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689
690
691
692
693
694
695
696
697
698
699

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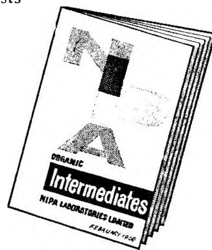

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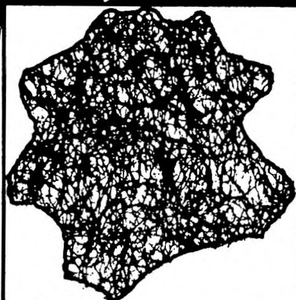

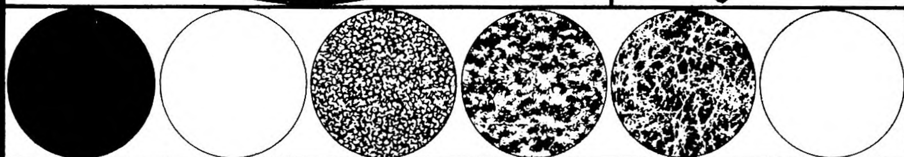

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Review Article

The role of calcium in neurohumoral and neurohormonal extrusion processes

LANCE L. SIMPSON

CALCIUM is a divalent cation, ubiquitous in its biological occurrence, important in its physiological significance. It is an ion of particular interest to investigators of neural and neuro-effector systems. Reviews have appeared that cite the role of calcium in excitation-contraction coupling of striate muscle (Sandow, 1965) and in the maintenance of the nerve membrane during nervous transmission (Abood, 1967). The intention of this review will be to summarize the various experimental evidences that implicate calcium in its role as a necessary factor in the processes of neurohumoral and neurohormonal extrusion. The diversity of physiological foci considered has meant that the discussion could not feasibly cite all primary references. Consequently, an attempt has been made, when applicable, to note recent and well prepared reviews.

The skeletal neuromuscular junction

Synaptology has made its greatest advances in the study of transmission from peripheral nerve to striate muscle. Several authors have made extended comments on the literature (McLennan, 1963; Eccles, 1964; Katz, 1966).

The morphology of the neuromuscular junction has been well described (Birks, Huxley & Katz, 1960; de Robertis, 1964). The nerve ending has a swelling at its terminal in which is contained numerous synaptic vesicles. The vesicles are generally believed to be the containers in which the transmitting substance, acetylcholine, is found. Acetylcholine is released quantally, in packets, from the nerve terminal to produce postsynaptic endplate potentials (epp) and miniature endplate potentials (mepp) (Martin, 1965; Katz, 1966).

As early as the work of Locke, there was recognition that the effectiveness of transmission from nerve to muscle was dependent upon the concentration of calcium ions within the bathing medium. More contemporary work has shown that the amplitude of the endplate potential is affected by calcium (Eccles, Katz & Kuffler, 1941; Kuffler, 1944), and that there is, within a limited range of concentrations, an approximately linear relation between the amplitude of the endplate potential and the concentration of calcium ions in the bathing solution. In the absence of sufficient amounts of calcium, neuromuscular transmission fails (del Castillo & Katz, 1954b).

Even more numerous are the observations on the effect of calcium on

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spontaneous activity at the motor endplate. Spontaneous subthreshold activity was initially described in frog skeletal muscle by del Castillo & Katz (1954b) and Fatt & Katz (1952), and subsequent reports of spontaneous miniature endplate potentials have been noted in mammals (Boyd & Martin, 1956a,b; Liley, 1956b; Hess & Pilar, 1963; Elmqvist, Hofmann & others, 1964), fish (Takeuchi, 1959), birds (Ginsborg, 1960), and invertebrates (Dudel & Kuffler, 1961; Usherwood, 1963). Only the frog and mammal have been subjected to extensive investigations of the relation between calcium and spontaneous activity. For the frog, Fatt & Katz (1952) reported that the frequency of occurrence of mepp's is independent of calcium ion concentrations of the bathing fluid. The calcium independence of spontaneous mepp's, coupled with the calcium dependence of epp's (see above), led to the notion that there exist in the frog two separate processes of acetylcholine release, only one of which was mediated by calcium (del Castillo & Katz, 1954a). Work on the frog involved the toe muscle, m. ext. longus dig. IV. Investigations on *in vitro* mammalian preparations stand in marked contrast to those on amphibian preparations. For both the tenuissimus muscle of the cat (Boyd & Martin, 1956a) and the diaphragm of the rat (Liley, 1956a; Hubbard, 1961), the frequency of mepp's is accelerated by increasing the bathing solution concentrations of calcium. Boyd & Martin (1956a) attempted to reconcile the species difference by suggesting that the alkaline earth metals (group II elements) can accelerate mepp frequency, the mammal being sensitive to calcium and other group II ions higher in the series, the amphibian being sensitive only to strontium and those ions increasing in series number. The notion is consistent with observations made by del Castillo (cf. Boyd & Martin, 1956a) that strontium and barium increase spontaneous potential activity, with barium, the higher of the two ions in the group II series, being more effective.

In addition to work involving increases in calcium, observations have been made on preparations bathed in minimal, or no, calcium. At concentrations below 0·20 mM calcium, no epp is detectable at the frog neuromuscular junction (del Castillo & Stark, 1952). Early work indicated that if calcium were removed from the bathing medium by treatment with ethylene diamine tetra-acetic acid (EDTA), most spontaneous activity at the mammalian neuromuscular junction ceased, but a fraction of spontaneous potentials persisted (Hubbard, 1961). Again, two separate release phenomena were suggested, in this instance both dealing with spontaneous mepp's, but only one requiring calcium as a prerequisite to release. However, more recent work (Elmqvist & Feldman, 1965a) has indicated that if the preparations were given prolonged treatment with EDTA, all mepp production is abolished. Apparently the nerve ending is capable of binding a small store of calcium, and this store, in addition to the free ion in the medium, must be chelated before miniature potentials will cease.

All reports agree that changes in calcium ion concentration do not markedly alter the mepp amplitude unless the change is several-fold above physiological levels, and the effect observed then, is due to a reduction

in the sensitivity of the postsynaptic membrane to acetylcholine (del Castillo & Engbaek, 1954; del Castillo & Katz, 1954a; Boyd & Martin, 1956a; Manthey, 1966).

Thus calcium appears first, to be a necessary synaptic constituent for all neuromuscular transmission, and second, to augment the amplitude of the endplate potential, and third, to increase the rate of occurrence of spontaneous miniature potentials in mammals. At least two sites have been proposed as loci at which calcium could be exerting the listed effects (cf. Katz & Miledi, 1965b). According to one alternative, calcium is intimately involved in the propagation of the nerve impulse (see Abood, 1967). Any alterations in the concentration of the cation would alter the facility with which the spread of depolarization could invade the nerve terminal (e.g., Frank, 1963). A second alternative arises from the combined observations of Fatt & Katz (1952, 1953) and of de Robertis & Bennett (1955) and Palade & Palay; Palay & Palade (1954). The detection of multimolecular yet quantal release of acetylcholine, as indicated by postsynaptic potentials, together with the recognition of subcellular vesicles, has led to the theory that the transmitter is stored in subcellular containers and that the containers spill their contents into the synaptic interspace during transmission. Calcium could be involved in the process in which the vesicles are made to extrude their contents. The evidence is highly in favour of the latter proposal. Three arguments are pertinent to resolving the problem. (i) It is possible to bathe nerve-muscle preparations in calcium-deficient media, and thereby cause severe reduction of the epp (del Castillo & Stark, 1952). If a micropipette is introduced into the calcium deficient endplate environment, and if calcium ions are electrophoretically propelled from the pipette, an enhancement of the endplate response occurs (Katz & Miledi, 1965b). The interval between calcium efflux from the pipette and increase in epp seems insufficient to allow diffusion of the ion to axonal sites of transmission. Rather, the nerve terminal seems the site of activity. (ii) Tetrodotoxin is a biological poison that suppresses bioelectrical potentials in both nerve and muscle, presumably by immobilizing sodium passage across the membrane (Nakajima, Iwasaki & Obata, 1962; Narahashi, Moore & Scott, 1964). Frog sartorius preparations bathed in the poison continue to display spontaneous mepp's, and locally applied current will result in depolarization of the nerve terminal plus a subsequent postjunctional epp. No current spread occurs in the nerve axon, yet addition of calcium facilitates the epp and removal of calcium results in failure of transmission (Katz & Miledi, 1967b). Again, it is the bouton that is implicated as the site of action. (iii) The third argument is perhaps the most germane. The vesicle theory purports that the mepp is the postsynaptic event following the release of acetylcholine from the vesicle content, and the epp is the summed effect of numerous mepp's. It has been reported that appropriate manipulations of the ionic species in the bathing medium of nerve-muscle preparations, particularly of calcium, will produce small but constant increments in the epp (Martin, 1965). Apparently calcium acts to facilitate the release of acetylcholine, and the incremental steps are the result of discrete

increases in the number of vesicles secreting their transmitter substance.

Data on mammalian preparations suggest that both spontaneous and nerve impulse-induced release of acetylcholine are calcium mediated, so both may operate via a common mechanism. The nerve impulse simply increases the probability of the spontaneous, random mepp's occurring synchronously. For the frog, the spontaneous and nerve-induced release are reported to depend on separate mechanisms, only the second being calcium mediated. However, it would be interesting to test the effect of prolonged EDTA treatment of amphibian preparations for possible changes in the frequency of spontaneous activity. Until such work is done, one should be cautious about assigning two processes of acetylcholine release to the frog myoneural junction. The persistence of spontaneous activity by the frog in low calcium media may mean that the creature is capable of binding significant stores of the divalent cation, and only an extended observation in the presence of a chelating agent would uncover depression of spontaneous activity. In the frog, the absence of an accelerating effect by calcium could be explained by the animal relying more upon its stores of bound ion than upon the freely circulating ion.

There is a finite amount of time within which calcium can act in facilitating the neurogenic release of acetylcholine. Measurements of transmission delay at the frog sartorius have produced a modal value of 0·75 msec (Katz & Miledi, 1965a). A short lag time intervenes between nerve terminal depolarization and the subsequent release of acetylcholine. During the lag a sequence of reactions presumably occurs, one step of which involves calcium, that allows the release of transmitter substance (Katz & Miledi, 1965c; 1967c). By bathing nerve-muscle preparations in calcium-deficient solutions, Katz & Miledi (1967c,d) have determined with some precision the point in time of junctional transmission during which calcium is most effective in mobilizing the neurohumoral releasing apparatus. Iontophoretically applied calcium is most effective if it is propelled into the preparation very shortly before a depolarizing pulse, but it is largely without effect if applied during the period of transmission delay. Thus the depolarizing pulse seems to allow the movement of calcium to its reactive site; after the depolarizing pulse wanes the sequence of molecular events transpire that will result in acetylcholine extrusion (Katz & Miledi, 1967a).

Of the various ions that are found in the extracellular space, only magnesium, sodium and potassium have been explored in depth for their interaction with calcium. Magnesium has long been known to interfere with neuromuscular transmission (Jolyet & Cahours, 1869). When preparations are bathed in 20–50 mM magnesium solutions that are otherwise physiological in their content, junctional transmission fails (Engbaek, 1952). The action of magnesium is reported to be the depression of acetylcholine release, and its action can be completely antagonized by the addition of calcium (del Castillo & Engbaek, 1954). Kinetic studies indicate that the amount of acetylcholine liberated from the nerve terminal is directly related to the calcium concentration and inversely related to the magnesium concentration. Hence, quantal emission is

appropriately described in terms of the calcium to magnesium ratio (Jenkinson, 1957).

The frequency of spontaneous activity at the skeletal neuromuscular junction of the frog has been noted as being unaffected by gross changes in the levels of magnesium (del Castillo & Katz, 1954a). A similar suggestion was made for the mammal (Boyd & Martin, 1956a), but subsequent experimentation showed the spontaneous activity at the neuromuscular junction to be depressed (Hubbard, 1961). The latter work also showed the magnitude of depression to be related to the frequency of activity. Consequently, magnesium has a marked effect in the presence of excess calcium and a minimal effect in diminished calcium solutions. Apparently calcium and magnesium compete for some intermediate in the release mechanism of acetylcholine (del Castillo & Katz, 1954a; Jenkinson, 1957; Hubbard, 1961).

Sodium has been discussed as an important factor in the liberation of acetylcholine, the data being based principally upon observations of preparation treated with cardiac glycosides (Birks, 1963). There are two significant drawbacks to such preparations. Firstly, it is difficult to extrapolate evidence from sodium pump inhibitor situations to physiological situations because of the diverse number of actions in which sodium is involved, many of which are not intimately related to junctional transmission. Secondly, sodium pump inhibitors sometimes behave as mobilizers of calcium (Govier & Holland, 1964; Elmqvist & Feldman, 1965b). Other workers have noted a depression of the epp in low sodium solutions, but the lowered response is the result of a reduction in the sensitivity of the postsynaptic element (del Castillo & Katz, 1955). Studies involving the spontaneous release of acetylcholine and sodium levels have been more definite. The frequency of spontaneous mepp's is inversely related to the sodium concentration, and sodium-induced depression is antagonized by calcium (Birks & Cohen, 1965; Kelly, 1965; Gage & Quastel, 1966). Gage & Quastel (1966), in examining the dynamics of the antagonism, proposed either that sodium and calcium compete for a common molecule, or that sodium reduces the ability of calcium to form a complex necessary for acetylcholine liberation. The site of antagonism appears different from that of the calcium-magnesium interaction. Excess potassium has a depolarizing action on the nerve terminal and consequently increases spontaneous activity (Liley, 1956c). The ion has an additional effect of mobilizing the interneuronal stores of acetylcholine (Gage & Quastel, 1965; Parsons, Hofmann & Feigen, 1965). In the presence of raised potassium, increases in magnesium exert a strong depressant effect on spontaneous activity. In comparable situations, calcium is without notable effect (Hubbard, 1961). The absence of an accelerating action by calcium may be due to a masking by the dual actions of potassium.

The adrenergic neuro-effector cell junction of smooth muscle

The juxtaposition of nerve and smooth muscle is somewhat dissimilar

from that of nerve and striate muscle. The cholinergic junction is characterized by a well-defined nerve terminal in which are located the synaptic vesicles (Birks, Huxley & Katz, 1960). The adrenergic nerve, in addition to its terminus, possesses significant numbers of varicosities that impinge upon the adjacent muscle tissue (cf. Burnstock & Holman, 1966). Both the nerve terminal and the individual varicosities contain synaptic vesicles, so both may be able to release catecholamines in transmission (Richardson, 1962; Merrillees, 1963; Burnstock & Holman, 1966). Preparation and analysis of subcellular fractions have provided evidence that noradrenaline and its precursors are associated with vesicles (Potter & Axelrod, 1963; Taylor, Chidsey & others, 1966; Austin, Chubb & Livett, 1967).

Bioelectrical phenomena associated with junctional transmission from nerve to smooth muscle was first described by Burnstock and his coworkers using the guinea-pig isolated vas deferens preparation (Burnstock & Hollman, 1961). Subsequent work has been done on such varied mammalian preparations as the bladder (Ursillo, 1961), the retractor penis (Orlov, 1961), the intestine (Gillespie, 1962), and blood vessels (Speden, 1964). In brief, the potentials recorded at the adrenergic nerve–smooth muscle junction are somewhat similar to those visible at cholinergic neuromuscular junctions. Stimulation of the nerve produces a postjunctional electrical change called an excitatory junctional potential (ejp), which in turn triggers the action potential. Spontaneous electrical activity, termed spontaneous excitatory junctional potential (sejp), can be recorded in the absence of nerve stimulation. Apparently the sejp's summate to produce the ejp, but quantal release of transmitter at the nerve-smooth muscle junction has been difficult to demonstrate. A portion of the problem lies in the inconsistent amplitude of the sejp. The variance may be due to a wide range in the size of the gap between adrenergic nerve terminals or varicosities and the underlying muscle. Such variance in the width of the synaptic interspace would modify the amounts of transmitter diffusing to postsynaptic reactive sites. Presumably the catecholamines are released in quantal form from the prejunctional vesicles.

The effects of calcium on adrenergic nerve transmission to smooth muscle have been described by Kuriyama (1964). If the medium bathing a hypogastric nerve–vas deferens preparation contains less than 0·5 mM calcium, nerve stimulation fails to produce a response in the muscle. Increases in the cation concentration up to 25 mM cause progressive increases in the amplitude of the ejp, as well as prolonging the falling phase of the potential. Repetitive nerve stimulation results in enhancement of the ejp. Elevating the calcium concentration has the effect of further enhancing the facilitatory response during the first few minutes of exposure, while continued bathing causes a diminution of the ejp amplitude. Preparations soaked in a medium with low calcium, with the other ions appropriately adjusted, demonstrated potentials that fluctuated incrementally. Stepwise fluctuations are indicative of quantal release of transmitter. High calcium concentrations first accelerated, then decelerated the frequency of sejp, though the appearance of the spontaneous

activity never entirely ceased. Low calcium concentrations consistently diminished the frequency of the spontaneous potentials.

Other work has shown alterations in the gross behaviour of postjunctional elements in response to changes in calcium levels in the perfusion fluid (Burn & Gibbons, 1964a,b). Stimulation of the periarterial nerves causes inhibition of the pendular movements of the isolated rabbit ileum. Increasing the calcium concentration in the solution from below normal to normal facilitates the inhibitory response produced by shocks to the periarterial nerves. Excess calcium continues to facilitate the inhibition until the cation reaches concentrations of about 17·6 mM. At this concentration calcium no longer magnifies the inhibition of contraction, but it does prolong its duration. Tests with exogenous noradrenaline showed the effect not to be the result of a sensitization of the ileum to the transmitter. Continued repetitive stimulation of the periarterial nerve produces a diminished though constant response in the muscle; addition of calcium provokes further reduction from the contractile response. A noteworthy observation was that preparations bathed for 20 min in medium lacking calcium, upon readmission of calcium, immediately showed inhibition of contraction independent of nerve stimulation. That this event is linked to calcium mobilization of transmitter was shown by pretreating nerve–ileum preparations with reserpine. Those preparations treated with the drug, and thus partially depleted of their catecholamine content, were sluggish in showing the response. Results qualitatively similar to those reported for the isolated ileum have also been noted in isolated rabbit atria (Burn & Gibbons, 1965).

Additional work (Kirpekar & Misu, 1967) described a quantitative relation between the amount of calcium in the perfusion fluid and the amount of noradrenaline released from stimulated splenic nerves. Absence of calcium ions results in near failure of the nerve to liberate any transmitter, whereas replacement of the ion allows normal output. The ratio of noradrenaline output to stimulus could be expressed as a linear function of the logarithm of calcium perfusion levels. Alterations in the calcium ion content of the media appeared to act directly upon the releasing apparatus of the neurotransmitter rather than upon impulse conduction in the nerve fibre. Similar work on the cat colon *in situ* has detected an absence of response in the absence of calcium, plus the spontaneous release of transmitter when the ion is restored to calcium free medium (Boullin, 1966).

Determination of the site at which calcium is active in mediating the release of adrenergic transmitters has not been accomplished. The task is complicated by the presence in the literature of two opposing proposals regarding the liberation of adrenergic neurotransmitters. According to the "classical" theory (see Ferry, 1966), invasion of the nerve terminal by depolarization leads to an influx of calcium. The in-moving cation participates in the sequence of events leading to transmitter release. Opposed to the classical theory is the hypothesis offered by Burn & Rand (1959, 1965). These authors suggest that acetylcholine is released first by the nerve impulse, then in turn provokes the release of catecholamine.

Calcium could act either to promote the release of acetylcholine, as in the cholinergic system, or to promote the release of catecholamine. The authors favour the latter possibility and believe that acetylcholine increases permeability of the nerve terminal membrane to calcium. The choice of the latter rests largely on an analogy with the adrenal gland (see below) rather than on empirical evidence. Nevertheless, the weight of observations appears not to favour the Burn–Rand hypothesis. In two recent reviews, one by Burn & Rand (1965), the other by Ferry (1966), the suggestion is made that the most important evidence for the cholinergic-link hypothesis is the blocking action of adrenergic transmission by botulinum toxin and by hemicholinium-3, both cholinergic blocking agents. Neither observation seems pressing. There are several reports that contest the universal blocking action of botulinum toxin on adrenergic transmission (Ambache, 1949; Vincenzi, 1967), and even the original positive report did not find paralysis of all preparations (Rand & Whaler, 1965). In addition, the cholinergic blocking effect of hemicholinium-3 does not necessitate a similar action at the adrenergic synapse. As Paton has pointed out, hemicholinium-3 is structurally similar to a phenylethylamine, and thus may exert a blocking action peculiar to adrenergic junctions (Paton, 1963). The literature seems to support the assumption that calcium acts at the adrenergic nerve–smooth muscle junction like it does at the cholinergic junction. Nerve depolarization causes calcium to move to some site at which it can greatly increase the probability of synchronous release of transmitter packages.

An interaction between calcium and magnesium has been noted at many of the adrenergic neuro-effector systems studied. Excess magnesium reduces the frequency of spontaneous electrical activity at the vas deferens. Reduction in the amplitude of the ejp follows elevation of the magnesium concentration, and the effect is countered by calcium (Kuriyama, 1964). The response of the ileum to stimulation of sympathetic fibres is antagonized by magnesium (Burn & Gibbons, 1964a); output of noradrenaline by both the splenic nerve and neural plexus of the colon is diminished in perfusion fluids with high magnesium levels (Boullin, 1966; Kirpekar & Misu, 1967). All the effects are reversed by calcium.

Little work has been done in relating the action of other common physiological ions to that of calcium. Altering the levels of sodium and potassium does not modify cateholamine output at the splenic nerve terminal until such time as nerve conduction is blocked (Kirpekar & Misu, 1967).

Autonomic mediators at cardiac muscle

Reports that elucidate the mechanism of action of calcium at sites of cardiac innervation are few (Midrio & Sperti, 1963; Vincenzi & West, 1965). The negative chronotrophic, or cholinergic, response is augmented by increasing the calcium ion concentration of the bathing fluid. That the effect is cholinergic was proved by reserpinization and resultant inactivation of adrenergic influence. On the other hand, atropinization removed cholinergic influences and showed the positive chronotrophic,

or adrenergic, response to be enhanced by calcium. Apparently, release of transmitter from both the vagus and sympathetic cervical nerves is increased by calcium. Also, results indicated that spontaneous release of neurotransmitters was accelerated with high concentrations of the cation. Huković & Muscholl (1962) have introduced specific evidence favouring a noradrenaline release that is calcium influenced.

Autonomic ganglia

Both the morpology (de Robertis & Bennett, 1955; Taxi, 1961; Elvin, 1963a,b; Hunt & Nelson, 1965) and the pharmacology (Volle, 1966) of autonomic ganglia have been described. The frog and the cat sympathetic ganglion have been the subject of much study. In the mammal, the terminal arborizations of the preganglionic fibre intertwine with the dendritic processes of the postganglionic cell, and numerous functional contacts are obvious. In the amphibian, the postganglionic cell possesses few dendritic ramifications. The principal contact is between preganglionic boutons and postganglionic somas. Several terminals from one fibre may impinge upon a single soma. In all instances, the presence of synaptic vesicles in the prejunctional terminals have been witnessed.

Bioelectrical events at the ganglion are closely analogous to those noted at nerve-striate muscle and nerve–smooth muscle junctions. Both synaptic potentials and spontaneous miniature synaptic potentials have been recorded in the frog (Nishi & Koketsu, 1960; Blackman, Ginsborg & Ray, 1963b,c; Hunt & Nelson, 1965) and the squid (Miledi, 1966). Release of transmitter appears to be quantal, and observations on the ganglion are in keeping with the vesicle theory. Eccles failed to detect miniature synaptic potentials in the mammalian sympathetic ganglion (1955), but Blackman & others suggest the absence of activity results from an insufficient depolarization of the presynaptic terminals. Others have reported that transmission at the mammalian ganglion is quantally composed (Martin & Pilar, 1964). Like the various neuromuscular junctions, ganglionic synapses are markedly affected by fluctuations in surrounding calcium. Synaptic potentials vary directly with the levels of bathing calcium; transmission fails at low levels (Blackman & others, 1963c).

Work on the cat superior cervical ganglion was the first in which the depression of acetylcholine output in low calcium perfusion fluids was reported (Harvey & MacIntosh, 1940). Removal of calcium from the environment produced two distinct effects. Firstly, there ensued a period of rapid and spontaneous firing of the ganglion cells which appeared to be independent of the action of any transmitter. Secondly, synaptic transmission failed. Stimulation of the preganglionic fibres, whether by external pulses or by addition of potassium, failed to produce a peripheral action. Bioassay of perfusion fluid showed the sympathetic trunk did not release acetylcholine whenever transmission was blocked by calcium deficient medium. An additional interesting finding was that the ganglion cells showed transient behaviour that was dependent upon the previous ionic environment. Changes in calcium did not produce constant and

absolute differences in the contractility of the nictitating membrane; rather, differences were relative and a function of the previous concentration of the ion in the bathing solution. Perhaps the cells were binding some amounts of the cation, or, since the experiments were made *in situ*, there may have been a delay in reaching equilibrium with other tissues. Hutter & Kostial (1954) have extended the observations made on the cat ganglion. These workers have noted that increases in calcium from 2·1 to 6·3 mM result in a 40% enhancement in acetylcholine output; raising the cation concentration to 8·4 or 10·5 mM doubled the amounts of transmitter released.

A particularly important set of experiments was reported by Lipicky, Hertz & Shanes (1963). Working with radioactive calcium, they compared the influx of the ion in vagal and superior cervical trunks both at rest and during depolarization. The evidence indicates that calcium enters the nerve from the external environment rather than from binding sites in the membrane. Furthermore, the magnitude of calcium influx can be augmented by adding depolarizing levels of potassium; acetylcholine release is directly proportional to the magnitude of calcium influx. Addition of magnesium to the treatment medium partially prohibits the movement of calcium into the nerve during depolarization. Resting influx of calcium is unaffected by magnesium. These experiments present one of the clearest relations between calcium movement and the mobilization of transmitter. Also, the work indicates that magnesium exerts its depressant action on neurohumoral release not by interfering with the actual release mechanism, but rather by preventing calcium from reaching its reactive site within the nerve.

Several investigators (Hutter & Kostial, 1954; Blackman & others, 1963b,c) have likewise noted ganglionic depression by magnesium. Concentrations of 25 mM magnesium frequently reduced the acetylcholine output to levels too low for bioassay. Transmitter release suppressed by 15 mM magnesium was nearly relieved by 6·3 mM calcium. Low calcium–magnesium ratio fluid diminishes the synaptic potential. Raising magnesium decelerates miniature potential frequency, the magnitude of deceleration being relative to the recurrence frequency. At high recurrence frequencies, magnesium exerts a maximal depression. Potassium depolarization facilitates calcium-induced transmitter release (Lipicky & others, 1963), but by itself potassium is unable to mobilize the releasing apparatus (Harvey & MacIntosh, 1940). Treatment of ganglia with ouabain or digoxin results in increased intracellular stores of sodium and augmented spontaneous release of acetylcholine (Birks, 1963). Whether results like these directly implicate sodium in the extrusion of neurohumoral substance is difficult to decide (see p. 893).

The central nervous system

Though not as accessible to experimentation as the peripheral nervous system, the central nervous system (CNS) has been penetrated by several investigators. Reports of the presence of synaptic vesicles within brain structures are well documented (de Robertis, 1964; Whittaker, 1965).

Although several substances have been suggested as CNS transmitters, none has fulfilled the criteria for acceptance as a brain transmitter. Acetylcholine appears to be the neurohumour secreted onto Renshaw cells in the spinal cord (cf. Kuno & Rodomin, 1966). Electrical activity in the brain has been recorded by authors too numerous to cite; much credit goes to Bremer for his initial recognition of EEG activity. Spontaneous activity has been noted in both the brain (Li, 1959) and the spinal cord (Brock, Coombs & Eccles, 1952; Katz, 1966; Kuno, 1964). Transmission in the cord seems quantal in nature (Katz, 1966; Kuno, 1964).

