REVIEW ARTICLE

BIOLOGICAL ASSESSMENT OF TRANQUILLISERS. PART II*

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2. Behavioural Methods (not involving conditioning)

The methods of measuring sedation described under 1H and 1I might also have been described under behavioural methods, and form a link with this new section. We have grouped under behavioural methods those involving careful rather than superficial observation of responses of animals to their environment. These responses, though they may involve non-experimental conditioning, are spontaneous in the sense that experimental conditioning is excluded.

J. *Observational Methods*

Observational methods are often used as preliminary screening tests. Chen[203] discriminates between anaesthetic, hypnotic and sedative effects by the posture of rats and their reaction to tail-pinching, and Rubin and Burke[204] assay reserpine by grading the eye closure of mice. Similar methods combined with activity recording were used by Lim and co-workers[205]. Such techniques commonly detect sedative rather than tranquillising activity, and the effects of the sedative tranquillisers are the most impressive: thus reserpine, characteristically produces hunched posture and eye closure in laboratory animals[206]. The effects of tranquillisers have also been studied in fascinating experiments on the web-spinning behaviour of spiders[207], or on the fighting response of the Siamese fighting fish, *Betta splendens*[208,209], or its behaviour under stress[210], but it seems unwise to predict actions in man from results in these primitive creatures.

A more advanced technique for investigating the effects of tranquillisers on behaviour has been devised by Norton and co-workers using mainly the cat[211,212]: hamsters and monkeys are also suitable[213]. Briefly the method consists of observing the behaviour of untreated animals, selecting general patterns of behaviour, e.g., sociability, contentment, excitement, and aggressive or defensive hostility, and finding behaviour components that as far as possible are specific to one of the behaviour patterns. Five such components are chosen for each pattern, and for scoring purposes they are weighted, the least frequently occurring component being given the highest score. Each type of drug is said to have its own profile of action. Chlorpromazine predominantly reduces defensive hostility. Rauwolfia reduces sociability and, slightly, contentment. Meprobamate reduces hostility and sociability. Azacyclonol resembles rauwolfia in its effects but also increases excitement slightly[211]. Methamphetamine and LSD are found to have much in common; their effects being in general

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the converse of those produced by chlorpromazine[213]. There was not a perfect correlation of effects of drugs in different species, perhaps because the criteria chosen for each behavioural pattern were not the same in different species. This method, though involving a praiseworthy attempt to introduce subtlety of observation into a field where subtlety of action is frequently claimed but rarely demonstrated, appears extremely complex and time-consuming. Its value is not yet fully determined, but appears doubtful because of the rather similar profiles of action that have been recorded for some drugs the clinical effects of which are dissimilar (e.g., rauwolfia, azacyclonol).

The use of naturally aggressive animals should enable calming effects to be distinguished more readily from purely sedative effects. Rhesus monkeys are wild and aggressive and so make good subjects for this test. Chlorpromazine[214] and other phenothiazines[215,216], reserpine[217,218], meprobamate[148] and hydroxyzine[13] are all said to tame monkeys though their effects are not identical. Hendley and co-workers[219] claimed that chlorpromazine and reserpine insulate the animals from their environment most effectively by almost completely depressing reactivity to stimulation, but that meprobamate tames them more reliably while not affecting alertness. Hydroxyzine has similar effects to those of chlorpromazine[13]. Hosko and co-workers[215,216] also concluded that taming is not necessarily a function of sedation or resistance to arousal. Lysergic acid diethylamide and bufotenine also tame, probably due to sensory block as blindness, ataxia and analgesia accompany the tameness[220]. Benactyzine does not tame[132].

Chlorpromazine does not always tame; one calm monkey was made aggressive by a dose that calmed aggressive monkeys[214], and chronic administration of large doses of chlorpromazine caused major convulsions in four monkeys and produced apparent hallucinations in three of the four[221].

We have used these methods in Rhesus monkeys: our opinion is that the great variation in response of different monkeys, and of the same monkey on different occasions, and the difficulty of handling the large number that such variation requires makes these methods less attractive than they superficially appear to be. Also we are less convinced of the difference between tameness and sedation than some workers are, but we must admit that our experience is rather small.

K. *Direct Intracerebral Injection*

Drugs may be injected directly into the brains of conscious animals by using permanently implanted cannulae. The technique for implanting cannulae into the lateral ventricles of cats was described by Feldberg and Sherwood[222,223] and Haley and Dickinson[224] described a modification of this method for use in dogs. After recovery from the anaesthetic, the animals behave normally and show no ill-effects, appearing to be undisturbed by the presence of the cannula. Drugs can then be injected quickly and painlessly into the brain and their effects studied. Most drugs have pronounced autonomic effects, but more specific effects are

often observed as well. The actions of some psychotomimetics and tranquillisers have been investigated in cats. Mescaline produces bouts of violent scratching[225] and lysergic acid diethylamide (LSD) produces an initial restless state followed by drowsiness accompanied by slow waves in the EEG[225,226]. In the dog LSD has been said to produce a reversion from an adult to a puppy behaviour pattern[227]. In both animals a "fear complex" has been described[228]. Serotonin produces muscular weakness[225,227,229,230] and reserpine in suitable doses produces marked sedation[228,230,231] differing from that of serotonin especially in its time course[231]. The effects of the two drugs do not summate and the effects of reserpine cannot be duplicated by its metabolites[231]. Chlorpromazine in dogs also produces depression[232,233].

Haley observed similar results when the drugs were injected directly into the brains of mice[234,235]. Mescaline again produces scratching and, like LSD[235,236], causes aggressiveness at low doses and depression at higher doses[236]. Chlorpromazine produces alternating bouts of sedation and hyperexcitability in many animals[236].

Although these techniques were designed to eliminate peripheral drug effects, they seem to lack specificity and results have been disappointing. Many of the observed effects may be due to vascular changes.

L. *Sham Rage*

Normally friendly animals can be made aggressive by surgical interference with the brain, and the rage reaction found in such animals is usually called sham rage because it is elicited by slight stimulation and is inappropriate and purposeless. Chlorpromazine suppresses the symptoms of sham rage in decorticate and diencephalic cats[237] and rabbits[238], at doses that have no effect on the behaviour of normal animals. Reserpine also protects against the appearance of rage in similarly operated cats[239,240]. Rats made savage by septal lesions are tamed by meprobamate but not by chlorpromazine[241].

Rage reactions are also produced by electrical and chemical stimulation of the amygdala and surrounding areas of the brain[242]. Naquet, cited by Gloor[243], states that the behavioural effects of amygdaloid stimulation are only apparent during seizure-like discharge of the amygdala. However, chlorpromazine produces a marked increase in the spontaneous electrical activity of the amygdaloid complex amounting to seizure activity at high doses[244] and reserpine prolongs the duration of evoked seizures[245].

It has been reported that rage reactions in cats appear after lesions of the amygdala[246] but later work has shown that such lesions produce relative docility[247], and as similar lesions in rats[248,249] and monkeys[250] also produce docility, the balance of the evidence is in favour of taming by amygdaloid lesions. Weiskrantz and Wilson[251] pointed out the similarity between the effects produced by reserpine and those produced by lesions of the amygdala and adjacent structures in monkeys. However, by testing the effects of reserpine in amygdaloidectomised animals, these workers concluded that the amygdala is not a critical site of action of reserpine, and further observations have confirmed them in this view[252]. The exact

importance of the electrical changes induced by drugs at this site remains to be determined.

3. Neurophysiological Methods

The experiments last mentioned are part of a wider and still expanding branch of tranquilliser research summarised here as neurophysiological. The neurophysiological and biochemical approaches should eventually provide us with information first on the parts of the brain concerned with emotionality and other properties of mind, second on the chemical transmitters important at these sites. A more rational design of tranquillising drugs will then become possible. Currently we think that these extremely important researches have much investigative and doubtful predictive value. We therefore consider them only briefly.

M. *Arousal and Recruitment*

Understanding of consciousness and attention was greatly increased by the discovery of Moruzzi and Magoun[253] that electrical stimulation of the brain stem reticular formation produced changes in the EEG consisting of abolition of slow synchronous discharge and its replacement by low voltage fast activity. This response is now called the arousal reaction. The authors suggested that the cortical arousal reaction to natural stimuli is mediated by collaterals of afferent pathways passing to the brain stem reticular formation and thence through the ascending reticular activating system to the cortex. Other workers[254] had noted that sciatic stimulation produced a secondary diffuse cortical response as well as the primary response localised to the somatic sensory areas. Later work on afferent conduction showed that potentials in the classical lateral sensory pathways showed projection to specific areas of the cortex, segregation of modality, and rapid conduction, whereas medially conducted potentials displayed slower conduction, no segregation of modality and distribution in wide areas of the cortex by way of the diffuse thalamic system[255]. The response via the medial system is thought to provide a background of alertness upon which the sensory discrimination mediated by the lateral pathway can act effectively.

Confirmation of the importance of the reticular activating system in maintaining consciousness was provided by investigations of the effect of chronic brain stem lesions in cats[256] and monkeys[257]. Lesions interrupting this system were followed by chronic somnolence and EEG synchrony. The effects appeared to be more severe in the monkey. Temporary interference with the activity of the reticular activating system had early been postulated as the possible mechanism by which anaesthesia is induced, and indeed it was found that this system is extremely susceptible to the action of anaesthetics[258,259]. It was suggested that this susceptibility is due to the complex multisynaptic nature of the reticular formation[258]. However, some spinal interneuronal depressants have little or no effect on the reticular activating system[260,261] and therefore it was suggested that the pathway for cortical arousal may not depend on interneuronal connections but that there may be an extra-thalamic pathway involving the

direct passage of impulses from the reticular arousal system into the internal capsule[261]. It is, of course, difficult to visualise any central pathway that does not involve numerous relays: a particular type of interneuronal network may, however, display drug specificity.

In close functional connection with the midbrain reticular formation is the diffuse thalamic projection system[262,263]. Electrical stimulation of this system produces high voltage, slow wave recruiting responses over large areas of the cortex, the waves coming in "bursts" or "spindles"[264] and this response can be blocked by activating influences from the midbrain reticular formation[265].

Many drugs have different action on the "arousal" and "recruitment" systems; thus barbiturates block arousal but enhance recruitment, mephenesin and other interneuronal depressants depress recruitment without affecting arousal, whereas ether depresses both systems[260,261]. The action of barbiturates on cortical arousal is characteristically biphasic as an excitatory phase occurs before the depression; alcohol has a similar effect though the excitatory phase is less pronounced[266]. Since anaesthetics modify activity so profoundly, techniques have been evolved for chronically implanting electrodes in the brain of animals so that electrical recordings may be taken during consciousness[267–270]. Evoked potentials may be recorded if stimulating electrodes are also implanted[271].

Chlorpromazine induces EEG synchrony similar to that seen in sleep[272–280] and this has been said to be due to depression of the midbrain reticular system[272,277,280–285]. However, although the behavioural arousal response to auditory or sciatic nerve stimulation is depressed considerably[105,274,275,277,278], the threshold for electrical arousal by reticular stimulation is only slightly raised[286–288]. Chlorpromazine appears to enhance recruiting activity[285].

Like chlorpromazine, meprobamate[105,234,289,290], hydroxyzine[235], benactyzine[236,291] and methyprylone[105,289], all induce EEG synchrony. These drugs also block or depress the behavioural alerting response to auditory stimulation[236,289,291].

Reserpine is not generally reported to produce an electroencephalographic picture of sleep[217,292], though there are some reports of an initial "sleep" pattern[231,232,234] followed by an arousal pattern, but without behavioural arousal. There is also a report of EEG activation followed by depression[293]. The threshold for stimulation of the midbrain reticular formation is unaltered[232,287,288] or, more probably, lowered[294].

Although reserpine does not depress the midbrain reticular activating system, it produces behavioural non-reactivity[295]. The dissociation of behavioural and electrographic arousal shows that the two cannot be simply equated. The suggestion has been made[296] that in the normal animal, attention is selective, but reserpine, by making the activating system more sensitive, destroys the selective suppression of sensory input so that the mechanisms of attention no longer function. It is perhaps doubtful whether the effects of reserpine on the activating system are sufficiently striking to justify this view.

Other examples of dissociation are known. Physostigmine produces EEG arousal without a corresponding change in behaviour[297–303] and atropine produces a synchronised EEG without impairing behavioural alertness[298–303]. Such actions are responsible for the suggestion that cholinergic neurones are concerned in the function of the activating system[304,305], but adrenaline and related drugs also have powerful effects[306]. Substances such as adrenaline[297], ephedrine[307], amphetamine[297–299,308] and methyl phenidate[307] produce behavioural as well as EEG arousal, and so do the psychotomimetic drugs mescaline[309] and LSD[298,309]. Both these classes of drug also produce cerebral synaptic inhibition in the trans-collosal preparation of Marrazzi and Hart[310] as does serotonin[311]. It is suggested that the naturally occurring inhibitory synaptic transmitters are adrenaline, noradrenaline and serotonin, of which the last is the most powerful[312,313]. The psychotomimetic substances mescaline, adreno-chrome, adrenolutin, LSD and bufotenine resemble these transmitters in structure and in their inhibitory synaptic action[311,312]. Purpura[314] has suggested that the inhibitory action of LSD is due to an activation of inhibitory synapses. This property could be an important one in the production of "psychotic" states, especially as it has also been shown that the tranquillisers chlorpromazine, promazine, reserpine and azacyclonol prevent or reduce this inhibitory action in doses at which they have no effect alone on synaptic transmission[313,315].

These stimulating researches provide a variety of ideas for the synthesis of potential tranquillisers of novel type: for the present it would probably be wisest to test such drugs by methods described elsewhere in this review.

4. Antagonism to Psychotomimetic Drugs

Certain drugs induce behavioural syndromes which have been presumed by some authors to imitate neurotic and psychotic states, and the actions of tranquillisers on these syndromes have been investigated.

Morphine produces a characteristic state in cats[316], consisting of a variety of autonomic effects such as mydriasis, motor effects such as tremor, ataxia and hyperactivity, and "psychic" effects manifested as anxiety, negativism and apparent hallucinations. The state is sometimes termed mania but is sharply distinguished from human mania, particularly by negativism. The amount of hyperactivity and restlessness varies with the degree of restraint such as the size of the cage in which the cat is confined. Chlorpromazine reduces morphine excitement[317–319] but does not alter the autonomic effects[318,319]. Reserpine is also an antagonist when given after morphine[319] though when it is given before morphine sometimes it is reported to antagonise[320] and sometimes to enhance excitement[319]. It is confirmed that low doses of reserpine enhance morphine excitement[317]. Azacyclonol is variously reported as being an effective antagonist[130], having only a weak effect[320], and having no effect[319]. Meprobamate and benactyzine diminish the signs[320]. The difference in results may be due to the different ways of assessing the morphine effects but even where the

results are consistent they are of doubtful clinical significance because the actions of morphine in man are so different from those in the cat.

Mescaline, however, is a drug that produces "psychotic" changes in man. These properties have been known for a long time and the drug has been used as a ritual poison. Intravenous mescaline in dogs produces characteristic behavioural changes such as apparent anxiety, negativism and "catatonia" with autonomic symptoms such as mydriasis and salivation[321,322]. Premedication with azacyclonol prevents the catatonia[322]. Similar effects are observed in the cat when mescaline is administered intraperitoneally[322]. The "mescaline response" of cats given mescaline intraventricularly has been used as a basis for testing tranquillisers[322]. Predosing with reserpine prevented the symptoms from progressing to catatonia. Chlorpromazine suppressed the mescaline response but large doses deepened the catatonia. Azacyclonol changed the catatonic state to excitement[322]. These workers did not find that intraventricular mescaline caused violent bouts of scratching, though it has been reported by others[225]. In mice subcutaneous mescaline produces a marked psychomotor stimulation which is antagonised by chlorpromazine, reserpine, meprobamate, azacyclonol and serotonin, but enhanced by benactyzine and phenobarbitone[63,64]. Mescaline given daily to rats has a non-specific stressing effect[323]. In conditioned rats, mescaline interferes with the conditioned response so that it becomes disorientated and though the stimulus still keeps its significance it is said to produce a hallucinatory crisis[324], inhibited by chlorpromazine. Unfortunately a full account of this crisis has not been given.

Stoll in 1947[325] was the first to report the psychotic effects of lysergic acid diethylamide (LSD) in man, and since then this compound has been studied extensively, many of the experiments being done on human volunteers[326–329]. LSD, besides having subjective psychotomimetic actions in man, is said to produce a change in urinary phosphate excretion which is like that seen in schizophrenics. The phosphate excretion in schizophrenics and in volunteers after LSD administration is much lower than in normal controls, and in a stressful situation or following adrenocorticoid administration the phosphate excretion of both groups shows a marked increase, an effect not seen in the controls[330,331].

LSD reactions have been studied in various animals. The Siamese fighting fish (*Betta splendens*) has been used to study LSD since it gives characteristic responses to this drug[332], responses that are suppressed by crude beef brain extract[333] but not by a variety of amino acids[333] or by reserpine[334]. Similar responses are observed with methyl lysergic acid diethylamide[335,336] but not with some other closely related ergot drugs[337]. The effects of LSD on a stress response of these fish have also been investigated[338]. LSD produces a characteristic state in the guppy (*Lebistes reticulatus*) which can be antagonised by reserpine but not by chlorpromazine[339]. The behavioural changes can be prolonged indefinitely by exposing the treated fish to indole or tryptamine[339].

In cats LSD produces a state of co-ordinated aggression in response to visual and tactile stimuli[340], but of the tranquillisers chlorpromazine,

promazine, meprobamate, azacyclonol and reserpine, only chlorpromazine was consistently effective in antagonising the LSD response, and reserpine intensified the reaction[340]. In rats trained to climb a rope, LSD produces confusion and prolongs climbing time[341] and though meprobamate, benactyzine and serotonin have antagonistic actions, chlorpromazine and reserpine enhance the effects[342]. LSD produces excitation in mice[343] and causes them to walk backwards showing behaviour similar to that of normal mice facing down a slippery inclined plane[344,345]. Another behaviour pattern that appears in LSD-treated mice is a rapid head-shaking response or "twitch" to a light touch at the back of the head[346]. A similar response appears in a proportion of mice when they are kept in solitary confinement for three weeks. If it appears within two days of isolation, it remains permanently when the mice are returned to groups after the three weeks period. If the response only appears after a week of isolation, it is lost gradually when the mice are returned to groups[346]. The effect of reserpine on permanent "twitchers" is that the response is lost only during the period of drug administration, but in the less-permanent "twitchers" the lost response does not reappear when the drug is withdraw[346]. We have confirmed these results on the "twitch" that develops after solitary confinement, and consider that the technique is a promising one for the assessment of tranquillisers. The techniques involving antagonism of psychotomimetic drugs are in our opinion less useful than one would expect them to be. The results of attempted antagonism by tranquillisers are often feeble and variable and no single technique can be selected and confidently proposed as a particularly useful method of assessing tranquillising drugs. Further research on this type of method and on new psychotomimetic drugs is needed.

