercial preparations of vasopressin (Dicker and Greenbaum, 1958) they do not appear to inactivate oxytocin significantly (Dicker, 1960). This agrees with Sealock and du Vigneaud's (1935) observation that a reduction of the disulphide bond of the oxytocin molecule does not result in loss of activity, since the disulphide bridge of oxytocin is constantly reformed under the action of oxygen (du Vigneaud, Ressler, Swan, Roberts and Katsoyannis, 1954). If, however, no oxygen is present, cysteine will in fact reduce oxytocin (Audrain and Clauser, 1958). Other differences are that whereas trypsin destroys vasopressin, it does not attack oxytocin

(Lawler and du Vigneaud, 1953) and that, in contrast with vasopressin, the addition of asparagine, glutamine and glycineamide singly or together does not appear to influence the process of inactivation of oxytocin (Dicker, 1960).

*Influence of Sex, Pregnancy and Lactation*

Ginsburg and Smith (1959) showed that the half life of oxytocin was about the same in male rats, in female rats in oestrous, and in female rats during the last week of pregnancy but that in lactating animals the rate of disappearance of oxytocin from the blood was significantly faster than in any other group. Furthermore, while in female rats in oestrous or during pregnancy, as in male rats, the exclusion of both the renal and splanchnic vascular beds resulted in a steady plasma concentration of injected oxytocin, no constant level was achieved in lactating rats under similar conditions. Thus though there is no evidence of preferential uptake of oxytocin by the uterus of the rats, whether pregnant or not, the hormone is readily inactivated in the lactating animal, presumably by the contractile cells responsible for the let-down of milk.

Though plasma from men or non-pregnant women does not inactivate either vasopressin or oxytocin, both hormones are readily inactivated by plasma of pregnant women. This inactivation is of enzymic nature. As the inactivation of oxytocin by plasma of pregnant women takes place in the presence of oxygen, it is clear from the work of Sealock and du Vigneaud (1935), Audrain and Clauser (1958) and Dicker (1960) that this inactivation cannot depend on the reduction of –S–S–bond, and Tuppy and Nesvadba (1957) were able to show that it was due to an amino-peptidase which split the molecule between its cystine and tyrosine components. Whether this aminopeptidase can inactivate the anti-diuretic hormone in a similar way is still under discussion. Hooper and Jessup (1959) using homogenates of placenta found that the enzyme responsible for the inactivation of oxytocin is present in the soluble fraction of the homogenate, whilst the enzyme destroying vasopressin is located in the mitochondria and microsomes only. Copper inactivates both enzymes, but silver and zinc inactivate oxytoxinase only. TEPP and DFP inhibit the enzymic activity of oxytocinase but not that of vasopressinase. These results suggest that oxytocinase, the enzyme responsible for the destruction of oxytocin, is an esterase with peptidase activity and is different from vasopressinase, the enzyme responsible for the inactivation of vasopressin, which is a peptidase and not an esterase (Hooper, 1960).

It is interesting to note that oxytocinase exists in the plasma of pregnant women and some anthrapoid monkeys only (Caldeyro-Barcia and Poseiro, 1958), but does not exist in the plasma of pregnant rats (Sawyer, 1954), rabbits, dogs and rhesus monkeys (Dicker and Whyley, 1960; 1961). Plasma oxytocinase appears to originate from the placenta.

*Mechanism of Action of Vasopressin and Oxytocin*

Until recently the action of the antidiuretic hormone has been described as restoring "water balance by promoting the reabsorption of the

osmotically free water left by the distal reabsorption of Na. Under the action of the hormone this water is reabsorbed; in its absence this water is excreted" (Smith, 1956). According to Wirz (1956) and to Gottschalk and Mylle (1959) the fluid in the first half of the distal tubule is hypotonic to plasma under all conditions of hydration, but in dehydrated animals it becomes isotonic in the second half of the distal tubule and hypertonic in the collecting ducts. In the kidney producing a concentrated urine, the tubular fluid comes into osmotic equilibrium first with the cortical tissue and later, in the collecting tubules, with the medullary tissue which is known to be increasingly hypertonic towards the papilla. Thus, according to this theory, the essential action of vasopressin is to increase the permeability to water of the distal parts of the nephron and collecting ducts. In 1958, Ginetzinsky found that the urine of several mammals contained hyaluronidase: this disappeared during water diuresis, but was present during osmotic diuresis, in the dog. In a histological study in rats, Ginetzinsky observed that the cement substance between the cells of the collecting tubules reacted as hyaluronic acid when the animals were water-loaded, but as its depolymerisation products when they were dehydrated. He concluded that when stimulated by the antidiuretic hormone, the cells of the collecting tubules secrete hyaluronidase, which in turn depolymerises the mucopolysaccharide complex of the basement membrane of the tubules, hence making "the structures separating the tubule lumen from the interstitial tissue permeable to water. The hypotonic fluid in the tubules then follows the osmotic gradient and undergoes facultative reabsorption" (Ginetzinsky, 1958). This is a new approach to a process which had hitherto been difficult to envisage. In a series of recent papers Dicker and Eggleton (1960a,b; 1961a,b) have shown that (*a*) normal human subjects excrete hyaluronidase when their urine is concentrated, but fail to do so during a water or alcohol diuresis, (*b*) the intravenous administration of either lysine- or arginine-vasopressin at the peak of a water diuresis leads to the excretion of hyaluronidase, (*c*) the concentration of hyaluronidase in the urine is quantitatively related to the degree of antidiuresis produced, (*d*) in cases of nephrogenic diabetes insipidus in which the administration of vasopressin does not produce an antidiuretic response, there is no urinary excretion of hyaluronidase. This suggests that in this rare renal syndrome the lack of response to either exogenous or endogenous vasopressin may be attributed to a failure of the antidiuretic hormone to release hyaluronidase, the absence of which would prevent the urine in the collecting tubules to equilibrate with its hypertonic surroundings. While this is a step forward, it still does not explain how the antidiuretic hormone works. Confirming previous work (Dicker and Greenbaum, 1958; Dicker, 1960) Fong and others (1960) have shown that the antidiuretic hormone is attracted to a receptor in the renal tubules and have postulated that this would involve electrostatic interactions of opposite charges. The hydroxyl group of tyrosine and the amide group of glutamine, asparagine and glycineamide appear in this respect to have a prominent attractive force. When the interactions between attractive and repulsive forces

have produced a suitable alignment of the hormone with its receptor, the thiol-disulphide exchange reaction takes place. This results in a hormone-receptor disulphide, a bond which is ultimately cleft by a reductase in conjunction with one or more enzymes (Dicker and Greenbaum, 1958). The reaction is then reversed and the receptor-sulphydryl group regenerated. From this it would appear that it is the series of sulphydryl-disulphide reactions which underlies the mechanism of increased passive transport of water through the tubules. Whether this is mediated by the secretion of hyaluronidase is not yet known, but is under investigation.

As for oxytocin, it would appear that its primary action on uterine muscle is the lowering of the membrane potential (Jung, 1957) which is followed by a series of tetanic action potentials accompanied by mechanical contractions. The latent period before these discharges occur depends on the hormone concentration. With increasing doses of oxytocin, there is a decrease of both the amplitude of the action potentials and of the time that elapses before the peak potential is reached. As the muscle remains contracted a long time after the action potentials have disappeared, it has been suggested that what has been called uterine tetanus is a contracture. Recent studies by Evans, Schild and Thesleff (1958) however, indicate that the action of oxytocin does not necessarily depend on changes of membrane potentials: an isolated rat uterus, immersed in a Ringer solution in which sodium has been replaced by potassium has no measurable membrane potential. It will contract, however, in response to a number of smooth muscle stimulants including oxytocin.

Two questions which have not yet been answered satisfactorily are, what is the function of oxytocin in the male, and whether in the female it is really of importance in the process of labour? The action on renal functions has been discussed and investigated recently in both sexes by Cross, Dicker, Kitchin, Lloyd and Pickford (1960), with on the whole, negative results. The view that oxytocin plays a part in labour finds its support in the analogy between normal, spontaneous uterine contractions and uterine motility elicited by oxytocin administered in correct dosages (Caldeyro-Barcia and Poseiro, 1958) and in the observation of an increased sensitivity to the hormone as term approaches (Fitzpatrick, 1957; Nixon and Smyth, 1958). Against it are the facts that no increased concentration of oxytocin has been found in plasma of women in labour and that the fate of the hormone in animals does not appear to be affected by the presence of absence of the uterus since it is the same in pregnant females and males (Ginsburg and Smith, 1959). The sole action of oxytocin which has been established with certainty is that on milk ejection. In 1948, Gunther observed that in lactating women in labour each uterine contraction was accompanied by a spontaneous ejection of milk. The milk ejection reaction, which is accompanied by a measurable rise of pressure in the mammary gland is the result of the direct action of oxytocin on the myoepithelial cells (Cross and Harris, 1951; Folley, 1956; Labouche, 1957). The rise in pressure in the ducts converging on the nipple in response to oxytocin is so characteristic that it has been used for the estimation of small amounts of the hormone (Berde and Cerletti,

1957; van Dyke, Adamson and Engel, 1956). It will be noted that these facts are in good agreement with the finding of a decreased oxytocin content of the neurohypophysis of lactating animals (Dicker and Tyler, 1953a) and with the observation that the rate of disappearance of oxytocin is significantly accelerated in lactating rats (Ginsburg and Smith, 1959).

This short review makes no claim to completeness. It is hoped, however, that it will have shown the progress achieved in this field of research and that it will allow an unbiased reader to form an independent judgment devoid of theoretical and dogmatic beliefs on some of the problems under discussion. A few of these are in the process of elucidation, many more await further investigation, the most challenging of them all being the mechanism of action of vasopressin and the role of oxytocin in the male.

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# RESEARCH PAPERS

## STUDIES ON THE KINETICS OF FUNGICIDAL ACTION

### PART I. THE EFFECT OF CONCENTRATION AND TIME ON THE VIABILITY OF *Penicillium notatum* SPORES IN SOLUTIONS OF PHENOL

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About 98 per cent of *Penicillium notatum* spores produced germ tubes in liquid nutrient media within 12 hr. Viability was unaffected by storage in distilled water for 20 days at 4°. The sporicidal activity of phenol was measured by determining the percentage of germinated spores before and after contact with phenol solution. Spores were separated on membrane filters from fungicide by rapid filtration, and incubated in Horowitz fluid medium. The rate of death in solutions containing 0·5 to 1·25 per cent phenol followed a first order reaction. The values of the concentration exponent for 50 and 100 per cent mortalities were 12 and 10·5 respectively.

VARIOUS methods have been used for evaluating the activity of fungicides (Berry and Perkin, 1946; Cochrane, 1958; Gerrard, Harkiss and Bullock, 1960; Horsfall, 1956; Reddish, 1957). Those based on the measurement of turbidity, oxygen uptake or of dry mycelial weight are of limited use (Cochrane, 1958) and appear to be suitable for the determination of extinction times only. Complete correlation of the percentage of germinating spores and the number of colonies obtained by a roll-tube method has not always been realised (Brown and Bullock, 1960). The slide-germination method described by the American Phytopathological Society (1943) for evaluating fungicides determines fungistatic and not fungicidal activity.

The method now described gives quantitative and reproducible results and seems capable of use with a wide range of fungi and fungicides. It facilitates rapid separation of spores and fungicide after reaction, permitting the determination of both the number of viable cells and the amount of fungicide taken up by the spores.

## EXPERIMENTAL

### *Preparation of Spore Suspensions*

*Penicillium notatum* strain 15378 from the Commonwealth Mycological Institute was used. Stock cultures were prepared by incubating slopes of Oxoid Sabouraud glucose agar inoculated with a small quantity of mycelium for 4 weeks at 28°. The slopes were then stored at 4°.

Agar slopes for the preparation of spore suspensions were made from 30 ml. of Sabouraud agar in 8 oz. emulsion bottles. These were inoculated with a loopful of spores from a stock culture and incubated for 10 days at 28°. 10 ml. of water was pipetted on to the surface of the slopes

which were then lightly scraped with a platinum loop to dislodge the spores. The resulting suspension was strained through muslin, centrifuged at 2,000 r.p.m. for 2 min. and the supernatant fluid containing mycelial debris rejected. The spores were suspended in 5 ml. of water. The suspension was transferred to a 30 ml. McCartney bottle containing ten glass beads, five of diameter 8 mm. and five of diameter 4·5 mm., and shaken with a Microid shaker at a moderate speed for 10 min. to break up the spore clumps. The resulting suspension was centrifuged, the supernantant fluid removed and the spores dispersed in 10 ml. of water.

TABLE I

RATE OF GERMINATION OF *P. notatum* SPORES IN WATER, WORT, SABOURAUD, AND HOROWITZ FLUID MEDIA AND ON WORT, SABOURAUD, AND HOROWITZ AGAR

| Incubation time (hr.) | Per cent germination | | | | | | |
|---|---|---|---|---|---|---|---|
| | Water | Wort | | Sabouraud | | Horowitz | |
| | | F | A | F | A | F | A |
| 6 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| 7 | 2 | 7 | 2 | 6 | 6 | 9 | 9 |
| 8 | 0 | 21 | 14 | 42 | 20 | 42 | 42 |
| 9 | 3 | 63 | 28 | 58 | 47 | 86 | 65 |
| 10 | 7 | 94 | 52 | 96 | 81 | 97 | 92 |
| 11 | 13 | 99 | 84 | 99 | 96 | 98 | 94 |
| 12 | 20 | 98 | 92 | 99 | 97 | 98 | 97 |
| 24 | 78 | Not countable | | | | | |

F = Fluid medium
A = Agar medium

This was repeated once more. A portion of the suspension was suitably diluted and the spore concentration determined by counting in a haemocytometer cell. The bulk suspension was then diluted with water to give $10^8$ spores per ml. Sterile apparatus and sterile distilled water were used throughout.

*Preparation of Phenol Solutions*

A stock solution containing 4 per cent w/v of Phenol B.P. in distilled water was prepared and stored at 4°. Dilutions were prepared as required using volumetric flasks and burettes.

*Choice of Recovery Medium*

The suitability of the growth medium was assessed by slide-germination and surface-plating methods.

