RESEARCH PAPERS

THE SECONDARY CONDITIONED RESPONSE OF RATS AND THE EFFECTS OF SOME PSYCHOPHARMACOLOGICAL AGENTS

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The secondary conditioned response has been studied in rats in an experimental avoidance situation. The activity of seventeen drugs has been tested upon the secondary conditioned response developed on a stable basis after an appropriate period of training. Chlorpromazine, promazine, reserpine and morphine block the secondary avoidance conditioned response as well as the usual avoidance conditioned response, in doses not affecting the motor function. Meprobamate, hydroxyzine, azacyclonol, phenaglycodol and phenobarbitone sodium have no specific inhibitory action on the avoidance conditioned response, but suppress the secondary conditioned response. A specific depression of conditioned behaviour is also produced by mescaline and iproniazid. Barbitone sodium, glutethimide, L1458 and mephenesin inhibit the conditioned responses only at neurotoxic doses. On the basis of these findings a new classification of "tranquillising agents" is proposed. It is also suggested that the systematic study of the secondary conditioned avoidance response of rats, may provide a useful experimental approach for studying the specific behavioural action of drugs.

Conditioned responses in rats have been used to evaluate the effects of central nervous system (CNS)-active drugs[1,2].

The conditioned avoidance response is a fear-motivated behaviour easily reproduced and widely used to investigate the effect of drugs. A noxious stimulus occurring contiguously with a warning stimulus can elicit anticipation of the noxious stimulus. In avoidance conditioning techniques a conditioning stimulus (buzzer) is associated with a "punishment" (electric shock) commonly defined as an unconditioned stimulus. The animal can avoid the shock by making a fixed response.

Rats are trained with repeated exposures to the experimental situation until the avoidance response is stable, when it is assumed that a conditioned avoidance behaviour has been established. This response is defined as the conditioned response (C.R.). With further training, some rats take up the position to avoid the unconditioned stimulus *before* the presentation of the conditioned stimulus.

Here it is assumed that the animals have developed a secondary conditioned reponse (S.C.R.) induced by environment, and its occurrence in rats has been described by others. It is usually regarded as a disturbing factor which is overcome either by excluding affected animals from further tests[1], or by prevention. In the latter instance rats are exposed to the experimental situation by placing them in a box containing the grid at the same time as they receive the conditioned stimulus. In

some circumstances the S.C.R. may be reinforced by allowing the buzzer to become a secondary negative stimulus that the animal can avoid or remove by making the correct response.

The present work is a study of the S.C.R. in the rat as well as the effects of many CNS-active drugs upon this conditioned behaviour in rats.

Experimental

Method

Male rats of the Carworth strain, weighing from 180 to 250 g. were used.

The experimental techniques were basically those of Cook and colleagues[3]. The animals were first conditioned to avoid an electric shock by placing them in a box the floor of which is a grid of steel rods. Shocks may be delivered to the grid from a stimulator. In all the experiments a shock of 45 ma, 250 V. was employed. The box is in a soundproof enclosure, which contains a buzzer. A wooden pole in the centre of the box provides the safety area. Rats escape the shock by climbing the pole (unconditioned response or U.R.) and by climbing the pole in response to the buzzer alone (conditioned avoidance response or C.R.). Long-trained rats climb the pole "before" the buzzer is activated and when this response becomes stable, the rat is considered to have developed a secondary conditioned response (S.C.R.). To assist this development and to stabilise the S.C.R., the conditioned stimulus was omitted when the rats made the correct response.

To provide enough conditioned rats, groups of 100–150 animals were trained. About twenty conditioning trials were given within five days to elicit a stable C.R. Ten to fifteen further trials were necessary to establish the S.C.R., in 85–90 per cent of the animals. For each experiment, rats showing a correct S.C.R. in ten consecutive trials were used.

On the day of the experiments the rats, in groups of ten, were tested to check the S.C.R. The drug to be studied was then administered either orally or intraperitoneally. One group received only saline to provide controls. Every thirty minutes each animal was placed in the experimental box. No stimulation was given during the first 15 seconds, when secondary conditioned animals react by climbing the pole usually within five-ten seconds. When this occurred the operator returned the rat to the floor grid. The animals responding for the second time were considered to have retained their S.C.R. unaltered.