Reports of cationic influence on CNS synaptic transmission are scarce. Distortions in body fluid levels of calcium and magnesium provoke a variety of disturbances that may be associated with disruption of synaptic activity, though alterations in membrane stability may also be implicated (see Katzman, 1966). Hypocalcaemia frequently results in seizures and modification of the normal EEG. Hypercalcaemia may provoke mental imbalance, and less often seizures and cerebellar ataxia. Magnesium is well established as a CNS depressant (Engbaek, 1952), and excessive amounts will suppress the EEG as well as producing an anaesthetic state. Administration of calcium acts as an antidote to hypermagnesia. Krnjević, Randić & Straughan (1966) have described an inhibitory process in the cerebral cortex that is impervious to iontophoretically applied magnesium, though Randić & Padjen (1967) have shown the release of acetylcholine from the exposed cerebral cortex to be strongly influenced by the presence of calcium.

In the spinal cord of the frog, synaptic transmission between dorsal roots 7–9 and the associated motoneurons is depressed by magnesium; high magnesium Ringer solution prohibits reflex activity (Katz & Miledi, 1963). Elevation of calcium to 6–10 mM was "partially successful" in ameliorating the magnesium block. It is intriguing that in the frog spinal cord, as at the frog neuromuscular junction, raising the magnesium concentration does not alter the frequency of spontaneous activity. The effects of calcium have not been reported.

Glandular secretion

The adrenal medulla is the paradigm of a sympathetic neuron and consequently is a liberator of catecholamines. Catecholamine storage vesicles have been obtained upon differential centrifugation of the homogenized organ (Blaschko & Welsh, 1953; Hillarp, Lagerstedt & Nilson, 1953). A simplified version of junctional events seems to be: (i) depolarization of the adrenal nerves and subsequent release of acetylcholine, (ii) stimulation of the adrenal gland by acetylcholine, and (iii) release of catecholamines from the chromaffin cells of the adrenal medulla. Calcium is the necessary factor for chromaffin cell extrusion of sympathomimetic agents.

Work describing the action of calcium in mobilizing the release mechanism of adrenal catecholamines has been presented largely by Douglas & Rubin (1961, 1963), Douglas & Poisner (1962) and Douglas

(1966, 1967). The investigations show mainly the amounts of catecholamine released from the adrenal medulla as a function of the type of fluid with which the organ is perfused. Glands were typically treated either by acute denervation or by hexamethonium to insure that effects were the result of direct actions rather than indirect actions via the nerve. Output of catecholamine by the resting gland bathed in Locke solution is between 0·05–0·1 μg/min. Addition of acetylcholine to a concentration of 10^{-5} μg/ml may evoke up to a 200-fold increase in amine output. The augmented output by acetylcholine is unaffected or enhanced by the removal of potassium or sodium from the perfusion fluid. Removal of calcium results in a dramatic loss by acetylcholine in its ability to evoke secretion of catecholamines. Maintaining acetylcholine at a concentration of 10^{-5} μg/ml while elevating calcium to 17·6 mM doubled the adrenal output. Addition of potassium to levels sufficient to cause depolarization of the chromaffin cells acted like acetylcholine in promoting the output of amines, but only if calcium were also present. Although both acetylcholine and excess potassium excite large secretions into otherwise normal Locke solution (tonicity adjusted), the excitatory effect is transient and eventually output falls to lower levels. Doubling and redoubling calcium ion concentration in the medium once output has stabilized will again provoke increases in medullary secretion. Finally, addition of calcium, even in the absence of any stimulation, to a preparation that had previously been calcium deprived, results in the spontaneous release of amines.

Studies with ^{45}Ca have indicated that there is a movement of calcium into the gland during activity. The influx of calcium in the resting medulla is 0·50 μmole/mg/sec. Stimulation of the organ (10^{-5} μg/ml acetylcholine) causes an eightfold increase in calcium influx.

The summed data of the various experiments have led the authors to propose that calcium is a stimulus-secretion coupler. Acetylcholine stimulates the chromaffin cells and thereby causes an increase in the permeability to calcium. Calcium moves to a reactive site at which it promotes the extrusion of catecholamine; thus the cation serves as the link between chromaffin cell stimulation and chromaffin cell secretion. The hypothesis had been advanced that calcium acts directly upon the storage granules to cause release of the granule contents into the cell and then diffusion outward (Philippu & Schumann, 1962), but the idea has been contested (Greenberg & Kolen, 1966). The granules apparently release their amine directly to the exterior of the cell.

Exposure of the adrenal glands to magnesium results in suppression of medullary output whether the stimulus is acetylcholine or excess potassium. Addition of significant amounts of calcium overcomes the suppression. Observation on the movement of ^{45}Ca in the presence of magnesium showed the movement into the gland to be severely depressed (Rubin, Feinstein & others, 1967). The observation closely parallels that made by Lipicky & others (1963) (see p. 898) and suggests that magnesium interferes with the movement of calcium rather than the mobilization of hormone. When magnesium is added together with calcium to previously calcium-deprived glands, spontaneous output of catecholamines is

reduced; when magnesium is in the calcium deprived preparation, reintroduction of calcium provokes no sponataneous output (Douglas & Rubin, 1963).

Secretory phenomena in the hypothalamo-hypophysical system may be similar to that of the secretomotor-adrenal system. Granules that probably contain the secretory substance have been reported (Palay, 1957; Gerschenfeld, Tramezzani & de Robertis, 1960). More particularly, the secretion of vasopressin appears to be calcium dependent (Douglas & Poisner, 1964a,b). Experiments on the isolated posterior pituitary show that vasopressin is secreted from the gland after electrical or excess potassium stimulation, providing that calcium is in the preparation. Acetylcholine is without effect, but since the isolated gland contains only the neurosecretory terminals rather than the somas, the absence of an acetylcholine action does not remove the possibility of the cells being cholinoceptive. However, several disparities do exist between calcium-provoked hormone extrusion in the adrenal and in the posterior pituitary. In contrast to the former, there is a definite limit to the secretory augmenting effect of calcium in the pituitary. Vasopressin release first increases, then decreases as calcium levels are elevated. A second disparity is that the reintroduction of calcium into calcium-free Locke solution does not cause the spontaneous release of vasopressin. Additional dissimilarity appears in noting the movement of ^{45}Ca. As might be expected, depolarization of the cells by potassium leads to ^{45}Ca influx. Addition of magnesium slightly depresses the influx of calcium, yet it markedly depresses vasopressin secretion. The conclusion drawn by the investigators is that calcium acts at the pituitary in the same way as it does at the adrenal to promote the secretion of hormone, but other mechanisms must also be involved.

The submaxillary gland parallels the behaviour of the adrenal and the pituitary glands sufficiently to warrant brief comment. The gland receives both adrenergic and cholinergic innervation. Solutions devoid of calcium failed to evoke the normal secretory response to either acetylcholine or noradrenaline. The magnitude of glandular output upon cholinergic stimulation was directly related to the calcium concentration within a 0·8 mM limit. Magnesium did decrease the response to cholinergic stimulation, but the effect was not impressive (Douglas & Poisner, 1963).

Proposed mechanisms

The available literature seems overwhelming in evidencing that chemical extrusion following excitation, whether from gland, neurosecretory terminal, or ordinary nerve bouton, is calcium dependent. In a number of instances, even the spontaneous release of chemical mediators is modified by the amounts of calcium within the environment. The level of existing awareness has moved from one of attempting to implicate calcium in extrusion processes to one of attempting to decipher the nature of the calcium link in the processes. At least two calcium-linked mechanisms have been sufficiently often witnessed to warrant proposals regarding the molecular activities involved: (i) the mode of action of calcium in

evoking, upon its reintroduction, spontaneous release of catecholamines from preparations that had previously been bathed in calcium-deficient medium, and (ii) the mode of action of calcium in promoting, after excitation, the synchronous release of large numbers of preformed packages of transmitter.

The first mechanism has been reported to be operative at both the adrenergic nerve terminal (see pp. 893–896) and at the adrenal medulla (see pp. 899–901). That these two sites demonstrate a similar behaviour may be more than coincidental. All peripheral adrenergic neurons are postganglionic sympathetic structures, and indeed, the adrenal medulla is a structural analogue of a postganglionic sympathetic neuron. Consequently, the mechanism proposed by Douglas (see Fig. 1) for the adrenal chromaffin cell may be very similar to those that function at the adrenergic nerve terminal. As Fig. 1 indicates, calcium is located both free in the

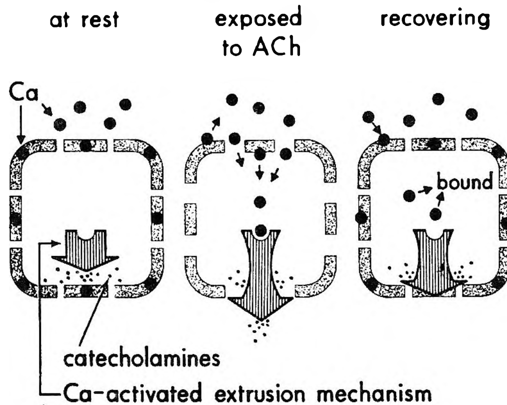

FIG. 1. Proposed role of calcium in catecholamine release. When calcium is located in the membrane, the membrane is stabilized and little transmitter substance is released. Upon exposure to ACh, the membrane loses calcium, thereby allowing the outward passage of transmitter. Recovery involves a relocating of calcium in the membrane. (After Douglas, 1967.)

extracellular space, and bound in the chromaffin cell membrane. Acetylcholine released from secretomotor nerves acts upon the membrane in such a way that the bound calcium is loosened. The result of the breakdown in membrane calcium binding is that the membrane subsequently becomes highly permeable to extracellular calcium. The in-moving cation proceeds to the reactive sites at which it can promote the extrusion of hormone. Recovery involves the rebinding of calcium by the membrane and accompanying loss of cell permeability to the cation. During those unusual experimental periods when no calcium surrounds the gland, some of the ion is lost from the membrane. Thus, when normal medium is reintroduced, the membrane is "leaky" until it can resequester its quotas of calcium. During the interim, the ion moves through the leaky membrane to evoke hormone extrusion. The proposal is compatible with the observation that readmission of calcium to deficient medium

promotes a transient, spontaneous release of catecholamines. It also explains the interaction between calcium and magnesium in relation to the observed phenomenon. When both ions are added to deficient medium, the amount of amine released is less than if only calcium is added. In this instance, magnesium acts by prohibiting calcium from participating in amine release. When magnesium is included in the deficient medium, readmission of calcium causes no spontaneous release. In the second case, magnesium simply substitutes for calcium in stabilizing the membrane. The ability of magnesium to act as a substitute in the membrane has been noted by others (Frankenhaeuser & Hodgkin, 1957).

Though the mechanism may be similar to that evoking release of amines from adrenergic nerves, it would be unwise to extend the notion to other structures. At the cholinergic myoneural junction, insufficient experimentation is available for drawing conclusions. Investigation of the hypothalamo-hypophysial system has revealed no such mechanism (Douglas & Poisner, 1964a).

A statement of the molecular events by which calcium links excitation and extrusion has not yet been elaborated. In fact, slight modification of the middle diagram in Fig. 1 adequately describes all systems thus far investigated. Alter the phrase "exposed to ACh" to "exposed to synaptic transmitter", and redesignate the catecholamines as being any neurohumour or neurohormone, and one has a universal scheme. Synaptic transmitters promote the influx of calcium, and calcium promotes the efflux of another chemical mediator. For the cholinergic neuromuscular junction, several diagrammatic variations on the scheme have been advanced. Del Castillo & Katz (1954a) have proposed that within the sequence of reactions leading to neurohumoral release, there is a reactive site or carrier molecule for which calcium and magnesium compete. Only the Ca complex is effective in transmitter release. To account for the calcium-independence of spontaneous mepp's in the frog, they further proposed that the hypothetical reactive mechanism could spontaneously revert to an active form which would promote extrusion. The work of Gage & Quastel (1966) introduced another variation to the basic scheme. Besides the antagonism shown by magnesium, calcium is also antagonized by sodium, at least in terms of the recurrence frequency of mepp's. And even excess calcium will depress the mepp frequency. The model intended to explain the additional antagonism indicates that the interaction is of a second or higher order. Consequently, Na_2Y or Ca_2Y (Y being the hypothetical site or molecule) will depress quantal release. One easily sees that either excess calcium or sodium could depress output. Another point to note is that the authors allow that the hypothetical mechanism for which calcium and magnesium compete may be distinct from the hypothetical mechanism for which calcium and sodium compete.

It was remarked above that no molecular scheme has been advanced to explain the specific mechanism by which calcium effects extrusion. However, if various lines of research tangential to that being reviewed are brought into perspective, a feasible mechanism appears. The mechanism is offered within the context of the assumption that extrusion from

all neural and glandular structures considered is roughly equivalent. The common appearance of vesicles, of quantal release of substance, and of excitation-extrusion dependence on calcium, make the idea not too presumptive.

The first line of research to be considered is that dealing with the structure of synaptic vesicles. Electron microscopic evidence has been presented that the vesicle is composed of an orderly array of osmiophilic and osmiophobic units, but the array does not conform to the unit membrane design (di Carlo, 1967). The observation lends credence to a hypothesis offered earlier by Burton, Howard & others (1964) and Howard & Burton (1964). Studies on the subcellular distribution of bound acetylcholine and ganglioside showed the two to be localized similarly in the brain, both within the nerve ending. This work, together with their observations on the physical characteristics of ganglioside, led Burton & others to propose the hypothesis illustrated in Fig. 2. The vesicle is

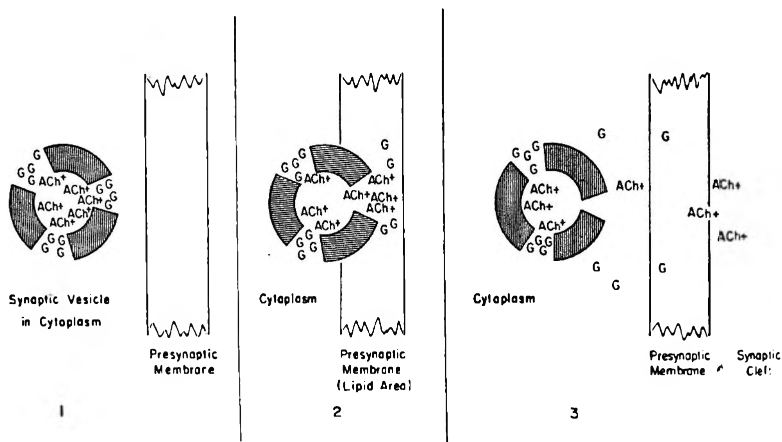

FIG. 2. Proposed mechanism for the vesicular release of ACh. 1. The resting state: the nerve membrane and a vesicle before release of the transmitter. 2. After the arrival of the nerve impulse: the vesicle has migrated to the nerve membrane in which it is partially soluble allowing transmitter release. 3. Recovery: migration of the vesicle away from the nerve membrane. (From Burton, Howard & others, 1964.)

built of ganglioside aggregates about which is a rigid structure, perhaps protein. As a result of the nerve impulse, the vesicle migrates towards the internal surface of the presynaptic membrane. The ganglioside of the vesicle is soluble in the lipid of the terminal membrane, so the ganglioside aggregate serves as a potential pore through which the transmitter can diffuse.

Quarles & Folch-Pi (1965), in a different set of experiments, have reported the effects of several cations, including calcium, on the distribution of gangliosides in an organic-aqueous biphasic system. The data show that when Ca is in the upper (organic) phase at concentrations below

5 or above 160 mM, all the ganglioside is found in the organic phase. At calcium concentrations between 5–160 mM, the ganglioside partitions to the aqueous phase. Sodium, potassium and magnesium have virtually no effect on the distribution of ganglioside. The most intriguing finding was that the ganglioside, when contaminated with protein, was induced by calcium to localize at the interface of the aqueous–organic phases.

Finally, some scant evidence is available to suggest the site at which calcium may be physiologically active. Botulinum toxin suppresses the release of acetylcholine from the cholinergic nerve terminal, and the poisoning can be partially antagonized by calcium (Thesleff, 1960; Simpson & Tapp, 1967a). Toxin and calcium act at separate steps in the release mechanism of acetylcholine (Simpson & Tapp, 1967a), and the toxin localizes within the synaptic interspace (Zack, Metzger & others, 1962), which suggests that calcium must act at the membrane or within the neuroplasm. In fact, papers have been cited in this review that reported the inward movement of calcium associated with subsequent chemical extrusion. Owing to the frequently noted release of transmitter in packages, it seems appropriate to assume that calcium acts at the membrane to promote release of transmitter from the vesicles.

The various pieces of research listed suggest the following scheme. The synaptic vesicles, composed of ganglioside and protein, are in constant intracellular movement. Collision with the internal surface of the membrane, be it neural or glandular, is frequent. On the occasion of a nerve impulse, calcium moves into the cell. For a brief time, the concentration of calcium at the surface of the membrane is high enough to allow the collision between ganglioside and the organic component of the membrane to persist. In addition, the presence of protein in the vesicle promotes the ability of calcium to orientate the vesicle at the interface of the neuroplasm (aqueous phase) and the membrane (lipid phase). Thus calcium evokes the extrusion of transmitter. The mechanism can be expanded to explain other events. During periods when the nerve or gland is at rest, only small amounts of calcium would reside near the internal surface of membranes. Again, at very low concentrations of calcium the effectiveness of ganglioside-lipid contact is facilitated. Spontaneous secretory activity could be viewed as a consequence of low intracellular, or low intramembranous, concentration of calcium. Those intermediate concentrations of calcium at which ganglioside is partitioned into the aqueous phase also have a physiological counterpart. Katz & Miledi (1967c) have applied lengthy depolarizing pulses to nerve terminals in studying the release of acetylcholine (see pp. 889–893). If the duration of the pulse is not excessive, the amount of acetylcholine released is a function of the pulse length. However, as the pulse is lengthened, there is an increasing time lag before liberation of acetylcholine. Apparently lengthening the pulse augments the influx of calcium, the ion moving down both concentration and electromotive gradients. According to the proposed mechanism, as long as the pulse persists, calcium can flow through the membrane. Its concentration may be such that it promotes the stabilization of vesicles in the aqueous neuroplasm. Upon cessation of the

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pulse, the concentration of calcium at the membrane increases, thereby enhancing extrusion as depicted above.

In view of the earlier scheme, namely, that of the calcium–magnesium and calcium–sodium interactions, the proposed mechanism would be too simple if it relied only on the physical presence of calcium to evoke extrusion. Obviously calcium must be situated in some critical relation to a reactive site on the membrane. During periods of rest, there are a finite number of transmitter releasing sites available (Gage & Quastel, 1966). During activity, the number of releasing sites must increase. Calcium is known to bind to artificial membranes composed of phosphatidylserine and phosphatidylethanolamine (Rojas & Tobias, 1965). Both phospholipids are accepted as constituents of membrane. It may be conjectured that temporary fixation of calcium by a lipid component of the membrane exposes calcium for its augmentation of secretion, and the calcium-lipid complex allow the conformational changes in the membrane that will promote outward diffusion of the transmitter.

An attempt can now be made to consider the implications of the ionic interactions listed earlier. The suppression of calcium influx by magnesium indicates competition for a carrier molecule. 5-Hydroxytryptamine has been suggested as a calcium carrier at smooth muscle (Woolley, 1958) and perhaps at the nerve terminal (Boroff, DasGupta & Fleck, 1963). Work in our laboratory (Simpson & Tapp, 1967b) has not favoured the latter idea. In any event, the principal action of magnesium is to prevent calcium from reaching the site at which it promotes transmitter release, and the hypothetical molecule remains unidentified. In addition, it should be recalled that magnesium only slightly reduces calcium influx in the posterior pituitary, but the ion dramatically reduces vasopressin output. A discussion of this phenomenon will be momentarily deferred. Work at the frog myoneural junction introduced the possibility that sites for spontaneous transmitter release in the frog can promote release in the absence of calcium. This is not an unreasonable proposition. The sites at which calcium is temporarily fixed during nervous activity may spontaneously make the conformational changes necessary for transmitter diffusion. Also, there is little difference between low-calcium and no-calcium in terms of effective ganglioside retention in the lipid. The only difference would be in the ability of calcium to bind with the ganglioside-protein moiety at the neuroplasm–membrane interface. Yet, before attempting extended explanations, the experiments with EDTA offered on page 892 should be undertaken to insure that spontaneous events in the membrane could actually occur.

Two loci readily suggest themselves as potential areas in which calcium and sodium could compete. The locus could be extracellular, and competition between sodium and calcium would determine influx of calcium. Or, alternatively, the locus could be the releasing site in the membrane. The latter is suggested by various observations. If sodium and magnesium do compete with calcium at sites of intracellular movement, then a resultant sequence would be: magnesium prevents calcium from moving to the site at which sodium prevents calcium from moving to a releasing site,

or vice versa. The sequence seems teleologically unsatisfactory. Some experimental support is offered by the fact that phospholipids which bind calcium also bind other inorganic ions, sodium and magnesium included (Rojas & Tobias, 1965). Sodium fixed to the appropriate membrane structure could represent a nonfunctional releasing site. This could also explain the action of magnesium in the pituitary. A portion of its action is to depress influx of calcium; a supplementary action could be to fix to the releasing sites and thereby render them nonfunctional. Caution has been exercised thus far in not using the term "reactive site". Rather, it seems wise to assume that there are a number of sites from which release can potentially take place, but only when calcium is fixed there is the site reactive. This caution allows the possibility that when one calcium ion fixes to the site, the site is functional. When more than one calcium ion, i.e., Ca_2Y, or some other inorganic ion becomes bound to the site, release is prevented because the site is not reactive. In this context "reactive" means the ability to lodge the vesicle for a sufficient time for humoral or hormonal release.

Recent observations by Rahaminoff (Dodge & Rahaminoff, 1967; Rahaminoff, 1968) indicate that the rate of packet release in a single epp is nearly proportional to the fourth power of CaY. Consequently, there may be as many as four, or some multiple of four, effective CaY bindings involved in the emission of a single packet of transmitter.

Admittedly an assumption was made in assuming that all extrusion processes are roughly comparable. However, the universal excitation–extrusion effect of calcium, the calcium influx antagonism by magnesium, and the quantal release of transmitter, all make the assumption tempting. They also encourage the comparison of data from dissimilar preparations. Gage & Quastel delineated a calcium–sodium antagonism at the mammalian myoneural junction (Gage & Quastel, 1966), and the mechanism drawn here from the available literature attempts an explanation involving the releasing site. Douglas & Rubin (1961, 1963), Douglas & Poisner (1962) and Douglas (1966) showed that removal of sodium facilitated catecholamine release from the adrenal medulla. Certainly a common mechanism at both sites is suspect. The proposed mechanism also facilitates the explanation of several physiological observations. One pertinent to the discussion will be considered here. In examining the kinetics of the calcium–sodium interaction Gage & Quastel (1966) suggested that the resting nerve must have available only a small number of sites for calcium-ion action. Increasing calcium to high levels depresses spontaneous secretion. The constant release of small amounts of transmitter is necessary for maintaining the functional integrity of receptor structures (see Katz, 1966). Were more than a limited number of releasing sites available, the level of calcium at the membrane could reach a concentration sufficient to stabilize the vesicles in the neuroplasm. Obviously this situation would not promote release. The existence of few rather than many releasing sites is actually necessary for maintaining the constant secretion of mediators. Only on the arrival of a nerve impulse does the

number of releasing sites increase enough to allow adequate calcium for promoting vesicle-in-membrane stabilization.

A summing-up

There can be no question of the involvement of calcium in the release of several chemical mediators. The ion has been implicated at various neural and neuro-related structures. Evidence presented thus far has been largely quantitative, i.e., modifying the calcium concentration to a specific degree will result in measurable alterations in humoral or hormonal output. Explanations to account for the action of calcium are few and unspecific. An attempt has been made here to collect from the literature a mechanism that is amenable with the quantitative observations. The mechanism explains processes of extrusion in terms of critical calcium concentrations at or within the cell membrane. Both the presence of calcium and its physical relation to phospholipid in the membrane are necessary for effective extrusion. In view of the relative naivety of the literature with respect to molecular procedures and membrane architecture, the mechanism discussed may well be largely of theoretical value only. It therefore seems appropriate to indicate at least one possible course of investigation into the model. Since it is possible to isolate synaptic vesicles in a fraction of some purity, and since phospholipid monolayers are constructed without great difficulty, it would be appropriate to expose vesicles to an artificial system in which there is a phospholipid monolayer. Application of a modest electrical bias could be used to mobilize the vesicles. It would be important to determine whether calcium, when fixed to the artificial lipid membrane, does in fact have the capacity to stabilize vesicles at the interface of an aqueous-lipid biphasic system. Even if such a demonstration were not forthcoming, study of the system would possibly suggest alternatives to the mechanism proposed herein.

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Lack of inter-action between propranolol and mebanazine

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It has been suggested that treatment with monoamine oxidase inhibitors should be withdrawn 2 weeks before commencing treatment with an adrenergic β-receptor blocking drug e.g. propranolol. Experiments in anaesthetized cats have failed to unmask any undesirable property of propranolol following amine oxidase inhibition. Furthermore, combined treatment with both types of drug reduced the cardiovascular responses to intravenous tyramine and amphetamine. The pressure response to intraduodenal tyramine was no greater in animals treated with both drug types compared with that in cats receiving amine oxidase inhibitor alone, whereas the tachycardia was much reduced.

POTENTIATION of the pressor responses to indirectly acting sympathomimetic amines is a well recognized phenomenon following inhibition of monoamine oxidase. As a consequence of a number of clinical catastrophes, many warnings have been given about the dangers of concomitant administration of other drugs (particularly those affecting the cardiovascular system) and the ingestion of certain foodstuffs. Recently it has been suggested that monoamine oxidase inhibitor therapy should be withdrawn for two weeks before the institution of treatment with propranolol, a specific antagonist of adrenergic β-receptors (Frieden, 1967). The experiments on cats reported here were undertaken to determine whether or not the contra-indication of this particular combination of drugs was truly justified. Mebanazine (α-methylbenzylhydrazine) was chosen as the monoamine oxidase inhibitor because of its potency and lack of sympathomimetic activity (Spinks & Whittle, 1966).

Experimental

METHODS

The experiments were made using cats (1·8–2·8 kg) anaesthetized with chloralose. Blood pressure was recorded from a femoral artery using a pressure transducer and heart rate with a cardiotachometer throughout the experiments. A saphenous vein was cannulated for the intravenous injection or infusion of drugs and in some animals a cannula was also inserted into the duodenum via a midline incision. Five cats were used in each group. Pretreatment with mebanazine at a dose level of 5 mg/kg i.p. was made for 3 days before experiment in those cases where other drugs were given intravenously. For those experiments in which tyramine was given intraduodenally, the cats received 5 mg/kg 18 hr before experiments with an additional 2·5 mg/kg i.d. 2 hr beforehand.

Results

EFFECT OF MEBANAZINE ON RESPONSES TO PROPRANOLOL

The intravenous infusion of propranolol at 5, 10 or 50 μg/kg/min produced a significant bradycardia and a slight reduction in mean blood pressure. The bradycardia, which averaged 21%, was not dose dependent,

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there being no difference between the 5 and 50 μg/kg/min effects. In cats pretreated with mebanazine there was no significant alteration in heart rate or blood pressure as compared to the control cats nor in the responses to propranolol infusion. There was no evidence of any increase in blood pressure following propranolol in any mebanazine treated animal.

EFFECT OF MEBANAZINE AND PROPRANOLOL ON THE RESPONSES TO INTRAVENOUS TYRAMINE AND AMPHETAMINE

Intravenous tyramine over the dosage range of 20–2000 μg/kg produced a dose-dependent series of increments in blood pressure and heart rate (Table 1). In another group of cats which received an intravenous

TABLE 1. CHANGE IN MEAN BLOOD PRESSURE ($\pm$S.E. MM HG) AND HEART RATE ($\pm$S.E. BTS/MIN) TO ASCENDING DOSES OF TYRAMINE IN UNTREATED CATS AND THOSE RECEIVING EITHER PROPRANOLOL OR MEBANAZINE SEPARATELY OR IN COMBINATION

Dose of tyramine (μg/kg)	Pretreatment							
	None		Propranolol		Mebanazine		Mebanazine and propranolol	
	BP	HR	BP	HR	BP	HR	BP	HR
20	+8±3	−4±2	+2±3	−3±2	+22±12	0±15	+10±10	+1±2
60	+17±3	+7±5	+11±3	−4±5	+42±13	+36±7	+35±4	+18±5
200	+32±5	+10±9	+28±6	−9±10	+78±17	+55±15	+55±7	+21±6
600	+79±17	+29±17	+44±11	+4±10	+92±15	+65±10	+69±4	+31±4
2,000	+107±8	+66±19	+94±12	+39±8	+161±12	+100±9	+78±12	−30±12

infusion of propranolol (5 μg/kg/min) for 30 min before and throughout tyramine dosage there was a smaller increase in blood pressure and heart rate at each dose level. Cats which had been pretreated with mebanazine

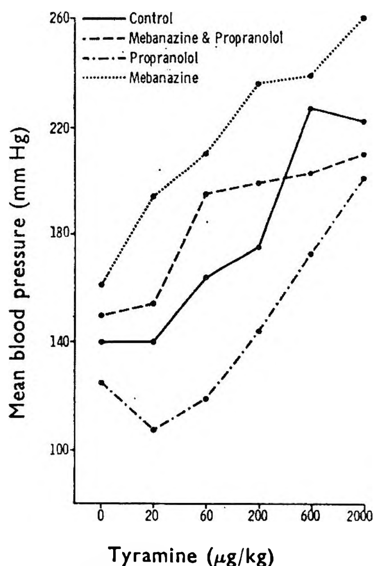

FIG. 1. Final mean blood pressure after intravenous tyramine in cats which had received no medication, propranolol or mebanazine separately or a combination of both drugs.

exhibited potentiated responses throughout the dose range. In those animals receiving a combination of both drugs the responses were larger than those in control cats but lower than in cats pretreated with mebanazine alone. The results have also been presented in a manner comparing the dose of tyramine with the peak blood pressure observed (Fig. 1). It is apparent that the degree of hypertension produced under the influence of both mebanazine and propranolol was at no time greater than that with mebanazine alone.