20 ml. quantities of Oxoid Wort and Sabouraud agar and Horowitz (1947) agar were poured into Petri dishes. Two one drop volumes of a spore suspension of *P. notatum* containing $10^8$ spores per ml. were placed on each plate with a standard dropping pipette. Immediately afterwards, one drop volumes of water and double-strength Oxoid Wort broth, Oxoid Sabouraud fluid medium and Horowitz (1947) fluid medium

were placed on separate microscope slides, with standard dropping pipettes. The procedure was duplicated. One drop of the *P. notatum* spore suspension was added to each slide and mixed with the medium; a square of side about $\frac{3}{4}$ in. was previously outlined on each slide by smearing with Canada balsam to prevent the liquid spreading. The plates and slides were incubated at 28°, the latter in humid chambers prepared by placing glass tubes as supports within Petri dishes containing absorbent cotton wool moistened with water.

The slides and plates were examined microscopically at 1 hr. intervals and the percentage of germinated spores determined by a differential count of 100 spores. The mean of two experiments (Table I) shows that the rate of germination was most rapid in Horowitz fluid medium.

TABLE II

EFFECT OF STORAGE OF *P. notatum* SPORE SUSPENSION AT 4° ON THE RATE OF GERMINATION IN WATER AND HOROWITZ FLUID MEDIUM

| | Per cent germination | | | | | |
|---|---|---|---|---|---|---|
| | Water | | | Horowitz medium | | |
| | Storage period (days) | | | | | |
| Incubation time (hr.) | 0 | 10 | 20 | 0 | 10 | 20 |
| 6 | 1 | 2 | 2 | 1 | 1 | 2 |
| 7 | 2 | 1 | 1 | 9 | 5 | 8 |
| 8 | 0 | 2 | 3 | 42 | 42 | 50 |
| 9 | 3 | 0 | 5 | 86 | 84 | 75 |
| 10 | 7 | 7 | 8 | 97 | 95 | 90 |
| 11 | 13 | 5 | 8 | 98 | 96 | 98 |
| 12 | 20 | 14 | 17 | 98 | 97 | 99 |
| 24 | 78 | 76 | 70 | Not countable | | |

To determine the effect of storage upon the viability and rate of germination of an aqueous spore suspension, counts were made as described above on a fresh spore suspension using water and Horowitz fluid medium, and after it had been stored for 10 and 20 days at 4°. The results are shown in Table II.

*Effect of Phenol on Viability and Rate of Germination*

60 ml. quantities of a suspension containing $10^8$ spores per ml. and a solution of phenol of twice the required strength were mixed after equilibrating at 25°. 20 ml. amounts of the resulting suspension were immediately distributed into each of six test tubes. 10 ml. of water was mixed with 10 ml. of the parent spore suspension to provide a control aqueous suspension containing no phenol.

Five min. after mixing, one of the reaction suspensions was filtered under reduced pressure through a membrane filter (Oxoid) supported in a Seitz filter unit. The filtrate was stored at 4° for subsequent determination of phenol concentration. $2 \times 10$ ml. quantities of water were

immediately passed through the filter to remove the remaining traces of phenol solution.

The filter was removed from the Seitz unit and the spores washed off into a test tube with 2 × 5 ml. quantities of water. A drop of the resulting suspension was added to a drop of double-strength Horowitz fluid medium on each of two microscope slides and incubated at 28°. Differential spore counts were made after 6 hr. and subsequently at 1 hr.

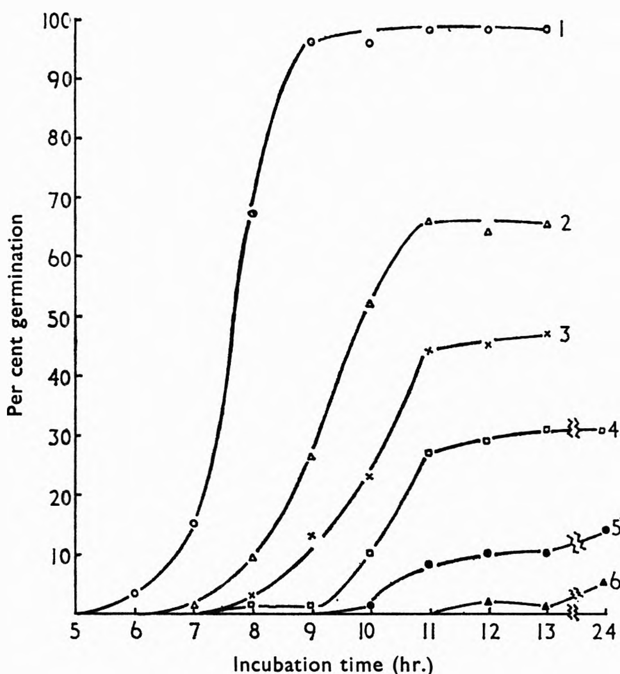


FIG. 1. The effect of time of contact on the viability and rate of germination of *P. notatum* spores treated with 1 per cent phenol.
1. Control (spores in distilled water).
2–6. 5, 10, 20, 40 and 60 min. contact with phenol respectively.

intervals up to 13 hr. and again, where indicated, at 24 hr. The remaining reaction mixtures were treated similarly after 10, 20, 40, 60 and 80 min. respectively, and the control suspension as soon as possible after mixing to obtain the initial number of viable spores.

The experiment was repeated using different concentrations of phenol.

## RESULTS

### *Effect of Medium on the Germination of* P. notatum *Spores*

Germination began in each nutrient medium and in water within 7 hr. but the rate of germination in the following 5 hr. differed markedly and was more rapid in the nutrient liquid media than on the surface of the corresponding solid media; it was most rapid in Horowitz fluid medium

TABLE III

EFFECT OF CONCENTRATION OF PHENOL ON THE VIABILITY AND RATE OF GERMINATION OF *P. notatum* SPORES

| Phenol concentration (per cent) | Contact time (min.) | Per cent germination | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Incubation time* (hr.) | | | | | | | | |
| | | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 24 |
| 0·5 | 0 | 3 | 22 | 73 | 96 | 96 | 97 | 97 | 97 | — |
| | 5 | 2 | 14 | 59 | 86 | 96 | 96 | 96 | 96 | — |
| | 10 | 1 | 8 | 44 | 84 | 88 | 90 | 90 | 91 | — |
| | 20 | 0 | 6 | 37 | 83 | 93 | 90 | 90 | 89 | — |
| | 40 | 0 | 7 | 27 | 76 | 86 | 93 | 92 | 91 | — |
| | 60 | 0 | 2 | 19 | 64 | 77 | 87 | 88 | 87 | — |
| | 80 | 0 | 0 | 15 | 62 | 80 | 88 | 87 | 86 | — |
| 0·75 | 0 | 4 | 26 | 69 | 96 | 95 | 95 | 97 | 97 | — |
| | 5 | 1 | 9 | 48 | 86 | 90 | 92 | 93 | 89 | — |
| | 10 | 0 | 7 | 45 | 82 | 80 | 86 | 89 | 85 | — |
| | 20 | 0 | 6 | 34 | 76 | 79 | 81 | 86 | 84 | — |
| | 40 | 0 | 4 | 36 | 71 | 72 | 80 | 80 | 83 | — |
| | 60 | 0 | 2 | 9 | 58 | 65 | 68 | 69 | 68 | — |
| | 80 | 0 | 1 | 8 | 37 | 60 | 65 | 65 | 68 | — |
| 0·875 | 0 | 3 | 22 | 73 | 96 | 96 | 97 | 97 | 97 | — |
| | 5 | 0 | 6 | 40 | 77 | 80 | 78 | 80 | 80 | — |
| | 10 | 0 | 2 | 26 | 67 | 70 | 71 | 78 | 78 | — |
| | 20 | 0 | 0 | 17 | 41 | 70 | 70 | 73 | 73 | — |
| | 40 | 0 | 0 | 2 | 25 | 43 | 54 (65) | 67 | 66 | — |
| | 60 | 0 | 0 | 0 | 1 | 11 | 26 (41) | 50 | 52 | — |
| | 80 | 0 | 0 | 0 | 0 | 0 | 2 (20) | 32 | 28 | — |
| 1 | 0 | 3 | 15 | 67 | 96 | 96 | 98 | 98 | 97 | — |
| | 5 | 0 | 1 | 9 | 26 | 52 | 66 | 64 | 65 | — |
| | 10 | 0 | 0 | 3 | 13 | 23 | 36 (44) | 45 | 47 | — |
| | 20 | 0 | 0 | 1 | 1 | 10 | 15 (27) | 21 (29) | 31 | 31 |
| | 40 | 0 | 0 | 0 | 0 | 1 | 3 (8) | 10 | 10 | 14 |
| | 50 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 2 | 5 |
| | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1·125 | 0 | 3 | 28 | 80 | 94 | 93 | 95 | 96 | 96 | — |
| | 5 | 0 | 0 | 0 | 2 | 4 | 3 (12) | 21 (28) | 20 (28) | 24 |
| | 10 | 0 | 0 | 0 | 0 | 0 | (3) | 4 (9) | 7 | 10 |
| | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| | 40 | Nil | | | | | | | | |
| 1·25 | 0 | 3 | 28 | 80 | 93 | 95 | 95 | 96 | 96 | — |
| | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 |
| | 10 | Nil | | | | | | | | |

Figures within brackets indicate germinated spores and spores swollen before germination.
* Spores washed free from phenol solution and incubated in Horowitz fluid medium.

(Table I). The slide-germination method using this medium was therefore selected for subsequent experiments.

The percentage germination after 12 hr. incubation was approximately 98 per cent in all the nutrient media except Wort agar in which it was 92 per cent. In water, however, it was only 20 per cent. After 24 hr. incubation the growth of the germ tubes in the nutrient media had produced a dense mycelial mass in which the parent spores were barely visible, whereas in water the germ tubes, now produced by 78 per cent of the spores, were still in a very early stage of growth.

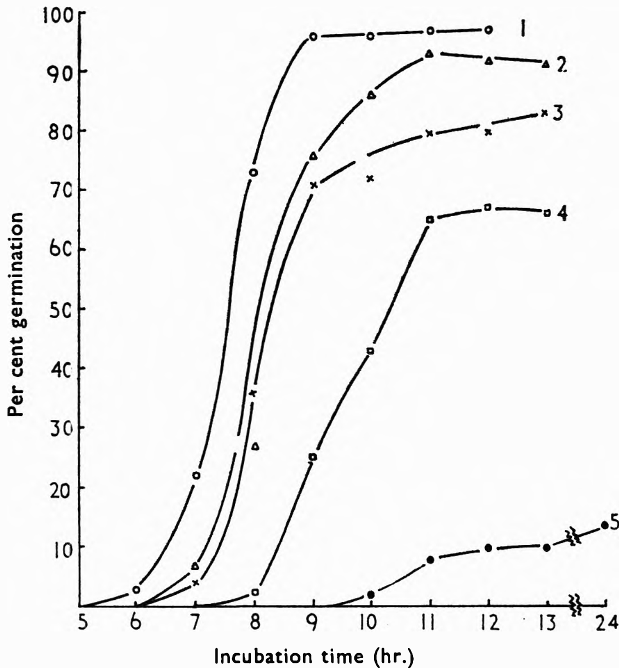


Fig. 2. The effect of concentration of phenol on the viability and rate of germination of *P. notatum* spores. Time of contact = 40 min.
1. Control (spores in distilled water).
2–5. 0·5, 0·75, 0·875 and 1 per cent phenol respectively.

### *Effect of Storage at* 4°

Table II shows that the viability and the rate of germination of a freshly prepared suspension of spores in distilled water was unaffected by storage for 20 days at 4°.

### *Effect of Phenol*

In the weaker concentrations with short contact times the effect of phenol was mainly to increase the duration of the lag phase before germination began; the percentage of spores killed was small (Table III) The duration of the lag phase and the number of spores killed increased with the phenol concentration and the time of contact. This is shown in Figs. 1 and 2, which are drawn from data in Table III.

## Discussion

The method employed for preparing the aqueous spore suspensions was satisfactory as the spores were uniformly distributed and at least 96 per cent were found to be viable on all occasions after incubation in

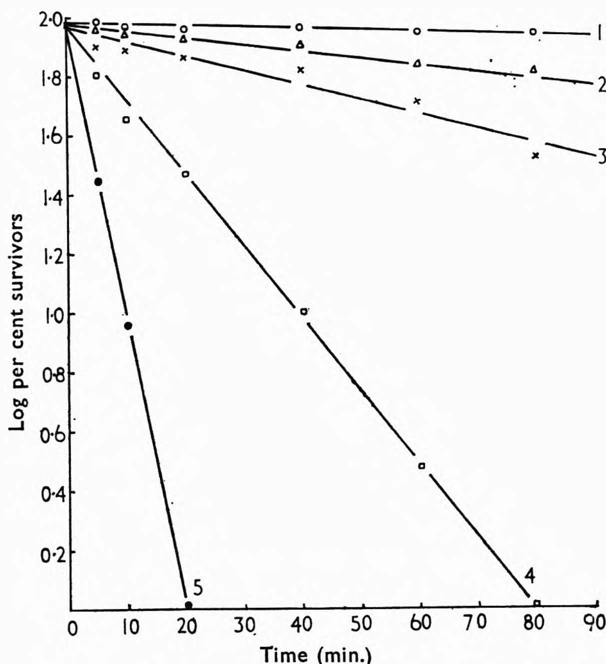


Fig. 3. Log per cent survivor-time curves for *P. notatum* spores in solutions of phenol.
1–5. 0·5, 0·75, 0·875, 1 and 1·125 per cent phenol respectively.

Horowitz medium for 12 hr. About 85 per cent of the spores in suspensions containing $10^8$ per ml. occurred as single cells and the remainder as clumps of 2 to 5 spores.

There was no increase with longer incubation periods than 12 hr. in the number of either untreated or phenol-treated spores germinating in

TABLE IV

Concentrations of phenol required for 50 and 100 per cent death of *P. notatum* spores in 5, 10, 20, 40, 60 and 80 min.

| Contact time (min.) | 5 | 10 | 20 | 40 | 60 | 80 |
|---|---|---|---|---|---|---|
| Log LD 100 .. | 0·11 | 0·097 | 0·0512 | 0·0325 | 0·0150 | 0 |
| Log LD 50 .. | 0·03 | $\bar{1}$·9925 | $\bar{1}$·9775 | $\bar{1}$·9575 | $\bar{1}$·945 | $\bar{1}$·92 |

Horowitz fluid medium (Tables I and III). The percentage of germinated spores after 12 hr. incubation could therefore be regarded as the percentage survivors.