Any rat not making the correct response within the 15 seconds received the buzzer for 30 seconds. The C.R. by normally conditioned rats occurred within two seconds of the sounding of the buzzer. If the animal did not respond within the 30 seconds, shocks were delivered by the grid floor.

In this experimental design the deconditioning effect of a drug may be shown by: (i) the loss of the S.C.R. only (C.R. is retained); (ii) the loss of the S.C.R. and C.R. (U.R. is retained); (iii) the loss of the S.C.R., C.R. and U.R., usually as a result of a marked impairment of motor function.

For the evaluation of the activity of a drug, the maximal effect was always considered. As the experiment consisted usually of many trials (every 30 minutes for four hours or more) in which the negative reinforcement was not given regularly, a group of controls was always tested concurrently to insure that the S.C.R. would not be extinguished spontaneously for lack of reinforcement. Of 207 control rats, only six showed a loss of the S.C.R. in one or more of the eight half-hourly trials.

We used 630 rats, all of which were rested for at least two weeks before being used again.

TABLE I

THE DEVELOPMENT AND EXTINCTION OF THE SECONDARY AVOIDANCE RESPONSE PERCENTAGES OF RATS SHOWING A S.C.R. AT DIFFERENT STAGES OF THE CONDITIONING SCHEDULE (TOTAL RATS USED 70)

Day	Cumulative number of		Responding rats per cent (S.C.R.)
	trials	reinforcements	
1	4	4	0
5	18	14	83
10	33	19	92
15	48	21	94
25	78	21	89
35	83	21	85
65	89	21	78

Development and Extinction of S.C.R.

From Table I it may be seen that over 90 per cent of rats develop and retain a S.C.R. after about 33 conditioning trials, 94 per cent being reached after 48 trials. A significant decrease of this percentage does not occur after five weeks. The same rats develop a stable C.R. within the first 15 trials, which is retained for a longer period than the S.C.R., on cessation of reinforcement.

The duration of the conditioning and of the extinction is perhaps one of the most important features and allows a quantitative differentiation of the two phenomena of secondary—and normal—conditioning.

RESULTS

Effect of Drugs on C.R. and S.C.R.

As the effects of many CNS-active drugs on avoidance C.R. are well known, their effectiveness on C.R. and S.C.R. has been compared. For this purpose, we determined for each drug the ED50 blocking the S.C.R. as well as the C.R. and the U.R. These ED50 estimates were made by plotting on probability paper the percentages of respondent rats for every log dose, and fitting the straight line according to the method of Litchfield and Wilcoxon[4]. The ED50's for S.C.R. block, C.R. block and U.R. block were then compared and tested for the significance of differences. By this procedure the quantitative evaluation of the specificity of drug activity upon the conditioned avoidance behaviour was made possible. Seventeen drugs were tested and the ED50 and confidence limits are given in Table II.

Chlorpromazine was given orally to groups of previously conditioned rats in twelve different doses—from 0·5 to 45 mg./kg. The S.C.R.

TABLE II

EFFECTIVENESS OF VARIOUS DRUGS IN SUPPRESSING AVOIDANCE CONDITIONED RESPONSES IN RATS

ED50 ACCORDING TO THE METHOD OF LITCHFIELD AND WILCOXON[4] IN mg./kg.