5. Conditioning Methods

The terminology of conditioning was devised by Pavlov though his rigid methods are very little used to-day except in Russia, and the conditioning techniques now commonly used elsewhere bear very little relationship to those originally put forward by the Pavlovian school.

Conditioning methods are often classified into classical and instrumental methods, and instrumental methods can be further classified into respondent and free-operant types. A classical conditioned response fails to secure reinforcement from the environment; an instrumental response secures reinforcement. A respondent conditioned response is elicited by an environmental stimulus; free-operant behaviour is emitted "spontaneously".

O. *Avoidance Conditioning*

In the search for a convenient conditioning technique, Warner[347], in 1932, described a method of conditioned avoidance that has been widely used. Rats are put individually into a box that has a grid floor and is divided into two compartments by a low fence. The unconditioned stimulus is an electric shock applied through the grid, and by terminating

the shock as soon as the rat scrambles over the fence, the crossing from one compartment to the other is soon established as a consistent response to the shock. Then a buzzer is introduced at a set interval before the shock, and provided this interval is not too long, crossing the barrier becomes established as a conditioned response to the buzzer and shock is avoided. This is not conditioning by Pavlovian standards because the response to the shock is not a naturally-occurring response and also because, as Warner pointed out, the responses to shock and buzzer, although in end-result the same, differ considerably, that to the shock being scrambling and inefficient whereas that to the buzzer is smooth and fast.

Warner's avoidance conditioning technique has been used by very many workers with very little modification[348–352]. Usually the experiments have been done with rats but a similar technique has also been used with monkeys[353,354]. The effect on conditioning of such variables as number of trials given per day and their spacing has been studied[355,356].

In the monkey, reserpine produces a decrement in performance[353,354] as do two other rauwolfia alkaloids, desmethoxyreserpine and rescinnamine[354]. Serotonin, like reserpine, produces a decrement in the performance of rats[357], and so does pentobarbitone: its action has been attributed to ataxia[354]. Azacyclonol has no effect[354]. Benactyzine, under the specialised conditions described by Jacobsen[349], improves the avoidance response. Chlorpromazine retards the acquisition of the conditioned avoidance response in the rat[352] and in trained rats it decreases resistance to extinction of the response[352,358,359]. Control experiments have shown that the effect of chlorpromazine on extinction is not due to a motor deficiency or to accumulation of the drug[359], but to relearning during the chlorpromazine treatment[359]. The effect of chlorpromazine on extinction of avoidance conditioning is so reliable with regard to dosage that it can be used as a bioassay technique[353].

A slightly different method of avoidance conditioning has been used by Courvoisier[324,360,361] and Cook[128,362]. In this method the rat clings to a vertical pole to avoid the shock. Chlorpromazine is said to produce a specific block of the conditioned response (C.R.), that is, it blocks the response to the warning signal but not to the shock[128,324,361,362]. Reserpine also has this effect[361,362]. Meprobamate, however, does not block the C.R. except in ataxic doses[362]. Other substances that do produce a specific block of the C.R. are morphine, serotonin and LSD[362]. Low doses of LSD antagonise the blocking effects of serotonin, reserpine, chlorpromazine and the non-specific action of meprobamate but have no effect on morphine action[362]. Barbitone and methylpentynol produce a non-specific block but pentobarbitone has a more specific effect though only at neurotoxic doses[362]. Pfeiffer and Jenney[363,364] tested the effects of various muscarinic drugs on the avoidance response while protecting the animal from unwanted peripheral effects by using methylatropine nitrate which does not pass the blood-brain barrier. Arecoline, pilocarpine and eserine inhibit the C.R. under these conditions, so the mixture of arecoline and methylatropine was tried clinically and was found to produce a striking lucid interval in schizophrenics.

Another type of avoidance conditioning has been tried in cats and suggested as a possible basis for testing tranquillisers[365]. Cats with a natural tendency to attack mice are conditioned by being given an electric shock every time they attempt to pick up a mouse. This is a very rapid conditioning procedure and an average of only three trials is required to produce mouse avoidance behaviour that persists for several weeks without reinforcement. Under the influence of chlorpromazine, benactyzine, and to a lesser extent meprobamate, cats so trained will continue to pick up the mouse again and again though dropping it when shocked[365]. This procedure is claimed to demonstrate the same relative order of activity of drugs as is obtained by more elaborate conditioning methods, and therefore appears promising.

P. *Free Operant Conditioning*

Avoidance conditioning is not the only type that can be used for testing the effects of centrally-acting drugs. Another that has been adopted successfully to the study of drug effects is the free operant response for a food or water reward. This technique is due to Skinner who conditioned hungry or thirsty rats to obtain pellets of food or drops of water by lever-pressing. The animals will work for a very small reward so that the experiment can be quite long. One of the reasons why this technique has become so important is that the rate of lever-pressing provides a quantitative objective measure of the animal's behaviour, which, under these conditions, is emitted quite spontaneously. In Skinner's early studies on discrimination the correct response was always rewarded, but this meant that the eating of the food pellet interfered with the rate of lever-pressing. Dinsmoor[366] showed that periodic reinforcement is a suitable procedure and that the rate of lever-pressing is a satisfactory index of learning. If the periodic reinforcement is at a fixed interval a temporal discrimination tends to be set up so that the reinforcement schedules must be carefully selected.

It has been shown that the rates of response are markedly affected by the schedule of reward[367]. Dews, using the pecking response of pigeons for a food reward, showed that a fixed ratio of reinforcement, for example, a reward for every fiftieth peck, produced a high and constant rate of pecking, whereas a fixed interval schedule produced a low rate of pecking which increased in rate throughout the interval[368]. The effect of drugs depends very much on the schedule of reinforcement used. For example, pentobarbitone on a fixed interval schedule causes a markedly reduced rate of pecking, but has no effect or even increases the pecks on a fixed ratio schedule[368]. In using this technique for screening purposes Dews[369] suggests that the ratio performance should be used as an indication of the physical capabilities of the animal, and that the effects to be noted on interval schedules are the total number of pecks made and the amount of pausing of more than 10 seconds that occurs. Reserpine has a characteristic effect of producing bursts of high rates of response interspersed with long pauses, and this is interpreted as the release of the pigeon from normally powerful stimulus control and an analogy is drawn between this

effect and the reduction of obsessive compulsive drives[369]. Not only the schedule but also its parameters alter the effects of drugs[370].

Pigeons form especially suitable subjects for studying the effects of drugs on discrimination because their vision is so good. Instead of having a single key to peck the pigeons are faced with two keys that can be illuminated separately and they have to learn to peck the lighted key when an intervening bar is dark and the dark key when the bar is lighted[371]. This provides a sufficiently fine discrimination task, and both correct and incorrect responses can be recorded. Drugs can be even more accurately differentiated by this method. Blough[372] showed that LSD elevates the visual threshold at doses which cause no disturbance in motor performance or discrimination and that although LSD and chlorpromazine both reduce the rate of pecking LSD improves discrimination whereas chlorpromazine impairs it. Pentobarbitone and alcohol, like chlorpromazine, also reduce accuracy but, unlike chlorpromazine, they increase the response rate. This technique probably provides one of the best means available for investigating subtle differences in central actions of drugs, but is, of course, elaborate and time consuming.

The mechanical set-up used in free-operant conditioning can be adapted to include study of avoidance conditioning. For example, lever-pressing behaviour can be maintained by having a high frequency auditory stimulus continuously present unless the lever is pressed to give a silent period[373]. More generally, however, an electric shock is employed as the aversive stimulus. This technique has been used with rats[374] and monkeys[251]. Shocks are given at regular intervals unless the lever is pressed, when the next shock is delayed. Sometimes a warning signal is given before the shock and the shock can then be avoided by responding during the warning period, or escaped by responding during the shock[375]. Reserpine depresses avoidance behaviour under these conditions, but pentobarbitone affects it only slightly or not at all[251].

On theoretical grounds it would seem that the screening of tranquillisers should be based on avoidance rather than on positive-reinforcement conditioning. However, an even better method might be to investigate drug effects on the emotional disturbance or "anxiety" caused by expectation of the unpleasant stimulus. This is a classical, not an instrumental response.

Q. *Conditioned Emotional Responses*

Jacobsen and co-workers[349] have studied the stress, partly an unconditioned and partly a conditioned emotional response, shown by rats subjected to repetitive buzzer-shock sequences. This stress is scored quantitatively by allotting positive marks for muscular tension, immobility, arched back, pilo-erection, etc., and negative marks for relaxed posture, and movements. At the same time conditioned avoidance responses may be studied though the circumstances are not ideal for this purpose. In our experience the scoring system may be much simplified, the score for "tense-immobile" alone showing a satisfactory slope against log-dose of benactyzine. Benactyzine, a known parasympathocolytic drug, was

selected by Jacobsen as the best of a series of compounds tested for their ability to reduce the stress score. It was also shown to increase the number of avoidance responses. This drug was one of the earliest, if not the earliest, to be selected by a planned programme of behavioural research based on an analysis of the type of action on conditioned responses to be expected of a clinically useful tranquilliser, and Jacobsen's contribution to methodology in this field has been outstanding. It is unfortunate that benactyzine, whose actions on conditioned responses, both emotional and avoidance, are more impressive than those of any other drug we have handled, should have made so little progress clinically. This fact suggests that a wider evaluation of benactyzine, particularly perhaps under free-operant conditions, is desirable so that correlation between its laboratory and clinical properties may be reassessed.

On Jacobsen's test a few other compounds such as hyoscine and high doses of reserpine can also "normalise" behaviour[376,377] though reserpine does not increase the number of avoidance responses. Meprobamate has an effect similar to that of benactyzine but at higher doses, and it does not make the animals more lively as benactyzine does[377]. We find meprobamate only feebly active at doses that just fail to cause ataxia. Chlorpromazine, alcohol, the barbiturates and other compounds tested do not improve performance or "normalise" behaviour[376–378].

R. *Conditioned Emotional Responses During Free-operant Behaviour*

A previously neutral stimulus such as a "clicker" signal can acquire an emotional significance by being paired with an electric shock. Usually the "clicker" is sounded for two or three minutes and is followed by a shock. If the conditioned emotional response to this signal is superimposed on a lever-pressing habit, lever-pressing is inhibited during the signal, and by comparing the rates of response before and during the signal a quantitative measure of the conditioned emotional response can be obtained[379,380]. Usually a variable interval schedule is used because this gives a high, constant rate of response. It is convenient to record the response cumulatively on a slowly-moving kymograph; the control period can then be seen as a steeply sloping line and the conditioned emotional period as a nearly horizontal one. The effects of drugs can then be noted both on the slope of the control part of the graph and on the difference in slope between emotional and non-emotional responses. As many drugs cause a change in overall rate of responding[381], Brady[382] expresses his results in terms of an "inflection ratio". The inflection ratio is the ratio of the difference between response rates during and before the stimulus to the rate before the stimulus. Complete cessation of lever-pressing during the conditioned emotional periods gives a ratio of -1 and a constant rate of responding before and during these periods shows as a ratio of 0.

Reserpine is the drug that has been most widely tested by this technique. Brady reported that reserpine reduces the overall rate of lever-pressing by rats and monkeys but abolishes the distinction between "clicker" and "non-clicker" periods[381,382]. Weiskrantz and Wilson[383], supplementing

the objective measurement with observational ratings, and testing monkeys for acquisition, extinction and retention of the conditioned emotional response also concluded that the reserpine-treated animals showed considerably less disturbance during the emotional stimulus than did saline controls. However, Stein[384], using slightly different techniques, found that rats drugged with reserpine almost to the point of inactivity, still acquired an intense conditioned emotional response. The discrepancy between the results is unexplained. Brady[382] has studied the point of action of reserpine on his conditioned emotional response by introducing a behavioural control. Rats are trained on a schedule identical to that described for emotional conditioning except that the electric shock is only given if the lever is pressed during the "clicker" signal. Under these conditions reserpine does not restore lever-pressing during the signal. Its action can therefore not involve failure to hear the signal or indifference to the shock, and may reasonably be supposed to involve reduction of anxiety associated with an expectation of unavoidable shock. Chlorpromazine does not block the conditioned emotional response but weakens it so that the behaviour of treated animals is like that of animals conditioned with weaker shocks[385]. Chlorpromazine blocks extinction of the response[385]. Meprobamate does not block the conditioned emotional response though observationally the rats are calmer and more relaxed[385]. Morphine has been shown to restore bar-pressing inhibited by anticipation of shock in in rats, and the effect is proportional to the dose until doses causing confusion are reached, so the conditioned emotional response superimposed on bar-pressing in response to positive reinforcement has been proposed as a suitable test for analgesics[386–388].

The conditioned emotional methods described in this section are among the most impressive yet proposed and further work with them must be eagerly awaited.

S. *Conflict Neurosis*

Another study has developed from Pavlov's work on conditioning, that of "experimental neurosis". In Pavlov's experiments the neurotic state was brought about by forcing the animal to make increasingly fine discriminations until discrimination became impossible. A technique has been described for inducing this type of neurosis in the rat by training it to discriminate between light signals securing on the one hand positive and on the other negative reinforcement in the Skinner box[389–391]. It appears not to have been used for drug evaluation. The essential element in production of neurotic behaviour by such methods is not the increasing difficulty of discrimination *per se*, but the associated conflict. Masserman[392] produced approach-avoidance conflict in cats by training them to secure food at a bell-signal by switch-pressing, and then intermittently superimposing an aversive air-blast stimulus. This approach-avoidance conflict gave rise to a form of behaviour designated as "neurotic". The cat showed signs of fear and tension, feeding behaviour was disrupted, displacement activities appeared, and some of the behavioural abnormalities persisted out of the experimental situation. Masserman[392]

studied means of making the neurosis more severe and long-lasting, such as forcing the animal to the food box by reducing the size of the cage, and he also studied possible methods of curing the neurosis; methods such as rest and the display of a normal cat. The effect of drugs on this type of conflict behaviour in cats has been studied by Jacobsen and co-workers[377,393,394]. Of the drugs tested, benactyzine gave the best results[377,393,394] though meprobamate also had a considerable normalising effect but only at ataxic doses[377]. Alcohol also produced some improvement in behaviour but at ataxic doses[377,393,394]. Chlorpromazine[377,393,394] and reserpine[377] were ineffective and it was suggested that this is possibly because of the stupor they caused which masked any return of feeding to normal. This is an interesting method but it requires further study: the activity of tranquillisers does not correlate well with their usefulness in clinical psychoneurosis.

T. *Motivation*

Any conditioning procedure involves the use of appropriate motivation. Avoidance conditioning makes use of aversion to noxious stimuli such as electric shock; free-operant conditioning makes use of appetitive drives; and the conditioned emotional techniques of Brady and Hunt and approach-avoidance conflict methods use both types of motivation. In interpreting the effects of drugs on these tests, therefore, it is important to consider the effects of the drugs on motivation. As Miller has pointed out[395] various methods have been described and used for measuring "fear" or "emotional" responses without there being much work on the relationship between the measures obtained by such different techniques. There has been some work on the differences in drug effects when different motivations are used during free-operant conditioning[396,397]. In tranquilliser work it is particularly important to determine drug effects on appetitive as well as on fear drives. For example, tetraethylammonium was reported to reduce fear in rats but experiments by Brady[398] showed that this drug depressed hunger- or thirst-motivated behaviour as well as that due to fear.

But the effects of psychotropic drugs on motivation are important in their own right. In a situation of conflict, either experimentally produced or as found clinically, reduction in intensity of one of the conflicting drives may well be a satisfactory means of resolving the conflict. Miller[395,399] has emphasised the need for using a number of diverse techniques for evaluating the effects of drugs on motivation. He[399] cites the work of Conger[400] on the effect of alcohol on approach-avoidance conflict in rats. Hungry rats are discouraged from running down an alley for food by means of electric shocks, the intensity of which is increased until the rat refuses to go to the food box. After alcohol administration the animal resumes eating at the box. This effect was shown to be due to a reduction in the fear of shock rather than to an increase in hunger by testing the strength of each motivation separately. However, it was also found that altering the width of illumination of the alley produced the same changes as injection of alcohol, so that the effect of the alcohol might simply be

due to change in perception of the situation. Carefully devised experiments permit analysis of such complex effects, which exemplify the need for caution in interpreting the actions of tranquillising drugs in any single experimental situation.

U. *Neurophysiology and Conditioning*

Neurophysiological evidence indicates that there are two stages in conditioning, the first involving more general and diffuse effects in the brain, and the second more specific and localised effects[401,402]. An intermediate stage has also been described in which the visual cortical rhythm tends to follow the frequency of the intermittent photic stimulation used as the unconditioned stimulus, but during the presentation of the conditioned stimulus[402]. Recordings from the reticular formation suggest that it is of great importance in the elaboration of the conditioning processes[402,403] and therefore that drugs which affect the activity of this structure must also affect the expression and acquisition of conditioned responses[403]. The work on and the evidence for the action of tranquillisers on the midbrain reticular formation is reviewed in the section on neurophysiology (M). An investigation on the differential action of chlorpromazine on reflexes conditioned to central and peripheral stimulation[404] showed that this drug produced a greater depression of the peripherally-induced conditioned response and it was suggested that this differential action might be due to the action of chlorpromazine on the arousal system.

As tranquillisers affect conditioning, it is interesting to note the effects on conditioning of various procedures used clinically in the treatment of mental disorder. Gellhorn[348,405] found that leptazol, insulin hypoglycaemia and electrically-induced convulsions cause recovery of an inhibited conditioned avoidance response in rats. A similar effect is produced by benactyzine[406]. Hunt and Brady have shown that a series of electro-convulsive shocks will diminish or eliminate a conditioned emotional response[380,407,408] in the Skinner box and that if the convulsions are prevented by giving the electroshock under ether anaesthesia the effect of the electroshock is not nearly so marked[409]. Later experiments showed that audiogenic convulsions also eliminate the conditioned emotional response whereas auditory stimulation of animals that are not susceptible to the convulsions has no effect[410]. Neither auditory stimulation nor audiogenic seizures destroy memory as indicated by the number of errors made in running a maze[411]. Therefore it is possible that convulsions affect emotional behaviour selectively, though it is probable that care must be taken in applying results on the so-called emotional conditioning of laboratory animals to the treatment of emotional disorders clinically.

V. *Self-stimulation of the Brain*

Delgado observed that animals showed anxiety in a situation which had been previously associated with electrical stimulation of parts of the brain[412,413] and this anxiety could be used to motivate avoidance and operant conditioning[414]. Olds and Milner[415,416] found that stimulation of other parts of the brain acted as a positive reinforcement or "reward" effective in operant conditioning and in maze running. There is some

suggestion that increase in amperage of the stimulating current increases the reward value of the stimulation[417]. The reinforcing value of the stimulation and effects of drugs can be assessed by the rate of bar-pressing in a Skinner-box[418]. Drug effects vary with different electrode sites. Chlorpromazine and reserpine depress response rates for hypothalamic stimulation but have less effect when the electrodes are implanted in the septal area[418]. Azacyclonol depresses hypothalamic and septal stimulation rates but its effects are only of short duration[419]. Pentobarbitone has little effect[419]. Amphetamine increases rates of responding but pipradrol only does so with laterally placed electrodes[419] and LSD is a non-selective depressant[420].