Fig. 3 shows a linear relationship between the log per cent survivors and time for phenol concentrations of 0·5 to 1·125 per cent which indicates that the rate of death of *P. notatum* spores in solutions of phenol follows a first order reaction.

The concentrations of phenol required for 50 and 100 per cent mortalities after 5, 10, 20, 40, 60 and 80 min. respectively, were obtained by plotting the log per cent survivors against log concentration of phenol (Table IV). The calculated regression lines for the relation log concentration: log time for these percentage mortalities are shown in Fig. 4. The slopes of these lines give values for n of 12 for 50 per cent and 10·48 for 100 per cent mortality. Calculation of n from data in Table III using the equation $C^n t$ = a constant, give values of 13·4, 11·8, 11·8 and 13·1 for 50, 70,

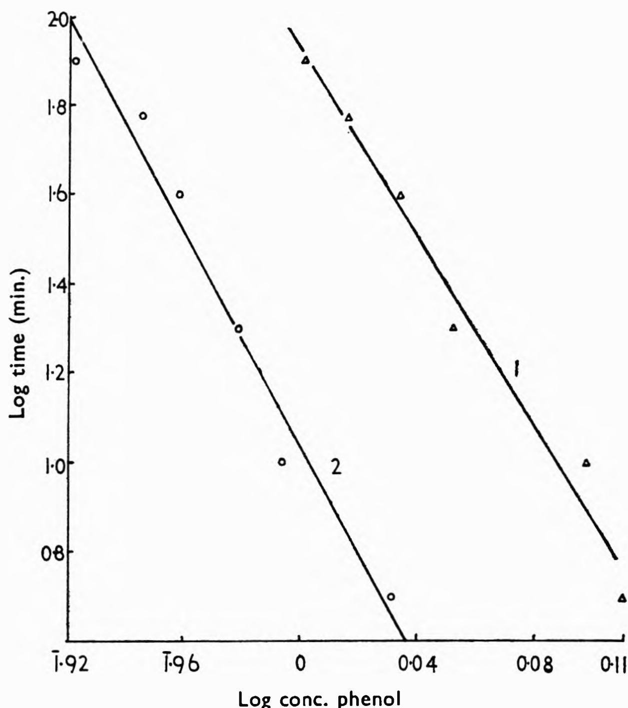


FIG. 4. Relationship between log time and log phenol concentration for 50 and 100 per cent death of *P. notatum* spores.
1. 100 per cent death. 2. 50 per cent death.

90 and 98 per cent mortalities respectively. These values are considerably greater than those of 4·4 and 5·6 obtained for phenol against *E. coli* by Withell (1942) and Jordan and Jacobs (1945).

These high values of n may be explained on the basis that a certain critical concentration of phenol must be adsorbed on to the spore surface for death to occur. Below this concentration there will be a rapid fall in the percentage of spores killed. The relation between the amount of phenol taken up by the spores and the percentage kill is under investigation.

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# THE EFFECT OF FASTING, COLD STRESS AND ACTH ADMINISTRATION ON THE BLOOD SUGAR AND LIVER GLYCOGEN LEVELS OF NORMAL AND ADRENALECTOMISED RATS AND MICE

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Fasting or exposure to cold lowers the blood sugar and liver glycogen levels of rats and mice. These decreases are not affected by adrenalectomy but they are retarded by pretreatment with ACTH.

The rôle of the adrenal cortex in carbohydrate metabolism is not clear, although it has been known for a long time (Britton, 1931) that carbohydrate changes follow adrenalectomy. Britton and his colleagues (Britton and Silvette, 1932; 1934; Britton, Kline and Silvette, 1938; Britton, Silvette and Kline, 1938a,b) concluded that death after adrenalectomy was due to a failure of carbohydrate metabolism. Parkins, Hay and Swingle (1936), however, considered that adrenalectomy led to no disturbance of carbohydrate metabolism. Further, in 1937, Deuel, Hallman, Murray, and Samuels reported that, if rats were maintained on sodium chloride, glycogen deposition in the liver was not impaired by adrenal insufficiency.

Long, Katzin and Fry (1940), in a review of the influence of the adrenal cortex on carbohydrate metabolism, agreed with Deuell and others that adrenalectomised rats and mice maintained on sodium chloride and glucose had no abnormalities of carbohydrate metabolism; when fasted, however, a rapid fall of liver and muscle glycogen occurred.

Animals become more susceptible to stress after adrenalectomy. For example, adrenalectomised mice exposed to cold die more quickly than sham-operated controls. Further, D'Arcy (1957) showed that administration of adrenocortical hormones enabled adrenalectomised mice to survive in the cold as long as, or if the dose was sufficiently high, even longer than, untreated intact mice. A number of adrenal steroids possessing glucocorticoid activity gave this protection, whereas deoxycortone was relatively ineffective (D'Arcy, 1956). Some workers, like Winternitz, Dintzis and Long (1957), have found that the liver glycogen is lowered by adrenalectomy, whilst others, like Young (1946) state that it is unchanged. However, workers agree that the liver glycogen and blood sugar levels of adrenalectomised animals fall rapidly when fasted.

The work reported here was undertaken to examine the effect of adrenalectomy on the carbohydrate metabolism of rats and mice subjected to stress.

## Experimental

### *Materials and Methods*

Wistar rats and mice of the Swiss strain were fed on a cube diet and water, and maintained at a constant temperature of 70°F. Adrenalectomised animals were kept on a normal diet but given 0·9 per cent saline to

drink. Groups of 6 male rats (150–170 g.) and groups of 10 male mice (15–18 g.) were used.

A long-acting preparation of ACTH, Cortrophin Zn (Organon), was injected subcutaneously in volumes of 0·1 ml. (4 units) into mice and 0·5 ml. (20 units) into rats. Injections of normal saline were given in similar volumes to control animals.

*Experimental Procedures*

Adrenalectomy under ether anaesthesia was by the usual lumbar approach. Adrenalectomised mice were used 48 hr. after, and adrenalectomised rats 5 days after, removal of their adrenal glands. Fasted animals were deprived of food but allowed free access to water or saline. Exposure to cold was effected by placing the animals for varying times in a cold room maintained at $2 \pm 0{\cdot}5°$. Mice were placed in pairs under inverted food hoppers and rats were kept in cages containing three animals. The

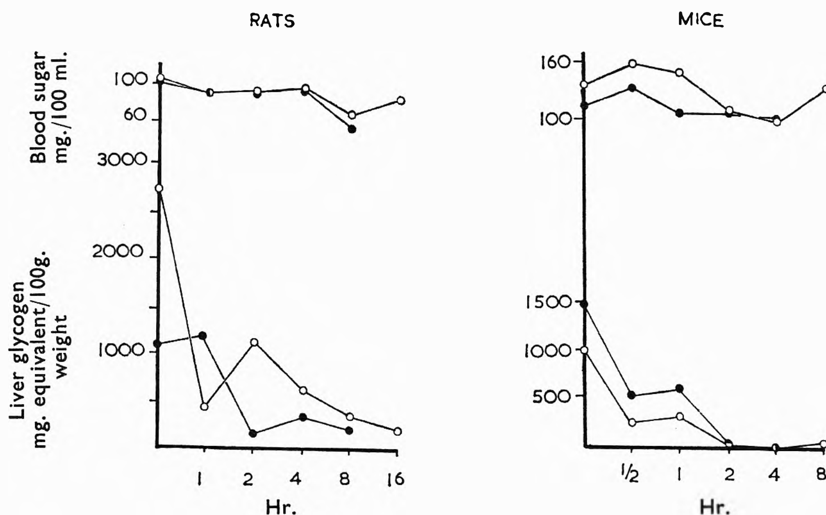


FIG. 1. The effect of cold stress on the blood sugar and liver glycogen levels of intact (○) and adrenalectomised (●) rats and mice.

floors of all the cages were covered with paper to absorb urine. Cold-stressed animals were killed, and their tissues removed, inside the cold room. ACTH was administered 15 hr. before commencement of fasting or of exposure to cold, and a similar dose of ACTH was given 24 hr. later if the animals survived. Liver glycogen was determined by the method of Block and D'Arcy (1958) and blood sugar by the method of Hagedorn and Jensen (1923).

## RESULTS

The results of the experiments are summarised in Figs. 1–4.

*Effect of Cold Stress and Fasting on Carbohydrate Levels*

In both rats and mice, exposure to cold produced a rapid fall in the levels of liver glycogen. Although adrenalectomy did not alter the rate

of fall of liver glycogen, the adrenalectomised animals died earlier. For example, the adrenalectomised mice were dead after 8 hr. exposure and the adrenalectomised rats, with one exception, were dead after 16 hr.,

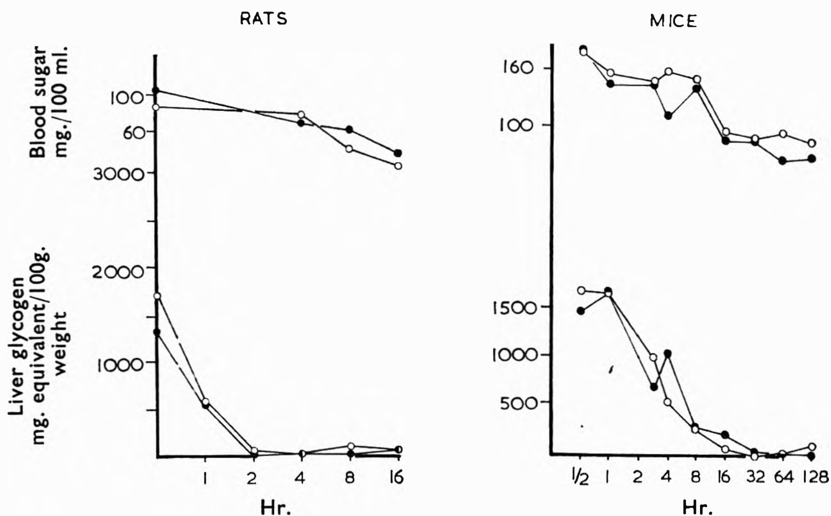


FIG. 2. The effect of fasting on the blood sugar and liver glycogen levels of intact (○) and adrenalectomised (●) rats and mice.

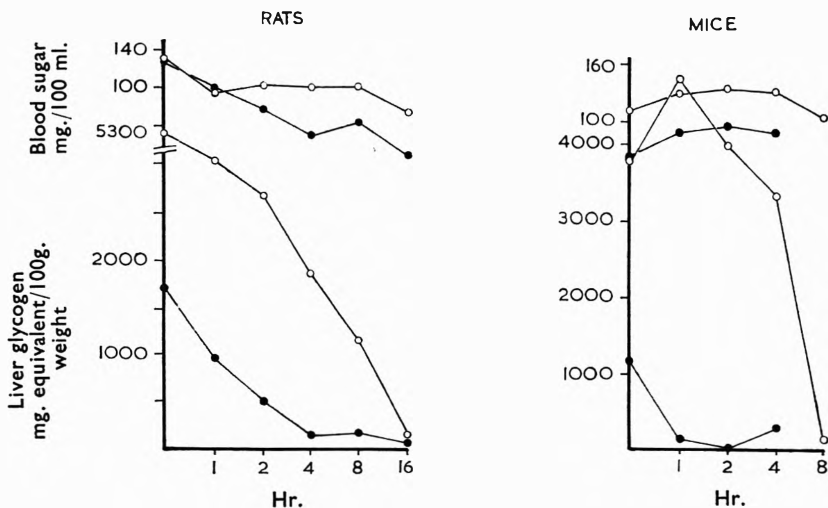


FIG. 3. The effect of ACTH administration on the blood sugar and liver glycogen levels of intact (○) and adrenalectomised (●) cold stressed rats and mice.

whereas the unoperated animals in each case were alive at these times. Fasting produced a similar fall of liver glycogen and a marked lowering of the blood sugar in both intact and adrenalectomised rats and mice.

*Effect of* ACTH *Treatment on Carbohydrate Levels After Cold Stress or Fasting*

ACTH administration had no effect on the liver glycogen levels in adrenalectomised animals whereas the levels in unoperated animals were markedly raised. For example, whereas the mean liver glycogen level in untreated mice was 1,500 mg. glycogen equivalent per 100 g. weight, after ACTH administration it was nearly 4,000; similarly, the level in untreated rats was less than 3,000, and after ACTH it was more than 5,000. Moreover, after fasting or exposure to cold, the raised levels remained high for a long period of time; for instance, after 4 hr. exposure to cold, the liver glycogen level in mice treated with ACTH was still more than 3,000

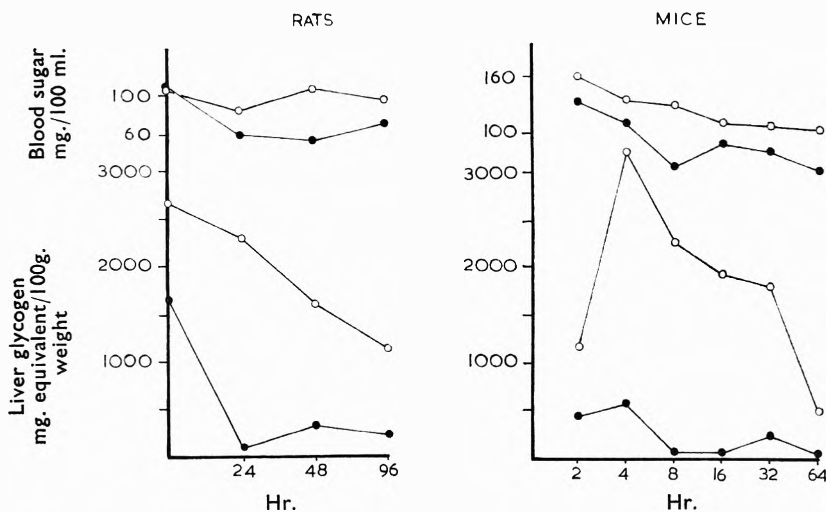


FIG. 4. The effect of ACTH administration on the blood sugar and liver glycogen levels of intact (○) and adrenalectomised (●) fasting rats and mice.

mg. equivalent glycogen per 100 g. weight whereas the level in the uninjected counterparts was almost zero. The blood sugar levels of the ACTH-treated unoperated animals of all four experiments were only slightly higher than those of the ACTH-treated adrenalectomised animals.