TABLE 2. CHANGE IN MEAN BLOOD PRESSURE AND HEART RATE AND DURATION OF RESPONSE TO AMPHETAMINE IN UNTREATED CATS AND THOSE RECEIVING EITHER MEBANAZINE ALONE OR BOTH MEBANAZINE AND PROPRANOLOL

		Dose of amphetamine			
		100 μg/kg		200 μg/kg	
	Pretreatment	Change	Duration (min)	Change	Duration (min)
Blood pressure (mm Hg)	None	+39± 8	6±4	+58±10	10± 3
	Mebanazine ..	+87± 9	15±4	+110±11	17± 2
	Mebanazine and propranolol	+11± 2	8±3	+60± 9	5±3
Heart rate (bts/min)	None	+18±16	8±4	+42± 9	19± 6
	Mebanazine ..	+47± 9	15±3	+82± 9	30±10
	Mebanazine and propranolol ..	+ 4± 4	<1	+15± 4	10± 4

The results of similar experiments in which amphetamine was used as the pressor agent are summarized in Table 2. Once again the responses after combined propranolol and mebanazine were appreciably smaller than in cats pretreated with mebanazine alone. It was also apparent that the duration of the responses was reduced to one third that of the mebanazine group in those animals with adrenergic β-blockade.

EFFECT OF MEBANAZINE AND PROPRANOLOL ON THE RESPONSES TO INTRA-DUODENAL TYRAMINE

Tyramine was administered intraduodenally at a dose of 5 mg/kg, 45 min after beginning an infusion of saline or propranolol (10 μg/kg/min).

TABLE 3. BLOOD PRESSURE AND HEART RATE IN CATS PRETREATED WITH MEBANAZINE BEFORE AND AFTER INTRADUODENAL TYRAMINE FOLLOWING INFUSION OF SALINE OR PROPRANOLOL

		Blood pressure (systolic/diastolic mm Hg)				Heart rate (beats/min)			
Infusion	Cat. No.	Initial	45′ after infusion	Peak response to tyr.	Change in systolic	Initial	45′ after infusion	Peak response to tyr.	Change
Saline	1	155/125	140/120	250/200	+110	150	162	278	+116
	2	140/95	137/92	235/140	+ 98	225	225	315	+ 90
	3	110/60	92/65	252/166	+160	153	165	294	+129
	4	120/105	120/100	223/200	+103	160	170	280	+110
	5	70/40	60/30	144/124	+ 84	155	150	235	+ 85
	Mean	119/85	110/79	221/166	+111±16	169	174	281	+106±9
Propranolol	1	110/96	93/77	160/143	+ 67	188	123	163	+ 40
	2	105/75	75/45	200/165	+125	135	115	160	+ 45
	3	128/60	122/60	266/195	+144	185	165	210	+ 45
	4	147/105	127/87	220/120	+ 93	202	162	240	+ 78
	5	155/125	160/130	220/190	+ 60	195	148	208	+ 60
	Mean	129/92	115/80	213/163	+98±17	181	143	196	+54±8

The infusion was continued until the end of the experiment. The results are reported in Table 3. Following tyramine there was a steady rise in blood pressure which reached a peak at about 20 min. Heart rate also increased but the peak value was not observed until the peak pressure change was passed. The animals receiving mebanazine alone showed a slightly higher pressor response than those which also received propranolol [systolic values + 111 (84–162) *vs* + 98 (60–144) mm Hg] but the difference was not statistically significant. The changes in heart rate were statistically smaller in the propranolol group (P < 0·01). The essential differences in the two groups are illustrated in Fig. 2.

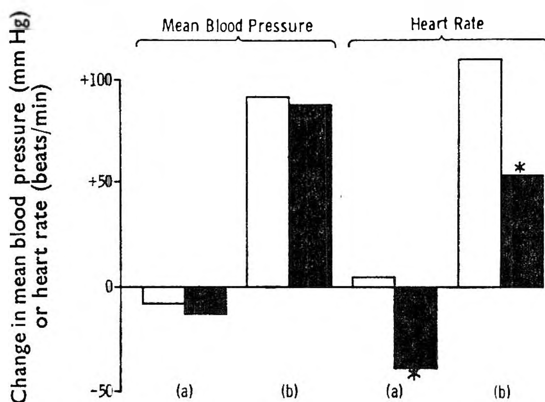

Fig. 2. Average changes in mean blood pressure and heart rate in cats pretreated with mebanazine following the infusion of saline (open columns) or propranolol (solid columns) before (a) and after (b) the intraduodenal administration of tyramine. An asterisk denotes a significant difference between saline and propranolol infused animals (P < 0·05).

Discussion

The mechanism by which monoamine oxidase inhibitors potentiate the pressor actions of indirectly acting sympathomimetic amines is now well understood. Substances such as tyramine and amphetamine are good substrates for this particular enzyme which provides the principal means for their metabolism. As a consequence, inhibition of monoamine oxidase leads to both a potentiation and prolongation of their actions. In addition, amine oxidase in the gut wall plays a significant role in the detoxification of such substances following their administration orally as pure drugs or their ingestion as a constituent of certain foodstuffs, notably cheese. Inhibition of amine oxidase leads, therefore, to large amounts of potentially pressor amines passing into the blood stream. In contrast, monoamine oxidase inhibition does not potentiate the hypertensive effects of the natural adrenergic transmitters adrenaline and noradrenaline since these substances are predominantly metabolized by catechol-*O*-methylation.

The present experiments did not provide any evidence that monoamine oxidase inhibition altered the responses to propranolol itself. The only other possibility was that combined administration of both types of

drug would exacerbate the risk of hypertensive crisis, if the contra-indication was truly justified. It is recognized that propranolol will potentiate the pressor responses to adrenaline as a consequence of the blockade of its vasodilator component. Under normal conditions, however, there is no indication that indirectly acting sympathomimetic amines provoke a significant release of adrenaline. The pressor responses to intravenous injection of noradrenaline are reduced by propranolol, due to a reduction in the cardiac component of its action (Shanks, 1966). There is a suggestion that prolonged infusion of noradrenaline may produce a slightly greater pressor response after propranolol (Glover & Hutchison, 1965) but this has not been observed after shorter infusions (Brick, Glover & others, 1966). We have not observed any increase in either the pressor or tachycardia responses when both an amine oxidase inhibitor and propranolol are given together, compared with amine oxidase inhibitor alone, following intravenous injection of tyramine or amphetamine. The reduction in duration of the responses to amphetamine after combined administration of both types of drug may well indicate that its hypertensive action has a major cardiac component following amine oxidase inhibition. Similarly, the pressor responses to intraduodenal tyramine, in a dose equivalent to approximately $\frac{3}{4}$ lb (350 g) of Canadian Cheddar cheese (Blackwell & Marley, 1966) for a 70 kg man, were not potentiated by combined administration of mebanazine and propranolol.

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Mutual suppression of cardiovascular effects of some β-adrenoreceptor agonists in the cat

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Observations were made of the effects of adrenaline, isoprenaline and orciprenaline on the blood pressure, heart rate and configuration of the ECG in cats. The effects of single injections of these amines in producing a rise in heart rate and a fall in blood pressure were reduced during the intravenous infusion of any one of them: the pressor response to injected adrenaline was enhanced during an infusion. Combinations of these amines did not produce changes in the configuration of the ECG. These experiments were motivated by allegations of acute toxic effects on the cardiovascular system of large doses, or of combinations, of sympathomimetic bronchodilators in asthma. No evidence of deleterious effects of interactions was obtained.

IN the treatment of asthma, sympathomimetic amines are commonly employed in aerosol inhalers for their bronchodilator action, this being an effect on the β-adrenoreceptors of bronchial smooth muscle. The safety of these preparations has been questioned in a number of articles over the last few years because of deaths among asthma patients known to have been using metered aerosol sprays, particularly those containing adrenaline, isoprenaline or orciprenaline as the active component. It has been suggested that excess exposure to these amines (Greenberg & Pines, 1967), or their use in combinations (Anon., 1965), can result in untoward effects arising from their cardiac activity. Greenberg & Pines (1967) think that the amines may induce ventricular fibrillation, and other workers have suggested that they cause cardiac arrest and that this is the cause of death. The evidence for these assertions has included a number of ECG records showing patterns varying from ventricular tachycardia (Greenberg & Pines, 1967) to atrial flutter (Exon, 1967) in patients using sympathomimetic aerosols.

Although much work has been done on the blood pressure effects of large doses of these amines and on interactions between them, there has been little experimental work done on their cardiac effects. Therefore, experiments were made on cats to investigate the cardiovascular effects of interaction between three sympathomimetic amines commonly supplied as aerosol preparations, namely adrenaline, isoprenaline and orciprenaline.

Experimental

METHODS

Cats of either sex, weighing between 1·7 and 4·5 kg, were anaesthetized with chloralose (90 mg/kg) and pentobarbitone (5 mg/kg) given intraperitoneally. Blood pressure was monitored from the right femoral artery with a Statham strain gauge transducer. The heart rate was measured through a cardiotachometer coupler connected to needle electrodes inserted under the skin. Both blood pressure and heart rate were recorded on an Offner Dynograph. The electrocardiogram was recorded on a

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Phillips electrocardiograph using electrodes corresponding to standard human limb lead II. Intravenous injections were given into the right femoral vein in a volume of 0·1 ml and washed in with 0·1 ml/kg of 0·9% NaCl solution. The left femoral vein was cannulated for infusions from a Palmer motor-driven syringe at a constant rate of 0·2 ml/min.

The interval allowed to elapse before giving another injection after injecting adrenaline or isoprenaline was 7 min. However, after an orciprenaline injection, the interval before giving another injection was 15 min, since the increase in heart rate produced by this drug was long-lasting. Sometimes, the rate did not return to the previous level even within the 15 min period; despite this, the next injection was given. Control responses were not less reproducible when given during a sustained response to orciprenaline than when given after one of the other drugs.

Results

The first part of each experiment was devoted to obtaining the control response to injections of each of the sympathomimetic amines. The doses of injections of these drugs were chosen to produce definitely measurable increases in heart rate, of approximately equal magnitude from one experiment to another. Table 1 shows the heart rate increases produced by control injections of the three amines. The effect of adrenaline injections (0·1 to 1·0 μg/kg) on blood pressure varied, the response being purely depressor, biphasic with depressor and pressor components, or purely pressor. Isoprenaline (0·05 to 0·5 μg/kg) and orciprenaline (0·5 to 5·0 μg/kg) produced only a fall in blood pressure.

TABLE 1. EFFECT OF CONTROL INJECTIONS OF ADRENALINE, ISOPRENALINE AND ORCIPRENALINE ON HEART RATE.

Drug	No. of experiments	Mean heart rate increase (beats/min)	Standard error
Adrenaline (0·1–1·0 μg/kg)	25	22·8	±2·2
Isoprenaline (0·05–0·5 μg/kg)	20	31·3	±2·8
Orciprenaline (0·5–5·0 μg/kg)	26	20·3	±1·6

The initial rate of infusion chosen for adrenaline and orciprenaline was 0·1 μg/kg/min and for isoprenaline it was 0·05 μg/kg/min. The drugs had no effect on heart rate or blood pressure at these rates of infusion. If the response to control injections was unchanged during the infusion at the initial rate, the rate of infusion was increased until an effect was observed, but the rates were limited to the minimum that affected responses to injection since it was desired to minimize the effect of infusions themselves on heart rate and blood pressure.

The cardiovascular responses induced by injections of the three amines were unchanged during saline infusions (0·2 ml/min) in 3 control studies. During infusions of adrenaline (0·1 to 0·5 μg/kg/min), isoprenaline (0·05 to 1·0 μg/kg/min), or orciprenaline (0·05 to 0·5 μg/kg/min), the tachycardia and depressor effects obtained with single injections of the amines were significantly reduced. Figs 1–3 illustrate the effects of infusions on the

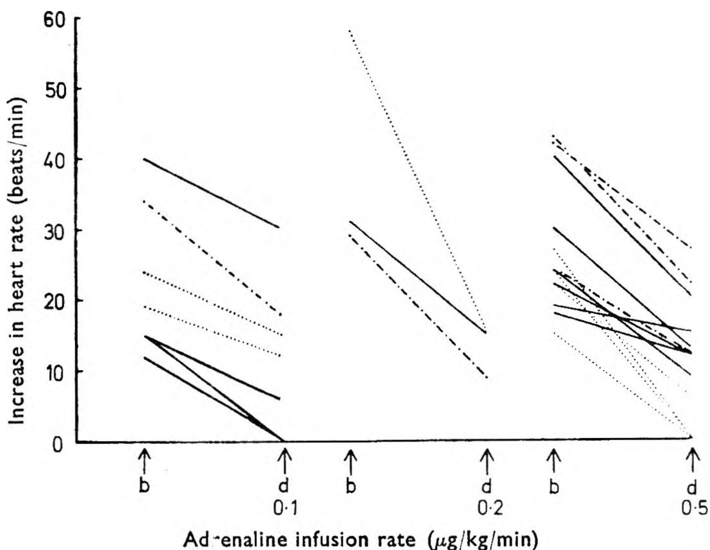

FIG. 1. Reduction in tachycardia in response to injections of adrenaline (· · · · · · ·), isoprenaline (·–·–·–·) and orciprenaline (———) produced by infusions of adrenaline at 0·1, 0·2 and 0·5 μg/kg/min. The control responses to the amines are indicated by b, and the responses during the infusions by d.

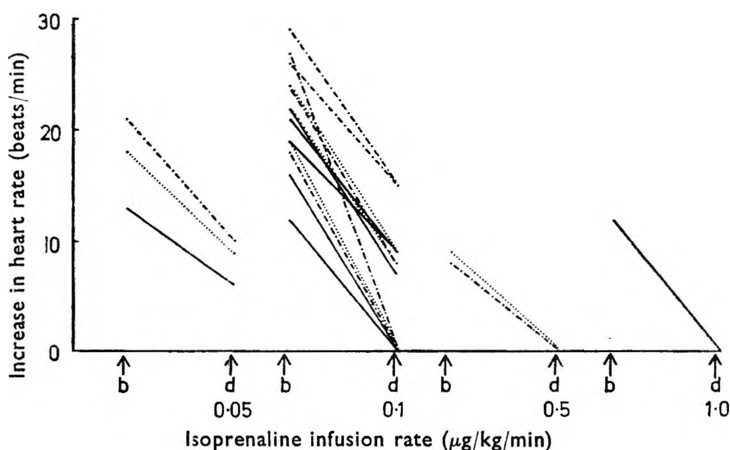

FIG. 2. Reduction in tachycardia in response to injections of adrenaline (· · · · · ·), isoprenaline (·–·–·–·) and orciprenaline (———) produced by infusions of isoprenaline at 0·05, 0·1, 0·5 and 1·0 μg/kg/min. The control responses to the amines are indicated by b, and the responses during the infusions by d.

tachycardia produced by injections of the three amines. The onset of this antagonism was fairly rapid (5–10 min), and a partial or complete return of the responses to control levels was often observed after the infusion was terminated (Fig. 5).

Effect of adrenaline infusions. Fig. 4 illustrates the effect of an infusion

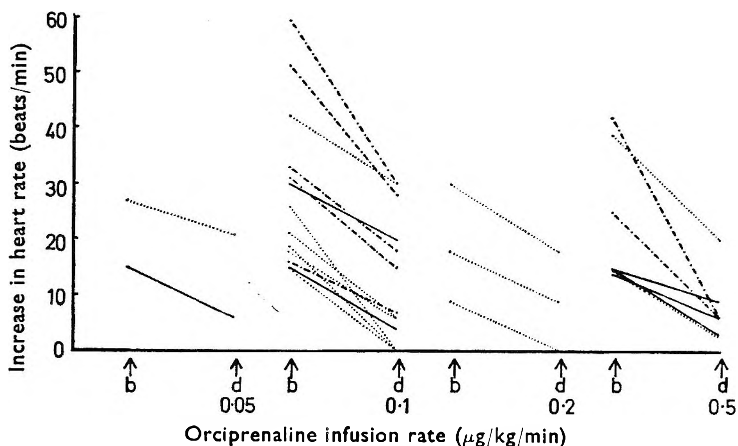

FIG. 3. Reduction in tachycardia in response to injections of adrenaline (· · · · · ·) isoprenaline (·–·–·–·) and orciprenaline (———) produced by infusions of orciprenaline at 0·05, 0·1, 0·2 and 0·5 μg/kg/min. The control responses to the amines are indicated by b, and the responses during the infusions by d.

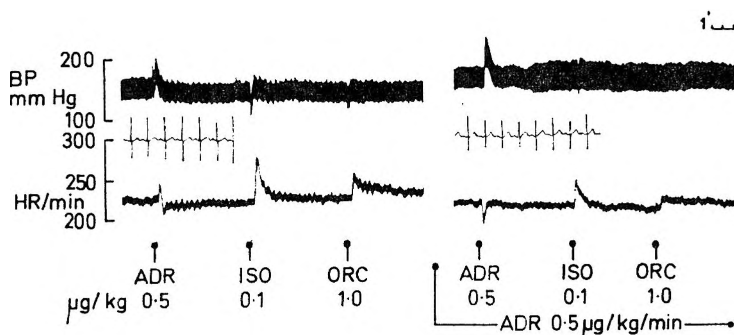

FIG. 4. Antagonism of responses to injected adrenaline (ADR), isoprenaline (ISO) and orciprenaline (ORC), during infusion of adrenaline in a chloralose anaesthetized cat (3·7 kg). The upper tracing is arterial blood pressure (BP), the lower is heart rate (HR). Intravenous injections are indicated by the arrows, and intravenous infusion by the horizontal line. The portions of ECG record were taken during the control period and during the infusion, in each case before injections were given.

of adrenaline on responses to single injections of adrenaline, isoprenaline and orciprenaline. During adrenaline infusions, the depressor component of the response to adrenaline was abolished and the pressor component was variably enhanced. The tachycardia previously obtained with injections of adrenaline was converted to reflex bradycardia in 4 of 10 experiments, and reduced by more than 40% in the remaining experiments. Depressor effects of isoprenaline (0·1 to 0·2 μg/kg) were less effectively blocked than were those of adrenaline. In 3 experiments, a complete block of isoprenaline vasodepression was seen during adrenaline infusions, with more than 40% antagonism in three others. The tachycardia produced by isoprenaline was reduced by 40 to 60% in each case. Infusions

of adrenaline reduced the depressor effects of orciprenaline injections more readily than those of isoprenaline, complete antagonism of the effect of orciprenaline being obtained in 9 of 12 experiments, with more than 50% reduction in the remaining three instances. The tachycardia in response to orciprenaline was abolished in 3 cats, reduced by 30 to 80% in 7 cats, and no effect was observed in the other two.

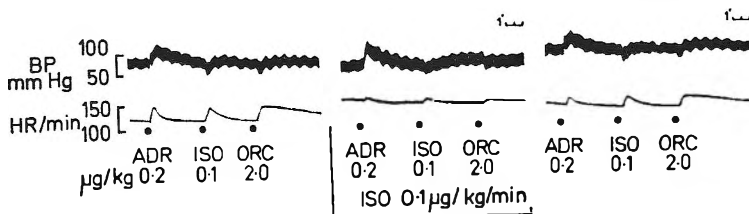

FIG. 5. Antagonism of responses to injected adrenaline (ADR), isoprenaline (ISO) and orciprenaline (ORC) during an infusion of isoprenaline in a chloralose anaesthetized cat (1·7 kg). The upper tracing is arterial blood pressure (BP), the lower is heart rate (HR). Intravenous injections are indicated by the arrows and intravenous infusion by the horizontal line.

Effect of isoprenaline infusions. Infusions of isoprenaline had a similar effect to those of adrenaline in modifying the responses to injections of the three amines, as shown in Fig. 5. The effects of adrenaline on heart rate were reduced to 50% of the preinfusion levels. The antagonism of depressor responses to isoprenaline was greater than 40% in 7 experiments, and the tachycardia produced by isoprenaline was reduced by more than 50%. Isoprenaline infusions even more readily antagonized orciprenaline depressor effects, producing 75 to 100% antagonism in 5 cats, with a 50 to 60% decrease in the cardiac response.

Effect of orciprenaline infusions. In one experiment, an infusion of orciprenaline converted the response to adrenaline from tachycardia to a reflex bradycardia, and in 8 other experiments there was a reduction in tachycardia of greater than 28%. The antagonism of vasodepressor effects of isoprenaline varied from zero to 50% in 8 experiments; the accompanying heart rate increases were reduced to 50 to 80% of the control value. Orciprenaline infusions resulted in a decrease in the chronotropic response induced by single injections of the same amine from 30 to 60%, but the depressor effects were variably affected, no antagonism being observed in 3 cats, whereas in 2 others there was complete abolition of the response. The results from one experiment are illustrated in Fig. 6.

OBSERVATIONS ON THE CONFIGURATION OF THE ECG

Infusions of the three sympathomimetics sometimes produced changes in the configuration of the T-wave, varying from depression to enhancement, but effects were inconstant in both occurrence and duration. When an infusion produced a decrease in the positive chronotropic response to the injection of an amine, the effect of the injection of that amine on the T-wave was also reduced. In only one experiment was an arrhythmia

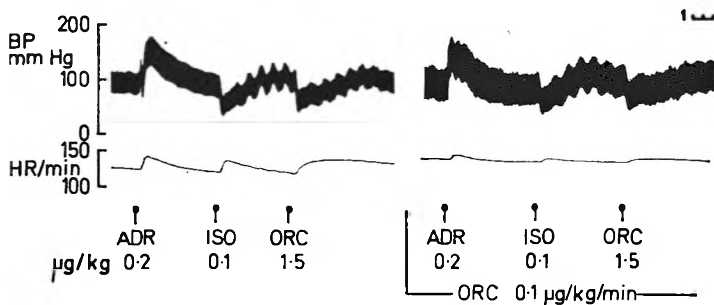

FIG. 6. Antagonism of responses to injected adrenaline (ADR), isoprenaline (ISO) and orciprenaline (ORC) during infusion of orciprenaline in a chloralose anaesthetized cat (2·8 kg). The upper tracing is arterial blood pressure (BP), the lower is heart rate (HR). Intravencus injections are indicated by the arrows and intravenous infusion by the horizontal line.

observed during an infusion, and this occurred when the positive chronotropic effect of adrenaline had been so effectively antagonized that the normal response of tachycardia was converted to a reflex bradycardia.

Discussion

These experiments demonstrate that in normal cats the interaction between adrenaline, isoprenaline and orciprenaline results in antagonism rather than potentiation of cardiovascular effects attributable to actions on β-adrenoreceptors. During infusions of the amines, the animal shows decreased sensitivity to cardiac and depressor effects of single injections of any one amine. Vasodepressor effects were more readily antagonized than were the positive chronotropic effects, particularly those induced by injections of orciprenaline, and some effect on heart rate generally persisted with injections of isoprenaline and orciprenaline although their effects were usually diminished. Reflex bradycardia was often seen when the depressor component of an adrenaline response was abolished during an infusion and the pressor effect of adrenaline was thereby increased. Reversal of the blood pressure effects of isoprenaline and orciprenaline was not observed, although this has been shown for isoprenaline by Gutman & Beyth (1964) and Beyth & Gutman (1965) using infusion concentrations of adrenaline and isoprenaline comparable to ours. Butterworth (1963) demonstrated that large doses ('priming doses') of isoprenaline blocked the depressor effects of smaller doses, and caused the appearance of pressor activity to subsequent administrations of the large dose.

Thus our experimental observations give no evidence of cardiovascular toxicity arising from the interactions of the amines. It has been suggested that the pathological changes associated with asthma may cause untoward drug effects. Lockett (1965) showed that when the heart is fatigued or under strain, it is more subject to arrhythmias precipitated by catecholamines, and Kerr (1967) considered that this may apply when the myocardium is anoxic. However, as has been pointed out (Herxheimer, 1965;

Kennedy, 1965), Lockett's observations with the heart-lung preparation may not be a true reflection of the behaviour of the human heart in the asthmatic state.

However, not enough is known of the effects of sympathomimetic drugs in the conditions associated with asthma. The possibility of reversal or blockade of the effects of sympathomimetic amines on the bronchi after giving large doses has not apparently been reported.

Our experiments have been concerned only with the possibility of the acute cardiovascular toxicity of sympathomimetic amines. The effect of long term therapy with sympathomimetic amines requires careful consideration, bearing in mind the possible development of changes in myocardial reactivity or tolerance to bronchodilator efficacy.

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The effect of some non-ionic surfactants and a polyoxyethylene glycol on the dissolution rate of griseofulvin

P. H. ELWORTHY AND F. J. LIPSCOMB

Measurements of the dissolution rate of griseofulvin in water, four non-ionic surfactants, and a polyoxyethylene glycol have been made. These results are analysed in terms of a zero order rate constant (k_1) for transfer of the drug from the crystal to the bulk of the solution, and a first order constant (k_2) for the reverse process. Surfactants greatly increase the dissolution rate, increasing k_1 and decreasing k_2. Polyoxyethylene glycol is not so effective as the surfactants at increasing dissolution rates. In an attempt to interpret k_1 and k_2, it appears that both chemical and transport processes are involved in the dissolution, the presence of surfactant decreasing the energy change for transferring griseofulvin molecules from the crystal to the solution.

ONLY a few studies on the effect of surfactants on the dissolution rate of solids have been published. Parrott & Sharma (1967) have shown that surfactants increase the dissolution rate of benzoic acid, while Wurster & Seitz (1960) and Levy & Gumtow (1963) also observed an increased dissolution rate in the presence of sodium lauryl sulphate. The dissolution rate of griseofulvin is increased by the presence of surfactants (Bates, Lin & Gibaldi, 1967, Bates, Gibaldi & Kanig, 1966).

As surfactants are commonly used in pharmaceutical formulation, it is of interest to study their action on the dissolution rate in detail, to gain some understanding of the underlying mechanisms. The dissolution rate can be the rate-limiting step in the absorption of a drug, and the possibility of increasing it by the use of surfactants may have applications in formulation work. In the present study, griseofulvin was chosen as the insoluble drug, and its dissolution studied in a range of non-ionic surfactants. The solubility of this compound in various solvents and in surfactant solutions has already been reported (Elworthy & Lipscomb, 1968a, 1968b).

Experimental

The physical properties of the griseofulvin used have been reported (Elworthy & Lipscomb, 1968a). The surfactants used were commercial samples based on hexadecanol with varying numbers of ethylene oxide units e.g. $Me[CH_2]_{15}[O{\cdot}CH_2{\cdot}CH_2{\cdot}]_xOH$ where $x = 22$, 38, and 50 (abbrev. to HEx); the octylphenol containing surfactant $Me_3C{\cdot}CH_2{\cdot}CMe_2{\cdot}C_6H_4[O{\cdot}CH_2{\cdot}CH_2]_{10}OH$, (Abbrev. to OE10), and polyxyethylene glycol 400 (Elworthy & Lipscomb, 1968b). Deionization of these compounds did not affect the results.

Viscosities of solutions relative to water were measured in Ostwald capillary viscometers.