Some electrode positions elicit a curious alternation of behaviour: when rats are trained to make one response to turn the stimulation on and another to turn it off, they alternate repeatedly between the two responses[399]. It was found[399] that methamphetamine and chlorpromazine both depress the response rates but whereas the main effect of methamphetamine is to delay turning the stimulation off, chlorpromazine causes a large increase in the time before the stimulation is turned on.

These interesting and ingenious techniques should provide information about both the motivational effects of drugs and their sites of action.

Discussion

We have already commented briefly on most of the methods that we have listed and classified, and now propose to discuss only their selection for screening purposes, and the directions in which improvement seems necessary or likely.

It is probable that at present no single method can confidently be used for the evaluation of novel drugs, unless prediction of their properties is unusually precise, and most workers in this field use a battery of tests. Tripod's interesting paper[63] provides an excellent illustration of the way in which such a battery can be used to discover the spectrum of activity of tranquillisers, sedatives, hypnotics and anticonvulsants. Obviously more reliance must be placed on some tests than on others and, if random screening is being used, economy will usually require that some sequential design be adopted. More informed searches for new drugs will probably require test patterns that differ according to the types of drug being studied: for example, meprobamate and chlorpromazine have very different activity spectra. Nevertheless, most useful test patterns will probably include methods from all, or nearly all, of the five classes we have reviewed. They should certainly include some of the more specific methods, and, if possible, some of the methods that are known, or believed, to correlate best with clinical usefulness.

It is this last respect in which improvement is most necessary. All of us should like to have methods of inducing in animals recognisable mental disorders simulating the main clinical disorders. We do not think that such experimental neuroses as have been described are completely satisfactory, and the problem of setting up model psychoses is almost wholly unsolved.

It is, of course, debatable whether the non-human brain, even of a primate, is sufficiently advanced to display that complexity of irrational behaviour which characterises psychosis. Rodents and carnivores are probably still less promising experimental subjects. Nevertheless there is much evidence that the neurophysiological events underlying the most complex human behaviour, rational or irrational, are unlikely to differ in kind from those of more primitive creatures. We think that advance is most likely to result from increased knowledge of the links between behaviour and such neurophysiological events. It is probable that much of this knowledge will be obtained from studies of tranquillisers in unified programmes of behavioural and neurophysiological research, and that such knowledge will allow the design of still better tranquillisers, and their recognition by improved methods.

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RESEARCH PAPERS

THE ACTIONS OF DIGITALIS LEAF PREPARATIONS AND OF CARDIAC GLYCOSIDES ON THE ISOLATED RIGHT VENTRICLE OF THE GUINEA PIG

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A method is described for recording kymographically the systolic contraction and the resting length of the electrically stimulated, isolated right ventricle of the guinea pig. Two samples of *Digitalis purpurea* leaf and a sample of *Digitalis lanata* have been assayed in terms of the Third International Standard of *D. purpurea* using metameters dependent on changes in the systolic contractions and resting length of the muscle and the time elapsing before the changes occur. In a similar manner digitoxin and ouabain have been assayed in terms of digoxin. The results are compared with those obtained by slow intravenous infusion of the preparations into guinea pigs. The potencies derived from the two methods are in close agreement in the case of the digitalis leaf preparations. Digitoxin, however, is more active than digoxin on the ventricle preparation irrespective of the metameter used and less active by the slow infusion method, while ouabain has the same activity as digoxin on the ventricle and twice the activity by the infusion method. The results for ouabain on the ventricle using the different metameters, are homogenous, those for digitoxin are not. There is little difference between the general actions of the three glycosides on the ventricle but each glycoside is capable of producing different effects on systolic contraction and resting length depending on the dose employed. The possible mechanisms responsible for these effects are discussed.

The relationship between the therapeutic and toxic actions of cardiac glycosides has long been a problem in animal and clinical pharmacology. While the toxic properties of these substances are easily defined their therapeutic properties, especially in animal experiments, are less easily recognised and open to a variety of interpretations.

Cardiac glycosides may act via the nervous system of the heart for example by inducing a return to normal rate in auricular fibrillation, or they may act directly on the heart muscle itself inducing a more efficient utilisation of energy in congestive heart failure.

Several workers have used isolated portions of the heart to study the direct actions of the cardiac glycosides on muscle. Trevan and Boock[1] first described the use of isolated rabbit auricles for the estimation of the activity of digitalis leaf preparations; the preparations were allowed to act on the auricles for 4 minutes and the increase in amplitude of contraction was recorded. Halpern and others[2] used the rabbit's left auricle only and calculated the increase in work done per second against a given load after the addition of convallotoxin to the organ bath containing the auricle.

Cattell and Gold[3,4] registered photographically the increase in amplitude of the isometric contractions induced by glycosides on the electrically

stimulated, isolated papillary muscle of the right ventricle of the cat. In one series of muscle preparations they determined the threshold concentration of glycoside resulting in an augmentation of amplitude in 50 per cent of the preparations, which they defined as the "therapeutic factor", and in another series they determined for given doses of the glycoside the time taken for the amplitude to decline to 80 per cent of its maximum augmented contraction. This was defined as the "toxic factor".

White and Salter[5], Sciarini, Ackerman and Salter[6], White, Belford and Salter[7], and Ipsen and White[8] using the same preparation in a manner similar to that of Cattell and Gold[3,4] have described statistical procedures which have permitted the evaluation of potency of one glycoside in terms of another. In one series of experiments they determined the minimum dose of glycoside which showed a positive inotropic effect, and in another the dose of one glycoside which could be substituted for another glycoside isodynamically at a given stage where the increase of the amplitude of contraction after substitution continued at the same rate as before. In a final series the dose required to produce toxic effects (decline in amplitude) was determined. The authors define the first two metameters as measurements of therapeutic activity.

Luisada and Diamond[9] studied the action of cardiac glycosides on diastolic and resting length of isolated ventricle strips, papillary muscles and atrial strips of cats and dogs and showed that whereas all glycosides examined induced an isotonic decrease in resting length there was a number which, at various concentrations of glycoside, induced an increase in resting length.

The increase in amplitude of the isotonic contractions of the electrically driven isolated right ventricle of the rat induced by ouabain has been observed by McDowall and Zayat[10,11].

Wedd and Blair[12] found that glycosides were capable of producing a shortening of the QT segment of an electrogram obtained from an electrically stimulated strip of turtle ventricle, and this shortening, which reflected an increased rate of recovery from systolic contraction, was not related to the mechanical contraction of the muscle.

In the present paper a study has been made of the influence of digitalis leaf preparations and of cardiac glycosides on the resting length and amplitude of contraction of the electrically stimulated isolated right ventricle of the guinea pig. Only one dose of the drug is allowed to act on each preparation so as to avoid the assumptions of previous workers regarding rates of action and metabolism of the glycosides.

The results are compared with those obtained for the same preparation administered by slow intravenous infusion to the guinea pig according to the method of biological assay described in the B.P., 1958, p. 943.

Method

A male albino guinea pig of 400–500 g. in body weight is killed by a sharp blow on the head. The heart is quickly removed and placed on a nylon-foam pad soaked in warm, oxygenated salt solution. The entire outer wall of the right ventricle is cut from the heart. The apex of the

ventricle and the muscle adjacent to it is tied to the glass hook in which is sealed a platinum electrode. A cotton thread is tied around the stump of the pulmonary artery and the ventricle suspended to form a triangular wedge as shown in Figure 1. The organ bath is a modification of that described by Green, Riley and White[13] where the nozzle through which the oxygen enters the fluid and the angle of the two arms connecting the two compartments lead to a greater efficiency of circulation and the prevention of static regions in the more remote parts of the fluid. The bath thus permits the muscle to be oxygenated without agitation, and prevents substances which would form a froth from producing a seal over the top of the fluid in the compartment in which the muscle is housed.

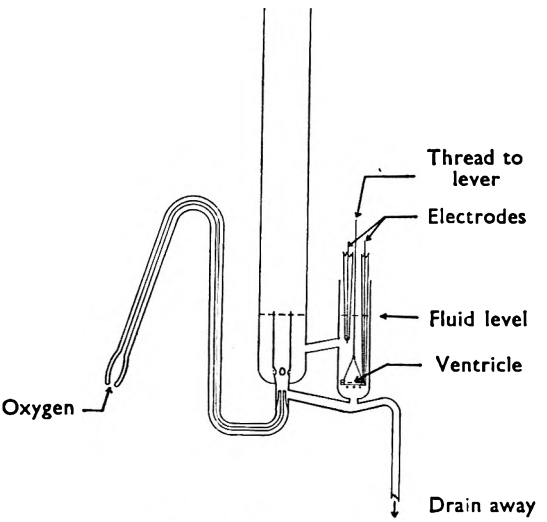

FIG. 1. Isolated organ bath

The platinum electrodes are 0·020 inches in diameter. The free electrode, 2 mm. in length, is placed in the chamber close to the muscle. The organ bath contains 68 ml. (34 ml. in one series of experiments) of salt solution of the following composition: NaCl 9·0, KCl 0·42, $CaCl_2$ 2·015, $MgCl_2$ 0·0025, Glucose 0·5, and $NaHCO_3$ 0·5 g., in one litre of distilled water. The $CaCl_2$ is added last as 1 ml. of a 20·15 per cent (w/v) solution, otherwise precipitation may occur. The final pH is 7·8.

The excess of $CaCl_2$ in this solution produces a very large augmentation of the amplitude of contraction of the muscle thus enabling the contractions to be recorded kymographically, obviating the need for expensive optical cameras and enabling one to observe the progress of the experiment without awaiting a developed photograph before the results can be observed.

The solution is oxygenated with pure oxygen at the rate of 200 ml. per minute. The apparatus is immersed in a water bath maintained at $35 \pm 0{\cdot}2°$. The muscle is stimulated from a square-wave stimulator as follows: duration 5 msec., volts 50, rate 2 per minute. The slow rate of stimulation allows a clear record of the resting length of the muscle to be made, as well as producing large contractions which are recorded isotonically with a spring loaded lever, the long arm of which is made of balsawood weighing 1 g. The fine nickel-silver spring coil is adjusted so that when a 6 g. weight is suspended at the point of attachment of the

cotton, the lever is horizontal. The lever gives a 32-fold magnification of the actual muscular contractions.

The muscle is allowed to settle in the bath for up to 3 hours before any drug is added since during this time the amplitude of contraction often becomes larger.

Preparations Examined

Digitalis leaf preparations. Two samples of *Digitalis purpurea* leaf, one of high and one of low potency as determined by the B.P. 1958 method of assay in guinea pigs, one sample of powdered *Digitalis lanata* leaf and the International Standard of Digitalis Leaf (1949) were extracted with 80 per cent (v/v) ethanol at room temperature according to the B.P. 1958 procedure. The two test extracts of *D. purpurea* leaf were then freeze-dried and taken up again in a similar volume of 80 per cent (v/v) ethanol so that the amount of alcohol administered was constant irrespective of the dose of digitalis. The tincture of the sample of *D. lanata* was diluted with 80 per cent (v/v) ethanol for the same reason. The highest dose of the standard preparation was 0·4 ml. (40 mg. of leaf).

Cardiac glycosides. Digoxin, digitoxin (assaying at 1,000 I.U./g. by the guinea pig infusion method against the International Standard of Digitalis Leaf), and ouabain were each used as a solution of 1 mg./ml. in 70 per cent (v/v) ethanol.

In any test the amount of alcohol administered to a given ventricle preparation was kept constant by using as diluent for lower doses the appropriate concentration of solvent.

Results

Assay of Digitalis Leaf Preparations on the Guinea Pig Ventricle

The three extracts of digitalis leaf were assayed against the International Standard using a randomised block design with three doses of each extract

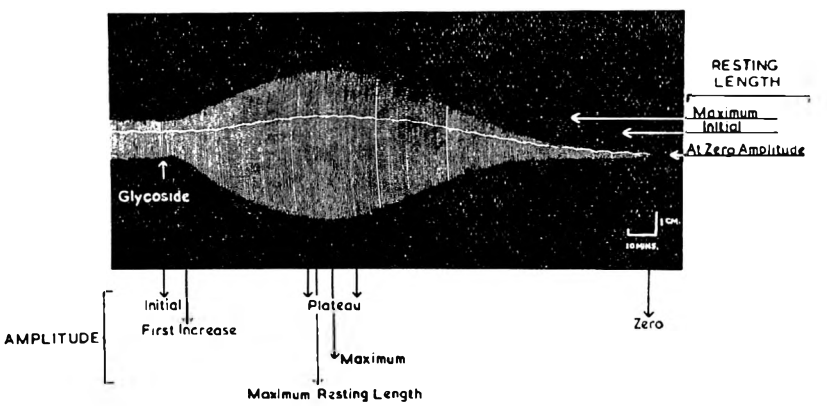

FIG. 2. Kymograph tracing of the electrically stimulated isolated right ventricle of the guinea pig. The arrows indicate the changes in systolic contraction and resting length induced by a cardiac glycoside.

in a dose ratio of 2 : 1. Each dose was administered twice to each bath and replicated in three different baths. Only one dose was administered to any ventricle. In this way each dose was tested six times. There were 72 preparations in all.

Figure 2 shows a typical kymograph record and the points marked on the tracing indicate the various metameters used to assess activity. The tracing registered above the resting length is due to a recoil of the spring following contraction of the muscle. A study was made of the changes in resting length and systolic contraction of the ventricle induced by the digitalis extracts, by conducting analyses of variance on the following metameters against $\log_2$ dose.

The $\log_{10}$ time to (*a*) the first increase of amplitude, taken as the first point at which the amplitude increases 1 mm. over the initial amplitude and thereafter continues to increase, (*b*) the beginning of plateau of maximum contraction, taken as the time to reach 95 per cent of the maximum contraction, (*c*) the maximum amplitude, (*d*) the duration of plateau, that is, the time embraced by the 95 per cent limits on maximum contraction, (*e*) the maximum length, that is the maximum relaxation of the muscle relative to initial length, and (*f*) the zero amplitude. Other metameters included (*g*) the $\log_{10}$ rate of increase of amplitude obtained by dividing the increase of amplitude from the first observable increase to the maximum amplitude by the time during which this change takes place, (*h*) the maximum resting length of the muscle minus initial resting length, (*i*) the initial resting length minus resting length at zero amplitude,

TABLE I

ASSAY OF DIGITALIS LEAF PREPARATIONS ON THE ISOLATED RIGHT VENTRICLE OF THE GUINEA PIG

POTENCY AS A PERCENTAGE OF THE 3RD INTERNATIONAL STANDARD

Sample	*Digitalis lanata*		*Digitalis purpurea*				
	B		C		D		
Assumed potency as percentage of standard	200		33·3		66·7		
Metameter	Mean potency	Limits of error (P = 0·95)	Mean potency	Limits of error (P = 0·95)	Mean potency	Limits of error (P = 0·95)	$\frac{b^2}{s^2[']^2}$
$\log_{10}$ time to/of							
(*a*) First increase in amplitude	222	174–283	33	31–36	67	52–85	40·9
(*b*) Beginning of plateau	227*	—	42†	—	71	61–82	108·4
(*c*) Maximum amplitude	209*	—	41†	—	69	61–78	164·0
(*d*) Duration of plateau	200†	—	42†	—	74	65–85	137·7
(*f*) Zero amplitude	188†	—	40*	—	71	64–80	202·2
(*g*) $\log_{10}$ rate of increase of amplitude (mm./min.)	177	145–216	34	28–41	65	54–80	61·4
(*h*) Maximum resting length—initial resting length, mm.	280*	—	57*	—	82	65–108	44·4
(*i*) Initial resting length—resting length at zero amplitude, mm.	243*	—	55*	—	72†	—	(130·4)
(*k*) Maximum increase in amplitude corrected for initial amplitude	334	106–2351	70	24–671	111	36–784	2·2

* Significant non-parallelism between test and standard ($P \leqslant 0{\cdot}01$).
† Significant non-parallelism between test and standard ($0{\cdot}01 < P \leqslant 0{\cdot}05$).

(*j*) the maximum increase of amplitude corrected for the initial amplitude in the phase of increase of maximum amplitude with increase in dose, and (*k*) the maximum increase of amplitude corrected for the initial amplitude in the phase of decrease of maximum with increase in dose.

The residual error after removal of sums of squares attributal to baths, doses, and the baths × doses interaction was used to calculate fiducial limits of error for each assay. The analyses also included tests for linearity, parallelism and quadratic components and the interactions of each with drugs and baths.

Table I shows the results obtained for each sample of digitalis leaf assayed against the International Standard using the various metameters. The column on the extreme right of the Table gives the index of precision, b^2/s^2I^2, where b = slope in $\log_2$ of assay, s^2 = variance attributable to error, and I is the correction factor for conversion of $\log_2$ to $\log_{10}$ with respect to the coefficients used in the analysis[14].

In the case of the sample of *D. lanata* only three estimates are valid, the remainder being invalid in virtue of statistical non-parallelism of the dose-reponse curves. Nevertheless the estimates of potency derived have been included, which, although strictly invalid, show the reproducibility of some of the estimates, particularly with those metameters dependent on time. A similar finding holds with the sample of *D. purpurea* leaf of low potency.

The estimates for the sample of *D. purpurea* leaf which has a potency closest to that of the Standard preparation are valid, apart from (*i*), the decrease in resting length. The index of precision is highest when time to zero amplitude is used as metameter, and least when the maximum amplitude corrected for the initial amplitude is used. In general the estimates dependent on time measurements are lower than those based upon the actual effects produced but the non-parallelism or low index of precision of the latter make a firm conclusion impossible.

TABLE II

ASSAYS OF THE DIGITALIS LEAF SAMPLES IN TERMS OF THE 3RD STANDARD FOR DIGITALIS BY SLOW INFUSION INTO GUINEA PIGS

Leaf	Sample	Potency as a percentage of the Standard	Limits of error (P = 0·95) as a percentage of the Standard
D. lanata	B	180·1	153·9–210·6
D. purpurea	C	32·4	23·9– 43·8
D. purpurea	D	67·9	64·7– 71·4

Table II gives the results obtained on the same digitalis leaf preparations assayed by slow intravenous injection in guinea pigs according to the method given in the B.P. 1958. The results are in close agreement to those shown in Table I, the best comparison being given by the $\log_{10}$ rate of increase of amplitude in the case of the sample of *D. lanata*, by the $\log_{10}$ time to first increase in amplitude, and $\log_{10}$ rate of increase in amplitude for the sample of *D. purpurea* of low potency, and by all the metameters involving time measurements in the case of the sample of *D. purpurea* of higher potency.

Investigation of the Actions of Three Cardiac Glycosides on the Right Ventricle Preparation of the Guinea Pig

The effects of digoxin on the isolated ventricle preparation were investigated by injecting into a 34-ml. bath all doses from 2·5 to 160 μg. in a geometric ratio of 2·0. Only one dose was allowed to act on each preparation; there were six preparations at each dose level. Using the same range of doses the effects of digitoxin and ouabain were also studied.