## DISCUSSION

*The Effect of Cold Stress*

In this present work it has been shown that when rats and mice die as a result of cold stress, death is independent of the rate of fall of their carbohydrate levels. Death under these conditions is not, therefore, the direct result of failure of carbohydrate metabolism.

Pretreatment of the animals with a large dose of ACTH some hours before the onset of the stress resulted in some alterations in the carbohydrate picture. In unoperated animals the levels were elevated above the resting level but there was no change in adrenalectomised animals. After 8 hr. in the cold, the liver glycogen levels of unoperated mice plunged to a level

reached by the adrenalectomised mice in 1 hr. In the rats, a similar but somewhat less precipitous fall occurs. It is interesting that administered ACTH has this effect, whereas the secretion of animal's pituitary gland does not produce a similar retention of carbohydrate. It may be that the stress is not sufficient to evoke an increased output of ACTH; or that the amount of ACTH produced is not enough to stimulate the adrenal cortex; or that the adrenocortical hormones, even if stimulated by the pituitary, are not produced in a sufficient quantity to influence carbohydrate metabolism.

The protection against cold is produced only by large doses of cortisone, about 1·25 mg/kg. (D'Arcy, 1956). However much ACTH is present, and however great its stimulus, the amount of steroid produced is nevertheless limited by the secreting capacity of the adrenal cortex. This maximum secretion may be sufficient to have a marked effect on carbohydrate metabolism but be insufficient to protect against stress. It may be that corticosterone—the main adrenal cortical steroid in the rat and mouse, and cortisone have two effects; one, to increase the survival time under conditions of stress and the other to exert a carbohydrate effect. A further possibility is that the action on carbohydrate is the more easily produced and the extended survival only occurs when a larger dose is administered. When a large dose of ACTH is administered, the secretion of corticosterone is increased and so the carbohydrate levels rise. Similarly, the protective mechanism of cortisone in cold stress may be explained by the dose being so high that the property of life maintenance becomes apparent.

*The Effect of Fasting*

The blood sugar and liver glycogen levels of normal and adrenalectomised rats and mice fell rapidly and steadily after fasting. Not only did many mice survive, but the survivors were equally distributed between the unoperated and adrenalectomised groups. This result is at variance with those of Britton and his colleagues and shows that adrenalectomised animals do not die because of a failure of their carbohydrate metabolism. However, Cox (1957) showed that the blood sugar of adrenalectomised rats fell to a lower level than that of normal rats when they were subjected to fasting.

The results reported here do not agree with those of other workers. Long (1942), for example, stated that the removal of the food from adrenalectomised animals depletes the liver of its glycogen whereas that of normal animals is unaffected. Further, Evans (1941) reported that adrenalectomised animals lost more carbohydrate than did unoperated animals in the early hours of fasting. However, in the present experiments, the liver glycogen levels of both normal and adrenalectomised animals fell at the same rate after fasting. The fall of carbohydrate in intact animals is difficult to explain, as ACTH administration delays the fall. The endogenous adrenal steroids secreted without ACTH administration may be either incapable of converting protein into carbohydrate faster than carbohydrate is being utilised, or there is no gluconeogenesis. The

results obtained from the experiments with ACTH support the former possibility. The large amount of adrenal steroid secreted as a result of the ACTH stimulus possibly enables the rate of gluconeogenesis to exceed that of glycolysis. The fall of the carbohydrate levels towards the end of the fasting period could be due to the diminishing effect of the ACTH: although the ACTH preparation used is long acting for man, it is not necessarily so for rats and mice, for the metabolism of these animals is faster.

An investigation into the blood corticosterone levels under the conditions of the present experiments may shed more light on this problem.

*Acknowledgements.* I wish to acknowledge the kind interest of Professor G. A. H. Buttle and the valuable technical assistance of Miss M. Roberta Yapp.

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# IPOMOEA—AN ANATOMICAL STUDY

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A detailed anatomical investigation has been made of the dried sliced tubercles of *Ipomoea orizabensis* (Pelletan) Ledanois which constitutes the drug known in commerce as Ipomoea. A list of the characteristic features of the powdered drug is given.

Ipomoea was imported into Europe early in the nineteenth century probably as a substitute or adulterant for Vera Cruz jalap. It occurred as irregular pieces which were sometimes sliced and sometimes roughly hacked from a very large tuberous root. In Germany it was called stipites jalap, jalap stalk and jalap tops and in France, fusiform jalap and woody jalap. In this country it has long been known as male, light and woody jalap.

The botanical name of the plant yielding the drug is *Ipomoea orizabensis* (Pelletan), Ledanois, (Fam. Convolvulaceae). The plant grows in Mexico near to Orizaba and this has led to the drug being known as Orizaba jalap.

Following the work of Power and Rogerson (1912) who declared the resins obtained from Ipomoea and scammony root to be almost identical, Ipomoea was used in place of scammony root as the source of scammony resin. The drug was included in the British Pharmacopoeia 1914 and given the synonym "Mexican Scammony." It has subsequently been included in the British Pharmacopoeias 1932 and 1948 and in the British Pharmaceutical Codices 1934–1959. However, apart from a brief note by Ballard (1911) when he compared its structure with that of scammony, Ipomoea does not appear to have been the subject of an original publication. Thus the descriptions given in the text-books are all brief and are either not illustrated or the drawings are not satisfactory. For this reason it was decided to make a careful anatomical study of the drug.

## Materials

Commercial samples were obtained from drug brokers, merchants and wholesalers and identified by comparison with specimens in the Museum of the Pharmaceutical Society of Great Britain.

## Macroscopical Characters

Most of the samples consist of dried, transverse slices of large tubercles, measuring from 5 to 10 cm. across and 1 to 3 cm. thick though slices less than 1 cm. thick may also occur. Some samples are almost entirely irregularly shaped pieces which may be wedge-shaped or even halves of smaller tubercles cut obliquely while occasionally portions of the narrow part of the root are also present.

The cork is greyish-brown to brown and coarsely wrinkled. Lenticels are not present. The transverse surface is grey or dark brown and

exhibits coarse fibrous protruding strands arranged in 3 to 8 irregular concentric zones. On some pieces of drug masses of dried latex which glisten in reflected light are visible in concentric zones which alternate with the zones of fibres. The drug is light and fibrous and the thinner pieces can be broken easily giving a short fracture. The larger pieces, however, are very difficult to break. It has only a very slight odour and a faintly acrid taste.

The smoothed transverse surface shows a small circular secondary cambium in the centre of the root and concentrically arranged around this are 2 to 7 roughly circular tertiary cambia. These are closer to each other the nearer they are to the periphery. Numerous black dots may be seen in the phloem on the outside of each cambium and on the inside of the cambia scattered xylem groups are readily visible (Fig. 1, A).

## Microscopical Characters

The cork consists of about 15 to 25 rows of cells, which in transverse and longitudinal sections appear brick-shaped and arranged in regular tangential, radial and longitudinal rows. In surface view they appear square or rectangular or polygonal or irregular in shape (Figs. 2, A). The individual cells measure 30 to 85 $\mu$ long, 20 to 45 $\mu$ wide and 5 to 15 $\mu$ high. The cell walls are thin, suberised and very slightly lignified.

The phellogen is not readily discernible but there is a distinct phelloderm. The outermost three or four layers of cells are arranged in a regular radial, tangential and longitudinal pattern but the inner phelloderm consists of irregularly shaped cells often elongated tangentially. Isodiametric sclereids and elongated sclereids are frequently present. The isodiametric sclereids occur either singly or in groups of two or three. They measure 80 to 175 $\mu$ long, 50 to 150 $\mu$ wide and the same in depth. They have lignified cell walls, 10 to 25 $\mu$ thick which show stratification and simple branched pits. The elongated sclereids occur either singly or in groups of two to six. They measure R* and T = 20 to 80 $\mu$ and L = 50 to 120 $\mu$. The cell walls are from 8 to 25 $\mu$ thick and are lignified with simple pits (Figs. 1, B; 2, D).

The most characteristic feature of the phelloderm, however, is the large number of latex cells, the walls of which are thin, brown and suberised. The cells occur in longitudinal rows of 2 to 8 or even 10 cells. The dimensions of the cells are R and T = 60 to 240 $\mu$ and L = 100 to 180 $\mu$. Frequently the radial and tangential are greater than the longitudinal dimensions (Fig. 2, E). The latex is granular and is soluble in ethanol, solution of sodium or potassium hydroxide, solution of chloral hydrate and partially soluble in ether. It is stained bright lemon yellow with solution of iodine. Cluster crystals of calcium oxalate measuring 8 to 48 $\mu$ in diameter are present in some cells and often appear to be concentrated in the outermost cells of the phelloderm. In the innermost cells

* In recording the measurements, the system adopted by Moll and Janssonius (i.e. the dimensions in the radial (R), tangential (T) and longitudinal (L) directions of growth) is used in the majority of cases for the sizes of cells. Where, however, this system might be confusing or ambiguous, the ordinary method of recording the longest and shortest axes irrespective of direction of growth, is given.

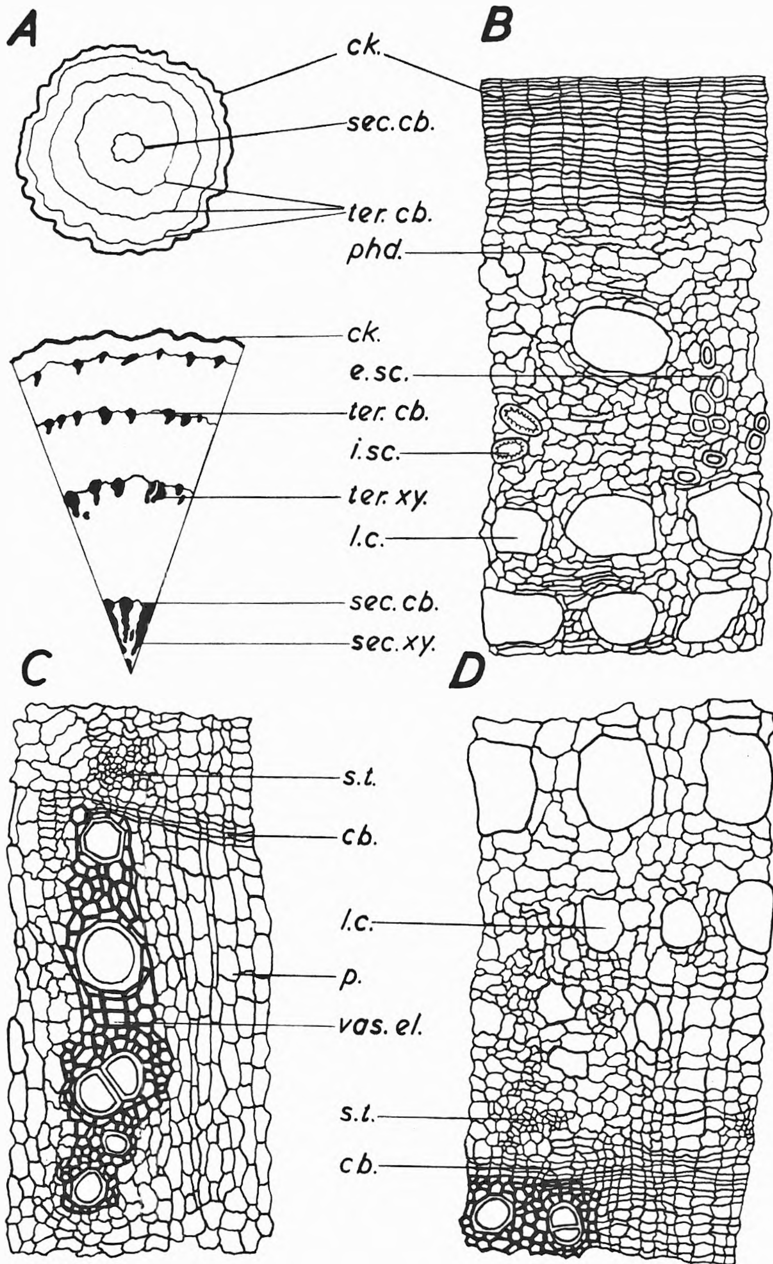


FIG. 1. *Ipomoea.* A. Transverse surface × 1/3–1 and segment of transverse surface × 1–3; B, transverse section showing cork and phelloderm × 90; C, transverse section showing tertiary xylem × 90; D, transverse section showing tertiary phloem × 90. cb., cambium; sec. cb., secondary cambium; tert. cb., tertiary cambium; sec. xy., secondary xylem; tert. xy., tertiary xylem; ck., cork; phd., phelloderm; e.sc., elongated sclereids; i.sc., isodiametric sclereids; l.c., latex cells; p., parenchyma; s.t., sieve tissue; vas. el., vascular elements.

they are arranged in longitudinal files of up to 30 crystals (Fig. 2, E). Prismatic crystals measuring from 10 to 20 $\mu$ are also present (Fig. 2, B).