The dissolution apparatus consisted of a thermostated 1 litre glass jar which contained the dissolution medium. All measurements were made at $25° \pm 0{\cdot}01°$ unless otherwise stated. A magnetic follower at the bottom of the jar was driven by a magnetic stirrer mounted below the thermostat.

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A series of synchronous motors provided stirring speeds of between 5 and 600 rev/min. A glass tripod, with a "gauze" made of fine glass fibres, supported the tablet of griseofulvin in the dissolution medium. The tablets were prepared on a hydraulic press, and were washed before use to remove any loose particles of griseofulvin dust.

An experiment was commenced by placing the tablet on its tripod in the dissolution medium. Samples (5 ml) of the medium were withdrawn at noted time intervals, and assayed by measuring their extinction in the 294–296 $m\mu$ region (the position of λ_{max} depended on the surfactant being used). When surfactants or polyoxyethylene glycol (PG) 400 was present, extinctions were measured against blank solutions containing the appropriate concentrations of these materials. After each sampling, an equal volume of fresh dissolution medium was added. Examination of solution in an ultramicroscope did not reveal any particles of griseofulvin to be present. Because of the small extinction measured in some experiments, it was necessary to check that there was no significant adsorption of griseofulvin on glassware. None could be detected.

Results and discussion

INTERPRETATION OF RESULTS

With the method adopted, the solubilities of griseofulvin in the dissolution media are so small that the surface area of the tablet does not change significantly during the experiment.

The dissolution is regarded as consisting of two processes, occurring simultaneously:

1. A zero order reaction for the transfer of griseofulvin molecules from the solid surface into the solution, with rate constant k_1.

2. A first order reaction for the deposition of solute from solution to the solid surface, with rate contsant k_2.

The rate of increase of concentration in solution:

$$\frac{dc}{dt} = k_1 - k_2c \quad .. \quad .. \quad .. \quad (1)$$

The solution to this equation with the condition that at $t = 0$, $c = 0$ is

$$c = \frac{k_1}{k_2} \cdot \left(1 - e^{-k_2t}\right) \quad (2)$$

Expanding the exponential term and rearranging gives

$$\frac{c}{t} = k_1 - \frac{k_1k_2t}{2} + \frac{k_1k_2^2t^2}{6} - \frac{k_1k_2^3t^3}{24} + \cdots$$

At fairly early times in the dissolution process, terms in t^2 and t^3 etc. can be neglected giving:

$$\frac{c}{t} = k_1 - \frac{k_1k_2t}{2} \quad (3)$$

A plot of c/t *vs.* t will have an intercept k_1 and a slope $k_1k_2/2$, enabling both constants to be evaluated. Trial calculations show that equation (3) gives 1% error in c compared to the exact equation (2) provided the k_2t term does not exceed 0·25.

Equation 2 reduces to the Noyes-Whitney equation (Noyes & Whitney, 1897). When equilibrium is reached, i.e. a steady state between dissolving and redeposition

$$\frac{dc}{dt} = 0 = k_1 - k_2 c_s$$

where c_s is the saturation solubility.

$$c_s = k_1/k_2 \quad (4)$$

and from equation (2)

$$c = c_s (1 - e^{-k_2 t})$$

or,

$$k_2 = \frac{1}{t} \cdot \ln\left(\frac{c_s}{c_s - c}\right) \quad (5)$$

which is the more usual form of the Noyes-Whitney equation. The rate constant of equation (5) thus appears to be the first order constant arising in the consideration of the dissolution-redeposition process. Equation (3) is useful if the saturation solubility is not known; when it is, equation (4) can be used to evaluate one constant when the other has been determined from (3) or (5).

The experimental results reported later are interpreted in terms of k_1 and k_2.

EFFECT OF EXPERIMENTAL CONDITIONS ON STUDY OF DISSOLUTION RATE

In the development of the apparatus already described, the effect of a number of experimental conditions on the observed dissolution rate was examined.

1. *Position of tablet.* Three tripods were constructed to hold the tablet, which in each experiment was placed in the centre of the gas jar; the lowest tripod gave a distance from the top of the stirrer bar to the bottom of the tablet of 0·3 cm, the medium tripod a distance of 1·6 cm, and the highest tripod one of 2·5 cm. (Thickness of stirrer bar = 0·76 cm.) Using a tablet prepared at a compaction pressure of 10 ton/inch^2, a stirring rate of 250 rev/min, and an initial volume of water of 300 ml, from which 5 ml portions were removed at timed intervals for the measurement of absorbance gave results which showed that at this stirring speed, the height of the tablet appears to make little difference to the amount of griseofulvin dissolving. The highest tripod was used in all subsequent experiments, as it placed the tablet equidistant between the surface of the water and the bottom of the jar, giving the minimum disturbances from rotation of the stirrer bar, or the development of a vortex at the surface (particularly when stirring rates exceeding 250 rev/min were used).

2. *Volume of water.* Using a stirring rate of 250 rev/min and a tablet compacted as before, the effect of using initial volumes of 200 and 500 ml of water was examined. In these experiments 5 ml portions were withdrawn for assay at timed intervals, and the results are shown in Fig. 1 in terms of the weight of griseofulvin per 100 ml of water released by the tablet.

Some authors, e.g. Bates, Lin, & Gibaldi (1967), have added an equal volume of fresh dissolution medium when each sample is withdrawn for

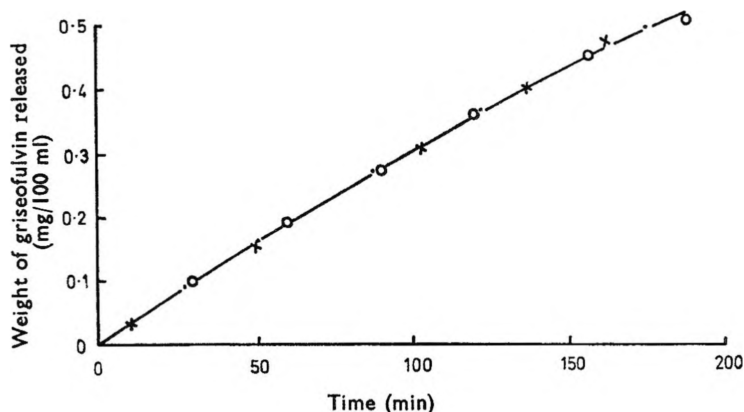

FIG. 1. Effect of different volumes of dissolution medium on the amount of griseofulvin dissolving. ○ = 200 ml. × = 500 ml. • = 300 ml, with replacement (see text).

assay. The results of such an experiment are shown in Fig. 1. All results have been corrected to allow for the amount of griseofulvin removed from the system for assay purposes. The different volumes and techniques of sampling do not appear to have a significant effect on the results. In all other experiments, an initial volume of 300 ml of water was used, together with replacement of withdrawn water with an equal volume of pure solvent.

3. *Rate of stirring.* Using a tablet compressed at 10 ton/inch², the effect of stirring rate of the rate of dissolution was determined. The results for k_1 and k_2 are given in Table 1. In both cases the rate constants increased with the rate of stirring.

According to Bircumshaw & Riddiford (1952) a plot of log k *vs.* log (stirring rate) should be linear with a slope of 0·5, if the dissolution process is diffusion controlled. Graphs of this type for both k_1 and k_2 are reasonably linear, considering the large experimental errors involved in measuring small extinctions in the 5 and 10 rev/min experiments, and have a slope of 0·54. 250 rev/min seemed to be a suitable stirring speed for further work, as the rate of solution was fairly high, and the tablet showed no signs of moving on its support, which was a difficulty in some experiments at 600 rev/min.

4. *Compression of tablet.* The mean values of k_1 were 1·07, 1·14 and

TABLE 1. EFFECT OF RATE OF STIRRING ON k_1 AND k_2

Rate of stirring* (rev./min)	$10^8 k_1$†	$10^4 k_2$‡
5	0·11	0·43
10	0·19	0·78
60	0·38	1·5
250*	1·12	4·6
600	1·58	6·5

* From standard runs.
† moles $litre^{-1}min^{-1}cm^{-2}$
‡ $min^{-1}cm^{-2}$

1·12 and of k_2 were 4·4, 4·6, 4·6 for 5, 10 and 15 ton/inch^2 using 300 ml water and a stirring rate of 250 rev/min and thus appear to show little variation when the tablets are compressed at these pressures.

5. *Other experimental conditions and the standard runs.* The following were adopted as standard experimental conditions: 300 ml initial volume of dissolution medium, withdrawal of 5 ml samples, replaced by 5 ml fresh medium; high tripod supporting tablet; rate of stirring 250 rev/min; compaction of tablets at 10 ton/inch^2; weight of tablets between 340 and 350 mg. Measurements on three representative tablets of weight 346·2, 348·0, and 341·8 mg, using a Cambridge Universal Measuring machine, gave diameters enabling the geometrical surface areas of 3·451, 3·450, and 3·463 cm^2 to be calculated. From these figures the apparent density of griseofulvin when compressed at 10 ton/inch^2 is 1·355. The diameter of the tablets will not vary with their weight, but their thicknesses will. Using weights of 340 and 350 mg, the density, and the observed diameter of 1·304 cm, the surface areas are 3·445 and 3·465 cm^2 respectively. Hence the weight of tablets was controlled between 340 and 350 mg, giving a maximum variation in geometrical surface area of about 0·6%. Values of k_1 and k_2 have been corrected to an area of 1 cm^2, using 3·45 cm^2 as the mean area of the tablet. The error introduced by using 3·45 cm^2 instead of the specific tablet area is considered to be small compared with other errors.

The results of five experiments made under the standard conditions detailed above are:

for 10^8k_1: 1·18, 1·21, 1·09, 0·99, 1·23, mean 1·14:

for 10^4k_2: 7·5, 3·8, 7·5, 9·6, 6·7. (From slopes.)

for 10^4k_2: 4·84, 4·79, 4·97, 4·06, 5·03, mean 4·63. (From solubility.)

The experimental data were treated as follows. Observed extinctions were plotted against time, and the best line drawn through the points using a french curve. Values of extinctions and time were read from the smoothed curve and plotted as (extinction)/time against time. The intercept of this graph gives k_1 (Fig. 2), which was converted to absolute units using the E(1%, 1 cm) of griseofulvin and the volume of the solution.

The column giving k_2 evaluated from the slope of the (extinction)/time *vs.* time plots shows much variation, so k_2 was evaluated using k_1 and the solubility data (Elworthy & Lipscomb, 1968b), giving much more reproducible results. The standard deviation of k_1 is 0·091, and that of the

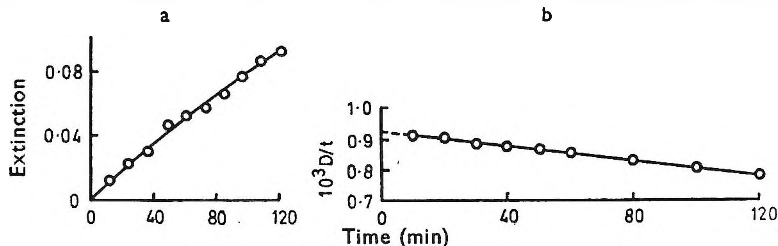

FIG. 2. (a) Extinction (in 1 cm cells) of solutions during dissolution of griseofulvin under standard conditions. (b) Extinction/time against time derived from the smoothed curve in (a).

second k_2 is 0·38 both corresponding to 8% of the mean value. In view of the precautions taken, this reproducibility was somewhat disappointing. The main part of the error probably arises in the measurement of small extinctions; under standard conditions the extinction reaches 0·1 (2 cm cells) in about 60 min. A further source of error may be the fracturing off of very small particles of griseofulvin from the tablet surface. Experiments in which the solution was filtered through 5/3 glass sintered disks, did not give any better reproducibility, so if particles become detached they must be small enough to pass through the filter. Also, we are interested in the value of k_1, which is the initial slope of the concentration-time graph, and which is not easy to estimate, due to the very small extinctions (at 10 min, extinction $\simeq$ 0·03 in 2 cm cells). The advantage of equation 3 is that it enables results taken at longer times to be used in an extrapolation to zero time.

No significant difference in the results was found when a tablet was used in three or four experiments. After this amount of use, the tablet was discarded in case repeated handling had affected the surface area. In all subsequent experiments, in view of the error of a single experiment, three repeat experiments were made, the results graphed on an extinction *vs.* time plot, and the best curve drawn through the points. The quoted results are thus a mean of three experiments.

6. *Effect of temperature.* The results of the determination of k_1 and k_2 at 25°, 35° and 45° were: 10^8 k_1 1·14, 2·29, 5·02 and 10^4 k_2 4·6, 6·5, 8·2 respectively.

Increase of temperature has a much larger effect on k_1 than on k_2, thus the total amount of griseofulvin going into solution in a given time is increased. Plots of log k_1 and k_2 *vs.* 1/T are reasonably linear, and activation energies (Ea) of 14·2 kcal and 5·4 kcal for the zero order and first order reactions respectively were obtained.

Edwards (1951) states that according to the Stokes-Einstein equation. the energy of a diffusion process (E_D) should depend only on the viscosity of the solvent. Over the temperature range 25°–45°, the mean E_D value is 4·4 kcal/mole. Considering the experimental error involved, the value of the activation energy for k_2, is fairly close to E_D, indicating that the control of this rate constant is very largely due to diffusion. The diffusion energy is only about one-third of the activation energy for the k_1 rate constant. As the rate of stirring experiments have shown an apparent diffusion control, it appears that other factors are involved in this dissolution step. The principal factor is probably the energy change in transferring a molecule of griseofulvin from the crystal to the solution.

EFFECT OF NON-IONIC SURFACTANTS AND POLYOXYETHYLENE GLYCOL ON DISSOLUTION RATE

Measurements of k_1 and k_2 were made as a function of concentration for cetomacrogol, HE38, HE60, OE10 and polyoxyethylene glycol-400.

Before discussing in detail the effect of surfactants on the velocity constants, an overall picture of their action in promoting the dissolution of griseofulvin is given. Fig. 3 shows the amount of drug dissolved at 100 min

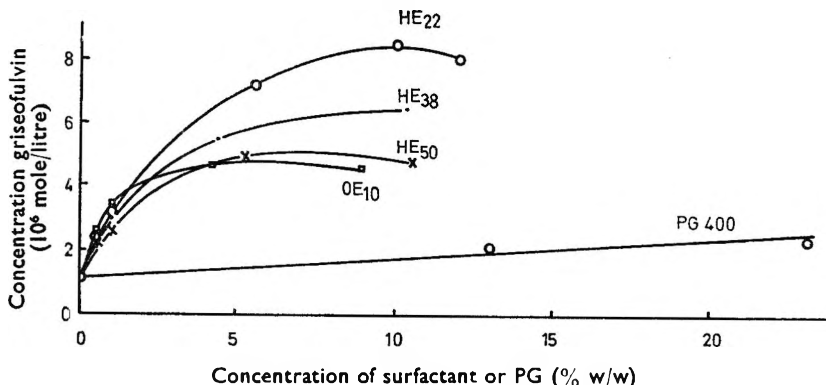

FIG. 3. Amount of griseofulvin dissolved after 100 min in contact with various surfactant and polyoxyethylene glycol solutions.

after the beginning of an experiment, calculated from the k values given in Table 2. There is a rapid increase of the amount dissolved with detergent concentrations, and 1% surfactant solutions give an increase of 2·5–3 times the amount dissolved in pure water. Higher surfactant concentrations give an increase of 5–8 times over the level in water. The polyoxyethylene glycol is not nearly so effective as the surfactants at increasing the dissolution rate, when the compounds are compared on a percentage basis.

All the surfactant concentrations used are well above the CMC, so that both wetting of the griseofulvin surface and adsorption of surfactant at it can be considered constant over the range of measurements made.

The effect of temperature on the dissolution rate was determined in a cetomacrogol solution. $10^8 k_1$ values were 6·90, 10·6, 18·58 and $10^4 k_2$ values were 0·841, 1·15, 1·87 for 25°, 35° and 45° respectively.

TABLE 2. DISSOLUTION OF GRISEOFULVIN IN SURFACTANT OR POLYOXYETHYLENE GLYCOL-400 SOLUTIONS AT 25°

Surfactant	Concentration % w/w	$10^8 k_1$	$10^4 k_2$
Cetomacrogol (HE22)	0·497	2·40	2·29
	0·959	3·16	1·71
	1·000	3·19	1·63
	4·654	6·9	0·84
	5·500	7·2	0·75
	10·00	8·5	0·49
	10·10	8·3	0·47
	12·00	7·7	0·37
HE38..	0·514	2·16	3·07
	1·047	3·22	2·48
	4·337	5·5	1·09
	10·36	6·4	0·55
HE50..	0·494	2·18	3·62
	0·985	2·68	2·36
	5·208	4·96	0·90
	10·53	4·52	0·33
OE10	0·501	2·66	2·08
	0·940	3·54	1·61
	4·148	4·52	0·50
	8·960	4·90	0·24
Polyoxyethylene glycol-400 ..	12·96	2·17	3·16
	23·10	2·38	1·51
	34·85	3·33	0·82
	45·03	3·77	0·40
	54·29	4·93	0·25

Both k_1 and k_2 increase with temperature, as they did in pure water. However, the activation energy for k_1 was 9·2 kcal/mole and for k_2 7·5 kcal/mole in 4·65% cetomacrogol solution, compared with values of 14·2 and 5·4 kcal/mole in pure water. Hence the presence of surfactant leads to a decrease in activation energy for the zero order reaction, and an increase for the first order one. This result will be considered further.

TABLE 3. EFFECT OF STIRRING RATE ON VELOCITY CONSTANTS IN CETOMACROGOL SOLUTIONS AT 25°

	Stirring rate rev/min		
	40	250	600
0·497% *w/w* cetomacrogol			
10^8k_1	0·68	2·40	4·49
10^4k_2	0·65	2·29	4·29
10·10% *w/w* cetomacrogol			
10^8k_1	2·04	8·32	17·5
10^4k_2	0·11	0·47	0·98

The effect of stirring rate on the velocity constants measured in 0·497% and 10·13% cetomacrogol solutions was evaluated (Table 3). Plots of log k_1 or k_2 against log of stirring rate gave a slope of 0·75 in both cases. This value differs from those found in pure water, and may indicate a different mechanism for dissolution in the surfactant solutions.

A further factor likely to influence the rate constants is the viscosity of the solutions. The relative viscosities are given in Fig. 4; the viscosities increase markedly at high concentrations of surfactants. When the rate constants for dissolution in surfactant solutions are plotted against concentration (Figs 5 and 6), at high surfactant concentrations both constants do not change sharply with concentration. This seems likely to be due to the rapidly rising viscosities of the solutions slowing the rate of diffusion of dissolved griseofulvin, and of griseofulvin being transported in a solubilized state. In the hexadecyl series of surfactants HE50 solutions have higher viscosities than HE22 solutions, while k_1 in HE22 is larger than k_1 in HE50 solutions. The viscosity of PG400 solutions increases in a roughly linear manner with concentration, while k_1 increases and k_2

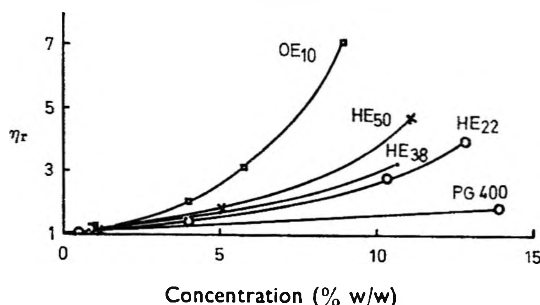

FIG. 4. Relative viscosities (η_r) of surfactant and PG400 solutions. (Experimental points at 0·5 and 1% cannot all be shown).

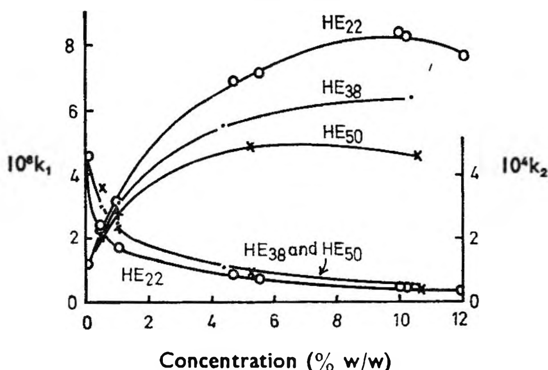

FIG. 5. Variation of k_1 (upper three curves) and k_2 (lower curves) with surfactant concentration for the hexadecyl containing surfactants.

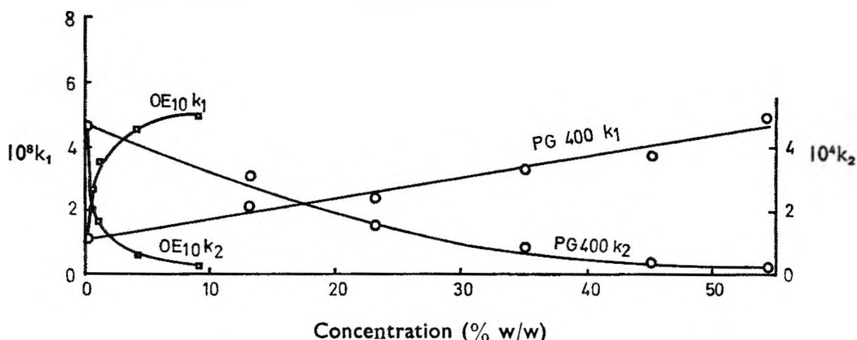

FIG. 6. Variation of k_1 and k_2 with solution concentration of OE10 and PG400.

decreases with concentration in the same way. The surfactants have a much more pronounced effect on the rate constants than the polyoxyethylene glycol.

It is not easy to interpret the rate constants in terms of fundamental quantities, and the interpretation given here is deliberately speculative.

It has been suggested (e.g. Bircumshaw & Riddiford, 1952) that for a dissolution controlled by a transport process, the velocity constant of the Noyes-Whitney equation (our k_2) can be written

$$k_2 = DA/V\delta \qquad (6)$$

where D is the diffusion coefficient of the solute, A the area of the surface in contact with a volume, V, of solution, and δ is the thickness of the diffusion layer. From the Stokes–Einstein equation, the diffusion coefficient will be inversely proportional to the solvent viscosity i.e. to $1/\eta$. There is no theoretical foundation for interpreting the zero order constant, k_1, but it seems reasonable to assume that this is proportional to DA/δ.

Using the solution viscosities (interpolated from graphs of log η_r against concentration), $k_1\eta$ and $k_2\eta$ were calculated. $k_1\eta$ gave a linear plot against

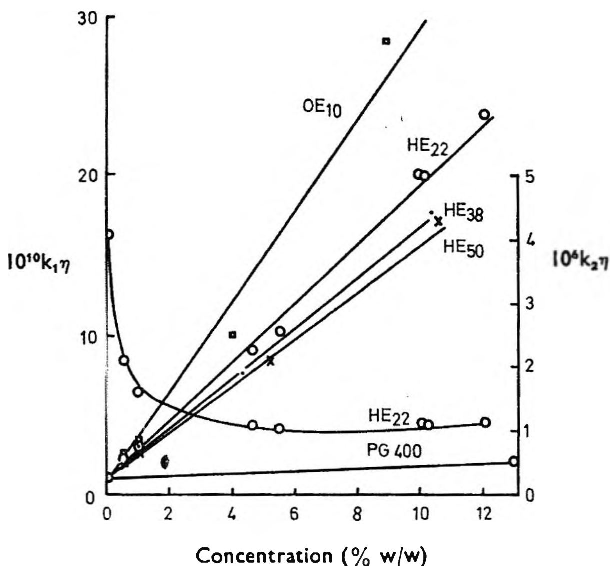

FIG. 7. Product of rate constant and viscosity of solution plotted against concentration. The straight lines refer to the $k_1\eta$ axis, while only one representative curve (for HE22) is shown as a plot of $k_2\eta$.

surfactant concentrations, instead of the type of graph shown in Figs 5 and 6. Also the different surfactants gave lines of different slopes (Fig. 7). Graphs of $k_2\eta$ against concentration are not linear. It seems necessary to account for the term V in equation 6. Although results have been expressed in terms of moles/litre, when we compare k_2 found in water with that in a surfactant solution, the capacities of the two "solvents" for griseofulvin are different. Hence the effective volume, Ve, was evaluated, being the volume of water to dissolve the amount of griseofulvin soluble at a particular surfactant concentration. Graphs of $k_2\eta$ Ve against surfactant concentration are linear, but again the slopes vary for the different compounds used. This treatment of k_2 is of course tentative.

Although there is some evidence from the rate of stirring experiments that diffusion does affect the velocity constant, there appears to be no sharp dividing line between those dissolution processes controlled by diffusion and those whose rate limiting step is a chemical reaction. A number of dissolution reactions seem to depend on both types of processes (see Bircumshaw & Riddiford, 1952), and on the relative magnitude of the energy changes involved. It seems likely that the presence of surfactants or polyoxyethylene glycol facilitates the transfer of a molecule of griseofulvin from the crystal surface into the solution, as the activation energy for this reaction is lower in cetomacrogol solution than in pure water. Also, the lowered energy of this process must be greater than any increase of activation energy due to increased viscosity effects.

In the case of k_2 the activation energy when surfactants are present is increased over that in water, which probably reflects the viscosity increase,

and also the possibility that the energy change involved in redepositing a griseofulvin molecule on a surface with an adsorbed film of detergent present may differ from that involved in removing it from the vicinity of other griseofulvin molecules.

To obtain an idea of the free energy charge on dissolution, ΔG, we can ignore activity coefficients and write

$$\mu \text{ (solid)} = \text{(saturated solution)} = \mu_0 + \text{RT ln m}$$

where m is the molality at saturation, hence

$$\Delta G = \mu_0 - \mu \text{ (solid)} = -\text{RT ln m}.$$

As we are only concerned with comparing the surfactants and polyoxyethylene glycol, molalities are used in place of mole fraction, and ΔG can be calculated from the solubilities already reported (Elworthy & Lipscomb, 1968b). ΔG has the value of −6·3 kcal/mole* in water, −5·4 and −5·1 kcal/mole respectively in 0·5 and 1% cetomacrogol solutions. Increase of cetomacrogol concentration to 4·7% decreases ΔG to −4·2 kcal/mole, while in 12% cetomacrogol solutions, ΔG has fallen to −3·7 kcal/mole. When these figures are compared with the value of k_1 given in Table 2, it seems likely that the rapid initial increase of k_1 with surfactant concentration may be related to the effect of the surfactant on the free energy of dissolution. There is a smaller effect (per unit of surfactant concentration) on ΔG at higher cetomacrogol concentrations, and also the viscosity of the solutions increased. The overall effect is a small variation of k_1 with surfactant concentration above the 4–5% region. It seems likely that the rate constants are related to both the transport properties of the solute, and to the energy changes in removing molecules of griseofulvin from the crystal to the bulk of the solution. A number of factors, like activity coefficients, the assumption that the diffusion coefficient of solute is governed solely by viscosity, and the fact that thickness of the diffusion layer can also be related to the viscosity (Bircumshaw & Riddiford, 1952), have been neglected in the above discussion.

The present results indicate that surfactants can significantly influence the dissolution rate of an insoluble drug.

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* ΔG is negative due to the choice of standard state.

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The optical rotatory dispersion of some *N*-decyl-*N*-*N*-dimethylalanine salts and their critical micelle concentrations

S. BONKOSKI AND J. H. PERRIN

The optically active salts of *N*-decyl-*NN*-dimethylalanine are shown to have anomalous optical rotatory dispersion curves and to not be fully ionized in aqueous solution. The position of the peak in the 225 to 230 mμ region varies with concentration of the surfactant as does the molecular rotation at a given wavelength. The positions of the peaks and the molecular rotations at these low wavelengths are pH sensitive. The change in molecular rotation with concentration enables the critical micelle concentrations of L and D *N*-decyl-*NN*-dimethylalanine hydrobromides to be determined by optical rotatory dispersion measurements, the results agreeing well with the values calculated from the literature. The change in rotation on micelle formation appears to be due to a change in the degree of ionization of these weak acids, a fact which is confirmed by pH measurements on these solutions.

OPTICALLY active betaines have been reported by Beckett, Kirk & Virji (1967) to have optical rotatory dispersion (ORD) curves of the "plain type" over the range they examined; however, plain curves are not to be expected for these amino-acids derivatives (Gaffield, 1964; Jennings, Klyne & Scopes, 1965; Cymerman Craig & Roy, 1964). Some optically active *N*-decyl-*NN*-dimethylalanine hydrobromides and hydrochlorides have been made, and further investigations of their ORD characteristics are reported here.