Figure 3 shows the maximum amplitude corrected for initial amplitude plotted against $\log_2$ dose for each glycoside. With each glycoside there are two distinct effects, namely a phase where an increase in dose produces an increase in the maximum amplitude, and a later phase where larger doses produce a decrease in maximum amplitude with an increase in dose.

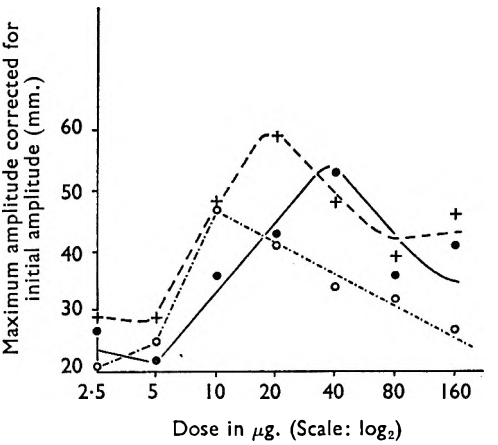

Fig. 3. The influence of the dose of cardiac glycoside on the maximum amplitude of systolic contraction of the electrically stimulated isolated right ventricle of the guinea pig.
●—● Digoxin. ○–·–·–○ Digitoxin.
+ — — + Ouabain.

Figure 4 shows for digoxin the $\log_{10}$ time for various metameters plotted against $\log_2$ dose. Between 2·5 and 20 μg. the time to zero amplitude remains constant, and between 20 and 160 μg. declines linearly with increase of dose. In the case of $\log_{10}$ time to maximum resting length, between 5 and 20 μg. there is a slight but significant decrease in the time taken to reach this point; thereafter, between 20 and 80 μg. there is a linear decline. With the three metameters $\log_{10}$ time to maximum amplitude, to beginning of plateau, and duration of plateau, the time remains constant up to 20 μg. and thereafter decreases linearly parallel to the curves for the other two metameters. In the case of $\log_{10}$ time to first increase in amplitude there is a linear decrease between 2·5 and 40 μg., thereafter it remains constant. The $\log_{10}$ rate of increase in amplitude does not alter significantly between 2·5 and 20 μg., but between 20 and 160 μg. the rate increases linearly.

With digitoxin and ouabain a similar picture obtains to that shown in Figure 3 for digoxin except that the point of inflexion for digitoxin occurs at a dose of 10 μg., and its $\log_{10}$ rate of increase of amplitude is linear between 2·5 and 160 μg. Also with doses above 20 μg. neither digitoxin nor ouabain produce an increase in resting length of the ventricle. Furthermore whilst for digoxin the $\log_{10}$ time to the first increase in amplitude decreases linearly with increase of dose the $\log_{10}$ time to this effect for digitoxin and ouabain decreases in a non-linear manner.

Figure 5*a* shows the changes in resting length of the ventricle plotted against $\log_2$ dose of glycoside. With digoxin between 5 and 80 μg. the increase in resting length varies inversely with increase in dose; the same holds for digitoxin between 2·5 and 40 μg., but for ouabain between 2·5 and 10 μg. the increase in resting length varies proportionally with increase in dose; above 20 μg. ouabain fails to produce an increase in resting length.

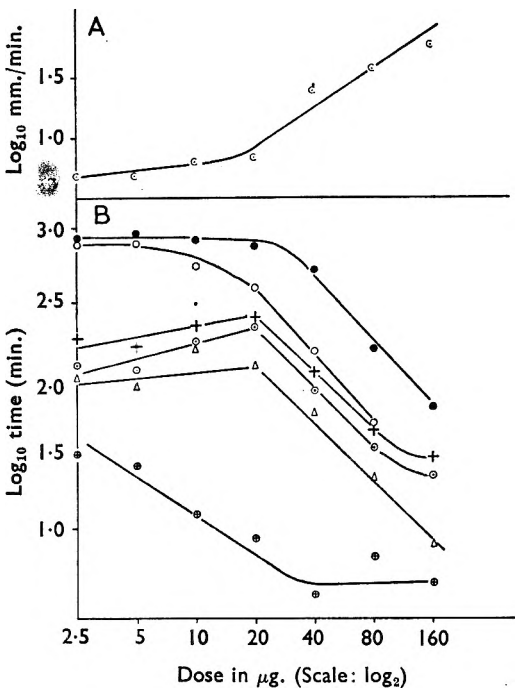

FIG. 4. The influence of the dose of digoxin on the time elapsing before various changes occur in the amplitude of systolic contraction and resting length of the electrically stimulated isolated right ventricle of the guinea pig.
A. Rate of increase of amp. × 100.
B. ●—● Zero amp.
○—○ Maximum resting length.
+—+ Maximum amp.
⊙—⊙ Beginning of plateau.
△—△ Duration of plateau.
⊕—⊕ First increase in amp.

Figure 5*b* shows the decrease in resting length at zero amplitude relative to the resting length initially. With ouabain in doses up to 10 μg., and digoxin and digitoxin up to 20 μg., the action causing an increase in resting length predominates so that, although contracture takes place, the resting length at zero amplitude is greater than that initially. Larger doses induce little or no relaxation of the resting length so that there is an overall shortening of the muscle.

In Table III are shown the potencies of digitoxin and ouabain calculated where possible in terms of digoxin for the various metameters in a manner similar to that described for Table I. Apart from $\log_{10}$ time to maximum resting length all the estimates involving time give greater indexes of precision than those with metameters involving measurement of the effects produced. Once again the index of precision is highest for $\log_{10}$ time to zero amplitude. Using this metameter digitoxin is 2·24 and ouabain 1·11 times as active as digoxin.

In the case of digitoxin, metameters *b*–*f* inclusive yield results which are heterogenous ($\chi^2 = 14{\cdot}222$, d.f. $= 4$, P $< 0{\cdot}01$), which is shown to be due to metameter *f*, i.e., $\log_{10}$ time to zero amplitude (χ^2(*f* v. *b* to *d*) $= 9{\cdot}307$, d.f. $= 1$, P $< 0{\cdot}01$). The metameters *g*–*j*, based on actual effects, yield results which are heterogenous ($\chi^2 = 8{\cdot}276$, d.f. $= 3$,

TABLE III

RELATIVE POTENCY OF DIGITOXIN AND OUABAIN IN TERMS OF DIGOXIN ASSAYED ON THE ISOLATED RIGHT VENTRICLE OF THE GUINEA PIG. DIGOXIN = 1

Metameter	Digitoxin†		Ouabain		
	Mean potency	Limits of error (P = 0·95)	Mean potency	Limits of error (P = 0·95)	$\frac{b^2}{s^2T^2}$
Log_{10} time to/of					
(*a*) First increase in amplitude	C	—	C	—	—
(*b*) Beginning of plateau	1·68	1·35–2·08	1·44	1·16–1·78	58·6
(*c*) Maximum amplitude	1·67	1·35–2·06	1·42	1·16–1·74	61·5
(*d*) Duration of plateau	1·54	1·18–2·00	1·08	0·84–1·39	37·5
(*e*) Maximum length	3·84	2·02–12·62	0·85	0·40–1·56	7·4
(*f*) Zero amplitude	2·24	1·94–2·59	1·11	0·96–1·28	177·1
(*g*) Log_{10} rate of increase of amplitude (mm./min.)	1·24	0·92–1·66	1·52	1·12–2·06	28·8
(*h*) Maximum resting length—initial resting length, mm.	2·30	1·42–3·92	*	—	11·9
(*i*) Initial resting length—resting length at zero amplitude, mm.	2·18	1·46–3·28	1·55	1·01–2·29	16·2
(*j*) Maximum amplitude corrected for initial amplitude (phase of increase/log dose)	1·58	1·07–2·25	1·39	0·97–2·07	19·4
(*k*) Maximum amplitude corrected for initial amplitude (phase of decrease/log dose)	NS	—	NS	—	—

† = Values should be multiplied by a factor of 1·4 for comparison with results on pure digitoxin.
C = Significant curvature of dose response lines.
* = Non-parallelism ($P < 0·001$).
NS = No slope.

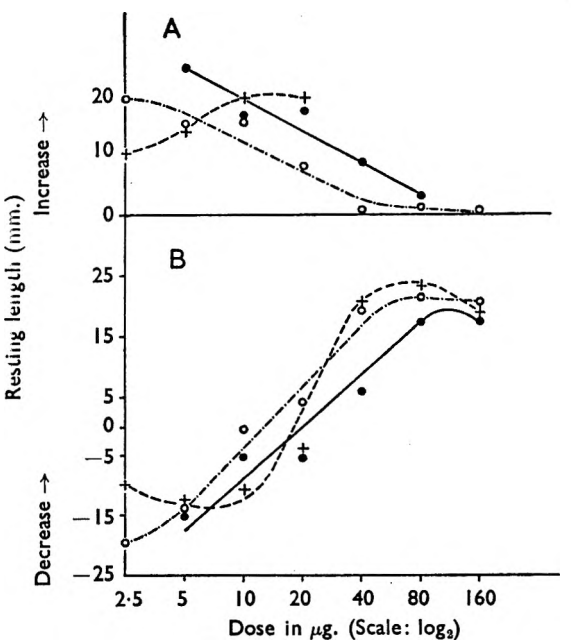

FIG. 5. The influence of the dose of cardiac glycoside on maximum resting length and resting length at zero amplitude of the electrically stimulated isolated right ventricle of the guinea pig. A, increase, B, decrease in resting length.

●—● Digoxin. ○-·-·-○ Digitoxin.
+ - - - + Ouabain.

$P < 0.05$). If, however, the results from metameter *f* are excluded from the rest, the results are homogenous ($\chi^2 = 13.233$, d.f. = 7, $P > 0.05$), and the mean potency ratio is 1·66. The results based solely upon time measurements, yield a statistical weight of 2331 for five metameters which is considerably greater than the weight of 673 attributable to the four metameters involving measurements of the effects produced.

In the case of ouabain none of the estimates is significantly different from each other. ($\chi^2 = 12.724$ for d.f. = 7, $P > 0.05$). The overall mean potency for ouabain is 1·26 times that of digoxin.

TABLE IV

THE MINIMAL LETHAL DOSE FOR DIGOXIN, DIGITOXIN AND OUABAIN DETERMINED BY SLOW INTRAVENOUS INFUSION INTO GUINEA PIGS

Glycoside	Minimal lethal dose, mg./kg.		Activity ratio	Activity ratio (digitoxin corrected on basis of pure glycoside, 1·4 I.U./mg.)
	Mean	Limits of error (P = 0·95)		
Digoxin	0·79	0·73–0·86	1·00	1·00
Digitoxin	1·54	1·36–1·73	0·51	0·71
Ouabain	0·39	0·36–0·43	2·03	2·03

Table IV gives the potencies relative to digoxin for digitoxin and ouabain as determined by slow infusion into guinea pigs. The result for digitoxin is in exact agreement to that of Brindle, Rigby and Sharma[15] (1·54 mg./kg.) using guinea pigs. Sellwood[16] has observed that samples of digitoxin may vary in potency from 655 to 1401 I.U./g. The potency of the sample used by Brindle, Rigby and Sharma[15] was 930 I.U./g.; that of the digitoxin used in the present study is 1000 I.U./g. which is close to the value of Brindle's sample. Since the sample examined by Sellwood contained 1·4 I.U./mg. the figures quoted for digitoxin should be multiplied by 1·4 so that the results can be compared with those of other workers using the U.S.P. Standard of digitoxin. The corrected ratio of activity for digitoxin to digoxin is that shown in the extreme right-hand column of Table IV. A similar correction should be applied to the results for digitoxin in Table III.

Table V is a compilation of results obtained from the literature of the activities of digitoxin and ouabain calculated in terms of digoxin. There is considerable variation in the activity ratios depending on the animal species and method used in the assessment of cardiac activity. The ratios shown in Table IV are in excellent agreement with those obtained in cats and pigeons[17–19] using the same assay technique. The lower activity ratio of digitoxin in the dog heart lung preparation[20] is the same as the uncorrected ratio shown in Table IV and may reflect the use of a less pure sample of digitoxin. The two results[7,8] obtained on the cat papillary muscle for ouabain are in good agreement with results from slow infusion assays in cats, pigeons and guinea pigs, whilst only one result is in agreement for digitoxin. Cattell and Gold[4] found ouabain and digitoxin to be equiactive on the papillary muscle preparation of the cat. In frogs[17,19] there is considerable variation in the activity ratios depending upon the method of assay. The ratio of activity for digitoxin using changes in

resting length of isolated ventricle strips and papillary muscles of the cat and dog heart[9] agree closely with the estimates given in Table III for the right ventricle of the guinea pig, though the high ratio of 67·0 for ouabain quoted in Table V for potencies based on the decrease of resting length is vastly different from that quoted in Table III.

TABLE V

THE ASSAY OF DIGITOXIN AND OUABAIN IN TERMS OF DIGOXIN BY VARIOUS INVESTIGATORS

Ref. No.	Method	Activity ratio, digoxin = 1·00		
		Digitoxin	Ouabain	
17	Cat infusion—Lethal dose	0·71	1·99	
18	„ „ —Minimal lethal dose (optimal infusion rate)	0·75	2·00	
19	Pigeon infusion—Lethal dose	0·77	2·22	
20	Infusion—dog heart-lung preparation, minimal dose for lowering right atrial pressure	0·55	2·24	
20	Infusion—dog heart-lung preparation, minimal dose for producing cardiac irregularities	0·52	2·37	
20	Infusion—dog heart-lung preparation, minimal lethal dose	0·59	2·46	
21	Embryonic chick heart, A-V block	—	3·07	
19	Frog heart-abolition of "staircase" effect, minimal effective dose	0·91	0·42	
17	Frog heart, minimal systolic dose	3·13	5·00	
12	Ventricle (turtle heart)—shortening of Q-T interval	1·00	1·00	
4	Papillary muscle (cat heart)—lowest concentration causing 50 per cent of preparations to respond	—	—	$\frac{\text{Digitoxin}}{\text{Ouabain}} = \frac{1}{1}$
7	Papillary muscle (cat heart)—isodynamic substitution	0·92	2·24	
8	„ „	0·79	2·30	
9	Ventricle strips and papillary muscle (cat and dog). lowest concentration producing a decrease in resting length	2·00	67·00	
9	Ventricle strips and papillary muscle (cat and dog). lowest concentration producing an increase	3·00	*	
22	Man—average digitalising dose (oral and I.V.)	2·21	5·36	
24	„ „ „ „ therapeutic	1·88	—	
24	„ „ toxic dose, daily divided dose	1·38	—	
24	„ „ „ „ undivided	1·55	—	
25	„ „ of range of I.V. dose for moderately complete initial digitalisation	0·7	2·19	
30	„ usual initial intravenous dose	2·00	2·00	
31	„ mean I.V. dose of range stated for initial digitalisation	1·36	4·05	

* Little or no increase in resting length with ouabain.

There is considerable variation for the activity ratios of digitoxin and ouabain in terms of digoxin quoted for man. Lown and Levine[23], and Herrmann[27] state that digoxin is rapidly and fairly completely absorbed from the alimentary tract, while Goodman and Gilman[26] state that 50 per cent or more is absorbed. Even allowing for incomplete absorption of digoxin when given orally digitoxin appears to be more potent in man and not less so as the results from slow intravenous infusion in animals might suggest. The activity ratio of digitoxin in terms of digoxin on the guinea pig right ventricle is therefore in good agreement with that obtained in man; the ratio for ouabain in terms of digoxin is higher in man.

DISCUSSION

The results of the assays of digitalis leaf preparations on the right ventricle of the guinea pig, particularly when obtained from metameters dependent on time, are in close agreement with those obtained by slow intravenous infusion into the same animal. From this it would appear

that the lethal dose determined by infusion is an assessment of the direct action on the heart muscle of the glycosides in the tinctures, or if it is not due solely to this, then any vagal or extracardiac actions must bear the same relationship to each other as the relative activities obtained from a direct action on the muscle.

The analyses of the assays on the ventricle of the crude leaf preparations revealed that the maximum amplitude decreased with increase in dose, and suggests that the doses induced predominantly toxic actions which may have minimised the therapeutic actions. The most precise estimates from the ventricle method were obtained by using $\log_{10}$ time to zero amplitude as metameter.

In contrast the relative potencies of digitoxin and ouabain to that of digoxin were different for the two methods of assay. Primary glycosides are extracted by the method used to prepare the digitalis tinctures, and Brindle, Rigby and Sharma[15] have found purpurea glycoside A to be three times as active as digitoxin, and purpurea glycoside B as active as digitoxin, by slow intravenous infusion into guinea pigs. It may be, therefore, that the other potent glycosides in the tinctures possess the same activity relative to each other by both methods of assay. Digitoxin is absorbed by plasma proteins more readily than the shorter acting glycosides such as digoxin and ouabain, and it may be because of this and its relative absorption by other tissues that it appears in acute tests less active than digoxin or ouabain in the intact animal.

Cattell and Gold[3] expressed the maximum systolic contraction as a percentage of the initial value and failed to find any relationship between the dose and the values so obtained. It is only when the maximum amplitude is corrected by covariance analysis for the initial amplitude and the effects over a wide range of doses are studied, that the biphasic relationship between dose and increase in systolic contraction becomes evident. This biphasic action occurs because with the lowest doses the appearance of the first increase of amplitude appears earlier the larger the dose, while the time to reach the maximum amplitude is constant, whereas with the higher doses the supervention of the toxic action results in an earlier negative inotropic response so that the time to reach maximum amplitude becomes shorter the greater the dose.

When the amplitude of contraction is declining there is a decrease in the resting length of the ventricle which is proportional to the dose. This decrease in resting length is not the cause of the reduction in systolic contraction since in a series of experiments in which the resting length was kept constant by mechanical adjustment the doses of glycoside produced a reduction to zero amplitude at the same rate as in ventricles in which the resting length was allowed to decrease.

It is presumably because of the variability of all these factors that the phase of decline of maximum amplitude is a poor metameter on which to base estimates of potency.

Thus each glycoside is capable, depending on the dose used, of producing different effects which are not directly correlated, though the three glycosides studied appear to have similar actions to each other.

With lower concentrations, the drug may be actively taken up by the muscle and utilised at a rate which reaches the optimum with the dose which produces the greatest increase in amplitude of contraction. With doses greater than the optimum there may be an additional passive diffusion which produces too high a concentration at the site of action which then results in a negative inotropic action. In addition the process controlling resting length must utilise the glycoside differently from that (or those) controlling the systolic contraction since the shape of their dose reponse curves are different.

The possibility that there are two mechanisms controlling systolic contraction should not be ruled out, one controlling increase of contraction dependent on low concentrations of glycoside, the other producing the negative inotropic effect dependent on toxic concentrations of glycosides. The rate of decline of amplitude is always greater than the rate of increase.