Apart from the sclereids and latex cells, all the cells of the phelloderm are packed with starch. The individual grains are round, oval or semi-faceted. The round and oval grains are single and the muller-shaped ones are compound, the compounds consisting of 2, 3, 4, 5 or occasionally

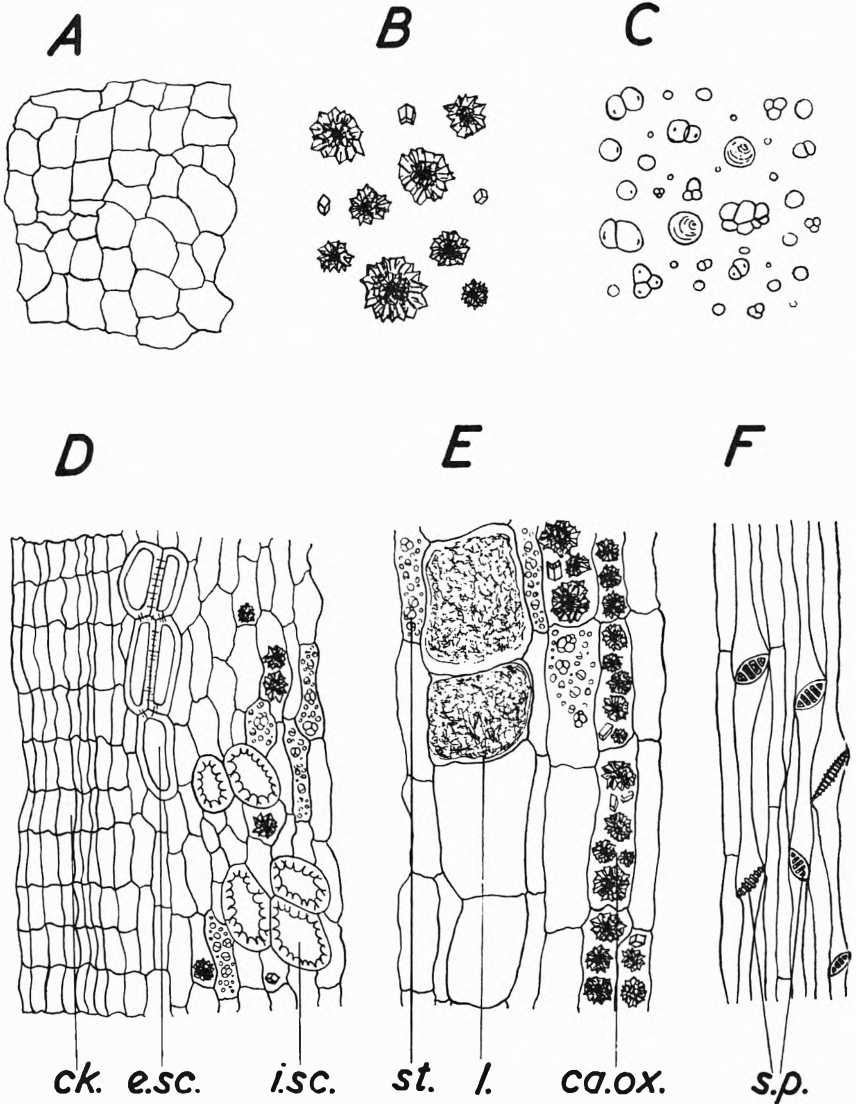


FIG. 2. *Ipomoea.* A, Cork, surface view × 120; B, crystals of calcium oxalate × 180; C, starch × 180; D, cork and phelloderm R.L.S. × 120; E, latex cells × 120; F, Sieve tissue × 120. ck., cork; ca. ox., files of calcium oxalate crystals; e. sc., elongated sclereids; i. sc., isodiametric sclereids; l., latex; s.p., sieve plates; st., starch.

up to 8 individual grains. The grains measure 2–**4·8**–**14·8**–33 μ in diameter but with some grains occasionally up to 40 μ. The hilum is slightly eccentric and cften appears as a slit or radiate cleft. Striations are faintly visible on the larger grains. No gelatinised grains appear to be present (Fig. 2, C).

No primary cortex is present so that the tissue abutting on to the phelloderm is tertiary phloem. This is formed from a tertiary cambium which arises in the previously formed tertiary tissue. The tertiary cambium also gives rise tc some tertiary xylem. There may be up to 7 such tertiary zones of tissue, the first tertiary cambium to have been formed having arisen in the secondary phloem. The tertiary phloem is quite extensive.

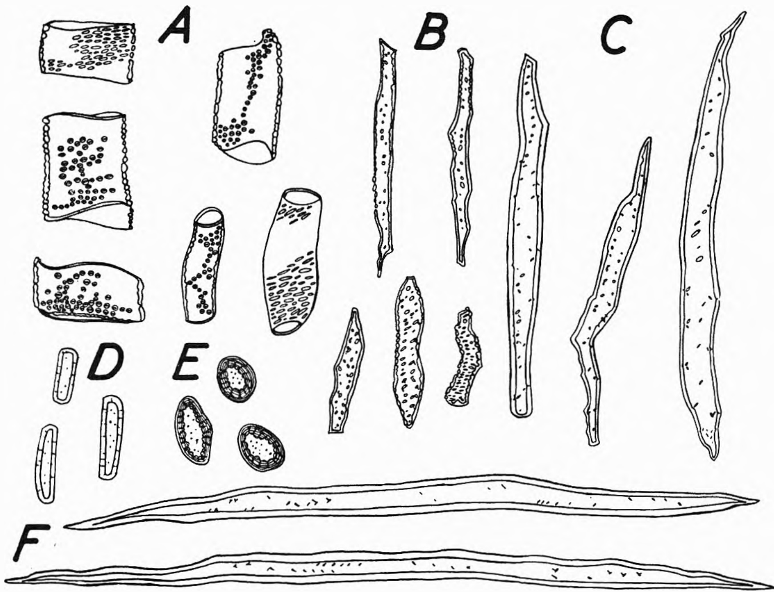


FIG. 3. *Ipomoea*. Isolated lignified elements all × 90. A, vessels; B, tracheids; C, fibre tracheids; D, elongated sclereids; E, isodiametric sclereids; F, fibres.

Although most of the cells are arranged in regular radial rows the tissue is characterised by the longitudinal rows of latex cells. Near to the cambium these are quite small but in the outer regions of the phloem they are large and similar to those in the phelloderm. Hemi-cylindrical groups of sieve tubes are present in the phloem opposite to the xylem element groups. The sieve tubes seen in longitudinal section have oblique end walls which are divided into several sieve areas (Fig. 2, F). Some of the parenchymatous cells of the phloem are slightly elongated tangentially while others are elongated radially. It is not possible to discern in the longitudinal sections any definite medullary rays though in the transverse sections some of the radial rows of cells give the appearance of being uniseriate rays. All the parenchymatous cells are full of starch and some contain crystals of calcium oxalate (Figs. 1, B, D).

The tertiary xylem is a band of tissue on the inside of each of the tertiary cambia. It is composed chiefly of thin-walled parenchymatous cells which are cut off from the cambium in regular radial rows and which are continuous with the radial rows of cells in the inner phloem.

The cells vary considerably in width between one radial row and another and many of them are elongated tangentially. As in the phloem it is not possible to discern any medullary rays in the longitudinal sections. The parenchymatous cells are packed with starch but only an occasional cluster crystal of calcium oxalate is present.

The xylem groups contain vessels, tracheids, fibre-tracheids and true fibres. No tracheidal vessels are present. The vessels have thick walls which are strongly lignified. The pits are usually round or oval bordered. The vessel elements may be long and narrow or they may be short and wide. The dimensions are R and T = 45 to 150 $\mu$, L = 70 to 210 $\mu$. The tracheids have thickened, lignified walls with elongated bordered pits. and measure R and T = 20 to 50 $\mu$, L = 140 to 400 $\mu$. The fibre-tracheids also have thickened, lignified walls with elongated, often slit-like pits. They measure R and T = 15 to 50 $\mu$, L = 250 to 650 $\mu$. The true fibres measure R and T = 15 to 50 $\mu$, L = 300 to 1500 $\mu$ and they have lignified walls up to 10 $\mu$ thick and with small slit-like pits. There are no latex cells in the xylem (Figs. 1, C; 3).

The secondary phloem and central secondary xylem consist of elements similar to those in the tertiary tissues.

## Characters of Powdered Ipomoea

1. Abundant starch, chiefly 2–**4·8–14·8**–33 $\mu$ in diameter, but with some grains occasionally up to 40 $\mu$. The smaller grains are rounded or oval and occur singly; the larger grains are muller-shaped and occur chiefly in groups of up to 8 components.

2. Portions of parenchymatous tissue packed with starch.

3. Numerous cluster crystals of calcium oxalate 8–**11·4–24·8**–48 $\mu$ in diameter. Some prismatic crystals of calcium oxalate 10–20 $\mu$ long.

4. Portions of parenchymatous tissue containing files of cluster crystals of calcium oxalate.

5. Masses of brownish granular latex which stains bright lemon yellow with dilute solution of iodine.

6. Brown cork in surface view, some cells of which have slightly lignified walls. The cells measure 30–85 $\mu$ long and 20–45 $\mu$ wide.

7. Fragments of lignified, thick walled vessels with round or oval bordered pits.

8. Tracheids, often entire, measuring 140–400 $\mu$ long by 20–50 $\mu$ wide and having elongated bordered pits.

9. Fibre-tracheids, often entire, measuring 250–600 $\mu$ long by 15–50 $\mu$ wide, and having oblique slit-like pits.

10. Fragments of fibres, 15–20 $\mu$ diameter with lignified walls up to 10 $\mu$ thick and having small slit-like pits.

11. Isodiametric sclereids, 80–175 μ long, 50–150 μ wide, with lignified walls, 10–25 μ thick and having simple branched pits.

12. Elongated sclereids, 50–120 μ long, 20–80 μ wide, with lignified walls 8–25 μ thick and having simple pits.

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# THE EXCRETION OF SOME AZO DYES IN RAT BILE

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A quantitative comparison of the biliary excretion of sixteen water soluble sulphonated azo dyes has been made to ascertain if any relation between the excretion pattern and molecular structure exists.

AFTER the discovery of the carcinogenicity of the fat soluble dyes 4-dimethylaminoazobenzene (D.A.B.) and 3′-methyl-4-dimethylaminoazobenzene (3′Me-D.A.B.) (reviewed by Miller and Miller, 1953) a number of countries have introduced regulations to control the use of food colours. Dyes which have been proved to cause tumours or to show acute toxicity have not been approved and where little information about toxicity has been available, the chemical nature of the molecule has been used as a guide to its safety.

For azo dyes, the tendency has been to approve those which are highly sulphonated since aromatic sulphonic acids are normally excreted unchanged (Williams, 1959) and because of their highly polar nature they are not so readily absorbed from the alimentary canal (Brodie and Hogben, 1957). Preference is also given to those dyes which are sulphonated in aromatic groups on both sides of the azo linkage so that, should reductive fission of the azo linkage occur, the sulphonated fragments will be readily eliminated. There is however little exact knowledge of the fate of azo dyes in animals, except for a preliminary report by Mellinger and Radomski (1959).

Observations in these laboratories on the metabolism of foreign substances (Cox and Wright, 1959; Slaytor, Pennefather and Wright, 1959) have shown the value of examining their qualitative and quantitative excretion in the bile after intravenous injection. In general, substances of relatively high polarity are poorly absorbed from the gut and excreted rapidly, and usually unchanged, in the bile; those which are readily absorbed are slowly excreted and then mainly as metabolites. We have now examined the biliary excretion of sixteen azo dyes sulphonated on at least one side of the azo linkage with the object of estimating the stability and persistence of the dye in the animal and also to obtain information which might reveal a relation between the chemical structure of the dye and its excretion and metabolism.

## EXPERIMENTAL

*Materials.* All the dyes were commercial samples with the exception of 3′-sulpho-4-dimethylaminoazobenzene, which was prepared by standard methods. Most were specially pure samples for use in foodstuffs, but each dye was recrystallised from water, or more generally, water-ethanol mixtures. Each dye gave one spot only when chromatographed on paper with the solvent systems pentanol : ethanol : ammonia : water (40 : 40 : 10 :

20) and ethyl methyl ketone: 4N acetic acid: water (120:40:25) (Verma and Das, 1956).

*3'-Sulpho-4-dimethylaminoazobenzene.* This dye was prepared by coupling diazotised metanilic acid with dimethylaniline according to the conditions described for the preparation of methyl orange (Vogel, 1956). It was recrystallised as the sodium salt monohydrate from hot water. Found: C, 48·6; H, 4·4; N, 12·4 per cent. $C_{14}H_{14}N_3O_3SNa.H_2O$ requires C, 48·8; H, 4·7, N, 12·2 per cent.

*Biliary excretion.* Albino rats (300 to 400 g. weight) were anaesthetised with urethane and the bile ducts cannulated with the shaft of a hypodermic needle attached to a length of polythene tubing. The dose of each dye injected in aqueous solution into a femoral vein was molecularly equivalent to 1 mg. of 4-dimethylaminoazobenzene per kg. Bile was collected

TABLE I

BILIARY EXCRETION OF WATER SOLUBLE SULPHONATED AZO DYES FROM RATS

| Name | Colour Index No. | Average excreted per cent (4 exp.) | Excretion range |
|---|---|---|---|
| *Azobenzenes* | | | |
| Methyl orange | 13025 | 55 | 38–59 |
| 3'-Sulpho-4-dimethylaminoazobenzene | — | 27 | 15–40 |
| Fast yellow | 13015 | 10 | 2–16 |
| *Phenylazonaphthalenes* | | | |
| Naphthalene fast orange 2GS | 15510 | 46 | 25–60 |
| Red 10BS | 17200 | 12 | 5–20 |
| Geranine 2GS | 18050 | 64 | 60–70 |
| Ponceau RS | 16150 | 15 | 10–20 |
| Orange GCN | 15980 | 23 | 10–40 |
| Sunset yellow | 15985 | 22 | 20–30 |
| Scarlet GN | 14815 | 0 | — |
| Ponceau SX | 14700 | 48 | 30–60 |
| *Azonaphthalenes* | | | |
| Carmoisine | 14720 | 38 | 30–40 |
| Brilliant scarlet | 16255 | 34 | 30–45 |
| Amaranth | 16185 | 53 | 43–79 |
| *Phenylazopyrazoles* | | | |
| Tartrazine | 19140 | 1 | 0–2 |
| Lissamine fast yellow 2G | 18965 | 96 | 95–100 |

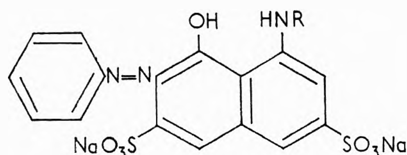
for 6 hr. and the whole sample used for analysis. To the bile was added 40 per cent zinc sulphate solution (3 ml.) followed by 11·2 per cent of potassium hydroxide (3 ml.). The solution was filtered and the precipitate washed with warm water until no more colour was eluted. The combined filtrates and washings were then adjusted to a suitable volume (50–100 ml.) and the optical density measured at the wavelength of maximum absorption. The dye concentration was read from a standard curve prepared with the original dye injected and the total amount of dye excreted then calculated. The identity of the dye was confirmed by spectra and comparative paper chromatography using the systems described above.

*Recovery Experiments.* Using the above methods of extraction and spectrophotometric analysis the recovery of each dye was not less than 95 per cent of the amount (0·5 mg.) added to the bile used as blank.

## RESULTS AND DISCUSSION

The results of the excretory experiments are shown in Table I. The biliary excretion of the dyes studied does not show a regular pattern.