The critical micelle concentration (CMC) of β-D-octyl glucoside has been measured by ORD (Mukerjee, Perrin & Witzke, unpublished), and the rotation at a given wavelength was found to be enhanced by a small but significant amount by micelle formation. Analysis of the plain dispersion curve of the glucoside by a single-term Drude equation (Drude, 1906), showed that on micelle formation the wavelength of maximum absorption was not significantly changed but that the "rotatory power" term was changed by a mere 8%. The change was thus considered to be due to the high concentration of glucoside head groups found at the micelle surface rather than any significant conformational restraint at this surface.

A CMC of slightly less than 1×10^{-2}M at 22° for *N*-decyl-*NN*-dimethylalanine hydrobromides can be calculated from the data of Beckett & others (1967) and the possibility of detecting this micelle formation by ORD is now reported.

Experimental

Materials. 1 Bromodecane (Eastman Organic Chemicals, Rochester, N.Y.), D-Alanine M.A. (Mann Research Labs., New York, N.Y., L-Alanine (Aldrich Chemical Co., Milwaukee, Wis.), palladium 30% on charcoal (Engelhard Industries, Inc., Newark, N.J.) were used as supplied by the manufacturer. All other chemicals were laboratory reagents. Doubly distilled water was used. Any pH adjustment was made using a Beckman Research pH meter and the pH against concentration plot was obtained from measurements made at 25 ± 0·02°.

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PREPARATION OF THE BETAINES

The optically active alanines were methylated by a slight modification of the method of Bowman & Stroud (1950). Alanine (25 g) was dissolved in water (1 litre) and palladium on charcoal (10 g of 30%) and aqueous formaldehyde (100 ml; 37%) were added. This mixture was hydrogenated under three atmospheres pressure until the theoretical quantity of hydrogen was taken up (2 hr). The mixture was then heated until boiling and filtered, and the catalyst extracted with hot water (200 ml) and the aqueous solution evaporated to dryness at 60°. After addition of the theoretical quantity of hydrochloric acid, the residue was re-evaporated twice from water to remove any paraformaldehyde. The residue was then dissolved in methanol and again evaporated to dryness. This dimethylalanine hydrochloride was then dissolved in more methanol and converted to *N*-decyl-*NN*-dimethylalanine hydrobromide or hydrochloride by the method of Beckett & others (1967). Each crystallization from anhydrous acetone was checked by melting point, optical rotatary dispersion, and nuclear magnetic resonance spectra. At least five recrystallizations were necessary to remove an optically more active impurity (probably unchanged dimethylalanine). The products were dried overnight at 50°. The yields were 1 to 5%.

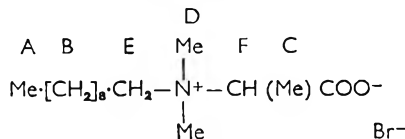
ANALYSIS OF DECYL BETAINES

Compound	Carbon		Hydrogen		Nitrogen		$[\alpha]_D^{22}$	m.p. °C
	Found	Theor.	Found	Theor.	Found	Theor.		
L (bromide)	53·0	53·2	9·6	9·5	4·1	4·1	−10·4	90–91
D (bromide)	53·1	53·2	9·5	9·5	4·1	4·1	10·4	92–93
D (chloride)	61·1	61·3	11·0	11·0	4·8	4·8	12·0	94–95

$[\alpha]_D^{22}$ were measured using 1% aqueous solutions.

NMR SPECTRA

Nuclear magnetic resonance spectra were obtained using a Varian Model A60A. A typical spectrum gave the following values:



(*N*-Decyl-*NN*-dimethylalanine hydrobromide) A, τ 9·14; B, τ 8·70; C, τ 8·44; D, τ 6·88, 6·84; E τ~6·5; F, τ 5·88; DOH, τ 5·42. A small trace of unreacted dimethylalanine was easily detectable on nmr integration. The D- and L-forms gave identical spectra.

MEASUREMENT OF OPTICAL ROTATORY DISPERSION

A Cary Model 60 spectropolarimeter (Applied Physics Corporation, Monrovia, California) was used. The measurements were made in either 2 or 5 cm cells taped to the cell carriage to aid reproducibility.

The concentrations for preliminary investigations were expressed as weight in volume and the scans made until the limits of the system and the instrument were reached. For the CMC determination, the concentrations were expressed as weight in weight and all solutions were scanned from 250 mμ until the peak in the region of 225–232 mμ was clearly defined. In all instances the opposite enantiomorph gave mirror image curves.

Results and discussion

(*a*) *General.* Fig. 1 shows scans of concentrations of *N*-decyl-*NN*-dimethyl-L-alanine hydrobromide above and below the CMC. This was estimated to be approximately $1{\cdot}0 \times 10^{-2}$ M from the data of Beckett & others (1967). Anomalous curves were obtained, the peak probably being the start of a Cotton effect around the carboxyl chromophore which has an absorption band in the region of 215 to 210 mμ (Schellman, 1960). The $1{\cdot}42 \times 10^{-2}$M solution had a peak at 229·5 mμ and a specific rotation, [α], of 256·0 at this peak, whereas the $2{\cdot}85 \times 10^{-3}$M solution had a peak at 228 mμ and a [α] of 283·4. The full significance of the peak shift and change in specific rotation on micelle formation will be discussed below. Fig. 2A shows a more detailed scan in the region of the rotational peak. At higher pH values the peak was shifted down to 226·5 mμ and at the acid pH it was shifted to 230·5 mμ, all the changes being reversible. These shifts suggest that there is a change in molecular species in moving the aqueous solution (pH 2·03) to more acid or more alkaline conditions. The nitrogen of the betaine is quaternary and so is always positively charged, leaving the betaine hydrobromide to behave as a monobasic acid. Fig. 2A suggests the acid to be only partially ionized; this is contrary to the finding of Beckett & others (1967). Similar observations were made with solutions of concentrations below the CMC and pH measurements on these solutions suggested a pK_a in the region of

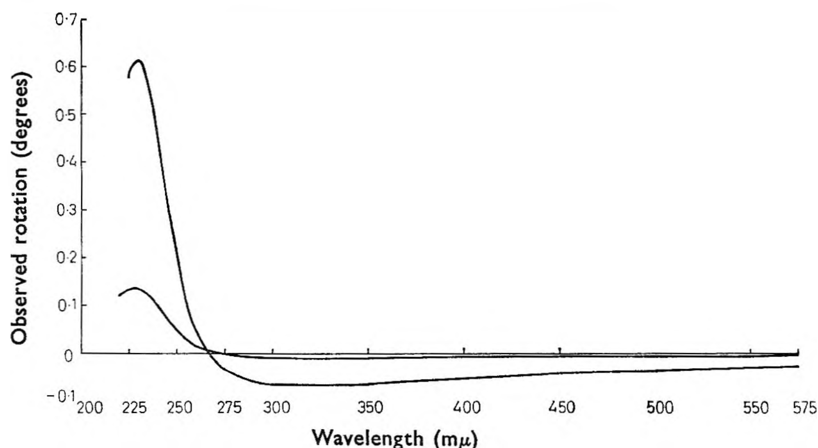


FIG. 1. ORD scan of L-decyl betaine hydrobromide. Upper curve concentration is $1{\cdot}42 \times 10^{-2}$ M and the lower concentration is $2{\cdot}85 \times 10^{-3}$ M. A 5 cm cell was used.

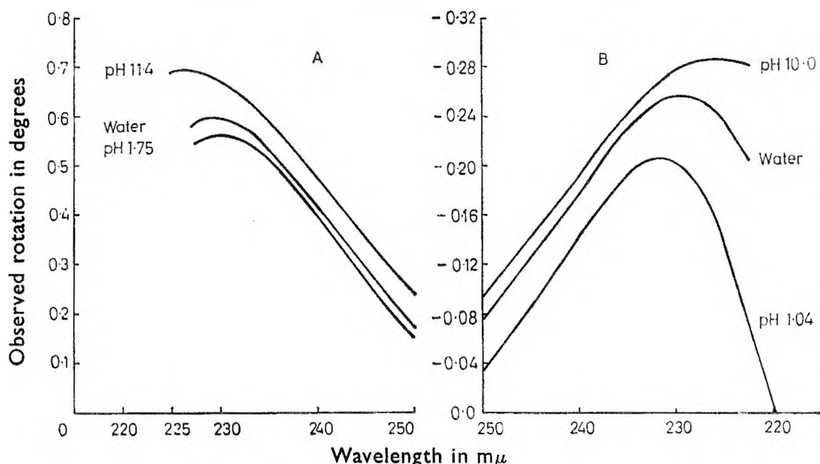


FIG. 2. A. Effect of pH on ORD curve of $1{\cdot}38 \times 10^{-2}$ M solution of L-decyl betaine hydrobromide. Measurements were made in a 5 cm cell. B. Effect of pH on ORD curve of $1{\cdot}65 \times 10^{-2}$ M solution of D-decyl betaine hydrochloride. Measurements were made in a 2 cm cell.

2·1 for the acid. When the betaine was in the fully ionized form the rotation was enhanced; this is not to be expected since the $-COO^-$ substituent has a plane of symmetry and decreasing the symmetry of acids by forming the symmetrical $-COO^-$ group decreases the rotation of amino-acids (Schellman, 1960). Fig. 2B shows the scan for *N*-decyl-*NN*-dimethyl-D-alanine hydrochloride, the replacement of the bromide by the chloride allowed scanning to lower wavelengths, but absorption prevented scanning below 205 mμ even with very dilute solutions, and so the second extremity of a Cotton effect was never obtained. The effects of pH change were similar to that observed for the bromide and a pK_a in the region of 2·2 was estimated from pH measurements on dilute solutions. The zero crossover of the pH 1·04 solution is at 219 mμ, in the region expected for a compound having a Cotton effect due to the absorption of the carboxyl group. The steeply ascending positive tail of the ORD curve at wavelengths between 225 and 200 mμ is characteristic of amino-acids having the absolute configuration of the D-series (Cymerman Craig & Roy, 1965).

(*b*) *Critical micelle concentration determination.* The full ORD scan in Fig. 1 suggested a change in rotation on micelle formation, and so the solutions were scanned from 250 mμ down to beyond the peak. A representative sample of the curves for various betaine concentrations is shown in Fig. 3. The CMC was approximately 0·325%, and it can be seen that the peaks at higher concentrations were in the region of 230–230·5 mμ whereas at concentrations below the CMC they were shifted to 228–229 mμ. The specific rotations [α] calculated at any wavelength were lower for concentrations above than for concentrations below the CMC (Fig. 4) where observed rotations are plotted against concentration at a fixed wavelength of 232·5 mμ). The line changes to a smaller slope after the

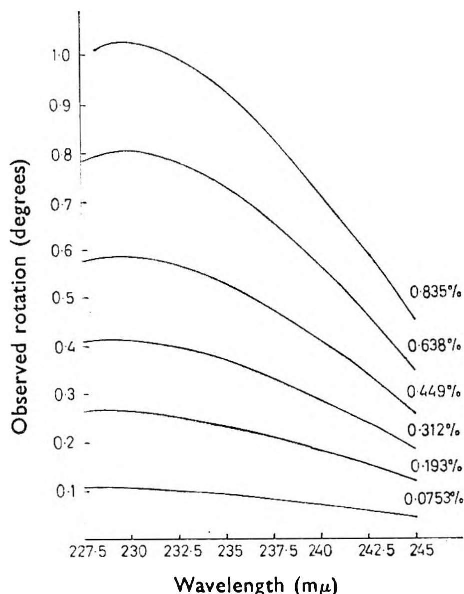


FIG. 3. Effect of concentration on ORD curves of the L-enantiomorph.

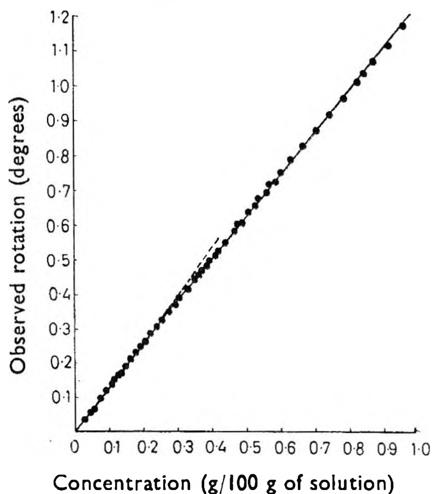


FIG. 4. Plot of observed rotation at 232·5 mμ against concentration for the L-enantiomorph.

CMC (0·325%) and the specific rotation calculated from the slope changes from a value of 259·6 below to one of 232·0 above CMC. A similar plot for the D-enantiomorph gives [α] values of −262·0 and −232·0 respectively and the same CMC. To emphasize the change in rotation on micelle formation, a deviation plot is shown in Fig. 5. The slope of the line below the CMC was calculated and the theoretical rotation for any concentration = slope × concentration, and the deviations observed from

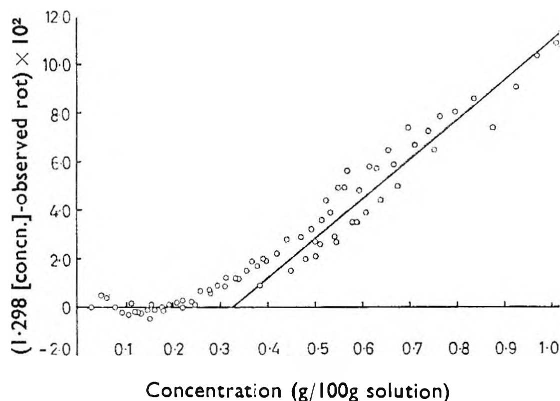


FIG. 5. Deviation plot showing the CMC for the L-compound.

this theoretical rotation are plotted against concentration in the diagram. The figure clearly shows a break in the region of the CMC and contains points from two different preparations of the betaine. The curvature near the CMC which was also observed with the octyl glucoside lends support to arguments against the phase separation model for micellar systems (Mukerjee, 1967).

The decrease in rotation and the shift of the peak to higher wavelength on micelle formation when compared with the shifts due to pH changes, suggest that the rotational changes are due to the change in degree of ionization of the betaine on micelle formation. The betaines are apparently less ionized in the micellar form and this change masks any head group concentration effects that may be present. That the change in rotation on micelle formation is due to a change in the apparent dissociation constant of the weak acid is confirmed by the plot of pH against log concentration shown in Fig. 6. A break occurs at the CMC and slope is diminished on micelle formation confirming that the betaine is less ionized when in the micellar form.

These surfactants probably form micelles with the polar head groups containing both the positive and negative charges exposed to water and the hydrocarbon chains forming an almost spheroidal core. The primary driving force for micelle formation is the tendency of the hydrocarbon portion of the monomer to associate with itself rather than to remain in close proximity to water. This "hydrophobic" bonding must overcome the repulsion forces in the ionic head groups of the ionic detergents. The surface of the betaine must always have a net positive charge due to the quaternary nitrogen and the fact that not all the carboxylic acid groups are ionized; however, the outer periphery of the micelle is negatively charged, being well orientated for capture of the hydrogen ions. This effect may be minimized by orientation of the negatively charged carboxyl group towards the positively charged nitrogen closer to the hydrocarbon core. If two adjacent carboxyl groups on the micelle surface are charged, then they will be repelled as far away from each other as possible, and

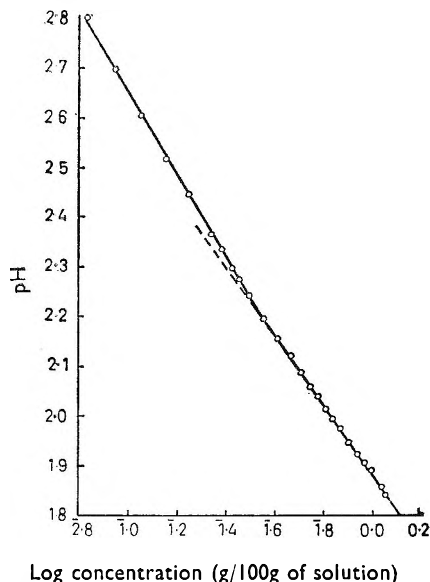


FIG. 6. Plot of pH against log concentration for the L-salt showing a break at the CMC.

micelle formation, or micelle stability, will be enhanced if one of the carboxyl groups captures a hydrogen atom and becomes uncharged, giving a possible reason for change in degree of ionization of the betaine on micelle formation. The lack of electrostatic theory for such systems, and the lack of further knowledge of the properties of the betaine micelles as well as the uncertainties of the glass electrode in such systems, makes further interpretation of the pH-log concentration plot impossible at this stage.

The authors would like to thank Mr. Robert Sandmann for obtaining the NMR spectra and Dr. Philip Hart for many helpful discussions.

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Aspirin and mucus

SIR,—Bleeding from the stomach may occur in about half the general population who take aspirin (Smith & Smith, 1966). The site of this untoward action of the drug has not been established, although several mechanisms have been suggested (Roth, 1963). One of these is that the drug diminishes the protective mucus barrier of the stomach, thus allowing abrasive food particles and the proteolytic enzymes and acid of the gastric juice to come into direct contact with the gastric mucosa. Erosions resulting in haemorrhage would then ensue. Aspirin could interfere with gastric mucus in one or more of several ways. The rate of secretion of mucus by the stomach may be affected and it has been reported (Menguy & Masters, 1965) that the administration of aspirin to the intact rat and to the dog with a denervated antral pouch resulted in a lowered volume of mucus. A decrease in the polysaccharide, particularly the sialic acid, content of the mucoproteins, was also observed, suggesting that aspirin inhibited the *in vivo* biosynthesis of these substances. Analogous experiments with an epithelial glycoprotein obtained from sheep colonic mucosa have shown that salicylic acid inhibits the *in vitro* biosynthesis of the protein (Kent & Allen, 1968). A further possible area of interaction is between aspirin and the formed mucoproteins. The topical application of the drug to the gastric mucosa of the cat has been stated to cause chemical denaturation of the mucus (Roth & Valdes-Dapena, 1963).

During the course of some experiments concerned with the incorporation of radio-active amino-acids into the proteins of gastric mucosa, we observed that the oral administration of aspirin caused an apparent aggregation and subsequent sloughing of gastric mucus in the rat and the rabbit. For this reason the effects of aspirin and salicylate on mucus were studied in mucoproteins separated by ultracentrifugation from pig mucus.

Mucin extracts were prepared by lightly scraping mucus with a glass microscope slide from the stomachs of freshly killed pigs, followed by gentle homogenization of the mucus in approximately four times by volume of 0·9% NaCl (pH 4·8) using an all-glass mortar and pestle. All operations were at 4°. The mixture was centrifuged at 3000 *g* for 10 min, the supernatant removed and concentrated twentyfold by ultrafiltration for 12 hr and the centrifugation repeated. The extracts were then dialysed for 10 hours against 0·15 M NaCl dissolved in either 0·01 M sodium phosphate buffer, pH 7·3, or 0·01 M sodium acetate buffer, pH 3·6. Portions (0·7 ml) of the dialysed extracts were mixed with 0·3 ml of the appropriate buffer-salt solution (control samples) or the buffer-salt solution to which either aspirin (acetylsalicylic acid) or sodium salicylate had been added in amounts to give final concentrations in the reaction mixtures of 50 mM. This concentration was chosen to approximate that expected to occur if 10 grains (equivalent to 650 mg) of aspirin was dissolved in 50 ml of water and mixed with 25 ml of human residual gastric juice. The sodium salicylate was easily soluble but the acetylsalicylic acid remained as a fine suspension in the reaction mixtures. In the salicylate-treated samples at pH 3·6 a large quantity of precipitate was observed. Sedimentation velocity experiments were made on these samples in single sector cells at 60,000 rev/min (250,000 *g*) and at 16·7 ± 0·1° using an Analytical Ultracentrifuge (Measuring and Scientific Equipment Ltd., London) fitted with a Schlieren optical system.

The results (Table 1 and Fig. 1) show that in the control samples, two components with approximate sedimentation constants of 10S and 4S were present. Salicylate at pH 7·3 caused no change but salicylate and acetylsalicylate at pH 3·6 caused a marked decrease in the 4S component and a slight increase in the 10S fraction.

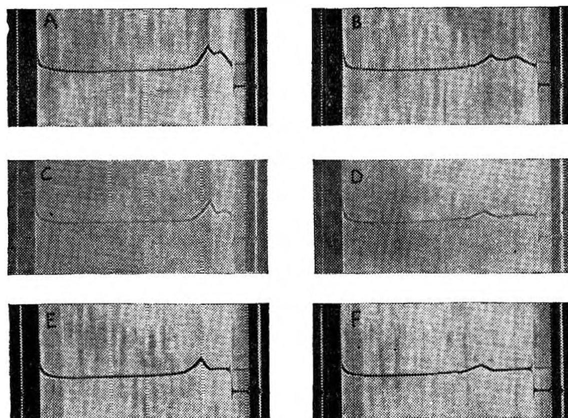

FIG. 1. Sedimentation patterns obtained of pig mucus pH 3·6 at constant speed of 60,000 rev/min. A, control at 750 sec; B, control at 1440 sec; C, with acetylsalicylate at 750 sec; D, with acetylsalicylate at 1440 sec; E, with salicylate at 750 sec; F, with salicylate at 1440 sec. Temperature 16·7 ± 0·1°. Phase angle 20° (M.S.E. Schlieren system). Protein concentration 10–12 mg/ml.

TABLE 1. SEDIMENTATION CONSTANTS OF PIG MUCUS FRACTIONS

pH of buffer-salt mixture	Treatment	Sedimentation constants ($S_{20w} \times 10^{13}$)	
		Slow component	Fast component
7·3	Control	3·59	Not determined
	Sodium salicylate	3·82	10·5
3·6	Control	4·48	9·62
	Sodium salicylate	Precipitated by salicylate	9·32
	Control	4·48	9·62
	Aspirin	Precipitated by acetylsalicylate	9·87

The sedimentation coefficients were calculated from the linear graphs of ln(x), where x is the distance between axis of rotation and the Schlieren peak plotted against time at constant speed. The effects of the salicylates on the viscosity of the solvents were measured and used in correcting the results to water values (S_{20w}) at 20°.

These data show that salicylate and acetylsalicylate change the sedimentation patterns of pig mucus preparations *in vitro* at pH 3·6. The principal effect is an aggregation of the 4S component to insoluble molecular species. If this effect occurs in man after the oral administration of aspirin, then the altered mucus may lack its normal protective properties. The cells of the gastric mucosa would then be directly vulnerable to toxic effects of the drug itself, as well as to damage caused by normal constituents of the gastric contents. Thus a physical interaction of aspirin and gastric mucus may contribute, at least in part, to the mechanism of gastrointestinal bleeding. Salicylate has no effect on the components of pig mucus at pH 7·3, and it may be relevant that either the incorporation of an excess of antacid, in the region of 20 m-equiv. per aspirin tablet (Wood, Harvey-Smith & Dixon, 1962) or the continuous administration of

calcium carbonate or aluminium hydroxide gel by mouth (Matsumoto & Grossman, 1960) reduces the gastrointestinal blood loss caused by oral aspirin in man.

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Distribution and metabolism of dopamine in guinea-pigs

Sir,—While the tissue distribution and metabolism of noradrenaline after intravenous injection has been extensively investigated, relatively little information is available concerning the fate of its precursor dopamine shortly after injection. While investigating the possible formation of vasoactive metabolites from [2-^{14}C]dopamine in guinea-pigs, we observed that the kidneys contained a proportionately larger amount of dopamine (μg/g) than all other tissues investigated in the 5 min after its intravenous administration. In those tissues examined, more than 50% of all the dopamine was metabolized to acidic metabolites within 1·5–2 min after its injection.

Guinea-pigs anaesthetized with urethane were killed either 1·5–2 min (maximum depressor effect) or 5 min (response completed) after the intravenous injection of 250 μg/kg (5 or 10 μc) of [2-^{14}C]dopamine (Halushka & Hoffmann, 1968). Tissue extracts of samples of the heart, liver, lungs, kidneys, spleen and plasma were chromatographed on thin-layer plates for separation of the radioactive components. The procedure involved an acidified acetone precipitation followed by a butanol–heptane extraction, drying of the final acid extract and the reconstitution in 250 μl of 0·01 N HCl–acetone (1:5). Fifty μl of the reconstituted solution was spotted onto either phosphate buffered silica gel or cellulose plates and developed with either water-saturated n-butanol–glacial acetic acid (6:1) or butanol–formic acid–water (15:3:2) (Holtz, Stock & Westerman, 1963).

At death the kidneys contained the highest concentration (μg/g wet weight) of radioactivity without regard to chemical species (dopamine equivalents) (Table 1). At the 1·5–2 min time interval this value was about three times greater than that in the tissue exhibiting the next highest concentration (lungs). The third highest concentration was in the liver, followed by the heart and then the plasma. In the animals killed at 5 min, the kidneys contained 6 times as much radioactivity as the lungs and the total radioactivity in the lungs and liver

TABLE 1. THE DISTRIBUTION AND METABOLISM OF [2-^{14}C]DOPAMINE INJECTED INTRAVENOUSLY IN GUINEA-PIGS

Tissue	Dopamine* equivalents	Dopamine	HVA + DPA	HVA
1·5–2 min post injection				
Kidneys	6·21 ± 0·44	†1·50 ± 0·30 (25%)	†3·53 ± 0·69 (69%)	‡0·25 ± 0·05 (4%)
Lungs	2·12 ± 0·45	0·31 ± 0·07 (15%)	1·48 ± 0·33 (78%)	0·18 ± 0·10 (10%)
Liver	1·30 ± 0·34	0·09 ± 0·01 (9%)	0·71 ± 0·22 (76%)	0·06 ± 0·01 (7%)
Spleen¶	1·25¶	0·30 (24%)¶	0·48 (71%)¶	0·15 (13%)¶
Heart	1·19 ± 0·14	0·51 ± 0·06 (44%)	0·54 ± 0·09 (50%)	0·05 ± 0·03 (5%)
Plasma	0·27 ± 0·04	0·06 ± 0·01 (19%)	0·21 ± 0·03 (55%)	0·08 ± 0·08 (5%)
5 min post injection				
Kidneys	6·88 ± 1·50	‡0·78 ± 0·49 (10%)	‡5·2 ± 1·0 (87%)	*f*0·37 (6%)
Lungs	1·16 ± 0·19	0·05 ± 0·01 (5%)	0·89 ± 0·19 (89%)	0·13 (17%)
Liver	0·68 ± 0·09	0·11 ± 0·02 (18%)	0·44 ± 0·09 (72%)	0·08 (11%)
Spleen	0·54*f*	0·14 (26%)*f*	0·33 (69%)*f*	0·08 (16%)
Heart	1·18 ± 0·17	0·32 ± 0·09 (27%)	0·67 ± 0·1 (64%)	0·10 (10%)
Plasma	0·34 ± 0·07	0·03 ± 0·01 (14%)	0·24 ± 0·06 (79%)	<0·002 0%

The results are expressed in μg/g ± s.e. The numbers in ( ) represent the % of the total radioactivity (d/min) in that tissue.

* Represents the total amount of radioactivity found in the tissue at the time of death, expressed as μg/g dopamine.

† Determined in 4 guinea-pigs. ‡ Determined in 3 guinea-pigs. *f* Determined in 2 guinea-pigs.

¶ Determined in 1 guinea-pig.

HVA = homovanillic acid.

DPA = dihydroxyphenylacetic acid.

had decreased to approximately one-half that at the 1·5–2 min interval, while that in the heart and plasma remained the same. The total amount of radioactivity in the kidney increased during the interval. Despite the rapid degradation of dopamine to acidic metabolites during the first 2 min, the kidney showed the highest concentration of unchanged dopamine (1·50 μg/g) which was 3 times greater than that of the heart (0·51 μg/g). At the 5 min interval the amount of unchanged dopamine (0·78 μg/g) in the kidney was 2½ times greater than that remaining in the heart (0·32 μg/g).

Dopamine (250 μg/kg) regularly elicited a vasodepressor response of approximately 10 mm Hg in the guinea-pig. Eble (1964) has shown in dogs that the vasodepressor response to dopamine is accompanied by dilatation of the renal vascular bed with increased renal blood flow. This increased flow could explain the higher concentrations of dopamine found in the kidneys. However, its preferential uptake by the kidneys was also seen in two experiments in which 25 mg/kg, given intravenously, produced only a pressor response. At the 1·5 to 2 min time interval, after this dose, the kidneys contained at least twice as much radioactivity as any other tissue and three times as much at the 5 min interval. Again, in dogs, McNay, McDonald & Goldberg (1965), have shown that such pressor responses to high doses of dopamine were accompanied by decreased renal blood flow. Thus, the higher concentrations of dopamine in the kidneys after a vasodepressor dose (250 μg/kg) do not seem to be solely the result of an increase in the proportion of the cardiac output delivered to the kidneys as a result of the pharmacological response, since this preferential distribution was also seen with doses producing renal vasoconstriction. As a consequence of these findings, the possibility of some type of enhanced uptake of dopamine by the kidney, not dependent solely on dose or vascular response, must be considered.