The glycosides were able to increase the amplitude of systolic contraction on the unfatigued guinea pig ventricle. A similar finding has been observed with ouabain on isolated strips of ventricle from the same species by Sanyal and Saunders[28], and by Cotten and Stopp[29] on the non-failing heart of the intact dog. Thus it is possible that glycosides act on the same mechanisms in the normal as well as the failing heart.

There is much work in the literature on the effects of glycosides on respiration, organic phosphates containing high energy bonds, carbohydrate metabolism and inorganic ion transport of heart tissue, and the actions of cardiac glycosides must eventually be explained at this level. The different mechanical effects produced on the guinea pig right ventricle show some of the stages at which the biochemical picture should be studied.

Acknowledgement. I wish to thank Mr. J. V. Smart for his advice concerning the statistical analyses.

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THE PHYSICAL PROPERTIES OF LYSOLECITHIN AND ITS SOLS

PART II. REFRACTIVE INDICES AND DENSITIES OF SOLS. MICELLE FORMATION

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The refractive index difference ($n_{sol}-n_{water}$) and density difference ($\rho_{sol}-\rho_{water}$) of lysolecithin sols indicate a change in the properties of the sols in the region of 0·02 per cent w/v, above this concentration the refractive index difference:concentration relation and the density difference:concentration relation are linear. Values for the partial specific volumes of lysolecithin in water, calculated from density measurements, are given. Surface tension:concentration measurements at four different temperatures indicate that the critical micelle concentration increases with an increase in temperature. Values for the change in heat content in the molecular aggregation process, calculated from the temperature coefficient of the critical micelle concentration are small and negative, increasing numerically from 3432 calories at 20° to 6100 calories at 40°. The associated entropy changes in this process are small and show a slight increase negatively with an increase in temperature.

STUDIES on the surface activity of lysolecithin previously reported[1,2] have been extended to include other properties of the sols of this biologically important compound. Experimental work reported here was made on the well-known β-(acyl)lysolecithin with the exception of a comparison with the α-(acyl)lysolecithin by refractive index measurements.

Refractive index measurements were made to find a relation between the refractive index difference ($\Delta n = n_{sol}-n_{water}$) and concentration of the lysolecithin sols to obtain a criterion for the determination of concentration of these sols. By comparing the measurements of an α-(acyl) lysolecithin with those of the β compound, the effect of the structural differences on the refractive index difference (Δn) could be examined.

The effect of concentration on the density difference ($\Delta\rho = \rho_{sol}-\rho_{water}$) of lysolecithin sols was examined at three different temperatures and values for partial specific volumes obtained.

We have shown elsewhere[1] that micelles commence to form in lysolecithin sols at a concentration of 0·001 per cent w/v. The variation of surface tension with concentration in this region has been investigated at four different temperatures to measure the effect of temperature on the molecular aggregation process and to obtain values for the change in heat content for the equilibrium: single molecule in solution $\rightleftharpoons$ single molecule in aggregate. Values for the associated entropy change (ΔS) in this equilibrium have been calculated.

EXPERIMENTAL

Preparation of Lysolecithins

β-(Acyl)lysolecithin was prepared by treating lecithin obtained from egg yolks with Russell viper venom according to the method described by

Saunders[3]. A sample of α-(acyl)lysolecithin was kindly given by Dr. G. M. Gray, Lister Institute of Preventive Medicine. Analysis of the α compound (made by the Lister Institute) and the β compound (the authors') gave the following:—

	α	β
Nitrogen (per cent on dry wt.) ..	2·76	2·77
Phosphorus (per cent on dry wt.) ..	5·9	5·95
N:P ratio	1:0·97	1:1·03
Iodine value	109	4·5

Preparation of Aqueous Sols

Sols of each lysolecithin compound were prepared according to the method previously described[1].

Apparatus

Refractive index. A Rayleigh interference refractometer (Hilger model M154) in combination with a 150 c.p. Pointolight light source was used to obtain difference readings (Δn) between pure water and the lysolecithin

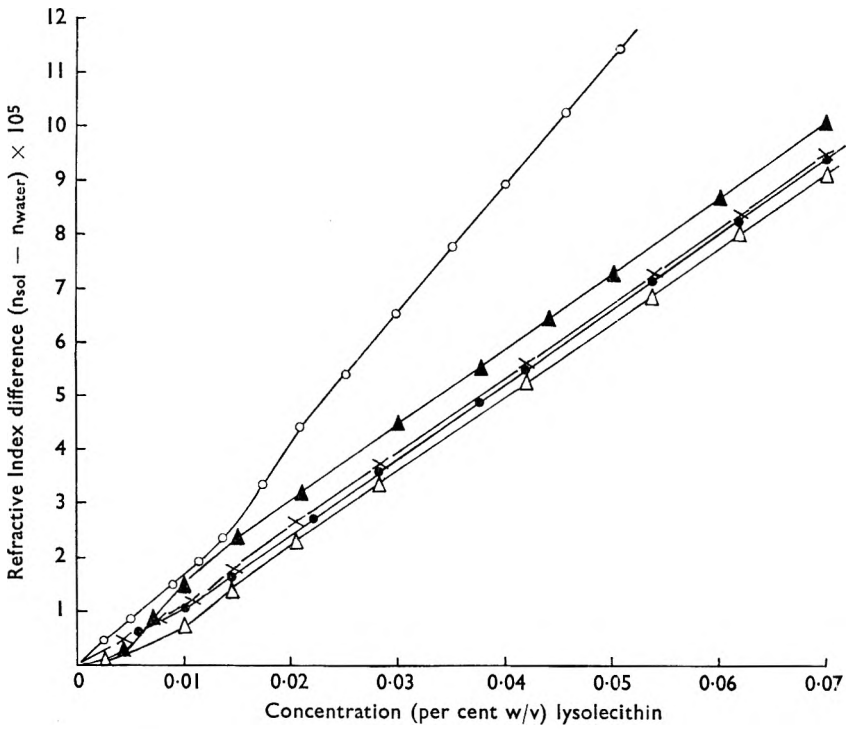

FIG. 1. Variation of refractive index difference (n_{sol} — n_{water}) with concentration of lysolecithin.

O—25° ▲—40° } α—(acyl) lysolecithin

X—20° ●—25° △—40° } β—(acyl) lysolecithin

sols. The liquids were contained in 1 cm. and 10 cm. fused silica cells enclosed in a thermostat controlled to $\pm 0{\cdot}01°$. The instrument was calibrated using a 1 per cent solution of maltose hydrate[4].

Density. Measurements to examine density differences (Δd) between pure water and lysolecithin sols were obtained by the sinker method described by Wirth[5]. The solutions were contained in a cylindrical pyrex glass vessel with a hemispherical base into which a sinker of similar shape and material was placed, allowing for a clearance of 0·5 cm. between sinker and container. The sinker contained sufficient mercury to give a pull of approximately 1 g. weight when completely submerged in water. It was suspended by a fine platinum wire, coated with platinum black where the wire passed through the liquid surface, and supported on one arm of a chainomatic balance assembly previously described[1]. A second sinker of similar pattern was used to enable duplicate readings to be taken successively. Volumes of the sinkers were about 220 ml. and weighings were determined to within $\pm 0{\cdot}1$ mg. Temperature control to $\pm 0{\cdot}001°$ was obtained using a lagged water bath and a Beckmann thermometer.

Surface tension. Surface tension measurements were made at 20°, 25°, 32·5° and 40° using the ring (dynamic) method described elsewhere[1]. Sols of concentrations up to 0·005 per cent w/v lysolecithin were examined.

Results

The refractometer used was the differential type which measured the refractive index difference between the sol and pure water. Measurements of α- and β-(acyl)lysolecithin sols below a concentration of 0·1 per cent w/v are shown in Figure 1. Values for sols of β-(acyl)lysolecithin at higher concentrations are given in Table I.

TABLE I

VALUES OF REFRACTIVE INDEX DIFFERENCES ($n_{sol}-n_{water}$) FOR INCREASING CONCENTRATIONS OF β-(ACYL) LYSOLECITHIN

Concentration (per cent w/v) lysolecithin	Refractive index difference ($n_{sol}-n_{water}$)		
	20°	25°	40°
5·00	0·0069626	0·0069677	0·0068357
4·60	0·006372	0·006341	0·006287
4·17	0·005743	0·005735	0·005673
3·70	0·005126	0·005121	0·005041
3·30	0·004458	0·004466	0·004477
2·50	0·003372	0·003369	0·003367
2·00	0·002753	0·002750	0·002691
1·40	0·001926	0·001923	0·001874
0·91	0·001251	0·001212	0·001214
0·50	0·000722	0·000708	0·000710
0·25	0·000332	0·000331	0·000313
0·10	0·000139	0·000137	0·000131

Variations of density difference between β-(acyl)lysolecithin sols and pure water for increasing concentrations of the sols are shown in Figure 2. The values for the partial specific volume of lysolecithin, calculated from density measurements, are 0·99827, 0·99836 and 0·99885 ml.g.$^{-1}$ at 20°, 25° and 40° respectively.

The variation of surface tension with concentration of lysolecithin at 20°, 25°, 32·5° and 40° is shown in Figure 3. The changes in heat content

associated with the molecular aggregation process were calculated using the equation developed by Stainsby and Alexander[6] (an approximate form of the equation was used taking the activity coefficient as unity). The entropy change (ΔS) associated with the equilibrium between lysolecithin molecules in solution and in the micelles was obtained from the equation $\Delta H = T\Delta S$. The curve relating log Cm and $1/T$ (where C_m is the critical micelle concentration at a given temperature) is shown in Figure 4. Values for the critical micelle concentration, the changes in heat content (ΔH) and entropy (ΔS) are shown in Table II.

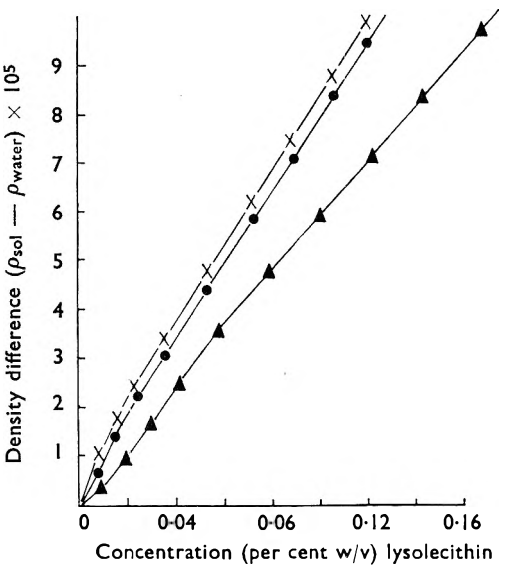

FIG. 2. Variation of density difference ($\rho_{sol} - \rho_{water}$) with concentration of lysolecithin. X—20°; ●—25°; ▲—40°.

DISCUSSION

Refractive Index

Above a concentration of 0·02 per cent w/v the refractive index difference bears a linear relationship to concentration for α- and β-(acyl)lysolecithins at the three temperatures of the experiment. The presence of double bonds in the α-(acyl)lysolecithin gives a higher specific refraction increment $\left(\frac{n_{sol} - n_{water}}{C}\right)$ than the saturated β compound. The rate of increase in the refractive index difference with concentration was less for β-(acyl)lysolecithin than for the α compound, an increase in temperature having the effect of lowering this rate of increase for both compounds.

The apparent physical change of the sols at a concentration of 0·02 per cent w/v is supported by studies on the surface activity of lysolecithin[1]. There it was shown that the rate of change of surface tension with concentration, greatest at 0·001 per cent w/v, rapidly diminished to almost zero at 0·02 per cent w/v where it was thought that the bulk phase consisted almost entirely of large micelles.

The break in the refractive index difference/concentration curves for each lysolecithin sol occurred at a slightly higher concentration with increasing temperature, a behaviour to be expected since the kinetic energy of the molecules is increased and hence the instability of the micelles is also increased.

Density

Measurements on the density difference between lysolecithin sols and pure water showed that below approximately 0·02 per cent w/v the

increase in density difference with increasing concentration does not obey a linear relationship; above this concentration, however, linearity becomes established at 20° and 25° although at 40° it is reached at 0·065 per cent w/v lysolecithin.

TABLE II

VALUES OF CHANGE IN HEAT CONTENT AND ASSOCIATED ENTROPY CHANGES OBTAINED FOR LYSOLECITHIN FROM THE SURFACE TENSION/CONCENTRATION RELATIONSHIPS

Temperature °C.	Range of critical micelle concentration	Mean value of critical micelle concentration		ΔH Calories	ΔS Calories/mole deg.
		Per cent w/v	Moles/l. × 10^6		
20	0·00045 to 0·00158	0·00101	1·95	−3432	−11·71
25	0·00044 to 0·00180	0·00112	2·168	−4233	−14·20
32·5	0·00021 to 0·00272	0·00136	2·628	−4955	−16·22
40	0·00012 to 0·00325	0·00168	3·256	−6100	−19·49

Below approximately 0·02 per cent w/v the rate of increase in density with increasing concentration appears to be greater than above this value. It is probable that the lysolecithin is present as small aggregates which are subjected to high compression by the strongly cohesive field of the water

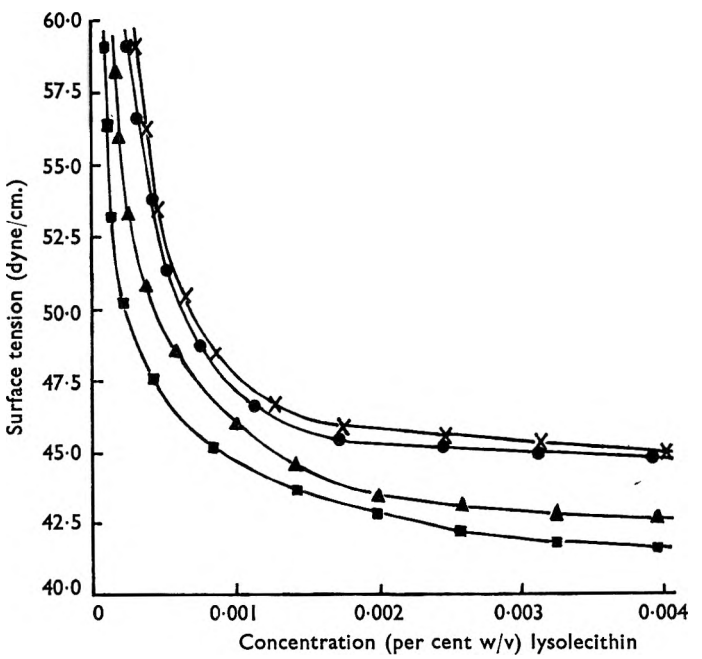

FIG. 3. Variation of surface tension with concentration of lysolecithin. X—20°; ●—25°; ▲—32½°; ■—40°.

molecules. Above 0·02 per cent w/v it is likely that large aggregates of lysolecithin molecules are present and the presence of these large particles reduces the cohesion between the water molecules surrounding the aggregates, resulting in less compression on the aggregates.

The effect of temperature on the point at which a linear relationship between density difference and concentration is established shows a similar pattern to the temperature effect on the refractive index difference/concentration relationship.

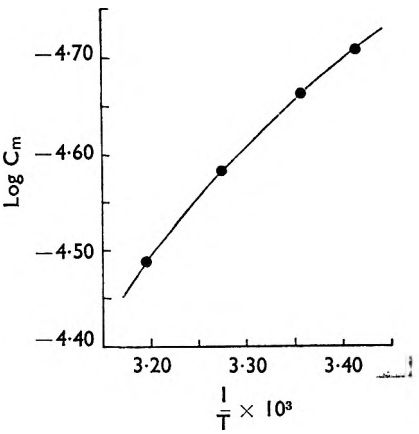

FIG. 4. Variation of logarithm critical micelle concentration (log C_m) with the reciprocal of absolute temperature ($\frac{1}{T} \times 10^3$).

The increase in density difference was considerably less for increasing concentrations at 40° than at 20° and 25°, which is probably due to a reduced strength in the cohesive field of the water molecules.

The value obtained for the partial specific volumes of lysolecithin indicate a slight increase in volume of the solute molecules for an increase in temperature.

Micelle Formation

The concentration region in which small aggregates are formed increases with an increase in temperature. This, a non-linear relation, showed that the increase in concentration at which aggregation takes place is greater at higher temperatures.

Values for the change in heat content in the equilibrium: single molecule in solution $\rightleftharpoons$ single molecule in aggregate, calculated from the temperature coefficient of the critical micelle concentration, increased numerically from 3432 calories per mole at 20° to 6100 calories per mole at 40° and were negative, that is, the heat liberated in the equilibrium increases with temperature. These figures are valid if the following main considerations are taken into account. Firstly, a critical concentration for micelle formation midway along the concentration range shown by the surface tension/concentration relationship was used in the calculation. Secondly, in their work on colloidal electrolytes, Stainsby and Alexander have shown that ions have little effect on the change in heat content and this has been an assumption we have made in obtaining values for lysolecithin sols which are essentially non-electrolytic in character. Thirdly, it has been assumed that the activity coefficient is unity since the values of C_m (critical micelle concentration) are quite small. Fourthly, the micelles formed by lysolecithin are approximately spherical, a probability substantiated by the low relative viscosity of the sols[3].

The changes in heat content for the lysolecithin system are smaller than the values obtained by Stainsby and Alexander for some fatty acids and larger than those for some typical soaps.

The entropy change in the aggregation process is small, showing a slight increase negatively, that is, a loss in entropy, as the temperature rises.

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APPLICATION OF A SPECTROPHOTOMETRIC METHOD TO THE DETERMINATION OF POTASSIUM PENICILLIN, PROCAINE PENICILLIN AND BENZATHINE PENICILLIN IN PHARMACEUTICAL PREPARATIONS

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A spectrophotometric method originally designed for the estimation of potassium penicillin has been applied to procaine penicillin and benzathine penicillin. A solution containing the penicillin salt is heated under controlled conditions with a sodium acetate-acetic acid buffer solution of pH 4·6 to which a trace of copper sulphate has been added. The optical absorption of the resulting penicillinic acid is measured on a spectrophotometer. The method has a standard error of 3 per cent and is applicable to a variety of penicillin preparations ranging in potency from 0·5 units/mg. upwards.

A method has been described by Herriot[1] for the determination of penicillin by its controlled degradation to penicillinic acid on heating with acetic acid-sodium acetate buffer of pH 4·6, followed by spectrophotometric measurement of the ultra-violet absorption at 322 mμ. It has since been shown by Stock[2] that traces of copper in the buffer solution play an important role in the reaction and that, in the absence of this element, reproducible results are unobtainable. Stock has successfully applied this method to the determination of penicillin in oral tablets. The object of this work was to study the extension of the method to formulated penicillin products other than oral tablets and to the procaine and dibenzylethylenediamine salts of penicillin, both of which are now widely used.