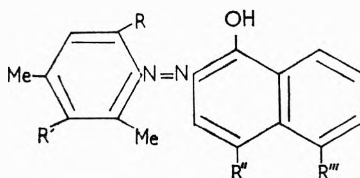
There does not appear to be any relation between the extent of excretion of unchanged dyes and the polarity of the molecule probably because polarity differences in highly sulphonated molecules are of little significance in relation to metabolism. Thus Red 10BS (I, R=H) is excreted only to the extent of 12 per cent whereas its *N*-acetyl derivative (Geranine




(I)

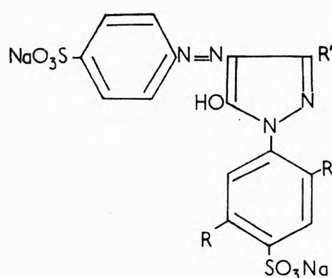
2GS) (I, R=COMe) which is less polar and less water soluble is excreted approximately 60 per cent unchanged.

It might be expected that if reductive fission of the azo linkage is responsible for low excretory rates of the unchanged dye, groups such as a hydroxyl ortho to the azo linkage could affect the stability of the molecule. However, Scarlet GN (II, R=R‴=$SO_3Na$; R′=R″=H) is not excreted




(II)

at all whereas its isomer Ponceau SX (II, R=R‴=H; R′=R″=$SO_3Na$) is excreted unchanged to about 50 per cent. Tartrazine (III, R=H; R′=COONa) which has a hydroxyl group ortho to the azo linkage is




(III)

almost completely absent from the bile, whereas the closely related dichlorinated dye Lissamine fast Yellow 2G (III, R=Cl; R′=Me) is quantitatively excreted.

There is some evidence to indicate that when large doses of azo dyes cause liver tumours the intact dye or some slight modification of it is the active carcinogen (Miller and Miller, 1953). It might be that dyes, such

as Tartrazine, which appear to be without any toxic or carcinogenic properties, are harmless because they are completely metabolised. It could also be inferred that dyes which are excreted quantitatively in the bile are less likely to be harmful than those which are retained. Further study of the metabolism of those dyes which are only partially excreted in bile is needed to determine whether the dye is retained unchanged or whether it is metabolised. The nature of any metabolites as well as the extent of urinary excretion must also be studied and work with these objects in view is proceeding in this laboratory.

*Acknowledgments.* We wish to thank Miss P. Milgate and Mr. J. Barrett for their careful technical assistance. This work is supported by the National Health and Medical Research Council of Australia and we are grateful to the Council of the Australian Food Technology Association for financial assistance.

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# ABSORPTION STUDIES WITH A SUSTAINED RELEASE TABLET

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The absorption of nitroglycerine, lobeline hydrochloride, radioactive ammonium bromide, creatinine, potassium penicillin V, and dihydromorphinone hydrochloride from sustained release tablets has been investigated in cats and in man. The substances were absorbed more slowly from the sustained release tablets than from conventional tablets. With the sustained release tablet fewer side-effects were seen with dihydromorphinone and a lower utilisation of creatinine and potassium penicillin V was observed. *In vitro* and *in vivo* conformity was good where a comparison could be made.

THE principle and *in vitro* control of a sustained-action preparation with the active substance compactly embedded in a porous plastic tablet have been described previously by Sjögren and Fryklöf (1960) and Sjögren (1960). The plastic material, which is insoluble in the digestive fluids, allows only the drug particles at the surface to dissolve. The fluids cannot reach the particles embedded beneath until the surface particles are dissolved. This action progresses successively through the tablet, giving sustained release. The rate of release can be varied over wide limits by modifications in the manufacturing process. The rate of release is relatively unaffected by varying amounts of liquid, mechanical treatment and changes in pH, digestive enzymes, viscosity, surface tension and electrolyte concentration.

Investigations with several substances in this kind of sustained release preparation is now described. Initially tests were made on cats. Later absorption investigations were made in man. In all tests the sustained release tablets were compared with conventional tablets or solutions of the drug or substance being examined. All *in vitro* results were obtained by the method described by Sjögren (1960).

## EXPERIMENTS IN CATS

Cats were anaesthetised with chloralose and urethane in doses of 60 and 100 mg./kg. weight respectively. Conventional tablets or sustained release tablets of nitroglycerine and lobeline hydrochloride were introduced directly into the small intestine with 20 ml. of normal saline through a cannula inserted in the duodenum. The doses were chosen so that a strong but not maximum biological effect was obtained. The absorption of the nitroglycerine was inferred from the induced fall in the mean arterial blood pressure, which was continually recorded from one of the femoral arteries. The absorption of the lobeline was estimated from the induced hyperventilation reflex and the respiratory irregularities. The respiratory movements were recorded by pneumatic tubes placed around the lower part of the thorax and connected to a piston recorder.

The mean values and general trends of the experiments with 10 cats are illustrated in Figs. 1 and 2. Nitroglycerine in the sustained release form gave a slower onset and a longer duration of action than when in

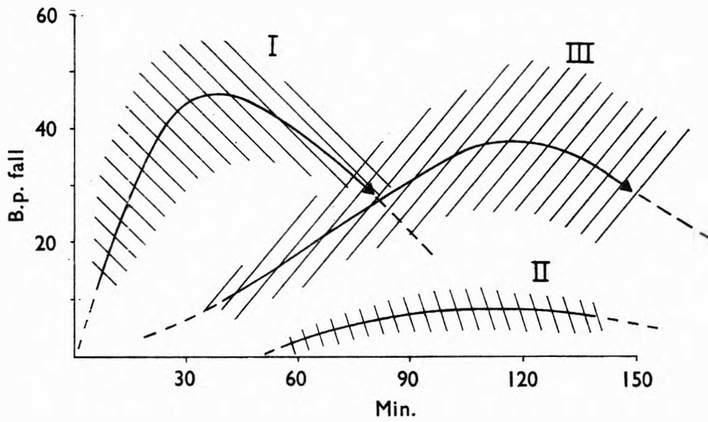


FIG. 1. Effect of nitroglycerine on blood pressure on cats.
I. 3 mg./kg. in conventional tablets
II. 3 mg./kg. in sustained release tablets
III. 10–15 mg./kg. in sustained release tablets.

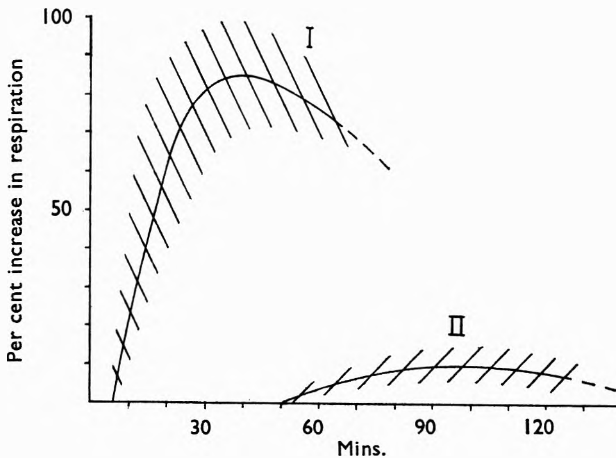


FIG. 2. Effect of lobeline on respiration on cats.
I. 30 mg./kg. lobeline hydrochloride in conventional tablets.
II. 30 mg kg. lobeline hydrochloride in sustained release tablets.

conventional tablets. To reach the same peak response a 3–5 times larger dose was required when administered in the long-acting tablets (Fig. 1). The trials with lobeline gave analogous results (Fig. 2).

## EXPERIMENTS IN MAN

### *Radioactive Ammonium Bromide $^{82}Br$*

Two groups of four subjects fasted before the experiment and received no food during the first 4 hr. They emptied the bladder immediately

before the experiment and two blood samples were taken to measure the radioactivity of the serum. Blood samples were taken $\frac{1}{4}$, $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, 3, 4, 6, 12 and 24 hr. after ingestion of 2 mg. radioactive ammonium bromide (activity 14 mc/g.) as a solution or as sustained release tablets. The blood samples were analysed by measuring the $\gamma$-radiation with a Well-crystal and scintillation detector and were compensated for decay and background. Urine samples were taken regularly during the experiment but the amount of radioactive bromide which was excreted in urine was found to be less than 1 per cent of the ingested activity and therefore negligible.

To obtain comparable values between the individuals, the radioactivity in the samples is given as per cent of the maximum value for each person,

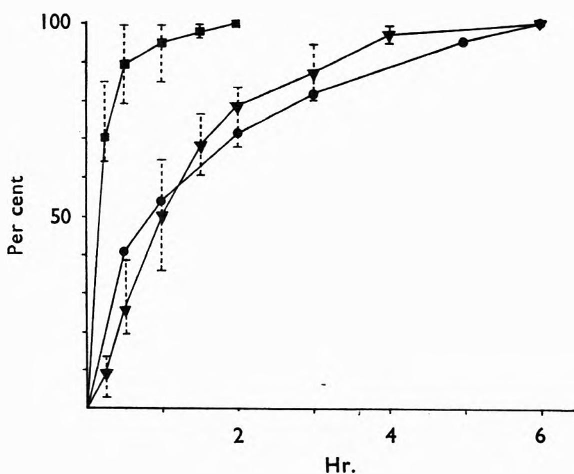


FIG. 3. Release *in vitro* and absorption of radioactive ammonium bromide.
● Amount released *in vitro* from sustained release tablets containing 2 mg. ammonium bromide.
■ Amount absorbed *in vivo* after administration of 2 mg. ammonium bromide in solution (mean value and variation range from 4 persons).
▼ Amount absorbed *in vivo* after administration of 2 mg. ammonium bromide in sustained release tablets (mean value and variation range from 4 persons).

who received either the solution or the sustained release tablets but not both, otherwise for safety it would have been necessary to allow an interval of at least 16 days between the series.

The mean values and ranges from the absorption trials are shown in Fig. 3 together with the *in vitro* release.

*Creatinine*

Five young healthy males fasted for 12 hr. before the experiment. Blood and urine samples were first taken and then the tablets were given with 300 ml. of water. 20 ml. blood samples were taken after 1, 2, 3, $4\frac{1}{2}$, 6, $7\frac{1}{2}$ and 9 hr. and centrifugated immediately. Urine was collected every 3 hr. A standard meal of two sandwiches and 300 ml. of water was taken after 3, 6 and 9 hr. The subjects were seated during the experiment. Each subject received on the first occasion, 24 placebo

tablets on the second 24 creatinine tablets of 125 mg. and on the third the same number of sustained release tablets of the same strength. Creatinine in the plasma and urine was determined according to a modification of the alkaline picrate method of Owen and others (1954).

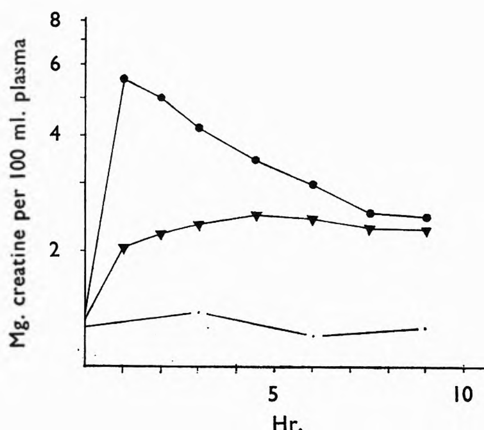


FIG. 4. Creatinine blood concentration (mean values from 5 persons)
● 3 g. creatinine in conventional tablets
▼ 3 g. creatinine in sustained release tablets
· Control

The resulting plasma concentrations are shown in Fig. 4 and the excretion in Table I. The placebo experiments show that the creatinine concentration was, on average, constant and so was the excretion. Conventional tablets showed a rapid absorption. The maximum concentration was reached after 1 hr. and was about 4 mg./100 ml. higher than the

TABLE I

URINE EXCRETION OF CREATININE

| | mg./hr. at hr. | | |
|---|---|---|---|
| | 0–3 | 3–6 | 6–9 |
| Placebo .. .. .. .. .. .. | 87 | 87 | 85 |
| 3 g. creatinine in conventional tablets .. | 312 | 276 | 167 |
| 3 g. creatinine in sustained release tablets .. | 127 | 176 | 112 |

basic value. The concentration then declined relatively rapidly and, after 9 hr., was about 1 mg./100 ml. above the basic value. The average creatinine excretion during the 9 hr. was 1·48 g. greater than in the placebo trials. The total amount absorbed was about 65 per cent of the ingested amount. The sustained release tablets gave a lower, but more constant, blood concentration of about 1 mg./100 ml. over the basic value from 1 to 9 hr. The excretion of the creatinine during 9 hr. was 0·45 g. greater than in the placebo experiments. The total amount absorbed can be estimated at about 33 per cent.

The sustained release tablets released the creatinine *in vitro* with a relatively constant rate for about 5 hr. (see Table II).

*Potassium Penicillin V*

The experiment was made on 36 fasting, healthy subjects, each served as his own control, conventional and sustained release tablets containing 500,000 I.U. of penicillin being taken on alternate occasions. A light standard meal was given 4 hr. after taking the antibiotic. Blood samples,

TABLE II

RELEASE *in vitro* FROM SUSTAINED RELEASE TABLETS USED IN THE ABSORPTION TRIALS

| Sustained release tablets containing | Per cent released at hr. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Creatinine 125 mg. | 25 | 45 | 63 | 77 | 90 | 98 | 100 |
| Potassium penicillin V 160 mg. | 46 | 90 | 99 | — | — | — | — |
| Dihydromorphinone hydrochloride 6 mg. | 48 | 57 | 66 | 74 | 82 | 90 | 96 |

from the finger tips, were taken immediately before the tablets and then after $\frac{1}{2}$, 1, 2, 3, 4 and 6 hr. The amount of penicillin in the samples was determined by the filter plate technique of Eriksson (1960).

The depot tablets released the penicillin dose in 2 hr. (Table II). Even this slight prolongation of supply gave a clear alteration of the blood concentration curves as shown in Fig. 5.