It is of interest to compare the distribution of dopamine with that of noradrenaline, administered intravenously. No comparable study of noradrenaline distribution is available in guinea-pigs, however, Whitby, Axelrod & Weil-Malherbe (1961) injected [^{3}H]noradrenaline intravenously into cats and killed them 2 min later. They found the greatest amount of radioactivity regardless of the chemical nature (noradrenaline equivalents), to be in the spleen followed by the lungs, heart, adrenal glands, kidneys, liver, small intestines and plasma.

To make a comparison with their figures, the uptake of dopamine by the tissues has been ranked in the order of total radioactivity/g (dopamine equivalents) at 2 min. We found the total radioactivity to be highest in the kidney followed by the lungs, the liver, heart and spleen (1 value) and the plasma. Thus, the tissue distribution of intravenously administered dopamine differs from that of noradrenaline. The spleen, an organ of high adrenergic activity, exhibited the largest uptake of noradrenaline, while our results show that the kidney contained the highest amount of dopamine (μg/g wet weight). The lungs, heart and plasma occupied the same rank order in the distribution of both dopamine and noradrenaline. If the uptake of dopamine into various tissues was related solely to the biosynthesis of noradrenaline, then a similar rank ordering of tissue uptake should have been observed for both amines. However, the kidney accumulated more dopamine than the heart or spleen, despite its failure to take up comparable amounts of noradrenaline. This indicates that the high renal uptake of dopamine is not explicable just on the basis that it serves as a precursor of noradrenaline in this organ.

The predominant metabolite of dopamine injected intravenously was dihydroxyphenylacetic acid (DPA) with small amounts of homovanillic acid (HVA) also being found. The deaminated products at the 1·5–2 min time period represented at least 50% of the total radioactivity in the heart and reached a maximum of 78% in the lungs. At 5 min, the deaminated products represented 64% of the total radioactivity in the heart, 89% in the lungs and 87% in the kidneys, the total amount (μg/g) increasing 1·5 times in the kidneys, only slightly in the heart, and decreasing in the lungs and liver while remaining the same in the plasma. This increase in the renal content of deaminated metabolites most likely represents uptake of DPA from the blood by the kidneys reflecting in part an excretory function (Werdinus, 1967).

Only 1 to 5% of the total radioactivity in all the tissues was found to be 3-methoxydopamine. Crout (1964) determined that [^{3}H]normetanephrine represented but 1 to 3% of the total radioactivity in the heart and plasma 1 min to 4 hr after intravenous injection of [^{3}H]noradrenaline in guinea-pigs. Together with our results, it is apparent that direct *O*-methylation of catecholamines does not play a significant role in their early metabolism in this species, compared to cats and mice in which the predominant metabolite of noradrenaline is normetanephrine (Whitby & others, 1961). In addition, dopamine is much more rapidly metabolized by monoamine oxidase than is noradrenaline in guinea-pigs. One min after completion of the injection of [^{3}H]noradrenaline, depending on the dose, Crout (1964) found that from 69–93% of the radioactivity in the plasma represented the parent amine. On the contrary, we found that at the same time only 19% of the plasma radioactivity was unchanged dopamine.

Only in the heart was the formation of noradrenaline from dopamine consistently noted, being approximately 5% of the total tissue radioactivity at 5 min.

The metabolism of dopamine has also been studied in rats and mice. Twenty-four hr after the intraperitoneal administration of 300 μg of [^{14}C]dopamine to rats, 39% of the dose appeared in the urine as homovanillic acid 3·4% as dihydroxyphenylacetic acid 6·5% as 3-methoxydopamine and 6·1% was unchanged dopamine (Williams, Babuscio & Watson, 1960). Symchowicz & Korduba (1967) found that the metabolism of dopamine administered intraperitoneally was much slower in the spleen than in the heart of the mouse. It is difficult to compare the distribution and metabolism of dopamine in these investigations with the present results because the time intervals, the organs studied, the doses of dopamine and routes of administration were all different. However, it is apparent that there is a species difference in the metabolism of dopamine as well as differences in the rate of metabolism of dopamine by different organs in the same species.

It has been suggested that dopamine may have a physiological function in addition to being a precursor of noradrenaline (Wurtman, 1965; Hornykiewicz, 1966). It is the only endogenous catecholamine known to dilate the renal vasculature and occurs in concentrations of 0·02–0·04 μg/g in the kidneys of rats, guinea-pigs, rabbits, dogs and cats (Anton & Sayre, 1964) and 0·1 μg/g in dogs (Wegman, 1963). No function has been ascribed to the endogenous dopamine in the kidney. However, consideration of the fact that exogenous dopamine causes an increase in renal blood flow and glomerular filtration rate (McDonald, Goldberg & others, 1964), a sodium diuresis (Goldberg, McDonald & Zimmerman, 1963), as well as the uptake of large amounts of dopamine into the kidney with rapid metabolic degradation, is consistent with the idea that dopamine could mediate some physiological function in the kidney.

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Adrenergic effector mechanisms in the stomach of the sheep

SIR,—Miert & Huisman (1968) reported evidence for the presence of α-stimulatory and β-inhibitory receptors in the wall of the rumen of the sheep.

We, too, find similar adrenergic effector mechanisms in another part of the stomach of the sheep, the reticulo-omasal orifice. Our experiments were made in decerebrate preparations of wholly milk fed lambs (up to 10 kg in weight and 6 weeks of age) and in lambs and normally fed sheep anaesthetized with chloralose (60–70 mg/kg). In all experiments anaesthesia was induced by the inhalation of halothane. Access to the reticulo-omasal orifice was obtained through an incision made in the reticulum after a mid-ventral laparotomy. The activity of the reticulo-omasal orifice was recorded from a water filled balloon retained in it and connected to a transducer (Setekleiv, 1964) to obtain either isotonic or isometric recordings with a Siemens Mingograph jet writing recorder. The results obtained from both records were similar and were confirmed by direct observation of the reactions of the reticulo-omasal orifice in the absence of the balloon. The drugs used were: (−)-adrenaline (Parke Davis), (−)-noradrenaline (Winthrop), isoprenaline (Winthrop), the α-adrenergic blocking agents tolazoline (CIBA) and phentolamine (CIBA) and the β-blocking agent propranolol (I.C.I.). They were injected into a saphenous vein or into the aorta cranial to the origin of the coeliac trunk. Both vagus nerves had been cut in the neck in all the experiments.

After intravenous or intra-aortic injections of adrenaline or noradrenaline (in doses, as the base, of 1–10 μg/kg) there was a closure of the reticulo-omasal orifice. This effect was obtained in all the preparations and proved to be repeatable in all. Recurrent openings and closures of the orifice, such as were detected before the amines were injected, ceased for a period after the contraction. The effect of these amines is thus interpreted as being excitatory in causing a closure of the orifice and inhibitory in producing a reduction in tonic activity. Adrenaline was more potent in causing a closure of the orifice than were equal doses of noradrenaline, but was less potent in producing an inhibition of tonic activity and an opening of the reticulo-omasal orifice than was noradrenaline.

When the α-blocking agents phentolamine and tolazoline (0·2–1·0 mg/kg) had been given intravenously there was no longer a closure of the reticulo-omasal orifice after the injection of adrenaline or noradrenaline; however, the inhibitory effects of these agents remained, and noradrenaline again proved more potent in causing an opening of the orifice.

Isoprenaline (1–10 μg/kg) was found to have a purely inhibitory effect on the reticulo-omasal orifice. Its effects and the inhibitory effects of adrenaline and noradrenaline were abolished by the administration of the β-blocking agent propranolol (0·1–0·3 mg/kg).

Evidence (cited by Miert & Huisman, 1968) for adrenergic excitatory effects on different parts of the ruminant stomach now includes the rumen, omasum and abomasum, and confirms the effect on the reticulum reported by Comline & Titchen (1951), who also obtained effects with sympathetic nerve stimulation.

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Aggressive behaviour provoked by pargyline in rats pretreated with diethyldithiocarbamate

SIR,—We have previously described various forms of aggressive behaviour induced by drugs affecting the brain amines (Randrup & Munkvad, 1966; 1968).

An excitation characterized by fighting, vocalization together with sudden bursts of fast running was reported in experiments with rats treated with monoamine oxidase inhibitors + L-dopa, the physiological precursor of the catecholamines, dopamine and noradrenaline. Biochemically this behaviour was connected with high levels and turnover of dopamine and noradrenaline (Scheel-Krüger & Randrup, 1967).

The present paper deals with an aggressive behaviour induced by the monoamine oxidase inhibitor pargyline in rats pretreated with repeated doses of disodium diethyldithiocarbamate $2H_2O$ (DDC).

DDC inhibits the synthesis of brain noradrenaline by blockade of the enzyme dopamine-β-oxidase (Goldstein & Nakajima, 1966; Carlsson, Lindqvist & others, 1966).

Male Wistar rats weighing 225–275 g were used. Rats (22) were observed after four doses of DDC given subcutaneously; 18 hr after the first dose of 500 mg/kg, the rats received 500, 50 and 500 mg/kg subcutaneously at intervals of 2 hr.

Another 35 rats were given pargyline hydrochloride (150 mg/kg s.c.) 2 hr before the last DDC injection, while 21 rats received pargyline alone.

The brain content of dopamine, noradrenaline and their respective 3-*O*-methylated metabolites, 3-methoxytyramine and normetanephrine, was analysed by a method involving chromatographic separation of the amines followed by a fluorimetric determination (Scheel-Krüger & Randrup, 1967; Jonas & Scheel-Krüger, unpublished).

The rats treated with the four injections of DDC were already sedated (no spontaneous activity in the home cage) before the last dose of DDC. The sedation persisted for at least 6 hr.

A striking contrasting behavioural excitation of aggressive character was provoked by the additional dose of pargyline.

The aggressive behaviour began 4–5½ hr after pargyline and increased in intensity during the following 2–3 hr.

Bursts of spontaneous vocalization were sometimes heard from rats kept in individual cages. Two rats placed in the same cage reared face to face in defence posture striking at each other with their forelegs. Much vocalization was heard in this situation (26/35 rats showed this behaviour). Rats receiving pargyline only as a control did not show these aggressive features. Vocalization and stereotyped sniffing have been observed after monoamine oxidase inhibitors

(nialamide and pargyline) given alone but only after larger or repeated doses (Randrup & Munkvad, 1966).

Biochemical analyses were made $5\frac{1}{2}$ hr after pargyline. Each value (μg/g brain tissue) represents the mean $\pm$ the standard error of the mean of 5 experiments each performed on 3 pooled total brains.

The rats treated with DDC + pargyline show a significant decrease (Student's *t*-test) of the brain noradrenaline (P $<0{\cdot}001$) and normetanephrine (P $<0{\cdot}001$). The values were $0{\cdot}085 \pm 0{\cdot}01$ and $0{\cdot}039 \pm 0{\cdot}002$, respectively, compared with the rats receiving pargyline only: noradrenaline $= 0{\cdot}61 \pm 0{\cdot}04$ and normetanephrine $= 0{\cdot}086 \pm 0{\cdot}007$.

Furthermore, DDC seems to induce a slight increase in the level of dopamine (P $= 0{\cdot}05$) and a significant increase of 3-*O*-methylated dopamine (P $<0{\cdot}001$). The values were $1{\cdot}19 \pm 0{\cdot}05$ and $0{\cdot}41 \pm 0{\cdot}04$, respectively, compared with the pargyline controls: dopamine $= 0{\cdot}97 \pm 0{\cdot}09$ and 3-methoxytyramine $= 0{\cdot}18 \pm 0{\cdot}02$.

For rats receiving DDC only, 4 times, the corresponding values for the brain amines are (3 analyses): noradrenaline $= 0{\cdot}054 \pm 0{\cdot}01$, normetanephrine $<0{\cdot}01$, dopamine $= 0{\cdot}76 \pm 0{:}07$ and 3-methoxytyramine $= 0{\cdot}035 \pm 0{\cdot}003$.

The production of aggressive behaviour by a pharmacological treatment which lowers brain noradrenaline was unexpected since other evidence indicates an association of aggressive behaviour with increased activity of brain noradrenaline (Gunne & Lewander, 1966; Scheel-Krüger & Randrup, 1967; Reis & Fuxe, 1968).

As a likely explanation for the above results the possibility exists that dopamine mimics the action of noradrenaline in central noradrenaline neurons. However, according to Carlsson, Fuxe & Hökfelt (1967), dopamine does not accumulate in central noradrenaline neurons, after treatment with DDC plus the monoamine oxidase inhibitor nialamide.

It is thus possible that features of aggressive behaviour are produced by disturbances of the balance between the aminergic systems in the brain. It is also possible that the aggressive behaviour observed is produced by some still unknown biochemical effect of DDC.

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Failure of morphine dependence in rats to influence brain noradrenaline turnover

SIR,—Since Vogt (1954) showed that morphine depleted the level of noradrenaline in the cat brain there have been many studies in which an attempt has been made to show a relation between morphine dependence and catecholamine metabolism. The effects of single doses or chronic administration of morphine on brain noradrenaline levels are complex and depend on the dose of drug and on the species of animal used.

Chronic administration of morphine to rats usually produces a small increase in brain noradrenaline levels (Freedman, Fram & Giarman, 1961; Maynert & Klingman, 1962; Gunne, 1963; Akera & Brody, 1968), but in the dog there is no increase in brain catecholamine levels (Maynert & Klingman, 1962; Gunne, 1963). When rats are abruptly withdrawn from long-term chronic morphine administration there is a withdrawal syndrome which is not associated with a decrease in brain noradrenaline, but in the dog, which has a more excitatory type of withdrawal syndrome, a large fall in noradrenaline level is seen in the brain after morphine withdrawal.

The significance of the raised brain noradrenaline levels in the rat is not known. This effect of chronically administered morphine is not dose dependent as is the severity of the abstinence syndrome and does not occur with levorphanol or methadone (Akera & Brody, 1968). Maynert & Klingman (1962) and Gunne (1963) found that the injection of a monoamine oxidase inhibitor into control and chronically treated rats produced a greater increase of brain noradrenaline in the morphine-tolerant animals and they suggested that this might indicate an increase in the synthesis of brain noradrenaline.

In the present experiments, noradrenaline turnover in morphine-dependent rats and in animals during drug withdrawal has been estimated from the rate of disappearance of radioactive noradrenaline from the brain (Iversen & Glowinski, 1965).

Sprague-Dawley rats initially weighing between 190–210 g were given two daily injections of morphine hydrochloride at 9.00 and 17.00 hr for 4 weeks. The doses were increased at the end of each week starting at 20 mg/kg and increasing to 50, 150 and finally 250 mg/kg intraperitoneally, control rats were given equivalent volumes of 0·9% saline solution. After four weeks, noradrenaline turnover in half the chronically treated and half the control rats was estimated. The morphine injections in the remaining chronically treated rats were replaced by saline injections. The noradrenaline turnover in these animals and in the remaining controls was estimated 60 hr after the withdrawal of morphine.

The rats were lightly anaesthetized with ether and killed at various times after the intracisternal injection of DL[^{3}H]noradrenaline hydrochloride (specific activity 1·82 c/mmole), 5 μc in 50 μl of Merle's solution. The brains were rapidly removed onto a chilled surface, weighed and homogenized in 14 ml of 0·4 N perchloric acid containing 20% EDTA (1%) at 0–4°, and left to extract for 45 min. After centrifugation, 0·2 ml of the supernatant fluid was transferred into a vial containing 4·0 ml of ethoxyethanol and 10 ml of phosphor (0·01% POPOP and 0·4% DPO in toluene) and the total radioactivity estimated by liquid scintillation counting. The remaining supernatant fluid was assayed for [^{3}H]noradrenaline and [^{3}H]normetanephine after ion-exchange chromatographic separation using Amberlite CG-120, Type 2. ^{3}H-Deaminated metabolites were calculated by difference (Iversen, 1963). Endogenous noradrenaline was estimated in some samples by the fluorometric method of Euler & Lishajko (1961).

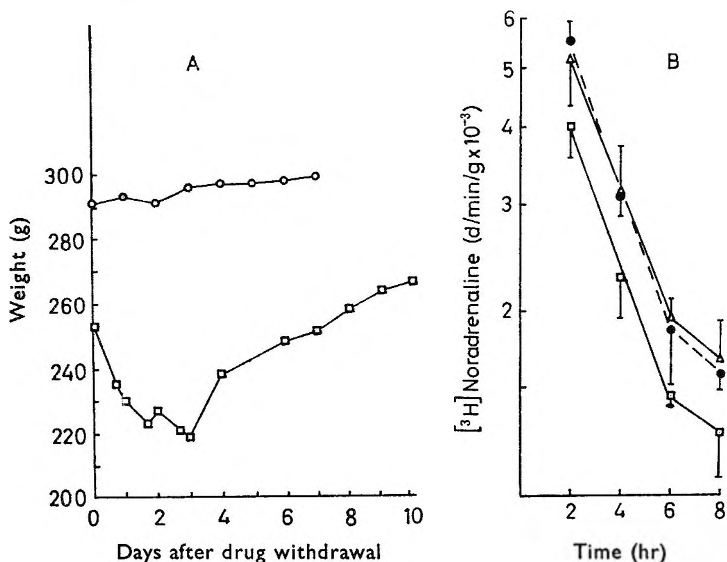

FIG. 1A. The effect of morphine withdrawal upon body weight of rats after chronic administration of an increasing dose of morphine twice a day. At day zero, saline was substituted for morphine (□). Controls (O). Each point is the mean body weight of 18 rats.

B. Disappearance of [^{3}H]noradrenaline from whole brain after intracisternal injection. Controls (●). Morphine dependent (△). Morphine withdrawn (□). Each point is the mean of four or five rats. The vertical lines show the s.e. of the mean.

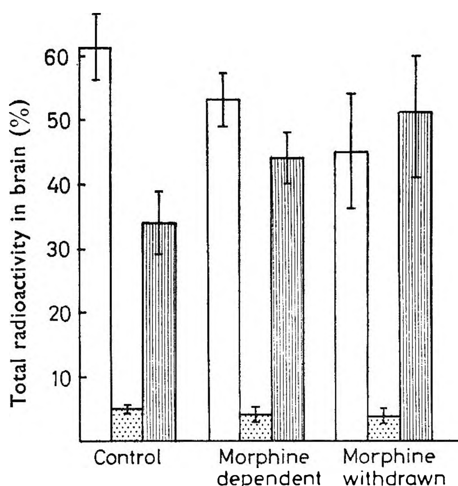

FIG. 2. Patterns of metabolites of [^{3}H]noradrenaline in the whole rat brain 2 hr after intracisternal injection. Open columns, [^{3}H]noradrenaline. Stippled columns, [^{3}H]normetanephrine. Hatched columns, ^{3}H-deaminated metabolites. Each value is expressed as a percentage of the total radioactivity in the brain and is the mean of three to five determinations. Vertical lines are the s.e. of the mean.

During the first week, injections of morphine into rats caused increased activity which lasted for about 1 hr. This effect was not apparent in the last week, by which time the rats had become somewhat vicious and difficult to handle. The withdrawal of morphine produced a pronounced abstinence syndrome in rats which was most obvious after 48 hr. The animals became very irritable, difficult to handle and showed hyperalgesia, piloerection and tremor. Diarrhoea and anorexia occurred with a subsequent mean loss of body weight of 13% after 3 days (Fig. 1A). Similar abstinence syndromes in rats have been described by Hanna (1960), Gunne (1961), and Akera & Brody (1968).

TABLE 1. ENDOGENOUS NORADRENALINE LEVEL OF RAT BRAIN

Group	Concentration μg/g ± s.e.	Number of rats
Control	$0{\cdot}43 \pm 0{\cdot}03$	6
Morphine dependent	$0{\cdot}47 \pm 0{\cdot}06$	7
Withdrawn	$0{\cdot}47 \pm 0{\cdot}03$	5

Noradrenaline turnover in rat brain was not affected by the chronic administration of morphine or by withdrawal of morphine from tolerant rats (Fig. 1B). The half-lives for noradrenaline turnover in control ($t\frac{1}{2} = 2{\cdot}3$ hr), morphine dependent ($t\frac{1}{2} = 2{\cdot}9$ hr) and "withdrawn" rats ($t\frac{1}{2} = 2{\cdot}6$ hr) did not differ significantly and are similar to the results of Glowinski, Kopin & Axelrod (1965) who found the half-life of [^{3}H]noradrenaline in whole rat brain to be about 3 hr. Although the rats were undoubtedly morphine dependent, the endogenous noradrenaline levels of morphine dependent and "withdrawn" rats were not significantly greater than in controls (Table 1).

There was no significant difference between control, morphine dependent or "withdrawn" rats in the pattern of [^{3}H]noradrenaline metabolites (Fig. 2). In all groups about 50% of the radioactivity in the brain after 2 hr was noradrenaline, this proportion increased to about 75% after 8 hr. As in the experiments of Glowinski & others (1965) the major fraction of metabolites was present as ^{3}H-deaminated products.

It appears from the present experiments that noradrenaline turnover in rat brain is not increased by morphine dependence or withdrawal of the drug. The increase in brain noradrenaline level reported by most authors is apparently not essential for the production of drug dependence in rats, for although the animals in the present experiments showed an obvious abstinence syndrome, there was no significant increase in brain catecholamine levels. Morphine dependence in rats produced by dose schedules which increase brain noradrenaline levels, does not increase brain 5-hydroxytryptamine levels (Maynert, Klingman & Kaji, 1962; Gunne, 1963) which presumably means that there is no inhibition of monoamine oxidase. This is supported by the present failure of chronic morphine treatment to significantly alter the pattern of catecholamine metabolism in the rat brain. As it seems unlikely that the increased levels of brain noradrenaline in morphine dependent rats are produced by increased synthesis or by inhibition of monoamine oxidase, it may be that chronic morphine administration affects the intraneural storage mechanisms of catecholamines. This suggestion is supported by the experiments of Freedman & others (1961) who found that morphine temporarily and partially reverses the noradrenaline releasing property of reserpine.

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An apparatus for the study of intestinal transfer of drugs

Sir,—An *in vitro* method for the study of intestinal transfer suitable for use with radioactive labelled compounds is described. An outline of the apparatus is illustrated in Fig. 1. A segment of small intestine is tied to a cannula at each end and suspended vertically in an organ bath with its oral end upwards so that any peristaltic contractions propel fluid in the direction of the circulation. Fluid to be perfused through the intestinal lumen (mucosal fluid) is added to the reservoir and its circulation is started by raising the reservoir initially to allow filling of the connecting tubings, and is maintained by a continuous stream of 5% carbon dioxide in oxygen. This gas mixture also serves to keep the pH constant at 7·4 and supply the oxygen requirements of the mucosal layer of the intestine. The serosal aspect of the intestine is bathed in fluid (serosal fluid) of composition similar to the mucosal fluid except that it contains the substance being studied. The serosal fluid is aerated with the same gas mixture introduced through a thin polythene tubing. The apparatus as illustrated is immersed in a thermostatic tank at 38°.

To mount the intestine, the lower cannula, consisting of a silicone tubing held by a rubber bung, is lightly coated with silicone fluid to facilitate its sliding within the lumen of the bung. The anal end of the intestine is then tied to the silicone tubing and the oral end of the intestine "threaded" into the organ bath. The silicone tubing is pushed into the organ bath until the oral end of the intestine protrudes from the bath and can be tied to the glass cannula. The silicone tubing is then retracted sufficiently to allow the preparation to be totally immersed in fluid. The reservoir can be raised or lowered to provide the desired intraluminal pressure as measured by the difference in level of the mucosal and serosal fluids (see Fig. 1). An intraluminal pressure of 2 cm or more will initiate peristaltic contractions (with guinea-pig small intestine). If peristaltic contractions are not required, the intraluminal pressure should be just high enough to allow a constant circulation of mucosal fluid to be maintained. The rate of the circulation can be adjusted by regulating the flow of the gas mixture and circulation time can be varied from 20 to 60 sec. A thin smear of silicone antifoam prevents frothing at the serosal and mucosal fluid surfaces in the organ bath and reservoir respectively.

This simple method permits the economical use of labelled compounds and also provides for efficient oxygenation which is vital in *in vitro* studies. We

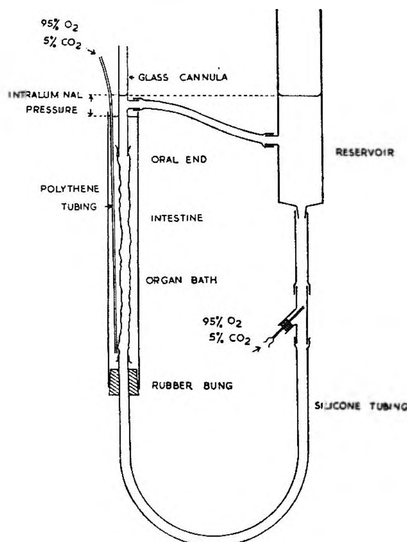

FIG. 1. Apparatus for the study of intestinal transfer by isolated pieces of intestine.

have used this apparatus to study the transfer of ^{14}C-labelled glucose by guinea-pig small intestine. Both the mucosal and serosal fluids have the following composition: (in g/litre) NaCl, 6·92; KCl, 0·35; $CaCl_2$, 0·28; $MgSO_4$, 0·14; KH_2PO_4, 0·16; $NaHCO_3$, 2·0. Labelled glucose containing 1 μc ^{14}C activity and 40 mg glucose were added to 20 ml of mucosal fluid. Glucose 0·01 g/litre was added to the serosal fluid. Glucose was estimated by the photometric method as described by Nelson (1944). ^{14}C activity was assayed in a liquid scintillation counter using a xylene scintillation mixture (Lambie, 1964). Serosal samples of 0·25 ml were used for the chemical and radioactive assay. The results from 6 experiments showed glucose appeared in the serosal fluid at 450 $\pm$39 μg/cm intestine/hr and ^{14}C activity at 15·4 $\pm$2·1 nc/cm intestine/hr. When the total amounts appearing in the serosal fluid are expressed as a percentage of the initial amounts in the mucosal fluid the values are 10·2 $\pm$0·9% for glucose and 11·1 $\pm$1·3% for ^{14}C activity. Application of the *t* test indicates the difference to be not significant. Isolation of the ^{14}C activity in the serosal fluid by chromatographic and autoradiographic techniques indicates that the activity is due to labelled glucose. Since labelled glucose can only have been transferred from the lumen of the intestine it is likely that glucose chemically assayed has the same origin.

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Brain desipramine level in relation to the anti-reserpine activity of imipramine

SIR,—The anti-reserpine activity exerted by imipramine (Garattini, 1959) is believed to be related to the formation in the body of a product of *N*-demethylation known as desipramine (Sulser, Watts & Brodie, 1962). However, Michaelis & Stille (1968) have recently postulated that the formation of desipramine would not be required to mediate the "antidepressant" action of imipramine. These authors have used for their studies the preventive effect exerted by imipramine on the tetrabenazine catalepsy and ptosis.

These findings prompted us to establish if the same conclusion would have been valid by using the test of the increase of body temperature elicited by imipramine in animals previously reserpinized (Garattini & Jori, 1967; Jori & Garattini, 1968).

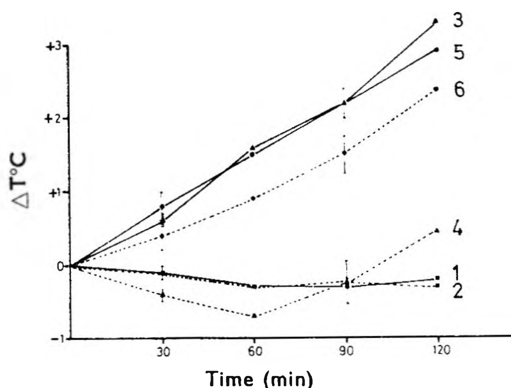

FIG. 1. Hyperthermic effect induced by imipramine and desipramine 16 hr after Reserpine (5 mg/kg i.v.). 1. Saline. 2. SKF 525 A (30 mg/kg, orally). 3. Imipramine (20 mg/kg i.p.). 4. SKF 525 A (30 mg/kg, orally) + imipramine (20 mg/kg i.p.). 5. Desipramine (15 mg/kg i.p.). 6. SKF 525 A (30 mg/kg, orally) + desipramine (15 mg/kg i.p.). The vertical bars represent the standard errors.