EXPERIMENTAL

Application to procaine penicillin. A standard solution was prepared containing 0·1 g./l. of Procaine Penicillin B.P. in 0·01M phosphate buffer. 20 ml. of this solution was diluted with 50 ml. acetate buffer (acetic acid-sodium acetate buffer of pH 4·6 containing copper sulphate equivalent to 0·45 p.p.m. of copper). Aliquots, each of 5 ml. of the diluted solution were heated in a water bath at 100° for varying periods of time, ranging from 5 to 35 minutes, cooled and made to volume in 10 ml. stoppered cylinders with acetate buffer. The optical absorptions of each of the solutions were measured at 322 mμ in 1 cm. silica cells with a spectrophotometer. A blank was done at the same time by proceeding exactly as in the preparation of the sample solution, but omitting the heating process. The results are shown in Figure 1. It was obvious from the outset that owing to the low solubility of procaine penicillin in water (0·4 per cent w/v) the method in its original form would be of little use in dealing with most procaine penicillin formulations because of the small weight of sample, and the uncertainty of securing complete extraction.

The above work was repeated using solutions covering the range of 5 to 25 per cent v/v methanol in water as solvent. The use of these concentrations of methanol did not produce any significant departure from the values obtained using water alone, and it was concluded that methanol, in which procaine penicillin is very soluble, would be suitable as an extracting solvent. Procaine absorbs strongly in the ultra-violet region of the spectrum exhibiting a maximum at 292 mμ. The absorption at 322 mμ (the wavelength maximum for penicillinic acid) is still significant and for pure procaine penicillin this irrelevant absorption amounts to 23 per cent of the total absorption at 322 mμ measured after heating on the water bath for 25 minutes. To ensure that the increase in absorption at 322 mμ was due solely to penicillin decomposition, a solution of the appropriate quantity of procaine base in M/100 phosphate buffer containing 20 per cent v/v methanol, was prepared and the absorption measured before and after heating with acetate buffer for 25 minutes. The optical densities of the two solutions were identical showing that, under the conditions of the determination, the contribution of the procaine component towards the total absorption is constant.

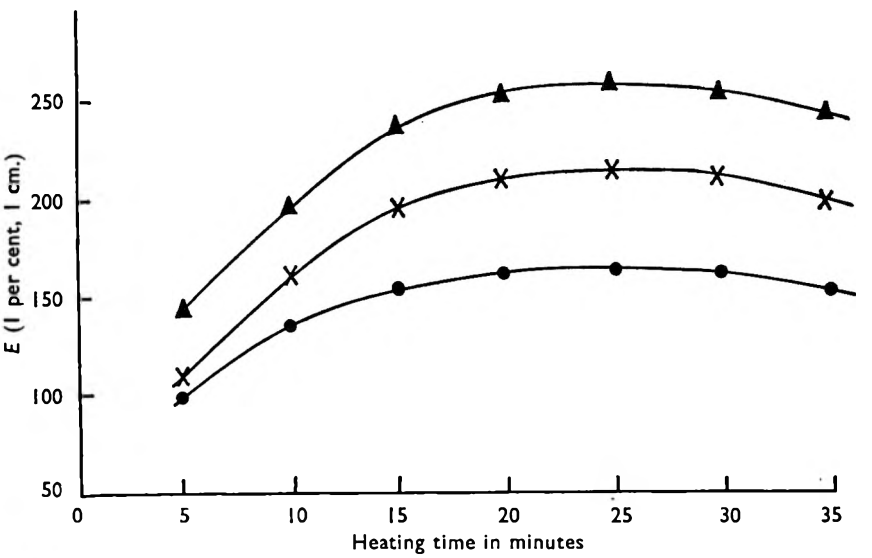

FIG. 1. Variation of E (1 per cent, 1 cm.) at 322 mμ with time of heating at 100°. ▲ Potassium penicillin, X benzathine penicillin, ● procaine penicillin.

Application to benzathine penicillin. This compound is virtually insoluble in water, sparingly soluble in most organic solvents, but significantly soluble in methanol and very soluble in dimethylformamide. A solution was prepared by dissolving 0·16 g. in 100 ml. of methanol and diluting to 500 ml. with 0·01M phosphate buffer and 20 ml. of the latter solution was diluted to 70 ml. with acetate buffer. Aliquots of this solution were heated for varying times as previously described. The results obtained were erratic, and this solvent was obviously unsatisfactory. Repetition of the work using aqueous 10 per cent v/v dimethyl

formamide (2·8 per cent v/v in the heated solution) as solvent gave good duplication of results, which are shown in Figure 1. The absorption of dibenzylethylenediamine at 322 mμ is negligible but to ensure that this obtained throughout the determination, a solution containing the appropriate quantity of the base was prepared in 10 per cent v/v dimethylformamide and its absorption measured at 322 mμ before and after heating for 25 minutes with acetate buffer. No difference in the absorption was observed showing that dibenzylethylenediamine at the concentration encountered in practice does not increase in absorption at 322 mμ. It has been shown[1] that the absorption produced at 322 mμ is a maximum when the pH of the solution is 4·68 and falls rapidly on either side of this value. It was confirmed (see Fig. 2) that the small quantity of copper in the buffer solution causes no change in this critical value and that for all practical purposes the results can be considered constant over the pH range 4·6–4·8. In the determination of benzathine penicillin the pH will fall between these limits, provided the concentration of dimethylformamide in the final solution does not exceed 5 per cent v/v.

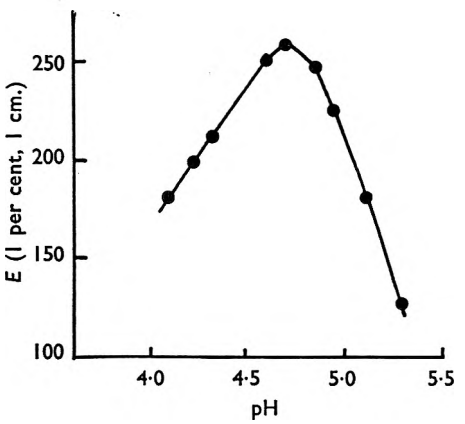

FIG. 2. Variation of E (1 per cent, 1 cm.) at 322 mμ with pH of the buffer solution.

RESULTS

The results shown in Table I are typical of many determinations carried out on pure samples of potassium penicillin, procaine penicillin, and samples of benzathine penicillin of known composition, and are compared with the microbiological potencies.

TABLE I

EXTINCTION COEFFICIENTS AT 322 Mμ COMPARED WITH MICROBIOLOGICAL POTENCIES

Compound		E (1 per cent, 1 cm.) 322 mμ	Microbioloigcal Potency u./mg.
Procaine penicillin	1	158	1000
	2	159	1000
	3	161	1000
	Average	159	
Benzathiene penicillin	1	212	1290
(Results calculated	2	218	1310
to Mol. Wt. 909)	3	214	1300
	4	215	1300
Average calculated to potency of 1300 u./mg.		214	
Potassium penicillin	1	253	1600
	2	255	1600
	3	256	1600
	Average	255	

Discussion

The spectrophotometric method previously developed for potassium penicillin can be applied to both the procaine and dibenzylethylenediamine salts provided a suitable solvent is employed, and the pH of solution is carefully controlled by suitable buffer solutions. The absorption at 322 mμ reaches a maximum after 25 minutes heating for these two salts, and this was subsequently found to be so for potassium penicillin itself. Earlier workers have used a heating time of 15 minutes, but in view of the above findings it was decided to increase this time to 25 minutes for all future work. It was also decided to use 0·01M phosphate buffer in place of water wherever possible as penicillin is more stable in this medium. A general method is described below and this is followed by applications of the technique to formulated preparations.

General Method

Reagents

Copper sulphate. Dissolve 0·392 g. of copper sulphate ($CuSO_4 \cdot 5H_2O$) in 100 ml. of distilled water. This solution contains 1 mg./ml. of copper. *Acetate buffer.* Mix equal volumes of 0·4 M acetic acid and 0·4 M sodium acetate and add 0·9 ml. of copper sulphate solution per 2 litres of mixed solution (equivalent to 0·45 p.p.m. of copper as Cu). 0·5 *M Phosphate buffer.* Dissolve 78 g. of sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$) and 179·1 g. disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$) in distilled water and make to 2 litres. Adjust the pH to lie between 6·45 and 6·55 by the addition of 2N sodium hydroxide or 2N phosphoric acid. 0·01*M Phosphate buffer.* Dilute 10 ml. of 0·5 M phosphate buffer to 500 ml. with distilled water.

Procedure

Prepare an extract of the preparation under examination by an appropriate method so that it will contain between 160–190 units/ml. Take a 20 ml. aliquot of this solution and add, by pipette, 50 ml. of acetate buffer. Pipette 5 ml. aliquots of the above solution into each of five 6 × 1 inch boiling tubes and simultaneously place four of these into a 1500 ml. beaker of boiling water in which the level of water is above the level of liquid in the boiling tubes, and the bases of the tubes are protected for example by a wire gauze supported by 1 inch legs, from direct contact with the base of the beaker. Allow the tubes to remain in the beaker for 25 minutes and maintain the water at boiling point during the whole of this period.

Dilute the contents of the fifth tube to 10 ml. by the addition of 5 ml. of acetate buffer and immediately read the absorption of the resultant solution in a 1 cm. silica cell at 322 mμ against a cell containing distilled water. The figure so obtained represents the blank.

After exactly 25 minutes heating, remove the four tubes from the water bath, cool rapidly and transfer the contents of each in turn to four 10 ml. stoppered cylinders, rinsing out each tube with 3 ml. of acetate buffer and adding the washings to the appropriate cylinder. Adjust the volume

of each cylinder to 10·0 ml. by the addition of acetate buffer and measure the absorption of each solution at 322 mμ against a cell containing distilled water. Calculate the average of the four readings and subtract from this the figure obtained in the blank determination. Calculate the E (1 per cent, 1 cm.) value for the sample and use this figure to evaluate the potency in the manner indicated.

$$\text{Potency} = \frac{E\ (1\ \text{per cent, 1 cm.})\ 322\ \text{m}\mu\ \text{sample}}{E\ (1\ \text{per cent, 1 cm.})\ 322\ \text{m}\mu\ \text{pure penicillin salt}} \times 10 \times \text{A units/g.}$$

Where A = 1600 for potassium penicillin
1000 for procaine penicillin
1300 for benzathine penicillin.

Applications

Preparations containing Potassium Penicillin

Penicillin Ointment B.P. and Penicillin Eye Ointment B.P. (1953). Transfer a weight of sample expected to contain about 8000 units of penicillin to a 150 ml. flask fitted with a ground glass stopper and add 20 ml. of carbon tetrachloride. Stopper tightly and shake the flask until all excipient material is in solution. Add, from a pipette, 50 ml. of 0·01 phosphate buffer and shake vigorously for 15 minutes. Allow the layers to separate and, by decantation, transfer the aqueous layer as completely as possible to a 100 ml. centrifuge tube. Centrifuge at 2000 r.p.m. for 10 minutes. Filter the aqueous layer through a No. 42 Whatman filter paper and determine the penicillin content of 20 ml. of the filtrate by the general method. *Note.* In order to test the reproducibility of the method in the hands of different operators, and to compare the results obtained with the microbiological method, a series of ointments of varying potencies were prepared in the laboratory from potassium penicillin of known purity and these were analysed on successive days by three operators. The results are shown in Table II.

TABLE II

Comparison of results obtained on laboratory prepared penicillin ointments

Sample No.	Theoretical potency u/g.	Spectrophotometric potency u./g.			Average spectrophotometric potency u./g.	Microbiological potency u./g.
		Operator 1	Operator 2	Operator 3		
1	750	730 740	740 750	750 700	735	760
2	1140	1140 1120	1130 1140	1120 1070	1120	1130
3	1250	1280 1230	1260 1210	1180 1190	1225	1220
4	1320	1390 1360 1380	1390 1300 1320	1280 1290 1350	1340	1300
5	1640	1650 1590 1660	1680 1650 1580	1690 1630 1580	1630	1600

Statistical evaluation of the results in Table II gives a standard error of 3 per cent for a single determination. The method is therefore comparable to the microbiological method in accuracy.

Penicillin Lozenges B.P. Transfer to a stoppered 100 ml. centrifuge tube a weight of powdered lozenges expected to contain 8000 units. Add 50 ml. of 0·01 M phosphate buffer and shake vigorously for 10 minutes. Centrifuge at 2000 r.p.m. for 10 minutes and filter through a No. 42 Whatman filter paper. Determine the penicillin content on 20 ml. of the filtrate by the general method. *Note.* With some formulations of lozenges the phosphate buffer extract is almost impossible to filter due to the presence of binding agents, tragacanth for example, which form viscous solutions. This difficulty can be overcome by adding 5 ml. of chloroform to the phosphate buffer extract, shaking vigorously and centrifuging at high speed (e.g., 4000 r.p.m.). The insoluble matter from the lozenge separates at the chloroform/water interface leaving the supernatant liquor sufficiently clear to use without filtration.

Procaine Penicillin and Preparations containing Procaine Penicillin

Procaine Penicillin, Injection of Procaine Penicillin, and Injection of Procaine Penicillin Fortified with Potassium Penicillin. Dissolve an accurately weighed quantity of between 0·13 and 0·14 g. of sample in 100 ml. of methanol and make to volume in a litre graduated flask with 0·01 M phosphate buffer. Determine the total penicillins on a 20 ml. aliquot of this solution by the general method.

Procaine Penicillin Oily Injection B.P. 300,000 *units/ml.* Accurately weigh between 0·3–0·4 g. of the sample into a 100 ml. beaker and add 50 ml. of light petroleum (b.p. 40° to 60°). Stir until all fatty material is in solution and filter with the aid of suction through a No. 4 sintered glass crucible. Wash the beaker and the residue on the filter with a further 50 ml. portion of light petroleum. Dissolve the residue in 150 ml. of methanol and transfer to a litre flask, washing the beaker and filter with a further 50 ml. of methanol and adding this washing to the bulk of the solution in the litre flask. Adjust the volume to 1 litre with 0·01 M phosphate buffer and determine the penicillin on a 20 ml. aliquot of this solution by the general method.

Procaine Penicillin Premix, Veterinary. (A mixture of Procaine Penicillin with an inert base). Transfer a quantity of sample, expected to contain about 16,000 units of penicillin, to a 250 ml. flask fitted with a ground glass stopper and extract the procaine penicillin by shaking for 5 minutes with 100 ml. of 0·01 M phosphate buffer containing 20 per cent v/v methanol. Filter through a Whatman No. 42 filter paper and determine the penicillin content of 20 ml. of the filtrate by the general method.

Benzathine Penicillin and Preparations containing Benzathine Penicillin

Benzathine Penicillin. Accurately weigh about 0·08 g. of sample; dissolve in 30 ml. of dimethylformamide in a 50 ml. volumetric flask and make to volume with water. Dilute 10 ml. of this solution to 100 ml. with 0·01 M phosphate buffer and, by the general method, determine the benzathine penicillin in 20 ml. of the solution so obtained.

Benzathine Penicillin Oral Suspension. Transfer a weight of sample, expected to contain about 100,000 units of penicillin, to a 50 ml. volumetric flask with the aid of 30 ml. of dimethylformamide, shake for 5 minutes and dilute to volume with distilled water. Using these quantities with the samples available, the sugars, suspending and dispersing agents remained in solution. Dilute 10 ml. to 100 ml. with 0·01 M phosphate buffer and, by the general method, determine the penicillin content of a 20 ml. aliquot of the solution so obtained.

TABLE III

COMPARISON OF RESULTS OBTAINED ON SAMPLES OF VARIOUS PENICILLIN FORMULATIONS

Sample		Nominal potency	Operator 1	Operator 2	Operator 3	Average spectrophotometric potency	Microbiological potency	Potency calculated from total base determination
Penicillin lozenges	1	5000 units/ lozenge	4840	5000	5030	5000	5200	
	2		5300	5200	5300	5300	5200	
	3		5970	5810	5790	5860	5700	
	4		5820	5850	5910	5860	5910	
	5		5740	5750	5920	5830	5920	
Procaine penicillin aqueous suspension	1	330,000 units/ml.	341,000	323,000	344,000	336,000	346,000	
	2		358,000	316,000	351,000	342,000	333,000	
	3		348,000	317,000	338,000	334,000	356,000	
	4		359,000	338,000	350,000	349,000	347,000	
	5		356,000	343,000	355,000	351,000	343,000	
	6		358,000	347,000	357,000	354,000	351,000	
Procaine penicillin oily injection	1	300,000 units/ml.	322,000	306,000	280,000	303,000	301,000	
	2		295,000	296,000	282,000	291,000	292,000	
	3		296,000	283,000	282,000	287,000	300,000	
	4		306,000	315,000	293,000	305,000	308,000	
	5		298,000	280,000	295,000	291,000	295,000	
	6		290,000	290,000	286,000	289,000	300,000	
Procaine penicillin fortified	1	1060 units/ mg.	1030	1020	1050	1045	1060	
	2		1070	1090	1060	1075	1085	
	3		1020	1075	1050	1050	1075	
	4		1080	1030	1060	1065	1065	
	5		1040	1070	1030	1050	1090	
	6		1050	1030	1060	1050	1095	
Procaine penicillin Premix	1	3000 units/ g.	2900	2900	—	2900	3100	2800
	2		2900	3100	—	3000	3000	2900
	3		2900	2900	—	2900	3200	3300
	4		2900	3100	—	3000	3100	2900
	5		3000	3000	—	3000	3200	3200
	6		2800	2900	—	2850	3000	2900
	7		3000	2900	—	2950	3200	2900
	8		3200	3100	—	3150	3100	2800
Benzathine penicillin oral suspension	1	65,000 units/ml.	70,800	70,200	70,300	70,400	69,300	67,500
	2		68,000	66,400	69,200	57,800	65,600	65,500
	3		75,400	76,400	77,100	76,300	72,600	75,300
	4		64,700	67,800	68,200	66,900	65,500	66,900
	5		65,400	67,600	67,500	66,800	65,200	67,300
	*6		55,100	57,600	56,200	56,300	55,700	64,400
	7		63,700	66,200	64,000	64,600	64,000	—

* This sample was originally of correct potency but decomposition, due to adverse storage conditions, had subsequently taken place. This decomposition is shown by both the spectrophotometric and microbiological determinations. The total base figure result indicates that the preparation originally contained the correct amount of benzathine penicillin.

RESULTS

Using the techniques outlined in the previous sections, the results shown in Table III were obtained. The estimations of Total Base were carried out by a modification of the method of Knight and Stephenson[3].

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THE *IN VITRO* EVALUATION OF A SODIUM POLYHYDROXYALUMINIUM MONOCARBONATE HEXITOL COMPLEX AS A GASTRIC ANTACID

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Techniques for assessing antacid activity are discussed. The commonly used antacids have been evaluated using two complementary *in vitro* techniques and appraised using as criteria the properties considered to describe the ideal antacid. In confirmation with previous workers most of the substances tested were shown not to meet the optimum requirements. In particular the results confirmed the loss of activity of aluminium hydroxide gel preparations on drying and their variation in neutralising capacity. A new substance, a sodium polyhydroxyaluminium monocarbonate hexitol complex, which is a white tasteless odourless powder, compared favourably with the best of the established preparations tested—a liquid aluminium hydroxide gel and was the only solid meeting the suggested criteria.