*Dihydromorphinone Hydrochloride*

The pain threshold was assessed in 11 untrained fasting subjects (age 23–26 years) initially and 1, 2, 4, 6 and 8 hr. after the ingestion

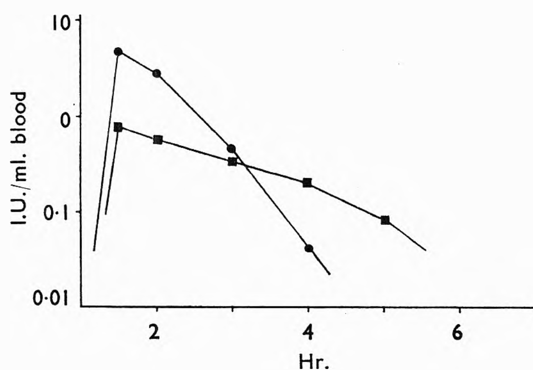


FIG. 5. Blood concentration of penicillin (mean values from 36 persons)
● 500,000 units penicillin V potassium in conventional tablets.
■ 500,000 units penicillin V potassium in sustained release tablets.

of the tablets. Placebo or 6 mg. dihydromorphinone hydrochloride in ordinary or sustained release tablets were given in turn to each subject with at least two days interval between experiments. No food was allowed during the first hr. Subjects were seated comfortably and did not smoke throughout the experiment. The pain threshold was determined by irradiation on the forehead (300 millical./cm.$^2$) with a Hardy-Wolff-Goodell Dolorimeter (Boreus and Sandberg, 1955, 1959).

Measurements were made at six places of the forehead and the mean value calculated. The analgesic effect was reflected in extended reaction time in comparison with the placebo experiments. The statistical calculation of the significance of the results was made as described by Boreus and Sandberg (1959).

Fig. 6 illustrates the results. Both kind of tablets containing dihydromorphinone hydrochloride gave, at all the evaluations from 1–8 hr., a higher pain threshold than placebo. The differences were statistically significant (P = 0·05–0·01). The sustained release tablets gave a higher

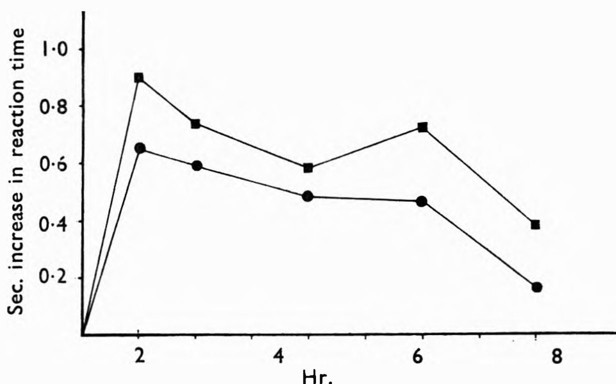


FIG. 6. Analgesic effect of dihydromorphinone hydrochloride expressed as reaction time in excess of the corresonding placebo results (mean values from 11 subjects)
■ 6 mg. in sustained release tablets
● 6 mg. in conventional tablets

pain threshold than the conventional tablets at all the evaluations. The difference was significant. (P = 0·05.)

The *in vitro* release from the sustained release tablets is shown in Table II.

## DISCUSSION

Investigations with this type of sustained release tablet containing sodium nitrite have previously been described by Sannerstedt (1960). He found that compared with conventional tablets larger doses were tolerated and a prolonged action could be attained. Two of the compositions investigated released most of the active substance *in vitro* within 4 hr. One released the main part in the first hr. and the other during the third and fourth hr. The first composition gave a rapid blood pressure fall which remained constant through the 4 hr. whereas the other also gave a rapid pressure fall, but did not attain maximum effect until the third hr.

The methods used in the present investigation with the nitroglycerine and lobeline preparations could not give an exact conception of the absorption rate or degree of utilisation, but they did show that the two drugs in the sustained release form had a slower onset of action and that to obtain the peak effect reached with conventional tablets required much larger doses of the sustained release form of the drug. The duration

of action was difficult to evaluate, a quantitative measure of duration was not obtained, but a qualitative difference was seen.

The experiment with radioactive ammonium bromide was intended to show the association between the release of the active substance *in vivo* and *in vitro*. It is known that bromide ions are easily absorbed and slowly eliminated. The measurements of radioactivity confirmed this. Excretion was insignificant compared with the absorption. The sustained release tablets clearly gave a more prolonged absorption than the solution. The absorption rate agreed well with the release rate for the preparation *in vitro*. The range of the results was similar for the solution and the sustained release tablets, which suggests that the release of activity from the tablets was similar from one individual to the other.

Creatinine has the advantage of being easily and accurately determined in blood and urine. It is absorbed quickly, but not completely, and, apparently, is not metabolised but almost entirely excreted in urine. The sustained release administration gave a lower peak and a more even concentration of creatinine in plasma than the conventional administration, which indicates a continual supply of the substance. The poorer absorption from the sustained release tablets may be caused by a decreasing absorbability along the alimentary canal or an effect of greater dilution. Dominguez and Pomerene (1945) found that absorption ceased 2 hr. after oral administration of creatinine in solution.

Penicillin is easy to determine in blood in therapeutic concentrations, but to maintain an even blood concentration is difficult as it is rapidly excreted. Oral penicillin also gives erratic blood levels because of poor absorption and variable inactivation in the gastrointestinal tract. The sustained release tablets gave a lower peak concentration than conventional tablets in all subjects but gave a longer duration only in a third. The results of the experiments show that potassium penicillin V is not suitable for this type of administration. A slower release of the antibiotic from the tablet is suggested by the results, but no definite conclusions can be drawn about the rate of release.

In the experiments with dihydromorphinone hydrochloride the pain threshold was always higher with the sustained release tablets than with conventional tablets. This unexpected result, with a lower grade of efficiency from the conventional tablets even initially, might depend on the side effects of these tablets (nausea and vomiting). There were no side-effects with the sustained release preparation. The results show that sufficient absorption is obtained from the sustained release preparation to prolong the effect of the drug and that with the drug in this form there is a much reduced risk of side-effects.

In the various trials the sustained release preparation gave a lower peak and a more even drug concentration or action, which indicates that the preparation releases the active ingredient continually as suggested by the *in vitro* analyses. The rate of release *in vivo* is difficult to estimate but seems to agree fairly well with the *in vitro* values in the experiments with radioactive bromide as well as in the investigations described by Sannerstedt (1960).

A lower availability from the sustained release tablets was found with creatinine and potassium penicillin V, and that might depend on rapidly decreasing absorption for these substances along the gastrointestinal tract.

The lower peak concentrations obtained suggest a possibility of reducing side-effects for certain drugs as was demonstrated in the case of dihydromorphinone as well as in the investigations by Sannerstedt (1960).

In earlier trials with this skeleton type of sustained release tablet uncertainty prevailed about discomfort that might be caused to the patient by the undissolved plastic skeleton. No discomfort has been reported over the 5 years that this preparation has been tested.

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# METHOD FOR THE QUANTITATIVE ESTIMATION OF STRYCHNINE AND BRUCINE IN NUX VOMICA BY PAPER ELECTROPHORESIS

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A method for the quantitative separation and estimation of strychnine and brucine in nux vomica seeds has been described. The method involves extraction of total alkaloids, electrophoretic separation in a buffer medium and subsequent estimation in spectrophotometer after elution in suitable solvent. The method is precise and accurate.

NUMEROUS methods have been published for the assay of strychnine and brucine. Waligora and Bylo (1953) used potentiometric chromatography with an antimony microelectrode. The pure alkaloids in admixture have been estimated from optical density values at their ultra-violet absorption maxima (Bhattacharya and Ganguly, 1952; Biggs, 1952). Chromatographic separation on a column of activated alumina (Fischer and Buchegger, 1950; Ridi and Khalifa, 1952) and kieselguhr (Jensen and Svendsen, 1950) have been reported, and separation and estimation have also been achieved by paper partition chromatography (Gore and Adshed, 1952; Briner, 1958).

We have applied horizontal zone electrophoresis on paper to separate and estimate the alkaloids from a crude extract of the drug. Since electrophoresis itself purifies the materials, the presence of impurities or other alkaloids of dissimilar mobilities does not interfere. On the basis of our observations we describe a method in which a simultaneous estimation of both the alkaloids is possible with an accuracy of $\pm 5$ per cent.

## EXPERIMENTAL

*Standard strychnine solution.* 98·9 mg. of strychnine sulphate N.F. was accurately weighed after drying and was dissolved in water to a volume of 50 ml.

*Standard brucine solution.* 98·8 mg. of brucine sulphate N.F. was similarly treated.

*Standard buffer solution.* 100 ml. of 0·1 N solution of citric acid (B.P.) neutralised with 20 ml. of 0·1 N solution of sodium hydroxide (B.P.) at room temperature (25°). The solution had a pH of 3·10 when measured with the glass electrode.

*Sodium nitrite solution.* 5 g. of sodium nitrite (pure) dissolved in water to make 500 ml. was used as electrolyte.

*Modified Dragendorff's reagent. Solution A.* Bismuth subnitrate 1·062 g. was dissolved in 50 ml. water and 12·5 ml. glacial acetic acid.

*Solution B.* Potassium iodide 25 g. was dissolved in 62·5 ml. water. The spray reagent was prepared from 1 ml. of solution A, 1 ml. of solution B with 2 ml. of glacial acetic acid and 10 ml. of water.

*Extract of Nux Vomica*

Powdered seeds of nux vomica (40 mesh) (10 g.) were shaken with 100 ml. of a mixture of ether and chloroform (2: 1) and 5 ml. of dilute ammonia for about 2 hr. The extract was transferred completely into a percolator and extracted with the same solvent mixture till the percolate became free from alkaloid (about 6 hr.). The percolate was filtered through paper and the filtrate was extracted with successive volumes of 20 ml., 10 ml., 10 ml. and 10 ml. of N sulphuric acid which was also used to dilute the extract to 100 ml.

*Apparatus.* Electrophoresis was conducted in an apparatus, locally made from Perspex sheet with an arrangement for adjusting the length of the paper strip according to needs.

Whatman No. 1 paper was used after washing with 1 per cent hydrochloric acid and a flush of water and subsequent drying in air.

Power was supplied from a stabilised constant voltage/current power pack (locally made) operating from 220V A.C. line with a maximum output of 1000V D.C. at 50 mA.

A Hilger "Univispek" spectrophotometer with a matched pair of quartz cells of path length 1·00 cm. was used for measuring optical density values.

*Method*

An aliquot of 0·05–0·1 ml. of a mixture of equal volumes of the standard solutions of strychnine sulphate and brucine sulphate containing 50–100 $\mu$g. of each of the alkaloids was spotted with an Alga syringe on a paper strip of 6 in. $\times$ 14 in. at two places along a straight line, about 4 in. from one end, one to locate the position of the substances after spraying, the other for the quantitative elution and estimation. The paper was then moistened with a fine spray of the buffer solution.

The whole arrangement was placed in an air-tight compartment and was allowed to saturate for 15 min. and then 250 V at 5–10 mA was applied. Electrophoresis was continued for 6 hr. The paper was then air dried and separated into two strips, one of which was developed by spraying with modified Dragendorff's reagent. Both strychnine and brucine were found to migrate to the negative pole with different mobilities, the former about 16 cm. the latter 13 cm. from the base line. The respective positions were then located on the second strip and the alkaloids were cut out and eluted separately in 10 ml. of 0·01 N sulphuric acid solution at 36° for 6 hr. A blank was run by cutting a similar portion from the same paper and eluting in the same quantity of the solvent. After elution, the solutions were centrifuged and the supernatants were assayed in the spectrophotometer against the respective blanks. The $\lambda_{max.}$ of strychnine and brucine being at 252 m$\mu$ and 262 m$\mu$ respectively, their $E$ (1 per cent, 1 cm.) values were calculated from the concentration density values of pure samples and were found to be as follows: strychnine, $E$ (1 per cent, 1 cm.) 252 m$\mu$ = 299·5; brucine, $E$ (1 per cent, 1 cm.) 262 m$\mu$ = 232·5 (in 0·01 N sulphuric acid).

Results obtained from the known quantities of the mixtures of standard strychnine and brucine solutions have been listed in Table I (A).

For the quantitative estimation of the alkaloids in nux vomica seeds, an aliquot of 0·05–0·1 ml. of the extract of nux vomica was similarly treated on the paper. Results have been shown in Table I (B).

A comparative study was conducted by estimating strychnine and brucine content of the samples according to the method as described in the B.P. (1958). The total alkaloid was first determined gravimetrically followed by strychnine assay by volumetric titration. The difference between these two values was considered to be the brucine content of the sample.

TABLE I

COMPARISON OF RESULTS OBTAINED BY ELECTROPHORESIS WITH THOSE OBTAINED BY CHEMICAL ASSAY

| | Strychnine | | | Brucine | | |
|---|---|---|---|---|---|---|
| Group | Present µg./ml. | Found by electrophoretic method µg./ml. | Found by chemical method µg./ml. | Present µg./ml. | Found by electrophoretic method µg./ml. | Found by chemical method µg./ml. |
| (A) | 9·88 | 9·40 | 9·56 | 9·45 | 10·05 | 9·41 |
| | 10·00 | 10·16 | 10·08 | 9·80 | 9·30 | 9·78 |
| | 4·92 | 4·85 | 4·90 | 5·00 | 5·15 | 5·06 |
| | 25·00 | 26·30 | 25·10 | 25·00 | 26·60 | 25·18 |
| (B) | — | 6·01 | 6·30 | — | 10·50 | 10·04 |
| | — | 12·10 | 12·32 | — | 15·90 | 15·00 |
| | — | 13·70 | 13·02 | — | 20·00 | 19·50 |
| | — | 7·93 | 7·50 | — | 13·15 | 13·30 |

The results of chemical assays are also recorded in Table I.

## DISCUSSION

We have observed that both strychnine and brucine obey Beer's law at their respective absorption maxima within concentrations of 5 to 40 µg./ml. of solution in 0·01 N sulphuric acid. The alkaloids are stable in acidic buffer medium. The absorption curve after electrophoresis showed no change in their physical properties.

The positions of the alkaloids on the paper can also be located by contact ultra-violet photography using a chlorine gas filter, when the same strip of paper can be eluted.