TABLE 1. HYPERTHERMIC EFFECT INDUCED BY IMIPRAMINE AND DESIPRAMINE IN RESERPINIZED RATS

No. of rats	Pretreatment (mg/kg/orally)	Treatment (mg/kg i.p.)	T.I. (° C) ± s.e.	Brain concentration (μg ± s.e.) Desipramine	Imipramine
7	Saline	Saline	−1 ± 0·4	—	—
5	SKF 525 A 30	Saline	−1 ± 0·6	—	—
6	Saline	Imipramine 20	+7·8 ± 1·2*	5·1 ± 0·9	9·9 ± 2·1
6	SKF 525 A 30	Imipramine 20	−1 ± 0·8	1·9 ± 0·4**	37·3 ± 2·2**
6	Saline	Desipramine 15	+7·5 ± 1·4*	8·5 ± 1·2	—
6	SKF 525 A 30	Desipramine 15	+5·4 ± 1·4*	8·9 ± 1·5	—

Reserpine (5 mg/kg i.v.) was given 16 hr before the pretreatment.

SKF 525 A was given orally 1 hr before the treatment (saline, imipramine, desipramine).

T.I. = Thermic index was calculated by adding the change of body temperature induced by saline or imipramine or desipramine after 30, 60, 90 and 120 min.

Desipramine and imipramine were determined in the whole brain 2 hr after their administration.

* $P < 0.01$ versus control groups.

** $P < 0.01$ versus saline + imipramine group.

Sprague-Dawley female rats weighing 150 ± 10 g were treated with reserpine (Serpasil CIBA) (5 mg/kg i.v.) 16 hr before the experiment and kept at a room temperature of 20° with a relative humidity of 60%. Fig. 1 shows that imipramine (20 mg/kg i.p.) or desipramine (15 mg/kg i.p.) produce a similar increase of temperature in hypothermic reserpinized rats. However if SKF 525 A, a known inhibitor of microsomal enzymes (Stitzel, Anders & Mannering, 1966), was given at a dose of 30 mg/kg orally 1 hr before imipramine or desipramine, an inhibition of the imipramine but not of the desipramine effect was observed. SKF 525 A itself did not affect the body temperature in reserpinized animals.

Measurements of imipramine and desipramine in brain according to the method of Dingell, Sulser & Gillette (1964) (Table 1) are consistent with the hypothesis that the presence of desipramine in brain would be necessary to obtain the increase of body temperature. These findings are thus at variance with the results of Michaelis & Stille (1968). The reasons for this are unknown, but three major differences in the experimental conditions should be considered.

Firstly, the use of tetrabenazine or reserpine has not been proved to be equivalent, particularly if the schedule of treatment is considered Secondly the end point used to evaluate the effect of imipramine or desipramine is different and may involve different mechanisms. In fact while the changes of body temperature induced by imipramine are currently considered as the result of an adrenergic interaction (Garattini & Jori, 1967; Ross & Renyi, 1967), symptoms such as catalepsy and ptosis seem to be more related to a cholinergic effect (Sulser, Bickel & Brodie, 1964). Imipramine shows a more pronounced anti-acetylcholine effect than desipramine (Lévy & Michel-Ber, 1965). It may be that for certain effects such as reversal of reserpine hypothermia, imipramine must be transformed into desipramine, while for other effects such as the anti-convulsant activity (Garattini, Giachetti & others, 1962) this *N*-demethylation may not be relevant because imipramine is more active than desipramine. Thirdly, the different strain of animals used by Michaelis & Stille may be a significant factor.

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On the mode of action of some immunosuppressant drugs

SIR,—I have investigated the influence of a number of immunosuppressant drugs of the alkylating group, such as nitrogen mustard, triazequone and the anti-metabolites methotrexate, azathioprine and 5-fluorouracil, on transplantation immunity in rabbits induced by allogenic skin grafts. Cytologic changes in the regional lymph node and a possible action on the activity of serum proteolytic enzymes were measured. The cytotoxic effect of the drugs was assessed by their influence on peripheral white blood cell counts.

The experiments were made with 177 young, randomly bred, male rabbits weighing about 2·5 kg each. The drugs were administered chronically in two doses related to the acute LD50 with the exception of methotrexate (Table 1).

In the experiments on enzymatic activity, the doses of the preparations were selected for their power to inhibit transplantation immunity.

Allogenic skin grafts were made on the dorsal surface of the ear of rabbits.

Seven days after making the allogenic skin graft, that is at the peak of blastic transformation and proliferative changes in the regional lymph node (André, Schwartz & Dameshek, 1962), the rabbits were killed by air embolism. A method elaborated by Zaleski, Rymaszewska & others (1964) was used to evaluate the morphologic changes in the regional lymph node, localized in rabbits at the base of the ear. The method consists in determining the percentage of blast cells in smears from a cellular suspension of the lymph node. The percentage of blast cells was calculated in the smears by the method of balanced fields described by Woolf (1950).

Serum proteolytic activity was assayed by the use of casein labelled with ^{131}I by a modification of the method of Henson (1959), giving a labelled substrate of pH 7·7 and content of free iodine less than 0·1%. Blood was drawn from fasting animals from the marginal ear vein, in amounts of 5 ml, into a test tube containing 100 units of heparin. The serum obtained by centrifugation was kept in a refrigerator at $-2°$ for 24 hr. Radioactivity of the serum was measured with a well scintillation counter. Results were expressed in terms of mg of digested casein/ml of serum.

The mean survival time of allogenic skin grafts in the control animals was 10·5 ± 1·7 days. The experiments on the influence of immunosuppressant drugs on transplantation immunity in rabbits demonstrated that azathioprine and nitrogen mustard gave the strongest suppression of the immunologic response to transplantation, markedly prolonging the survival time of the skin

TABLE 1. SCHEME OF DOSAGE OF THE PREPARATIONS AND THEIR INFLUENCE ON THE SURVIVAL OF ALLOGENIC SKIN GRAFTS AND THE BLASTIC REACTION IN THE REGIONAL LYMPH NODE IN RABBITS

Preparation (route of administration)	Doses		Method of administration in relation to the day of transplantation (= day 0)	Prolongation of survival time of skin allografts (days)	Decrease in the number of blast cells (%)
	Part of LD50	mg/kg			
Nitrogen mustard (i.v.)	1/10	0·16	−3, −1, 1	7·0	63
	1/4	0·40		7·0	68
Triaziquone (i.v.)	1/10	0·0055	−4, −3, −1, 1, 3	1·0	72
	1/4	0·014		4·0	89
Methotrexate	—	1·0	−4, −3, −2, −1, 1, 2, 3	1·5	78
		2·5		2·0	88
Azathioprine (oral)	1/20	25·0	−3, −2, −1, 0, 3, 4, 5, 6	13·0	69
	1/10	50·0		14·0	83
5-Fluorouracil (i.v.)	1/10	2·0	−2, −1, 0, 2, 3, 5, 6, 7	1·0	65
	1/4	5·0		2·0	80

grafts in doses which did not elicit any severe toxic symptoms. The effect of triaziquone was much weaker, and only after larger doses. Methotrexate and 5-fluorouracil were practically ineffective (Table 1).

In contrast to these results, the lymphnodal blastic reaction test showed that all the preparations exerted a strong antitransformative effect. However, distinct correlation between the survival time of the allografts and degree of inhibition of the blastic reaction was not observed. Contrary to expectation, the weakly active preparations triaziquone and methotrexate (a preparation devoid of an effect on transplantation immunity) inhibited the blast reaction more strongly than compounds which effectively suppressed the transplantation barrier like azathioprine or nitrogen mustard (Table 1).

The experiments on the influence of the immunosuppressant drugs on the activity of proteolytic enzymes in the serum revealed a distinct correlation of the degree of inhibition of the immunologic transplantation response and the reduction of proteolytic activity. Azathioprine and nitrogen mustard, both highly active in suppressing transplantation immunity in rabbits, specifically or nonspecifically inhibited activity of the serum proteolytic enzymes. On the other hand, triaziquone, methotrexate, and 5-fluorouracil, with weak or no effect on the transplantation reaction, exhibited a weak inhibitory component in relation to the serum proteolytic enzymes (Table 2).

TABLE 2. SERUM PROTEOLITIC ACTIVITY AFTER ALLOGENIC SKIN TRANSPLANTATION AND ADMINISTRATION OF IMMUNOSUPPRESSANT DRUGS

Preparation, mg/kg	Days before/after transplantation (= day 0)	Mean serum proteolitic activity (mg digested casein per ml serum)
Nitrogen mustard, 0·16	−4	0·039 ± 0·022
	9	0·055 ± 0·013
	16	0·034 ± 0·017
Triaziquone, 0·014	−5	0·038 ± 0·013
	8	0·094 ± 0·013
	15	0·119 ± 0·015
Methotrexate, 2·5	−5	0·051 ± 0·018
	7	0·079 ± 0·014
	15	0·101 ± 0·023
Azathioprine, 25·0	−4	0·050 ± 0·018
	8	0·046 ± 0·016
	18	0·083 ± 0·009
	23	0·039 ± 0·025
5-Fluorouracil, 5·0	−3	0·060 ± 0·016
	7	0·044 ± 0·007
	15	0·104 ± 0·011
Control	−1	0·037 ± 0·019
	8	0·083 ± 0·018
	15	0·115 ± 0·022
	25	0·046 ± 0·016

In addition, it was observed that azathioprine and nitrogen mustard also inhibit serum proteolytic activity in non-grafted animals.

Besides suppression of the blastic reaction, inhibition of proteolytic activity is an additional favourable factor in the complex mode of action of immunosuppressant drugs, contributing to pharmacologic transplantation tolerance.

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The continuous recording of arterial blood pressure in the conscious unrestrained rat

SIR,—Although indirect cuff methods are available to monitor blood pressures of large groups of animals, they suffer from the disadvantages that they give an interrupted record and the values obtained are influenced by the fact that the animals are restrained during the measurement. Insertion of cannulae into the aorta or carotid artery is time-consuming and difficult. Fujita & Tedeschi (1968) have recently reported blood pressure measurements using cannulation of the caudal artery with a fine polythene cannula, in rats of more than 300 g. We wish to report a similar method in smaller rats and without surgery.

Male or female Sprague-Dawley rats (Carworth Farm E strain), 80–200 g, were lightly anaesthetized with ether and a 20 s.w.g. needle connected by fine polythene tubing (Portex PP 60) to a Devices CEI transducer was inserted into the ventral caudal artery 2–2·5 cm from the base of the tail and retained in position with a strip of adhesive tape. A lateral tail vein was also cannulated with a 26 s.w.g. needle, inserted into polythene tubing (Portex PP 10), approximately 4–6 cm from the tip of the tail. A rigid plastic tube (internal diameter, 10–12 mm, length 3–4 cm longer than the rat tail) was slid over the two cannulae and anchored by a thread from the adhesive tape holding the arterial cannula (Fig. 1).

The rats may be trained to accept the tube over the tail, the length of the tube preventing the rat from chewing the cannulae, which may then be carried through the lid of a deep cage. Over a period of 5 hr no necrosis of the tail artery occurred and the patency of the cannulae is ensured by using heparinized saline (1000 u/ml) as the hydrostatic link to the transducer.

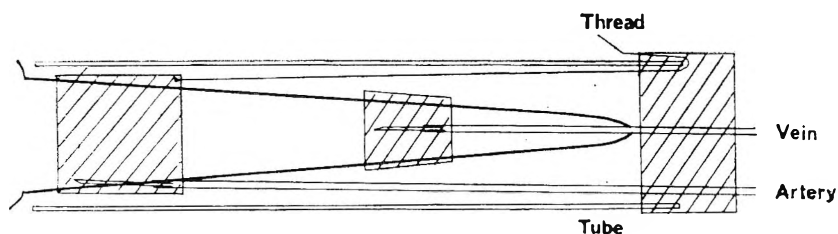

FIG. 1. Diagram showing the location of the cannulae and the protective tube for cannulation of a caudal artery and vein.

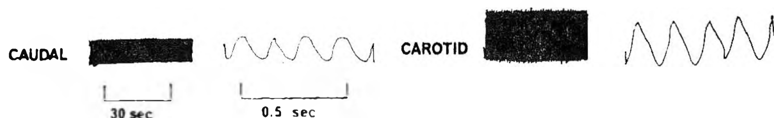

FIG. 2. Caudal and carotid arterial wave forms.

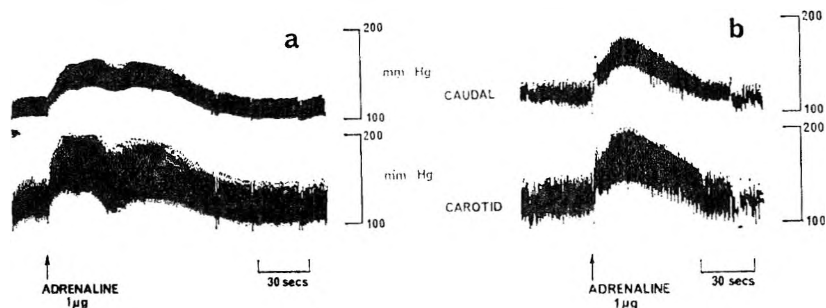

FIG. 3 (a). Simultaneous recordings of caudal and carotid arterial blood pressures showing response to adrenaline (5 µg/kg) in an anaesthetized rat.
(b) As (a), in a conscious, unrestrained rat.

Comparative studies with a caudal cannula and a carotid cannula, under light pentobarbitone anaesthesia showed similar wave forms and mean blood pressure (Fig. 2). Adrenaline (5 µg/kg) gave responses in both carotid and caudal pressure recordings which were similar in onset, magnitude and duration in anaesthetized and conscious animals (Fig. 3). Rats subjected to unilateral nephrectomy and maintained on saline with food *ad lib.* developed hypertension after 4 weeks (100·2 ± 1·4 mm Hg to 130 ± 2·5 mm) and an oral dose of guanethidine (4 mg/kg) reduced the blood pressure over 1–4 hr to 109 ± 5·1 mm.

Control groups of 8 animals were examined at weekly intervals with the caudal technique and there was no significant change in the mean blood pressure over 3 weeks (Week 1, 100·2 ± 1·4 mm Hg; Week 2, 105·5 ± 2·7 mm Hg; Week 3, 99·8 ± 1·6 mm Hg), demonstrating the value of this method in chronic experiments.

The advantages of the continuous recording of blood pressure in conscious, unrestrained animals are numerous, especially in long-term toxicological studies and in trials of potential cardiovascular drugs. In addition, the caudal artery cannula may be used for blood sampling, for instance in glucose tolerance tests.

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Reference

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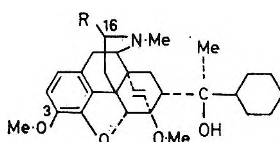
Structural requirements for antitussive activity in some novel 16-substituted derivatives of 6,14-*endo*ethenotetrahydrothebaine

SIR,—Powerful morphine-like analgesics and narcotic antagonists have been found in derivatives of 6,14-*endo*ethenotetrahydrothebaine (Bentley, Boura & others, 1965; Bentley and Hardy, 1967; Blane, Boura & others, 1967). Many of these compounds resemble other narcotics by causing depression of medullary centres mediating the cough reflex, and a search was therefore made within this series for an antitussive agent in which some dissociation of other morphine-like properties was apparent. The work led to the finding that substitution in the 16-position with an alkyl group resulted in the production of compounds possessing powerful antitussive properties, but having less prominent analgesic and other morphine-like actions than the unsubstituted compound (I; R=H) which is a highly potent analgesic.



I

Compounds having structure I (R = alkyl or aryl) were prepared from the parent carbinol via the 15-dehydro derivatives and reaction with Grignard reagents.

The antitussive activities of these compounds, as assessed by the method of Winter & Flataker (1954) after oral administration to guinea-pigs, were compared with those of methadone, morphine and codeine. The evidence (Table 1) indicates that the 16-methyl compound (R & S 6420-M) is the most active, being slightly more potent than methadone and 12 times more potent than codeine. Gradually increasing the length of the alkyl chain on R led progressively to decreased potency. The 16-phenyl derivative was inactive at the highest dose level used.

The 16-substituted compounds showed dissociation of antitussive and analgesic properties. Thus, whereas after oral administration the methyl and ethyl compounds respectively were approximately 12 and 4 times more potent than codeine as antitussive agents, they appeared 2–5 times less active as analgesics as indicated by the rat tail pressure method of Green & Young (1951). Demethylation of the phenolic ether group gave the corresponding oripavine which showed relatively more prominent analgesic properties (Table 1).

As R & S 6420-M was the most potent drug suppressing cough in the guinea-pig it was decided to compare its actions with those of several other antitussive agents on the cough reflex of cats, under pentobarbitone sodium anaesthesia, using the technique of Domenjoz (1952). The mean dose of each drug, when given intravenously, which was necessary to inhibit by 50% expiratory gasps elicited either by stimulation of the superior laryngeal nerve or by irritation of the trachea, is shown in Table 2. In this test situation R & S 6420-M, although less active than methadone, was about 8 times more potent than codeine. Comparison of the times during which cough was suppressed after injection of single equi-antitussive doses (2 × ED50) indicated that the duration of action of R & S 6420-M was intermediate between that of methadone and that of codeine (Table 2).

TABLE 1. RELATION BETWEEN THE DOSE LEVELS OF 16-SUBSTITUTED DERIVATIVES OF 6,14-*endo*-ETHENOTETRAHYDROTHEBAINE, CODEINE, MORPHINE AND METHADONE REQUIRED TO CAUSE AN ANTITUSSIVE ACTION IN THE GUINEA-PIG, AND AN ANALGESIC EFFECT IN THE RAT. ED50's are expressed in terms of the base and 95% confidence limits are shown in parentheses

Compound	3 subs.	16 subs.	N subs.	Antitussive ED50 (mg/kg P.O.)	Potency ratio (codeine = 1·0)	Analgesic ED50 (mg/kg) I.p.	Analgesic ED50 (mg/kg) Oral	Oral potency ratio (codeine = 1·0)
R & S 6420-M	MeO	Me	Me	1·97 (0·71–5·51)	11·6	11·2 (7·2–17·4)	54 (33·8–86·4)	0·4
R & S 6439-M	MeO	Et	Me	5·92 (1·94–18·1)	3·9	>100	>100	<0·2
R & S 6446-M	MeO	nPr	Me	>30	<0·8	27·0 (15·0–48·6)	44·0 (32·0–54·4)	0·5
R & S 6441-M	MeO	nBu	Me	52·8 (17·9–156·0)	0·4	>100	>100	<0·2
R & S 7721-M	MeO	Ph	Me	>100	<0·2	>100	>100	<0·2
R & S 8002-M	MeO	Me	H	56·0 (10·3–303·0)	0·4	11·2 (7·4–16·8)	>100	<0·2
R & S 7719-M	OH	Me	Me	2·97 (1·14–7·71)	7·7	0·11 (0·05–0·24)	2·7 (1·35–5·4)	8·2
Methadone	H	—	Me	3·04 (1·34–6·96)	7·5	0·57 (0·43–0·75)	1·9 (0·89–4·1)	11·7
Morphine	OH	H	Me	5·39 (2·74–10·6)	4·2	1·3 (0·92–1·79)	21·1 (10·0–44·4)	1·0
Codeine	MeO	H	Me	22·9 (11·7–44·8)	1·0	13·3 (10·3–17·1)	22·2 (11·6–43·0)	1·0

TABLE 2. RELATIVE POTENCIES OF R & S 6420-M, METHADONE, CODEINE, PHOLCODINE AND DEXTROMETHORPHAN AS COUGH AND RESPIRATORY DEPRESSANTS AFTER INTRAVENOUS ADMINISTRATION TO ANAESTHETIZED CATS. (Doses are expressed in terms of the base)

Drug	No. of cats	Mean dose required to reduce amplitude of cough by 50% (mg/kg)	Mean dose required to block respiratory movements (mg/kg)	Ratio	Duration of antitussive action: No. of cats	Duration of antitussive action: Time (min)
R & S 6420-M	5	0·43	7·9	18·4	3	116
Methadone	4	0·14	1·7	12·1	2	163
Codeine	6	3·4	21·2	6·2	3	20
Pholcodine	4	3·6	22·8	6·3	—	—
Dextromethorphan	3	2·3	3·0	1·3	—	—

The effects of R & S 6420-M on respiration of the anaesthetized cat resembled those of methadone rather than those of the other antitussive agents examined. After being given intravenously, at dose levels close to those depressing cough, both R & S 6420-M and methadone caused some decrease in the depth and frequency of respiratory movements whereas codeine, pholcodine and dextromethorphan usually reduced the depth but increased the rate of breathing. Nevertheless, after high doses had been administered, respiratory depression was the main toxic action of each drug and a measure of the relative prominence of this effect was obtained by comparing the mean dose required to cause cessation of respiratory movements to the dose required for production of an antitussive effect (Table 2). The ratio was found to be high for R & S 6420-M and methadone, significantly lower for codeine and pholcodine and very low for dextromethorphan.

It is of immediate interest that modification of the piperidine ring in the region of the basic nitrogen atom leads to the production of drugs in which antitussive actions are dissociated from some other morphine-like properties.

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Uptake of [³H]noradrenaline in the rat heart during increased sympathetic nervous activity associated with cold

SIR,—It is known that tissue with adrenergic innervation including the heart can take up noradrenaline from the blood or from the surrounding fluid. After administration, [³H]noradrenaline crosses the neuronal membrane of the sympathetic nerve endings into the cytoplasm. It is then taken up and retained within dense core vesicles where it gradually equilibrates with endogenous noradrenaline stores. [³H]Noradrenaline is released in response to sympathetic nerve stimulation, Hertting & Axelrod (1962), and is inactivated enzymatically or by re-uptake and binding in the sympathetic neuron. The physiological importance of noradrenaline re-uptake may consist not only in termination of its influence on the receptor sites of the effector organ, but also in the conservation of the sympathetic transmitter. Gillis, Schneider & others (1965) have shown that increased sympathetic nervous activity results in an increased retention of [³H]noradrenaline. They found, after labelling the endogenous stores of catecholamines 3 hr before the experiment, that continuous stimulation of the sympathetic nerves of the isolated atria for 50 min (at a rate of 10 shocks/sec) caused an increase in the specific activity of noradrenaline recovered from the atria. Similarly Chang & Chiueh (1968) observed that the intermittent stimulation of the cervical sympathetic trunk caused an increase of radioactivity in submaxillary glands. Less information is available about this mechanism *in vivo*. The present study was undertaken to determine whether or not there is an increased retention of [³H]noradrenaline in the rat heart during increased sympathetic nervous activity associated with cold. To gain insight into the mechanism involved, the effect of various drugs on the retention of [³H]noradrenaline during increased sympathetic nervous activity was examined.

Male albino rats of the Holtzman strain, 200–225 g, were used. To elicit sympathetic stimulation, rats placed in individual cages, were exposed to cold for 6 hr at 4°. Exposure to cold is known to result in increased sympathetic nervous activity and release of catecholamines (Euler, 1956).

($\pm$)-Noradrenaline-[7-³H]hydrochloride (specific activity 0·040–0·05 mc/mg) obtained from New England Nuclear Corporation was diluted to 6·5 μc/ml with isotonic saline before each experiment. To stimulate sympathetic nervous activity, rats were exposed to cold for 5 hr. Thereafter they were injected with 6·5 μc/100 g with [³H]noradrenaline. The animals remained a further 1 hr in

the cold. Thereafter the rats were killed by a blow at the base of the neck. The hearts were quickly removed, rinsed in ice-cold saline and homogenized in 10 ml 0·4N perchloric acid. The catecholamines were isolated from the clear supernatant fluid of the tissue homogenate by absorption on alumina and the eluates were assayed for tritiated (Whitby, Axelrod & Weil-Malherbe, 1961) and endogenous noradrenaline (Anton & Sayre, 1962).

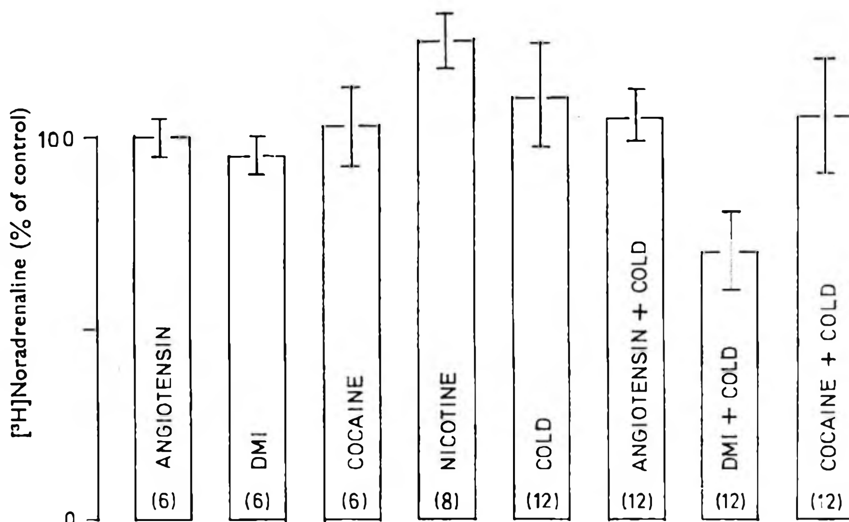

FIG. 1. Effect of various drugs on [^{3}H]noradrenaline content of rat heart during exposure to cold. Rats were exposed to cold for 5 hr. Thereafter, they were injected subcutaneously with 6·5 μc/100 g of [^{3}H]noradrenaline. Animals remained a further 1 hr in the cold. Some animals were administered subcutaneously angiotensin (5 μg/kg), desipramine (DMI) (2·5 mg/kg) or cocaine (10 mg/kg) 10 min after [^{3}H]noradrenaline. All animals were killed and their hearts analysed for [^{3}H]noradrenaline. Each bar represents the mean percentage of [^{3}H]noradrenaline in control animals $\pm$ s.e. Numbers in parentheses indicate the number of animals on which each mean is based.

The results (Fig. 1) indicate that there was no significant increase in accumulation of [^{3}H]noradrenaline injected during increased sympathetic nervous activity associated with cold, compared with that of controls. However, under similar conditions, there was a 20–30% release of [^{3}H]noradrenaline when endogenous noradrenaline stores were labelled 1 hr before the experiment. It is, therefore, suggested that retention of [^{3}H]noradrenaline is increased during increased sympathetic activity. In another series of experiments nicotine, a ganglionic stimulant, was used to stimulate sympathetic nervous activity. Rats were injected with 6·5 μc/100 g of [^{3}H]noradrenaline. Nicotine was administered subcutaneously every 2 min.

Animals were killed 1 hr after the injection of [^{3}H]noradrenaline and their hearts were assayed for radioactivity. The results indicate (Fig. 1) that there was a significant increase in retention of [^{3}H]noradrenaline. But nicotine has a dual action, initially it stimulates and then blocks the ganglion cell. Since ganglionic blockade results in increased retention of injected noradrenaline (Bhagat, 1963; 1967), results with nicotine may not be due to increase in sympathetic nervous activity, and therefore cannot be considered as conclusive.

The accumulation of [^{3}H]noradrenaline was unaltered by angiotensin or

TABLE 1. EFFECT OF TYRAMINE ON [^{3}H]NORADRENALINE CONTENT OF RAT HEART DURING EXPOSURE TO COLD

Treatment	Noradrenaline		Specific activity
	Endogenous μg/g	[^{3}H] counts/min/g	counts/min/μg
None	0·93 ± 0·03	2087 ± 60	2081 ± 181
Tyramine	0·61 ± 0·05	1174 ± 65	1825 ± 190
Exposure to cold	0·68 ± 0·04	1923 ± 233	2487 ± 203*
Exposure to cold + tyramine	0·39 ± 0·02	936 ± 75	2414 ± 210†

Animals (groups of 6) were exposed to cold at 4° for 5 hr before subcutaneous injection of [^{3}H]noradrenaline 6·5 μc/100 g of body weight, and remained in cold 1 hr more after the injection. Tyramine hydrochloride (10 mg/kg) was injected intramuscularly 30 min after labelled noradrenaline. One hr after the injection of [^{3}H]noradrenaline rats were killed and their hearts were assayed for endogenous and labelled noradrenaline.
* Specific activity of control vs. cold exposed rats $P < 0·05$.
† Specific activity of tyramine treated *vs.* cold + tyramine treated rats $P < 0·05$.

cocaine. Desipramine significantly decreased the accumulation of [^{3}H]noradrenaline during increased sympathetic nervous activity. While both cocaine and desipramine inhibit the uptake of noradrenaline, it seems that they act at different sites or in different ways. It is also possible that the increased retention of [^{3}H]noradrenaline during increased sympathetic nervous activity and the re-uptake of released noradrenaline are different processes. Iversen, Glowinski & Axelrod (1965) also found differences between the action of these two drugs on the uptake of noradrenaline in reserpine-pretreated rats.