IT has been recognised by many workers in this field that determination of merely the total neutralising value of an antacid is insufficient. Such a method[1] ignores certain important considerations, for example, the speed of action or the possibility of alkalisation with subsequent acid rebound. In our view, test methods should be capable of assessing each of the criteria required in the ideal antacid, and we consider these to be as follows. (i) It should show its maximum neutralising effect in the shortest possible time. (ii) It should neutralise an adequate amount of gastric hydrochloric acid and maintain its action during the normal period of gastric digestion. (iii) Any excess, however great, beyond the amount required to neutralise free gastric acid should not cause alkalisation. (iv) To be of value in ulcer therapy, it should raise the pH of the gastric contents to a level at which pepsin activity is significantly reduced but not totally inhibited. (v) Adequate and repeated doses should be palatable to the hyper-acid patient. Its use should not lead to laxative, constipating or other side effects, such as gastric irritation. Armstrong and Martin have pointed out that certain of these physiological properties can be determined "by theoretical consideration and known effects"[2].

The pH of gastric juice is normally quoted as 1·4–1·6[3,4] and the desirable value to be obtained by the use of an antacid is given variously as 3·5–4·0[5], 4·0–5·0[6], 4·0–5·0 +[7], 4·0–5·5 +[8], 4·0–6·0[4], and 4·0–8·0[9]. However acid rebound may occur if the pH of the gastric contents rises above 7·0[10]. In view of this risk, it seems desirable to raise the pH of the gastric contents only far enough to ensure adequate relief from hyperacidity, particularly if an ulcer site is present. A pH of 3·5–4·5 appears to attain this, and at the same time there is apparently sufficient residual pepsin activity to avoid secondary digestive disturbances[11].

In Vivo *and* In Vitro *Evaluation of Antacids*

Flexner and his various co-authors [12–14] compared results from the continuous recording of gastric pH *in situ* in dogs with those from *in vitro*

techniques and concluded that the latter gave adequately comparable values. Extension of their work to human subjects led them to draw similar conclusions.

This was generally confirmed by Brindle[15], who prepared an artificial gastric juice consisting of 0·05N hydrochloric acid containing 0·15 per cent each of pepsin, peptone and sodium chloride, and which had a pH (average) of 1·5 at 38°. A comparison of this with human gastric secretion by titration with 0·5N sodium hydroxide showed almost identical behaviour.

Clemow and Lowry[16] showed that 0·05N hydrochloric acid gave results strictly comparable with those for artificial gastric juice, and stated that this confirmed the work of Rossett and Flexner[17]. They also pointed out that *in vitro* experiments should be carried out at 37°. The omission of pepsin and other enzymes and buffers was, in their opinion, an advantage since differences in rates of reaction could be more readily detected.

For the *in vitro* evaluation of antacids two basic methods are available.

1. A therapeutic dose of the antacid is suspended in water and the volume of acid required to maintain an arbitrary pH is plotted against time. This curve is a function of the rate of neutralisation and neutralising capacity.

2. Simulated gastric juice is added continuously to an excess of the antacid, and the pH plotted against time. The resultant curve indicates duration of action (a function of the neutralising capacity), risk of acid rebound, probable degree of inhibition of pepsin activity and—to some extent—neutralisation rate. The detailed method, like that of most other workers, is essentially a modification of that of Rossett and Flexner[17], demonstrating the buffering ability of the antacid.

For a full *in vitro* evaluation of the efficiency of an antacid, both methods should be used, since the first indicates the rate at which neutralisation is achieved, and the second the duration of action at the desired pH.

Both approaches accordingly have been studied in the present work.

EXPERIMENTAL

1. *Acid Neutralised against Time (pH Constant)*

The antacid, if a tablet or powder, is reduced to a slurry with a little water in a mortar, and is transferred quantitatively to a 250 ml. jacketted glass reaction vessel. Liquid preparations (suspensions) are placed directly in the reaction vessel. Water at 37° is circulated through the jacket to bring the contents to normal body temperature. When temperature equilibrium has been attained, 0·1N hydrochloric acid is added to maintain the pH at the predetermined arbitrary level and the volume is plotted against time. The most important portion of the resultant curve is that covering the first 5–10 minutes.

2. *pH Against Time (Rate of Acid Addition Constant)*

The foregoing basic technique is used. 12·5 ml. of 0·2N hydrochloric acid are diluted with water to 75 ml. in the reaction vessel and the temperature brought to 37°. The antacid is added as a slurry in water, also

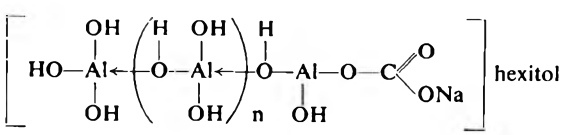
at 37°, followed after exactly one minute by the continuous addition of further 0·2N acid at the rate of 1·0 ml. per minute. This latter acid may be at room temperature without significantly affecting the temperature of the contents of the reaction vessel.

The variation between the rates of acid secretion in individuals is so great that an arbitrary rate of acid addition has to be adopted. Rossett and Flexner[17] suggested a secretion rate of 120 ml. of 0·1N hydrochloric acid per hour which is consistent with figures for the nocturnal secretion of patients with duodenal ulcer[18,19]. It is also similar tc the rate generally employed by others[5].

To ensure a constant rate of addition of acid Schleif[20] used a large number of small increments delivered from an automatic pipette (cf. Armstrong and Martin[2]). We have preferred a burette so modified that air is fed to the head space at constant hydrostatic pressure. The pH of the mixture is recorded continuously. The end point occurs when sufficient acid has been added to cause the pH to fall below 3, that is, when the neutralising capacity of the antacid under these conditions is exhausted.

The methods described above were used to study the following, sodium bicarbonate, magnesium carbonate, magnesium trisilicate, Mixture of Magnesium Trisilicate, B.P.C., bismuth subnitrate plus antacids (magnesium carbonate and sodium bicarbonate), milk solids plus antacids, aluminium glycinate, aluminium phosphate, aluminium hydroxide gel, dried aluminium hydroxide gel, and a new compound, sodium polyhydroxyaluminium monocarbonate hexitol complex.

This last compound is a white tasteless and odourless powder, decomposing without melting when heated strongly. It is insoluble in water but readily and completely soluble in dilute acids. At normal temperatures it may be stored indefinitely with no apparent change in its physical or chemical properties, including reactivity with acids. It probably has the structure:



where n = O or an integer, controlled by the preparative conditions.

Results

The results of these various studies are shown in the accompanying Tables and enable the division of these various antacids into the following categories.

(*a*) *Preparations which may produce acid rebound.* Reference to Table I shows that most of the older, rapidly acting antacids, for example, sodium bicarbonate, and magnesium carbonate, cause large variation in pH with dose. By increasing the dose it is possible to raise the pH above 7·0, producing alkaline conditions and the probability of acid rebound[10].

(*b*) *Preparations which may unduly inhibit pepsin activity* (Table I). It is generally accepted[17] that pepsin is inactivated at pH values greater than 5·0. Such widely used products as Mixture of Magnesium Trisilicate, B.P.C., and preparations of bismuth subnitrate with magnesium carbonate and sodium bicarbonate fall into this group.

TABLE I

EFFECT ON pH OF VARYING THE QUANTITY OF ANTACID WITH CONSTANT ACID ADDITION RATE
(see also Table III)

Material and weight used (as total active ingredients)	pH at (minutes)					
	1	5	10	30	50	70
Magnesium trisilicate .. .. 0·5 g.	1·75	2·57	2·67	2·31	1·95	—
" " .. .. 1·0 g.	2·10	4·50	5·07	4·48	3·90	3·22
" " .. .. 2·0 g.	3·25	5·43	5·57	5·31	5·07	4·81
" " .. .. 3·0 g.	4·80	6·52	6·35	5·95	5·73	5·48
Sodium polyhydroxyaluminium monocarbonate hexitol complex. 0·5 g.	4·05	3·90	3·88	3·80	3·72	3·68
" " " " " " 1·0 g.	4·07	3·95	3·94	3·88	3·83	3·78
" " " " " " 2·0 g.	4·13	4·02	4·01	3·98	3·97	3·94
Mixture of Magnesium Trisilicate, B.P.C. .. .. 0·5 g.	4·02	6·15	6·12	2·97	—	—
" " " " .. .. 1·0 g.	4·75	6·92	7·08	6·24	5·22	4·04
" " " " .. .. 2·0 g.	6·61	7·32	7·29	7·13	6·97	6·81
Bismuth subnitrate with antacids .. .. .. 0·5 g.	5·97	7·22	7·19	6·70	6·18	5·66
" " " " .. .. 1·0 g.	7·18	7·40	7·41	7·28	7·16	7·02
" " " " .. .. 2·0 g.	7·67	8·04	8·01	7·89	7·78	7·63
Magnesium carbonate .. .. 0·5 g.	5·50	7·16	7·17	6·54	5·91	5·25
" " .. .. 1·0 g.	7·10	7·41	7·33	6·92	6·48	5·95
Sodium bicarbonate .. .. 0·5 g.	5·33	6·28	6·22	3·95	—	—
" " .. .. 1·0 g.	5·58	6·61	6·57	6·12	5·68	—
" " .. .. 2·0 g.	7·07	7·32	7·28	7·13	7·05	—
Calcium carbonate .. .. 1·0 g.	4·88	6·31	6·23	5·97	5·70	—
" " .. .. 2·0 g.	5·30	6·42	6·52	6·43	6·41	—
Aluminium glycinate .. .. 1·0 g.	2·90	4·24	4·16	4·00	3·81	3·62
" " .. .. 2·0 g.	3·94	4·48	4·34	4·20	4·12	4·03
" " .. .. 3·0 g.	4·04	5·64	5·66	4·57	4·37	4·18
Aluminium phosphate .. .. 1·0 g.	1·85	2·49	2·48	2·34	2·20	—
" " .. .. 2·0 g.	2·44	2·69	2·66	2·49	2·32	—
" " .. .. 3·0 g.	2·62	2·89	2·84	2·67	2·49	—
Milk solids with antacids .. .. 1·0 g.	5·25	6·56	5·96	4·45	3·16	—
" " .. .. 2·0 g.	7·10	8·02	8·28	7·73	6·70	5·65
" " .. .. 3·0 g.	7·44	8·13	8·38	7·97	7·37	6·75
Milk solids (alone) .. .. 1·0 g.	2·39	2·13	1·86	1·31	—	—
" " .. .. 2·0 g.	2·74	2·50	2·22	1·66	—	—

pH at zero time : 1·50

TABLE II

COMPARATIVE RATES OF ACID NEUTRALISATION TO pH 3·5 FOR VARIOUS ANTACID PREPARATIONS

Material	Ml. 0·1N hydrochloric acid neutralised at (minutes)					
Basis of comparison: 0·5 g. active ingredient	1	2	5	10	15	20
Aluminium hydroxide gel (best available grade)	12	18	41	100	131	155
Magnesium carbonate	29	60	102	105	106	107
Milk solids with antacids	21	45	90	102	107	112
Sodium polyhydroxyaluminium monocarbonate hexitol complex ..	12	22	46	75	96	113
Bismuth subnitrate with antacids	16	31	69	81	84	88
Calcium carbonate	19	37	65	77	80	83
Mixture of Magnesium Trisilicate, B.P.C.	16	29	61	76	79	81
Sodium bicarbonate	57	58	59	59	60	60
Aluminium glycinate	4	8	21	36	47	55
Aluminium hydroxide tablets (best available grade)	3	5	14	30	48	67
Magnesium trisilicate	3	6	15	26	34	41
Milk solids	12	14	14	14	14	14
Aluminium phosphate gel	1	1	2	2	3	3

(*c*) *Preparations which do not raise the pH adequately* (Tables I and III). Aluminium phosphate, and milk solids alone or combinations of the last

with antacids fall into this group; it is appreciated that this method does not necessarily evaluate fully milk solid preparations. Most samples of magnesium trisilicate are also classed here; this compound not only gives a widely ranging pH according to dosage (Table I) but also differs in effect and effectiveness from one source to another[21,22], this accounting for the variable results reported in the literature.

TABLE III

VARYING EFFECT ON pH FOR ALUMINIUM HYDROXIDE PREPARATIONS FROM VARIOUS SOURCES
(Sodium polyhydroxyaluminium monocarbonate hexitol complex included as a reference substance)

Material Basis: Al content equivalent to 0·5 g. $Al(OH)_3$ (0·327 g. Al_2O_3)	Source of Sample	pH at (minutes)					
		1	5	10	60	80	90
Sodium polyhydroxyaluminium monocarbonate hexitol complex.	–	4·10	4·17	4·10	4·07	4·03	3·97
Aluminium hydroxide gel*	A	2·75	4·02	4·02	3·75	3·50	2·80
" " *	B	3·50	4·12	4·04	3·84	3·56	3·14
Dried aluminium hydroxide gel*	C	2·20	3·25	4·07	3·90	3·69	3·17
" " " " *	D	2·02	2·87	3·94	3·72	3·28	2·60
" " " " *	E	2·83	4·05	4·02	3·77	3·09	2·50
" " " " *	F	2·32	3·35	3·68	3·18	2·56	—
" " "	G	1·78	1·96	1·96	1·70	—	—
Aluminium Hydroxide, B.P.C. 1934	H	1·74	1·84	1·86	1·70	—	—
Aluminium hydroxide tablets*	A	2·03	3·54	3·62	3·43	3·28	2·74

* Described as complying with the B.P.C. (1954)
pH at zero time : 1·50

(d) Preparations which are slow in action. Certain of the preparations studied, aluminium glycinate, aluminium phosphate, and some samples of magnesium trisilicate, although raising the pH to the ideal range with adequate dosage, nevertheless have a delayed onset of action. This is particularly true for preparations of dried aluminium hydroxide gel. The marked loss of activity on drying aluminium hydroxide gels has previously been noticed (e.g.,[19]), and Tables III and IV illustrate the remarkably wide variations in reactivity of aluminium hydroxide preparations commercially available in this country.

TABLE IV

VARIATION IN SPEED OF ACID NEUTRALISATION TO pH 3·5 FOR ALUMINIUM HYDROXIDE PREPARATIONS FROM VARIOUS SOURCES
(Sodium polyhydroxyaluminium monocarbonate hexitol complex included as a reference substance)

Material Basis: Al content equivalent to 0·5 g. $Al(OH)_3$ (0·327 g. Al_2O_3)	Source of sample	Ml. 0·1N hydrochloric acid neutralised at (minutes)					
		1	2	5	10	15	20
Sodium polyhydroxyaluminium monocarbonate hexitol complex.	—	22	35	63	98	125	144
Aluminium hydroxide gel*	A	12	20	40	100	131	155
" " " *	B	14	22	42	65	83	100
Dried aluminium hydroxide gel*	C	10	16	28	47	66	85
" " " *	D	9	14	22	40	55	69
" " " *	E	9	14	25	39	50	55
" " " *	F	8	15	24	32	40	47
" " "	G	<1	<1	<1	<1	1	1½
Alumium Hydroxide, B.P.C. 1934	H	<1	<1	<1	<1	<1	<1
Aluminium hydroxide tablets*	A	3	5	14	30	48	67

* Described as complying with the B.P.C. (1954)

(*e*) *Preparations meeting the criteria of the ideal antacid.* Of the range of solid preparations tested, the only product fulfilling all requirements was sodium polyhydroxyaluminium monocarbonate hexitol complex. Of liquid preparations, only certain better grades of aluminium hydroxide gel gave a comparable performance.

Discussion

Using techniques similar to other workers our results in general agree with previous evaluations. In addition to established preparations, a new substance, a sodium polyhydroxyaluminium monocarbonate hexitol complex, has been tested. From the results it compares well with the best preparation examined—a liquid aluminium hydroxide gel—in rate and amount of acid neutralised and in maintenance of the pH at the optimum range of 3·5–4·5. In particular, its superiority over dried aluminium hydroxide gels is apparent. The loss of activity that may accompany drying and tabletting of these gels is well-known, and the results show the new compound to be at least twice as rapid in action as the best dried gel tested and to have superior powers of neutralisation and pH maintenance, which suggests that it might prove a clinically efficacious substance in the convenient form of tablets.

Acknowledgements. It is a pleasure to acknowledge assistance from Bayer Products Ltd., particularly from Dr. R. S. Inch, M.D., M.R.C.P.E., Miss B. Barrow, B.Pharm., and Mr. F. W. Whiting, B.Sc., in the preparation of this paper.

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A FLUORIMETRIC ASSAY FOR MINUTE AMOUNTS OF SOME THIOHYDANTOINS

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A fluorimetric assay sensitive to μg. quantities of certain thiohydantoins has been devised using dichloroquinone chloroimide as a fluorescence reagent. The nature of the buffer used is critical. Satisfactory results have been obtained for recoveries from urine, spinal fluid and plasma. 16 thiohydantoins and some related compounds have been examined by the suggested technique.

SEVERAL years ago, during an investigation of the chemical aspects of the suppression of tuberculous infection by a series of substituted 2-thiohydantoins, a search for sensitive assay methods was made. 5-*n*-Heptyl-2-thiohydantoin, the most active member of the series being studied, was used as the index compound in the search. Of the more promising chromogenic reactions, we chose to investigate the fine orange colour resulting from condensation with 2:4-dinitrochlorobenzene, and the deep purple-blue colour formed by condensation with 2:6-dichloroquinone chloroimide. The latter reagent, in pH 8 borate buffer, has been reported by McAllister to yield a yellow colour with 4-methyl-2-thiouracil and 4-propyl-2-thiouracil[1,2], and yellow to red colours with 2-mercaptoimidazole and its derivatives[3,4]. However, neither of these colour reactions turned out to have much advantage over simple spectrophotometry.

EXPERIMENTAL AND RESULTS

The Fluorimetric Estimation of 5-n-*Heptyl-2-thiohydantoin*

During the course of exploratory tests with dichloroquinone chloroimide in which the optimum pH for reaction was sought, it was observed that maximum colour development occurred only within a narrow pH range. For example, 100 μg. portions of the drug reacted as follows. In phosphate buffer 7·2—faint purple; in borate buffer 8·0—moderately strong purple; in borate buffer 8·4—much stronger purple; in borate buffer 8·8—maximum colour—fine deep purple-blue; in ammonia-ammonium chloride, pH 9·2—brownish violet, and in ammonia-ammonium chloride, pH 10·2—brownish violet.

The dirty brownish-violet colour in the NH_4OH-NH_4Cl buffer exhibited the faint dichroic colour which is so often a sign of fluorescence. On inspecting the reaction mixture under filtered ultra-violet light, an intense green fluorescence appeared. None of the purple-blue coloured reaction compounds show fluorescence. It appears that the nature of the buffer used is critical. Thus at pH 8·7, in borate buffer, a purple-blue colour develops, but no fluorescence. At pH 8·7, in NH_4OH-NH_4Cl, a brownish-violet colour develops, with strong green fluorescence under ultra-violet light. Presumably the borate interferes with the formation of the

fluorescent derivative, and permits the formation of the purple coloured derivative. With 5-*n*-heptyl-2-thiohydantoin, the utilization of dichloroquinone chloroimide as a fluorescence reagent made possible a method which is sensitive to one microgram, or less.