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# PHARMACOPOEIAS AND FORMULARIES

## THE DISPENSATORY OF THE UNITED STATES OF AMERICA 1960 EDITION*. VOLUME 2. NEW DRUG DEVELOPMENTS

REVIEWED BY G. R. BOYES

THE rapidity with which new substances are introduced into therapeutics has imposed a colossal task on the compilers of the New Drug Developments volume of the U.S.D., which contains monographs on 209 substances developed since the publication of the 25th Edition five years ago. This spate of new drugs also makes it essential for critical physicians and pharmacists to have access to reliable information on which to assess the merits or demerits of the drugs. The U.S.D. supplies this information together with references to reports of original work, from which statements have been abstracted, or where fuller details of methods of chemical synthesis, pharmacological properties, toxicity, therapeutic uses and side-effects may be found. Discoveries in chemistry, pharmacology and therapeutics have no national boundaries outside the iron-curtain; it is suggested that kanamycin which was discovered in Tokyo in 1957 and marketed in the United States in 1958 established some sort of a record for haste in international transmission from new laboratory discovery to available bedside treatment. Work in the Chester Beatty Research Institute in London led to the development of chlorambucil, a cytotoxic agent better tolerated and better absorbed than triethylene melamine. Chemical constitution is discussed with reference to that of analagous compounds, and attention is called to structure-action relationships. The enhanced pharmacological activity resulting from the introduction of fluorine into molecular structures is discussed for a number of compounds such as the corticosteroids, phenothiazine derivatives, saluretic agents and fluoxymesterone.

Antibiotics account for many of the additions and are discussed collectively, as well as individually under their generic names. The advantages and disadvantages of using corticosteroids simultaneously with antibiotics are clearly expounded; antibiolymphins—salts of antibiotics with polysaccharides or certain polycarboxylic acids—which have increased affinity for the lymphatic system, and methods which have been suggested for increasing blood-levels of tetracycline after oral administration are described. Varying opinions about the desirability of using antibiotics routinely for prophylaxis are reviewed. The importance of pH control in the assay of the antifungal activity of the amphotericins, the limitations of colorimetric methods of assay for oleandomycin, erythromycin and carbomycin and the numerous independent "discoveries" which led to the assignment of nine different names to the antibiotic now known as novobiocin are among the many other details to be found in this extensive survey.

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A review of the theories which have been advanced to explain the mode of action of hypoglycaemic sulphonylureas and biguanides; the pharmacology of spironolactone, a 17-spirolactosteroid which blocks the sodium-retaining action of aldosterone on the distal convoluted renal tubule, but which requires more extensive clinical study before its usefulness can be evaluated, and the specific anti-emetic action of trimethobenzamide hydrochloride are further examples of the information to be found here.

The use of enzymes in therapeutics is illustrated by desoxyribonuclease (pancreatic dornase) employed by aerosol inhalation to reduce the tenacity of pulmonary secretions, fibrinolysin given by intravenous injection to dissolve vascular clots in thrombophlebitis, and chymotrypsin used to promote the absorbtion of oedema fluid and blood in inflamed or traumatised areas. Uricosuric properties are possessed by sulfinpyrazone, the sulphoxide metabolite of an analogue of phenylbutazone and by zoxazolamine which also relieves spasm of skeletal muscle by an action resembling that of mephenesin. Antihypertensive drugs include trimethidinium methosulphate, syrosingopine and mecamylamine hydrochloride. Among the new psychotherapeutic compounds are imipramine hydrochloride, methaminodiazepoxide hydrochloride and $\beta$-phenylisopropylhydrazine hydrochloride.

Chemical compounds used as aids to diagnosis are mentioned with appropriate details of technique. There is a description of the use of radio-iodinated serum albumin for the determination of blood-volume, plasma-volume, blood circulation time and cardiac output and for the detection and localisation of brain tumours. A rapid simple *in vitro* test for estimating thyroid function measures the uptake of $^{131}$I-labelled liothyronine by a sample of the patient's blood. Azuresin, a dye coupled with a carbacrylic cationic exchange resin is used in the tubeless analysis procedure for the detection of achlorhydria and is better for this purpose than quinine carbacrylic resin. Bunamiodyl, a derivative of acrylic acid, is rapidly absorbed after oral administration and concentrated in the gall-bladder. It is used as a radio-opaque cholecystographic medium and, as the drug is excreted by the kidneys, intestinal opacities do not obscure the gall-bladder shadow.

Oxethazaine is a local anaesthetic used for the relief of gastritis; proparacaine hydrochloride is a surface anaesthetic for use in ophthalmic surgery and metabutoxycaine hydrochloride a local anaesthetic for use in dental surgery.

Drugs still in the stage of clinical investigation are not excluded but the available evidence concerning them is reviewed. This is a remarkably informative publication.

# LETTERS TO THE EDITOR

## An Interfering Glycoside in the Chemical Assay of Senna

SIR,—The chemical assay of senna by the method of Kussmaul and Becker (1947) and its modifications (Fairbairn and Michaels, 1950; Auterhoff, 1951) assumes that the sennosides are the only acidic anthracene glycosides present in significant amounts. The isolation by Fairbairn, Friedmann and Ryan (1958) of a primary glycoside of senna having a biological to chemical assay ratio of one and a half times that of the sennosides suggested the necessity for a re-appraisal of these assay procedures. An attempt was therefore made to develop an assay based on the fact that the primary glycoside is very soluble in water and the sennosides are almost insoluble.

During this work it was noted that alkaline solutions of aglycones obtained from a 70 per cent ethanol extract of senna pods were red-brown in colour, whereas those obtained from the sennosides were bright yellow. It was concluded that 70 per cent ethanol removes extraneous coloured material and in an attempt to eliminate this interference a 1 per cent sodium bicarbonate solution was used for the extraction of the total glycosides. Pure sennosides showed no decomposition when subjected to this treatment, but a reduction of from 10–20 per cent in the apparent sennoside contents of the pods examined was obtained. At the same time a marked increase in the apparent free anthraquinone content was noted and it was assumed that the reduction in apparent sennoside content was caused by decomposition of a glycoside present in the pod. Paris and David-Cuny (1955) have suggested the presence of significant amounts of anthraquinone glycosides in senna leaf, but their method of assay does not distinguish between acidic glycosides, which interfere in the Kussmaul and Becker assay and non-acidic glycosides, which are removed at the sodium bicarbonate extraction stage. Alexandrian senna pod was treated with a 1 per cent sodium bicarbonate solution and the liberated aglycone isolated. On recrystallisation from pyridine it gave orange-yellow needles of rhein; m.p. 326° (decomp.); acetyl derivative m.p. 246°; these were insoluble in water, but soluble in alkalis to give red coloured solutions; the infra-red spectrum and $R_F$ values obtained by paper chromatography were similar to those of an authentic sample of rhein.

A small quantity of a rhein-containing glycoside has now been isolated from Alexandrian senna pod as dull-yellow micro-crystals, insoluble in ether, but soluble in water and in dilute alkalis, the latter giving orange-red solutions. It is hydrolysed by heating with 10N sulphuric acid to give rhein in 60 per cent yield by a colorimetric assay, a value in accordance with rhein monoglucoside. When 10 mg. doses of the glycoside were administered orally to mice of mean body weight 25 g., no purgative effect was produced.

A quantity of the glycoside is being isolated for further examination.

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## Absence of Spasm in a Sensitive Assay for Acetylcholine

SIR,—One of the difficulties encountered by Blaber and Cuthbert (1961) in the use of di-isopropylphosphorofluoridate (dyflos, DFP) for increasing the sensitivity of the isolated guinea-pig ileum to acetylcholine is the tendency of the preparation to go into spasm at 20–30 min. intervals. Neostigmine and ethyl pyrophosphate (TEPP) have similarly been found to produce spasm.

Experience with the organophosphorus anticholinesterase mipafox (*NN'*di-isopropylphosphorodiamidic fluoride) in this department (Harry, 1961) has shown it to be almost devoid of this property and suggested its suitability for increasing the sensitivity of the guinea-pig ileum to small concentrations of

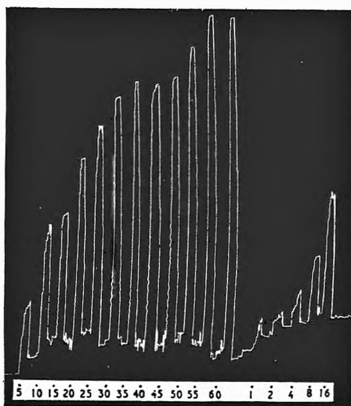


FIG. 1. Isolated guinea-pig ileum; mipafox $1 \times 10^{-5}$; morphine sulphate $5 \times 10^{-6}$; Krebs's solution at 37°; a dose-response curve to acetylcholine 5 to 60 pg. added to a bath of 10 ml.; followed by a dose-response curve of 1 to 16 pg.; 2 min. cycle, 15 sec. contact; total duration about 50 min. with absence of spasm.

acetylcholine. Our experimental conditions differed sufficiently from those of Blaber and Cuthbert to emphasise the general usefulness of their application of the use of morphine.

The ileum was suspended in 10 ml. of Krebs's solution (Gaddum, 1959) at 37° containing $5 \times 10^{-6}$ morphine sulphate. The tissue was incubated with $1 \times 10^{-5}$ mipafox for 1 hr., then excess mipafox was washed out. Using a 2 min. cycle and 15 sec. contact time the preparation regularly gave a good response to 100 pg./ml. of acetylcholine and occasionally responded to 1 pg./ml.

Slight fluctuations in baseline which occur are not troublesome, and spontaneous spasms are not seen (Fig. 1).

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## The Incorporation of $^{14}C$ from $NaH^{14}CO_3$ into the Soluble Metabolic Intermediates of Animal Tissues

SIR,—A considerable amount of information is available about the non-photosynthetic fixation of radioactive carbon dioxide by microorganisms (cf. Lynch and Calvin, 1953). However, relatively little is known about the incorporation of $^{14}CO_2$ into animal tissues. Katz and Chaikoff (1955) showed that rat liver slices incorporated between 4–10 per cent of the radioactivity from labelled bicarbonate and that the isotope was distributed amongst the metabolic intermediates as follows: 40–60 per cent in urea; 0–25 per cent in lactate; 10–20 per cent in glucose; 5–15 per cent in alanine; 5–10 per cent in glutamate and smaller quantities in organic acids. We have found that chopped preparations

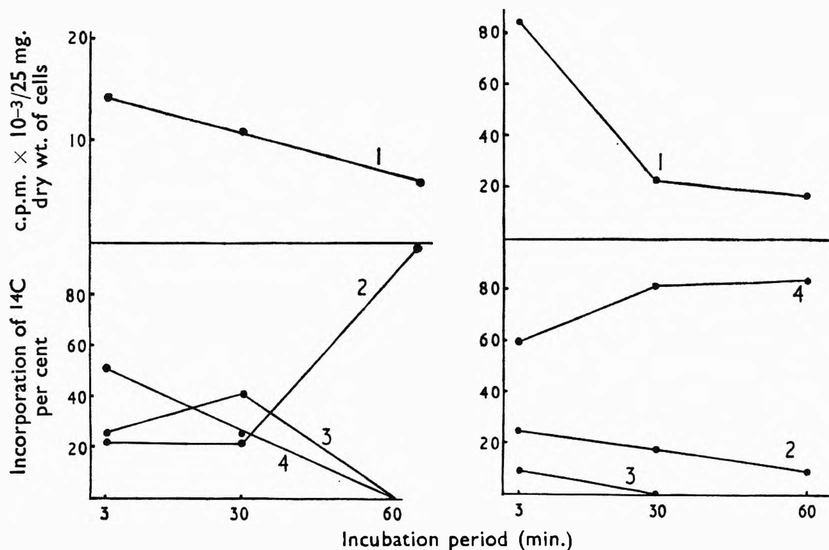


FIG. 1. Incorporaton of $^{14}C$ from $NaH^{14}CO_3$ into the soluble metabolic intermediates by HeLa cells.

FIG. 2. Incorporation of $^{14}C$ from $NaH^{14}CO_3$ into the soluble metabolic intermediates by transplantable mouse sarcoma cells.

1. Total $^{14}C$. 2. Lactic acid. 3. Tricarboxylic acid. 4. Amino-acids.

of rat liver (McIlwaine and Buddle, 1953) incubated with $NaH^{14}CO_3$ gave an almost identical pattern but that in rat liver homogenates most of the incorporated radioactivity appeared in the amino-acid fraction as follows: 40–50 per cent in glutamate; 10–20 per cent in $\gamma$-aminobutyrate; 5–10 per cent in aspartate and only 5–10 per cent in lactate. Suspensions of rat liver mitochondria incorporated over 80 per cent of the $^{14}C$ into the malate and citrate. Thus, the mitochondria incorporated the radioactivity principally into tricarboxylic acids, presumably via the carboxylation of pyruvate to yield oxaloacetate or malate, whereas in the homogenate the main end products were amino-acids derived by transamination reactions from the tricarboxylic acids. The absence of labelled glucose and urea in the mitochondrial and homogenate experiments suggests that the synthesis of these substances from bicarbonate needs a relatively intact cellular structure as is present in the liver slices and chopped preparations.

Other animal tissue preparations showed different patterns of incorporation. Chopped preparations of rat kidney incorporated 50–60 per cent of the isotope

into amino-acids (glutamic and aspartic acids) and 30–40 per cent into lactate; similar preparations of rat brain showed a 60 per cent incorporation into lactate and 30–40 per cent incorporation into di- and tri-carboxylic acids. Human foetal liver cells (Westwood, MacPherson and Titmuss, 1957) and HeLa cells in culture incorporated most of the isotope into lactate, tricarboxylic acids and amino-acids but the relative proportions of incorporation into these fractions changed with increasing time of incubation. With the HeLa cells, the extent of incorporation of radioactivity in amino-acids was substantially reduced as the incubation proceeded, that into the tricarboxylic acids reached a maximum at 30 min. and then rapidly decreased and lactate remained as the sole labelled end product after 60 min. (Fig. 1).

Transplantable mouse sarcoma 180 cells showed a different behaviour (Fig. 2). The radioactivity in the amino-acids increased with time of incubation whereas the reverse effect was observed to occur with the lactate and tricarboxylic acids.

5 mM salicylate or 0·5 mM 2,4-dinitrophenol reduced by 80–90 per cent the total incorporation of $^{14}C$ from the labelled bicarbonate into the soluble metabolic intermediates of the chopped tissue preparations except rat brain with salicylate where increases of 20–30 per cent were observed. A similar difference between brain and other isolated rat tissues with respect to the effects of salicylate on the incorporation of $^{14}C$ from labelled glucose has been reported by Smith and Moses (1960).

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