Tyramine (Table 1) was almost as effective in releasing noradrenaline in rats no matter whether rats were exposed to cold or not. However, there was an increase in the specific activity of noradrenaline recovered from the heart of rats exposed to cold and injected with tyramine. These findings indicate an increase in retention of [^{3}H]noradrenaline during continuous nerve stimulation.

It is concluded from these results that during increased sympathetic nervous activity there is increased retention of noradrenaline to meet the need for replacement of catecholamines in the nerve endings.

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Effect of protriptyline on the formation of [^{3}H]noradrenaline from [^{3}H]dopa

SIR,—Protriptyline, like other imipramine-like antidepressive agents, has been shown to block the amine-uptake mechanism at the level of the cell membrane of the peripheral (Carlsson & Waldeck, 1965; Malmfors, 1965) and central noradrenaline-storing neurons (Carlsson, Fuxe & others 1966). It has not been possible, however, to block the uptake mechanism of the dopamine neurons in the brain by members of this group of drugs (Carlsson & others 1966; Hamberger 1968). Further it has been shown that desipramine and protriptyline are able to reduce the accumulation of noradrenaline after dopa in both central and peripheral noradrenaline-neurons of reserpine, and nialamide-pretreated animals (Carlsson & others 1966). The present investigation was made to clarify the interaction of protriptyline with central and peripheral adrenergic mechanisms using [^{3}H]dopa in doses too low to influence the levels of the endogenous catecholamines.

Female mice, grouped six by six, were given 5 μg/kg [^{3}H]dopa with a specific activity of 33 c/mmole. In some experiments 10 mg/kg protriptyline was given 15 min before the [^{3}H]dopa, in others 15 min after. All injections were made intravenously. One hr after the injection of [^{3}H]dopa the animals were killed by decapitation, their hearts and brains removed and analysed for [^{3}H]noradrenaline and [^{3}H]dopamine as described elsewhere (Persson & Waldeck,1968).

When protriptyline was given 15 min before the [^{3}H]dopa, the yield of [^{3}H]noradrenaline from brain was three times lower than in the controls, whereas [^{3}H]dopamine increased about 60% (Fig. 1A). Pretreatment with protriptyline also reduced the yield of [^{3}H]noradrenaline from heart by about three times; [^{3}H]dopamine in this organ showed, if anything, a decrease. Protriptyline given 15 min after [^{3}H]dopa had little or no effect on [^{3}H]noradrenaline and [^{3}H]dopamine in brain or heart.

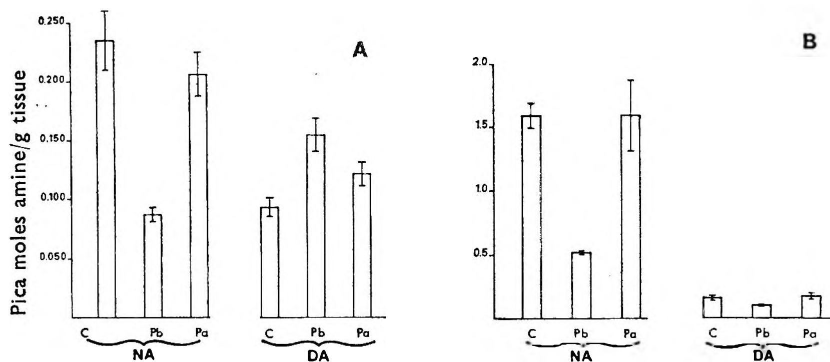

FIG. 1. Influence of protriptyline on formation in the mouse brain (A) and in the mouse heart (B) of [^{3}H]noradrenaline and [^{3}H]dopamine from [^{3}H]dopa. The controls C received [^{3}H] dopa (5 μg/kg i.v.) 60 min before being killed. The second experimental group (Pb) received protriptyline (10 mg/kg i.v.) 15 min before [^{3}H]dopa. The third group (Pa) received the same dose of protriptyline 15 min after [^{3}H]dopa. Each value is the mean of 3 experiments, each on 6 mice, whose brains and hearts, respectively, were pooled. Means $\pm$ s.e. are shown.

Reduced accumulation of noradrenaline with a concomitant increase in the dopamine level might indicate inhibition of the dopamine-β-hydroxylase. That protriptyline should block this enzyme does not seem very likely since the imipramine-like agents do not seem to possess dopamine-β-hydroxylase inhibiting properties *in vitro* (Creveling, Daly & others 1962). Furthermore, in the animals treated with protriptyline 15 min after [^{3}H]dopa, synthesis of [^{3}H]noradrenaline appeared to proceed undisturbed, the concentration of this amine being approximately doubled between 15 and 60 min after administration of [^{3}H]dopa (*cf.* Persson & Waldeck, 1968). This observation seems hard to reconcile with the assumption of an inhibition of dopamine-β-hydroxylase.

A more likely interpretation of the protriptyline effects observed seems to be interference by the drug with the transport of the dopamine serving as noradrenaline precursor. Carlsson & others (1966) proposed that in their experiments the "membrane pump blocker" desipramine prevented dopamine, formed after injection of dopa to animals pretreated with reserpine and nialamide, from re-uptake into the noradrenaline neurons after it had leaked out through the cell membrane. The now reported effects of protriptyline may be interpreted in similar manner. During the first minute after injection of [^{3}H]dopa the precursor will reach the dopa-decarboxylase near the cell membrane. The [^{3}H]dopamine formed has great opportunity to leak out but is taken up again by the "membrane pump". If protriptyline is given before [^{3}H]dopa this re-uptake is blocked and [^{3}H]noradrenaline levels in brain and heart will fall. The [^{3}H]dopamine outside the cell membrane of the central noradrenaline neurons cannot be transported away by the circulation, being prevented by the blood-brain barrier, but at the peripheral neurons this transport is possible. This may explain why [^{3}H]dopamine in brain, but not in heart, rose after protriptyline pretreatment. When protriptyline is given 15 min after [^{3}H]dopa, the [^{3}H]dopamine formed may be distributed more evenly in the cytoplasm, and leakage may thus be of less importance. Therefore the protriptyline effect on [^{3}H]dopamine and [^{3}H]noradrenaline is greatly reduced.

While studying the time-course of [^{3}H]catecholamine formation in mice from [^{3}H]dopa we have found an early accumulation of [^{3}H]dopamine with a maximum at 7½ min in brain and at 15 min in heart (Persson & Waldeck, 1968). As discussed in our previous paper, this accumulation might be extraneuronal, which would be in line with the interpretation given above. The recently reported findings by Nybäck & Sedvall (1968) that desipramine blocked the accumulation of [^{14}C]noradrenaline when given before but not after the precursor [^{14}C]tyrosine might be explained in similar manner.

The possibility of an extraneuronal decarboxylation of [^{3}H]dopa and an inhibition by protriptyline of the uptake of the [^{3}H]dopamine formed into the noradrenaline neurons may also be considered. However, against this possibility is the fact that most of the dopa decarboxylase activity seems to be located intraneuronally. For the central nervous system this has been demonstrated by Andén, Magnusson & Rosengren (1965), Heller, Seiden & others (1965) and Andén, Dahlström & others (1966). It is thus unlikely that the dopa decarboxylation occurring in the capillary walls (see Owman & Rosengren, 1967) is of any considerable importance for providing dopamine-β-hydroxylase with substrate.

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The effect of methylcellulose on the absorption of nitrofurantoin from the gastrointestinal tract

SIR,—Although many pharmaceutical suspensions are viscous, little attention has been paid to the relation between viscosity and the absorption of insoluble drugs from the gastrointestinal tract.

The relation has already been noted with soluble drugs. Malone, Gibson & Miya (1960), for example, noted that an increase in the concentration of sucrose in aqueous solutions of sodium phenobarbitone considerably lengthened the induction time for narcosis. Davison, Guy & others (1961) found that the plasma and brain salicylate levels after the oral administration of sodium salicylate solutions were significantly reduced when methylcellulose was added to the formulation. Recently, Levy & Jusko (1965) showed that methylcellulose reduced the uptake of salicylic acid from ethanol–water mixtures by the ligated rat stomach.

I have found that the insoluble urinary antiseptic nitrofurantoin when dispersed in therapeutically realistic volumes in methylcellulose and taken by mouth, is not excreted as rapidly in the urine as the drug in water suspension.

Freshly prepared suspensions of 0·5% w/v nitrofurantoin in water were administered (1·5 mg/kg) to 9 healthy male volunteers after an overnight fast. This was followed by 100 ml water. The bladder was emptied and urine was collected at hourly intervals. The procedure was repeated with 0·5% w/v nitrofurantoin suspensions in 5% w/v methylcellulose solutions. All urine samples were assayed immediately after collection by a polarographic method described by Jones, Ratcliffe & Stevens (1965).

The effect of methylcellulose on the excretion rate of nitrofurantoin is shown in Fig. 1. In the presence of methylcellulose, the concentration of nitrofurantoin in urine rises less rapidly and the peak concentration is delayed by 1 hr. The

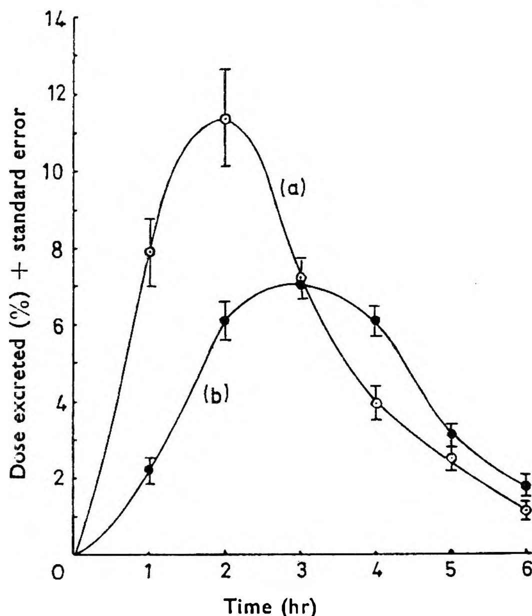

FIG. 1. Excretion rate of nitrofurantoin after oral ingestion of nitrofurantoin suspended in (a) water, and (b) methylcellulose solution.

amount of drug excreted in 6 hr is significantly reduced (P <0.01) and the biological availability of the drug is impaired.

The effect of methylcellulose as suggested by Levy & Jusko (1965) may be due to a modification of the gastric emptying or intestinal transit rates. Alternatively, the movement of drug molecules in the gastrointestinal fluids might be affected. Possible complex formation would reduce the availability of the drug and the presence of methylcellulose would markedly affect the dissolution rate.

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The inhibitory effect of subcutaneously administered degraded carrageenan on gastric secretion

SIR,— We have shown that subcutaneously administered degraded carrageenan has a pronounced inhibitory action on the acid secretory response of the intact guinea-pig stomach (Watt, Eagleton & Marcus, 1966b). This inhibitory action was observed in relation to histamine-stimulated secretion but not to the secretion stimulated by 3-(2-aminoethyl)pyrazole dihydrochloride (ametazole hydrochloride; Histalog) an analogue of histamine. We have now investigated the time course relative to this inhibitory action and, in addition, the effect of degraded carrageenan on fasting secretion.

Adult male albino guinea-pigs (550–750 g) were prepared for secretory studies by a preliminary fast of 15 hr, during which time they wore loosely-fitting Perspex collars to prevent coprophagy. Gastric secretion was then stimulated by the intramuscular injection of histamine acid phosphate (1 mg/kg) in aqueous solution (1 mg/ml). One hr later, the gastric juices were collected by intubation of the unanaesthetized animal. Total acid concentration was measured by titration using phenolphthalein as indicator.

Freshly prepared degraded carrageenan (5% aqueous solution) derived from the red seaweed *Eucheuma spinosum*, was administered as a single subcutaneous injection in a dose of 400 mg/kg. In the time course study, secretory tests were made on separate groups of animals at 2 hr intervals over the first 24 hr and at 2, 3, 9 and 14 days after the administration of carrageenan. Groups varied in size from 2 to 10 animals; the control group (at zero hr) comprised 20 animals. The effect of degraded carrageenan on the fasting secretion was studied in animals which had received the drug subcutaneously (400 mg/kg) 18 hr previously.

The time course study (Fig. 1) indicates a significant reduction (2 standard deviations below the mean of the control values) in both volume and total acid concentration of the histamine-stimulated juice from about 6 to 24 hr following a single injection of degraded carrageenan. By 3 days all values are within the normal range.

The effects of degraded carrageenan on the fasting gastric secretion are shown in Table 1. Degraded carrageenan lowers the volume significantly and tends to reduce the total acid concentration of fasting secretion.

It has been known for some time that orally administered degraded carrageenan, apart from possessing antipeptic activity, also causes a temporary reduction of gastric acidity in response to histamine (Anderson, Marcus & Watt, 1962). The results of the present investigation show that degraded carrageenan administered parenterally has a pronounced inhibitory effect on histamine-stimulated gastric secretion and that this effect is a prolonged one

TABLE 1. EFFECT OF 18 HR PRETREATMENT WITH SUBCUTANEOUSLY ADMINISTERED DEGRADED CARRAGEENAN ON FASTING GASTRIC SECRETION (MEANS ± ONE S.D.). NUMBER OF ANIMALS IN PARENTHESES

Treatment	Gastric secretion			
	Volume (ml)		Total acid (m-equiv/litre)	
Fasting (10)	3·3 ± 2·5	$P < 0·05$	98·5 ± 27·1	$P > 0·20$
Degraded carrageenan and fasting (10)	1·0 ± 1·0		85·8 ± 32·1	

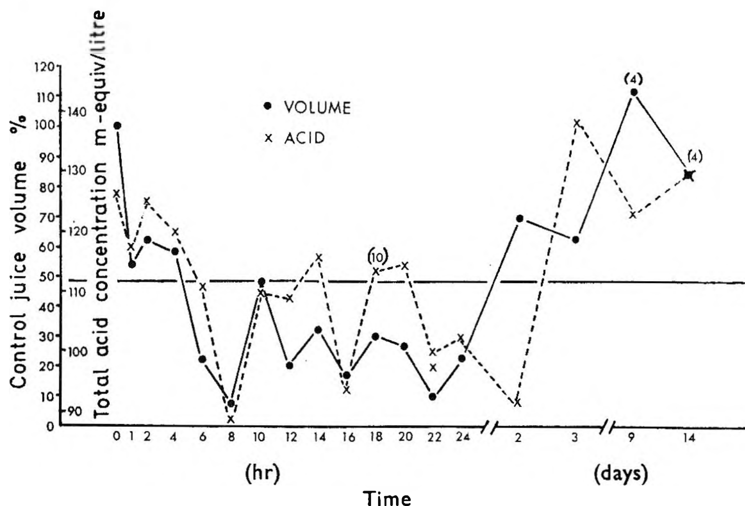

FIG. 1. Time course study. Effect on histamine-stimulated gastric secretion of degraded carrageenan administered subcutaneously. Two animals used at each point except as indicated at 18 hr, 9 and 14 days. The horizontal dark line represents two standard deviations below the means of the control values for both volume and total acid concentration.

lasting from about 6 to 24 hr. By the third day, the inhibition is no longer apparent. The results also suggest that degraded carrageenan suppresses the fasting gastric secretion.

We have previously suggested that the inhibitory effect of degraded carrageenan on histamine-stimulated gastric secretion in the guinea-pig may be due to complexing of the sulphated polysaccharide with histamine (Watt & others, 1966b). Another possible mechanism is the release of diamine oxidase from the intestine into the plasma, as occurs in the rat in response to heparin (Maudsley & Kobayashi, 1968). A prompt rise in plasma diamine oxidase also occurs in man after the injection of heparin (Dahlbäck, Hansson, & others, 1968).

Recently, the sulphated polysaccharide heparin has been shown to exert inhibitory effects on stimulated gastric secretion in experimental animals (Thompson, Lerner & others, 1966; Watt & others, 1966a). It has also been demonstrated that intravenous heparin inhibits both stimulated and basal gastric secretion in man (Thompson, Lerner & Musicant, 1966). Its use in the treatment of peptic ulceration, however, is inadvisable because of its anticoagulant properties. Such objection may not be applicable to degraded carrageenan. Anderson & Duncan (1965) have made a comparison of the anticoagulant properties of a variety of carrageenans and have shown that degraded carrageenan has only very slight anticoagulant activity. This is in accord with our own observation that no haemorrhagic diathesis was produced in any of our animals in the doses used. As with heparin, there is no granulomatous reaction in the subcutaneous tissues at the site of injection. It is possible that degraded carrageenan, or some other sulphated polysaccharide, administered parenterally may prove to be of therapeutic value in the management of peptic ulceration.

We thank Dr. G. B. Shirlaw of Laboratories Glaxo-Evans, Paris, for supplying degraded carrageenan.

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Effect of some psychotropic drugs on mice from a spontaneously aggressive strain

SIR,—The spontaneous or provoked aggressiveness of different animal species has often been used to study the antagonistic action of psychotropic drugs (Valzelli, 1967). In the mouse, a typical aggressiveness is manifested only after prolonged isolation of 3 to 4 weeks, which is technically difficult, or after painful stimulation.

A spontaneous aggressiveness is found in the male mice of the strain CF 1 (IFFA-CARWORTH) more than one month old, which is exhibited by tail wounds in mice grouped in a cage, only one animal, the "boss", remaining unwounded.

Preliminary tests were made to utilize this particular behaviour using the presentation of another animal to an aggressive mouse alone in his cage or in a new cage. However, in these conditions, prompt onset of fighting did not regularly occur.

To suppress the effect of a recent change of territory, we used the following conditions: preliminary isolation for 24 to 48 hr; the presentation of non-aggressive mice (male, Swiss strain, identical weight) according to the following protocol: every 30 min, two mice were successively presented, each animal being withdrawn after the first attack and being left, at the maximum, 5 min in the cage.

Under these conditions, 90% of mice behaved regularly in an aggressive manner, the others being easily eliminated. For the study of psychotropic drugs, we used groups of 8 aggressive mice for each dose. A repetition of the test every 30 min permitted the establishment of the kinetics of any anti-aggressive effect. In the control tests, we regularly obtained 16 aggressions for each animal; the effect of drugs was expressed as a percentage of the diminution of this aggressiveness.

The results are shown in Table 1. Efficacious doses are similar to those found by other experimenters using different methods (see Valzelli, 1967). This method seems to be better from two points of view: utilization of a natural aggressiveness and the technical facility of the test.

TABLE 1. PERCENTAGE OF DECREASE OF AGGRESSIVENESS IN MICE TREATED WITH SOME PSYCHOTROPIC DRUGS

Drug	mg/kg	Route of administration	% age of decrease of aggressiveness in mice treated after (time in min)					
			30	60	90	120	180	240
Diazepam	0·5	i.p.	0	0	0			
	1	,,	37·5	25	18·5	12·5	0	
	2	,,	25	31	37·5	31	18·5	0
	4	,,	56	31	18·5	25	18·5	0
	1	oral	0	0	0			
	2	,,	12·5	18·5	0			
	4	,,	37·5	12·5	0			
	8	,,	87·5	62·5	56	50	50	50
Meprobamate . .	64	oral	12·5	25	25	25	0	
	128	,,	6	6	0	0		
	256	,,	18·5	31	12·5	6		
Chlorpromazine . .	1	oral	25	25	12·5	12·5	12·5	
	2	,,	50	12·5	0			
	4	,,	56	50	50	37·5	25	
	8	,,	68·5	75	81	75	56	31
Haloperidol	0·125	oral	18·5	0				
	0·25	,,	12·5	12·5	6	0		
	0·5	,,	18·5	31	50	50		
Molindone	4	oral	25	12·5	0			
	8	,,	18·5	0	0			
	16	,,	68·5	75	68·5	25	0	
	32	,,	43·5	56	56	50	31	12·5
Imipramine	8	oral	25	31	25	25	37·5	37·5
	16	,,	18·5	12·5	31	31	0	
	32	,,	25	31	43·5	31	6	

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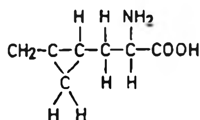
The isolation of L-leucine-free hypoglycin-A

SIR,—Hypoglycin-A, the toxic peptide from *Blighia sapida*, or ackee, a common article of Jamaican diet (Feng & Patrick, 1958), is known to have anti-sarcoma properties (Gaskin & Persaud, 1967). Hassall, Reyle & Feng (1954) and Feng & Patrick (1958) produced Hypoglycin-A (I) with L-leucine.



(I)

A new method has been found to prepare Hypoglycin-A free from L-leucine' a contaminant which antagonizes the anti-sarcoma properties (Gaskin & Persaud, 1967).

Material and method. 4·5 kg of mature seeds were ground and extracted with boiling water for 4 hr. The cooled filtrate was adjusted to pH 4 and proteinaceous material removed. The solution of mixed amino-acids was fractionated next on a Dowex 50W (x8) column (mesh 20–50, 60 cm × 5 cm). Elution was with N ammonia solution and the eluate was condensed to a small volume at 70° and then chromatographed on basic alumina Grade III (Merck) made up in acetone.

Development of this column was first with acetone and then acetone–water mixture, the latter solvent removing Hypoglycin-A from the column. The crystallized crude Hypoglycin-A was dissolved in warm water and further chromatographed on neutral alumina Grade III, from which 95% ethanol removed L-leucine, and a mixture of ammonia solution in ethanol removed Hypoglycin-A.

Hypoglycin-A was recrystallized from aqueous methanol and gave Rf 0·46 in a system of t-butanol–methanol–water (4:5:1). The yield was 0·03% w/w. The infrared spectrum in Nujol of the separated leucine was identical with that of authentic leucine, while that of the isolated Hypoglycin-A was identical with the synthetic material (Dr. S. R. Landor, private communications).

The nmr spectrum of Hypoglycin-A in D_2O at 25° is in agreement with the structure (I).

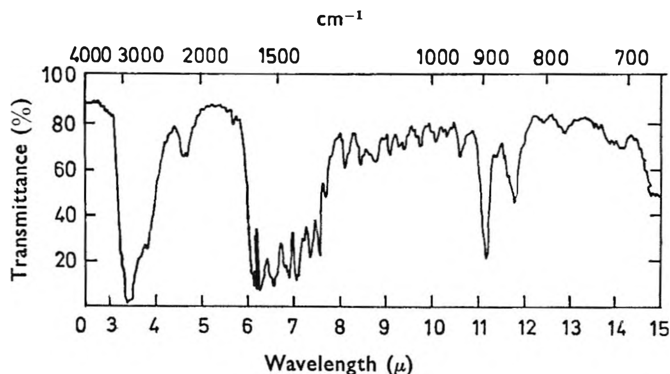


FIG. 1. Infrared spectrum of hypoglycin-A in Nujol.

Hypoglycin-A is one of the few natural products with this methylene cyclopropane ring system. The adsorption of both L-leucine and Hypoglycin-A on basic alumina took into account their respective solubilities in water, alcohol, acetone and pyridine. Both Hypoglycin-A and L-leucine are considered as neutral amino-acids, and should carry a negative change in 90% ethanol and be retained by acid alumina (Wieland, 1942).

However their behaviour on acid alumina indicated that Hypoglycin-A and L-leucine had very different physicochemical properties, and selective elution from neutral alumina of Hypoglycin-A with ammonia solution–ethanol was based on the amino-acids classification of Schramm & Primosigh (1944).

The yield from this method was 0·03% w/w as compared to 0·008 to 0·01% w/w in earlier methods.

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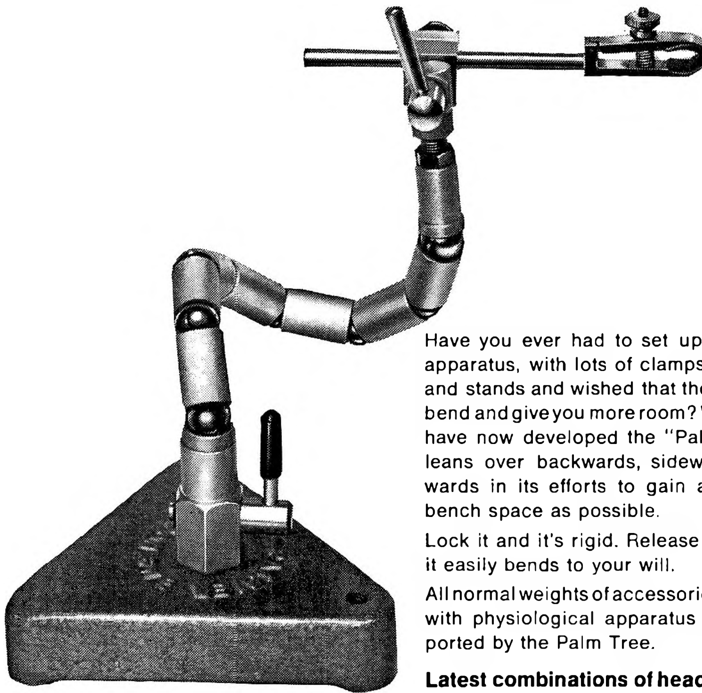

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Contents

889–910 LANCE L. SIMPSON
Review Article: The role of calcium in neurohumoral and neurohormonal extrusion processes

911–915 A. M. BARRETT, VALERIE A. CULLUM
Lack of inter-action between propranolol and mebanazine

916–922 JUDITH M. ATKINSON, M. J. RAND
Mutual suppression of cardiovascular effects of some β-adrenoreceptor agonists in the cat

923–933 P. H. ELWORTHY, F. J. LIPSCOMB
The effect of some non-ionic surfactants and a polyoxyethylene glycol on the dissolution rate of griseofulvin

934–940 S. BONKOSKI, J. H. PERRIN
The optical rotatory dispersion of some *N*-decyl-*NN*-dimethylalanine salts and their critical micelle concentrations

Letters to the Editor

941–943 K. D. RAINSFORD, J. WATKINS, M. J. H. SMITH
Aspirin and mucus

943–946 P. V. HALUSHKA, P. C. HOFFMANN
Distribution and metabolism of dopamine in guinea-pigs

947–948 D. A. TITCHEN, J. C. NEWHOOK
Adrenergic effector mechanisms in the stomach of the sheep

948–949 J. SCHEEL-KRÜGER, A. RANDRUP
Aggressive behaviour provoked by pargyline in rats pretreated with diethyldithiocarbamate

950–953 M. J. NEAL
Failure of morphine dependence in rats to influence brain noradrenaline turnover

953–954 T. S. YEOH, A. S. LEE
An apparatus for the study of intestinal transfer of drugs

955–956 A. JORI, D. BERNARDI
Brain desipramine level in relation to the anti-reserpine activity of imipramine

957–959 J. PATKOWSKI
On the mode of action of some immunosuppressant drugs

959–960 CONSUELO AGRELO, W. DAWSON
The continuous recording of arterial blood pressure in the conscious unrestrained rat

961–963 A. L. A. BOURA, D. I. HADDLESEY, E. J. R. HARRY, J. W. LEWIS, P. A. MAYOR
Structural requirements for antitussive activity in some novel 16-substituted derivatives of 6,14-endoethenotetrahydrothebaine

963–965 EITAN FRIEDMAN, B. BHAGAT
Uptake of [^{3}H]noradrenaline in the rat heart during increased sympathetic nervous activity associated with cold

966–968 TORGNY PERSSON, BERTIL WALDECK
Effect of protriptyline on the formation of [^{3}H]noradrenaline from [^{3}H]dopa

968–969 H. SEAGER
The effect of methylcellulose on the absorption of nitrofurantoin from the gastrointestinal tract

970–972 G. B. EAGLETON, J. WATT, R. MARCUS
The inhibitory effect of subcutaneously administered degraded carrageenan on gastric secretion

972–973 JACQUES-R. BOISSIER, SYLVIE GRASSET, PIERRE SIMON
Effect of some psychotropic drugs on mice from a spontaneously aggressive strain

974–975 MANLEY E. WEST
The isolation of L-leucine free hypoglycin-A

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