Reagents. *iso*Propanol, reagent quality. *iso*Butanol, redistilled. pH 10·2 buffer. Dissolve 23 g. ammonium chloride in 100 ml. water and add 27 per cent ammonium hydroxide to adjust pH to 10·2. pH 6·0 buffer. Prepare two solutions A and B. (A) Dissolve 675 g. KH_2PO_4 in 3000 ml. water. (B) Dissolve 150 g. $Na_2HPO_4 \cdot 12H_2O$ in 500 ml. water. Mix about 11 parts of A with 14 parts of B, varying the ratio slightly, if necessary, to adjust to pH 6·0.

Dichloroquinone chloroimide reagent. (DCQ) (2:6-dichlorcquinone chloroimide, Eastman). Purify as follows. Dissolve 1 g. in 50 ml. acetone. While stirring rapidly, add water slowly until a permanent precipitate is formed. Filter on a Buchner filter; dry briefly by suction, then in a vacuum chamber. Transfer to a glass-stoppered bottle and store in the refrigerator. Dissolve 50 mg. in 250 ml. of *iso*propanol. This solution, if kept cold, is stable for at least 2 weeks. *Standard solutions.* (*a*) Primary Standard. Dissolve 100 mg. of 5-*n*-heptyl-2-thiohydantoin in 200 ml. of 95 per cent ethanol. (Stable for at least 2 weeks in a cool dark place.) (*b*) Working Standard. Dilute 3 ml. of the primary standard to 250 ml. with water. Concentration is 6 μg./ml.; it must be prepared each day. Quinine reference standard. Prepare a solution containing 100 μg. of quinine sulphate per litre of 0·1 N sulphuric acid.

The Standard Curve

To each of a series of six fluorimeter tubes transfer, 0, 0·1, 0·2, 0·3, 0·4 and 0·5 ml. of the working standard. Add to each tube sufficient water to dilute to exactly 2·0 ml. To each add 4·0 ml. of a mixture of 3 volumes *iso*propanol and 1 volume of *iso*butanol. Then add 0·5 ml. of the pH 10·2 buffer and 0·5 ml. of the DCQ reagent. Mix and let stand for 2 hours. Read in the fluorimeter, using B-1 as the primary filter and P-C-1 as the secondary filter (Coleman photofluorimeter and Coleman filters) and setting the instrument at a convenient point with the quinine reference standard. Plot the net instrument readings against μg. of 5-*n*-heptyl-2-thiohydantoin. A linear plot results.

Extraction of Micro Amounts of 5-n-*Heptyl-2-thiohydantoin from Water*

To each of a series of five small separators, which must be scrupulously clean and stopcocks lubricated only with water, transfer 8·0 ml. of chloroform. Add, 0, 0·2, 0·4, 0·6 and 0·8 ml. of the working standard. Add to each separator 2 ml. of the pH 6·0 buffer and sufficient water to dilute the aqueous phase to 5 ml. Shake each funnel for two full minutes, let stand until the layers separate and draw off the chloroform layer into a clean centrifuge tube. Centrifuge only if necessary to clarify. Transfer 5 ml. by pipette to a fluorimeter tube and cautiously evaporate all of the chloroform on a steam bath. Cool and add 4·0 ml. of a mixture of 3 volumes of *iso*propanol and 1 volume of *iso*butanol. Add 2·0 ml. of water, then

0·5 ml. of the pH 10·2 buffer and 0·5 ml. of the DCQ reagent. Mix, let stand for 2 hours and read in the fluorimeter. The plot of net instrument readings against samples taken corresponds closely to that of the standard series, and demonstrates quantitative extractability of the compound from water.

Extraction from Urine, Spinal Fluid and Plasma

Precisely the same procedure given above may be used for extraction from urine, spinal fluid or plasma. Recoveries from human urine, 2 ml. samples with 0·6 to 1·2 μg. added drug per ml., are 100 to 104 per cent. One test using 1 ml. of monkey spinal fluid with 2 μg. added drug gave a recovery of 97 per cent. This technique has been found to yield 97 to 113 per cent recovery of amounts of drug of 6 to 30 μg. added to 2 ml. of human plasma*.

The *iso*propanol and *iso*butanol mixture is used to keep fats in solution. If the sample is known to be fat-free, it is possible to use a simpler solvent system. Thus, with water, urine or spinal fluid, extract with chloroform, evaporate, then take up the residue in 1 ml. 95 per cent ethanol and 5 ml. of water. Add 0·5 ml. of the pH 10·2 buffer and 0·5 ml. of the DCQ reagent. In this solvent fluorescence develops faster, so that only 1 hour of standing is required. The total intensity of developed fluorescence is also slightly greater in the ethanol medium than in the higher mixed alcohols.

Selectivity of the Fluorescenc Test

A start has been made on experiments which may eventually yield dependable information on the selectivity of the test, and the mechanism by which the fluorescent derivative is formed. Of the large number of compounds prepared in these laboratories by S. Archer and associates, 16 were selected for test. A few important related compounds were also tested. The figures in the last column of Table I refer to net fluorimeter readings equivalent to 2 μg. of the respective compound, by the technique already described. As noted, in the case of some compounds, much larger samples were taken (marked with star).

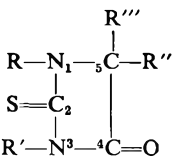
Some tentative generalizations may be drawn from the data in the Table. Hydantoins (i.e., oxygen instead of sulphur in the 2-position) develop no measurable fluorescence. (Tests 15, 16, 17). Neither do thiouracil or 6-propyl thiouracil. (Tests 21, 22). In the thiohydantoin series, it seems possible (Tests 1, 23) that, to achieve fluorescence, one of the 5-position hydrogens must be substituted. Apparently any monoalkyl substitution in the 5-position results in fluorescence. (Tests 1, 2, 3, 4.) But if both 5-position hydrogens are substituted, fluorescence is not measurable. (Tests 12, 13.) With the only dithichydantoin available to us (Test 20), the "monoalkyl-5" rule fails, and no measurable fluorescence results. Even the presence of one aromatic substitution in the 5-position seems to quench fluorescence. (Test 8.) When the benzene ring is separated from the nucleus by a methylene group (Tests 14, 9)

* Human plasma test by E. W. McChesney and associates of these laboratories.

fluorescence re-appears. However, a saturated cyclic substituent at the 5-position acts like an alkyl group (Test 5). In fact, note that 5-*cyclo*hexyl-2-thiohydantoin shows twice the fluorescence of the 5-alkyl compounds. It is equally interesting that when the *cyclo*hexyl group is insulated from the hydantoin nucleus by a single methylene group (Test 6) the fluorescence is drastically quenched; when the insulation is lengthened

TABLE I

COMPOUNDS TESTED SHOWING NET FLUORIMETER READINGS EQUIVALENT TO 2 μ μG. OF COMPOUND



Test No.	Compound	R	R'	R''	R'''	Instrument reading
1	5-*n*-Heptyl-2-thiohydantoin	H	H	H	*n*-heptyl	30
2	5-*n*-Butyl-2-thiohydantoin	H	H	H	*n*-butyl	33
3	5-*n*-Pentyl-2-thiohydantoin	H	H	H	*n*-pentyl	30
4	5-*n*-Hexyl-2-thiohydantoin	H	H	H	*n*-hexyl	31
5	5-*cyclo*Hexyl-2-thiohydantoin	H	H	H	*cyclo*hexyl	66
6	5-*cyclo*Hexylmethyl-2-thiohydantoin	H	H	H	*cyclo*hexylmethyl	6
7	5-(2-*cyclo*Hexylethyl)-2-thiohydantoin	H	H	H	2-*cyclo*hexylethyl	38
8	5-Phenyl-2-thiohydantoin	H	H	H	phenyl	0
9	5-(4-Methoxybenzyl)-2-thiohydantoin	H	H	H	4-methoxybenzyl	3
10	5-Propylmercaptomethyl-2-thiohydantoin	H	H	H	propylthiomethyl	0
11	5-Butylmercaptomethyl-2-thiohydantoin	H	H	H	butylthiomethyl	0
12	5:5-Dimethyl-2-thiohydantoin	H	H	methyl	methyl	0
13	1-Acetyl-5:5-dimethyl-2-thiohydantoin	acetyl	H	methyl	methyl	0
14	1-Acetyl-5-benzyl-2-thiohydantoin	acetyl	H	H	benzyl	22
15*	Hydantoin					0
16*	5-Heptyl hydantoin					0
17*	5:5-Diphenyl hydantoin					0
18*	Thiourea					0
19*	Tibione (4-acetamidobenzaldehyde thiosemicarbazone)					10
20*	5-Hexyl-2:4-dithiohydantoin					0
21*	Thiouracil					0
22*	6-Propylthiouracil					0
23*	Thiohydantoin					0

* Used samples of 100 μg.

NOTE: Most of the compounds indicated as showing zero fluorescence, do indeed fluoresce at much higher levels. However, in the latter cases, it remains to be established whether the compound itself is fluorescing, or some impurity.

to ethylene (Test 7) the fluorescence intensity returns to the range shown by the simple 5-alkyl derivatives in the Table. If the 5-alkyl substituent is interrupted by sulphur (Tests 10, 11) so that only one carbon insulates S from the nucleus, fluorescence is lost. It is possible that if two or more carbons separated S from the nucleus, the fluorescence would return, but this has not been checked.

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BOOK REVIEW

DRUGS OF CHOICE 1958–1959. Edited by Walter Modell. Pp. 931 (including Index). Henry Kimpton, London, 1958. 95s.

The shortage of good clinical trials and the multitude of new drugs demands not less than a man's whole labour properly to keep up and write successfully in the field. One, perhaps the only, means of providing the needed work of reference is to collaborate: what no one man can achieve, three or thirty may more easily manage. Thirty-seven American physicians have in fact just undertaken this great labour, and it is most interesting to see what sort of achievement has resulted. There is no doubt that this book has cost its contributors much labour; its bulk alone is suggestive, though it might have been still more laborious to write more briefly. It is not clear whether the book is the first of an annual series, as its date suggests. It contains much general matter which will not change from year to year, as well as opinion on new remedies which may well need revision as time passes, or may become unimportant if the remedies are superseded.

The statements made are generally worthy and appear to be reliable, but they are not supported by appropriate references. References are provided at the end of each chapter, and are admirably recent: but the lack of citations in the text is a serious inconvenience to a critical reader and makes it most difficult to trace the source of a particular opinion and decide what weight of evidence supports it. Perhaps it is unreasonable to desire such detail in what are mainly personal reports on experience. But it is often better to spend time looking at evidence than on hearing what people think. As a symposium of personal reports on clinical experience, this book is a large and generous collection.

MILES WEATHERALL.

LETTERS TO THE EDITOR

Catechol Amines in Bananas

SIR,—In a recent letter to this journal, West[1] suggested that the presence of catechol amines in bananas might give rise to false positive diagnoses of phaeochromocytoma if bananas were consumed during the collection of a 24 hour urine specimen for catechol amine estimation. This seems unlikely in view of the rapid destruction of orally ingested catechol amines, but the suggestion was put to experimental test.

Three normal adults collected 24 hour specimens of urine on 4 consecutive days. On the second day each volunteer consumed between the hours of 9 a.m. and 5 p.m. the pulp from 1 lb. of ripe bananas. A few grammes of pulp from each banana was weighed and dropped into N/100 HCl for estimation of catechol amines. Aliquots of the urine specimens were concentrated by selective adsorption on alumina. Noradrenaline and adrenaline were estimated in the concentrated extracts from urines and banana samples by differential assay on the blood pressure of the cat before and after phentolamine. The cat was anaesthetised with chloralose and pre-treated with atropine, mepyramine and hexamethonium.

The results show that the ingestion of relatively large quantities of banana pulp have no effect on the excretion of catechol amines and are quite unlikely to give rise to false positive diagnoses of phaeochromocytoma. Two of the three samples of banana pulp showed appreciable amounts of adrenaline as well as noradrenaline by the method of assay used. Unfortunately the amounts of amines present in the concentrated extracts were insufficient for chromatographic confirmation of this observation.

Subject	Banana pulp consumed (g)	Catechol amine excretion µg. per 24 hr.			Catechol amines in banana pulp µg. per g.	
		Day	NA	A	NA	A
P.B.M.	294	1	3·7	Nil	0·42	0·44
		2	0·6	5·8		
		3	2·9	3·7		
		4	10·7	3·7		
W.C.	232	1	18·9	Nil	6·25	Nil
		2	11·8	Nil		
		3	8·9	4·0		
		4	1·6	3·6		
R.C.	297	1	7·0	3·6	0·44	1·04
		2	6·0	3·1		
		3	8·3	4·4		
		4	2·6	Nil		

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October 20, 1958.

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The Functional Groupings of α-Elaterin (Cucurbitacin E)

SIR,—At the British Pharmaceutical Conference, Llandudno, 1958, Gilbert and Mathieson described[1] the functional groupings of α-elaterin (Cucurbitacin E) and omitted to refer to information already published[2,3]. Meanwhile we have obtained valuable degradation products which shed light on the position of some of the groupings referred[4,5].

The presence of the diosphenol system (enolised 1:2-diketone) has been [2,3] indicated by the bathochromic shift with alkali observed in the ultra-violet light; this shift of the maximum at 267 mμ to 318 mμ is accompanied by a characteristic decrease in the relative intensity of absorption. Specific bands in the infra-red at 1660 and 1413 cm.$^{-1}$ confirmed this grouping. Further, this system has been shown to be attached to a six membered ring and to react with *o*-phenylenediamine to form a quinoxaline derivative. Elaterin formed a red tris-2:4-dinitrophenylhydrazone indicating the three keto groupings present in the molecule (αβ-unsaturated ketone in addition to the diosphenol system).

The side chain of α-elaterin has now been elucidated. This side chain $Me_2C(OH)\cdot CH:CH\cdot CO\cdot C(OH)RR'$ excluded any possibility of a pentacyclic molecule. Further, during the alkaline degradation of α-elaterin three rearrangements occur thereby clarifying the different degradation products obtained. Hot alkali induces a benzilic acid type rearrangement of the diosphenol system leading to a ring contraction and the formation of an α-hydroxy acid; the αβ-unsaturated ketone in the side chain undergoes at the same time a reversed aldol condensation, with the subsequent formation of a methyl ketone (iodoform test of ecballic acid)[6], thereby 2-hydroxy*iso*butyraldehyde is liberated. In the presence of alkali 2-hydroxy*iso*butyraldehyde undergoes an acyloin rearrangement thus forming ultimately acetoin[4-6]. This aldehyde has also been obtained during the ozonolysis of α-elaterin. Elaterin as well as other compounds of this series have been found[2,7] to have strong antitumour activity on hard tumours, Sarcoma 37 and Black Sarcoma, in mice and are now under investigation. They have also been found to have a definite action on a transplanted tumour in the cheekpouch of the golden hamster. (Schwenk, private communication.)

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September 11, 1958.

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Assay of Acetylcholine on the Rat Blood Pressure

SIR,—The rat blood-pressure preparation does not seem to have been fully exploited for the assay of depressor substances, despite its use for the assay of pressor substances by Crawford and Outschoorn[1] and Dekanski[2]. By using sodium pentobarbitone with urethane as the anaesthetic and allowing the body-temperature to fall to about 28° it has proved possible to get a long-surviving, stable sensitive preparation.

Method. The method is a modification of that described by Dekanski. Male rats of about 250 g. are preferred. Anaesthesia is obtained by the intraperitoneal injection of a mixture of urethane (40 mg./100 g.) and sodium pentobarbitone (3 mg./100 g.) and the animal is placed in a supine position on an unwarmed table. The depth of anaesthesia can be increased by injecting more pentobarbitone (1-1·5 mg./100 g.). A short glass cannula is tied in the trachea and the animal is allowed to breathe naturally without artificial respiration. The right carotid artery is dissected carefully from the surrounding structures and tied high in the neck.

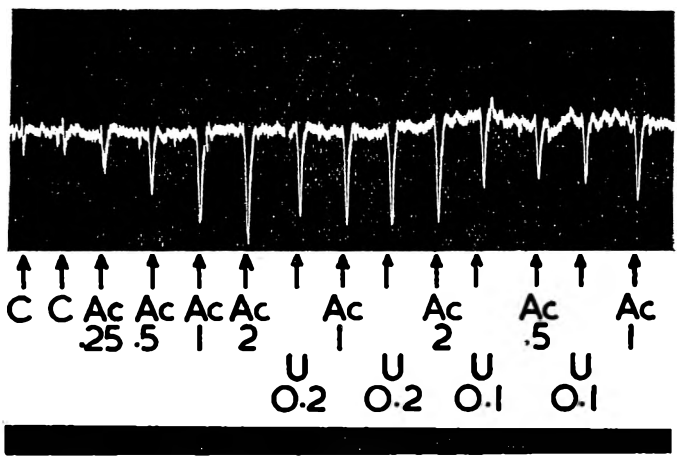

FIG. 1. Dose response of acetylcholine (Ac) in ng. of base. Volumes of unknown solution (U) in ml. (C) Injection artifact of 0·3 ml. Kreb's fluid with neostigmine 1×10^{-6}.

On the left side the femoral nerves are cut. The femoral vein is dissected from the artery up to the inguinal ligament and cannulated with a short fine bore polythene cannula connected by rubber tubing to a 1 ml. tuberculin syringe. The syringe is refilled with washing-in fluid through a side arm from a separating funnel. The venous cannula and a short section of the tubing just distal to it (into which injections are made) are held rigidly in a grooved perspex block clamped to the bench. This device facilitates the injection of fluid into the cannula and reduces injection artifacts. The dead space of the system is 0·03 ml. and thus only small volumes of fluid are needed for washing in injections. Before the anticoagulant (2000 units of dextran sulphate) is injected a check is made to see that there are no bleeding points. Ice cold saline swabs may be used if necessary to promote haemostasis. The artery is now cannulated and connected through polythene tubing to a Condon type manometer. It is more

satisfactory to inject several ml. of the washing-in fluid (which contains neostigmine methyl sulphate 1×10^{-6}), at intervals before beginning the assay, than to sensitise the preparation with a single injection of neostigmine.

The animal is left for 20 minutes and then the assay is begun, injections being made at 2 minute intervals for many hours.

The preparation shows a depressor response to as little as 0·5 ng. of acetylcholine, and the threshold may be as low as 0·25 ng. in the winter months (Fig. 1). Irregularities occuring in the course of an assay if not due to tracheal obstruction, may be reduced or abolished by the intravenous injection of 1 or 2 mg. of sodium pentobarbitone.

The successful use of this method for many months now suggests that it can replace the more conventional cat blood-pressure preparation, particularly in the estimation of the acetylcholine released by nerve stimulation from various nerve-muscle and ganglion preparations.

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October 28, 1958.

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