Substance	Route	ED50* for blocking S.C.R.	ED50* for blocking C.R.	ED50* for blocking U.R.
Chlorpromazine	oral	1·75 (0·92–3·32)	11·6 (8·99–14·96)	33 (25·38–42·9)
Promazine	oral	16 (10·0–25·6)	84 (62–113)	163 (153–173)
Reserpine	i.p.	0·46 (0·33–0·63)	1·15 (0·93–1·41)	†2·16
Hydroxyzine	i.p.	24 (15–37)	76 (56–103)	†120
Azacyclonol	i.p.	25 (13–46)	†155	†192
Meprobamate	oral	163 (116–230)	475 (380–593)	475 (380–593)
	i.p.	32·5 (13·0–81·2)	170 (139–207)	170 (139–207)
Mephenesin	i.p.	70 (57–84)	108 (99–118)	108 (99–118)
Phenaglycodol	oral	86 (59–126)	220 (191–253)	305 (246–378)
Barbitone Na	oral	85 (53–137)	112 (83–151)	134 (102–175)
Pentobarbitone Na	oral	9·6 (8·1–11·4)	18·3 (16·6–20·1)	19·5 (18·1–20·9)
Phenobarbitone Na	oral	25 (18–34)	80 (49–130)	97 (81–115)
Glutethimide	oral	163 (102–261)	255 (161–403)	255 (161–403)
L1458	i.p.	28·2 (18·8–43·3)	48·5 (36·7–64·0)	65·0 (58·5–72·1)
Morphine	s.c.	0·94 (0·70–1·26)	4·3 (3·23–5·72)	15·2 (11·6–19·9)
Iproniazid	i.p.	84 (63–111)	>200	—
Mescaline	i.p.	32·5 (2·12–4·97)	>50	—

* and 19/20 confidence limits. † Approximately.

was blocked in 50 per cent of treated rats (ED50) after 1·75 mg./kg. No animal on this dose showed any effect on C.R. which was inhibited with 11·6 mg./kg. (ED50). The U.R. was affected by higher doses (ED50, 33 mg./kg.).

We can thus affirm that chlorpromazine specifically blocks the S.C.R. and at higher doses the C.R. The differences in ED50 as shown in Table II were very significant.

Promazine shows closely similar activity, specifically blocking the S.C.R. and the C.R. However, its effectiveness was found to be lower than that of chlorpromazine, as higher doses were necessary.

With both substances the slopes of S.C.R.-blocking activity curves (Table III, Fig. 1) were found to be greater than those of C.R. and U.R. blocking activity; however, the differences were significant only in the case of chlorpromazine.

TABLE III

SLOPES OF LOG DOSE/ACTIVITY CURVES

Substance	Route	Slope of dose/activity and 19/20 C.L.* for blocking S.C.R.	for blocking C.R.	for blocking U.R.
Chlorpromazine	oral	5·16 (4·37–6·08)	1·84 (1·64–2·06)	1·61 (1·13 2·30)
Promazine	oral	2·03 (1·70–2·41)	1·62 (1·36–1·92)	1·11 (0·99–1·24)
Reserpine	i.p.	1·64 (1·09–2·46)	1·48 (1·20–1·82)	1·38
Hydroxyzine	i.p.	2·53 (1·48–4·30)	1·66 (1·12–2·32)	—
Azacyclonol	i.p.	2·2 (1·01–4·84)	—	—
Meprobamate	oral	1·90 (1·11–3·23)	1·43 (1·06–1·91)	1·43 (1·06–1·91)
	i.p.	2·84 (1·18–6·81)	†1·26	†1·26
Mephenesin	i.p.	1·24 (1·01–1·51)	†1·10	†1·10
Phenaglycodol	oral	2·10 (1·39–2·17)	1·36 (1·07–1·72)	1·42 (1·10–1·81)
Barbitone Na	oral	1·54 (0·46–5·08)	1·82 (1·25–2·63)	1·71 (1·31–2·22)
Pentobarbitone Na	oral	1·21 (1·01–1·44)	1·17 (0·78–1·74)	1·12 (1·06–1·19)
Phenobarbitone Na	oral	2·35 (1·58–3·48)	1·47 (0·98–2·20)	1·49 (1·24–1·78)
Glutethimide	oral	1·47 (0·41–5·26)	1·45 (0·83–2·54)	1·45 (0·83–2·54)
Morphine	s.c.	2·13 (1·21–2·72)	1·82 (1·40–2·36)	1·34 (1·04–1·71)
L1458	oral	2·2 (1·18–3·47)	1·37 (1·12–1·67)	1·12 (0·79–1·58)
Iproniazid	i.p.	1·43 (1·15–1·77)	—	—
Mescaline	i.p.	2·18 (1·01–4·68)	—	—

* According to Litchfield and Wilcoxon[4]. † Approximately.

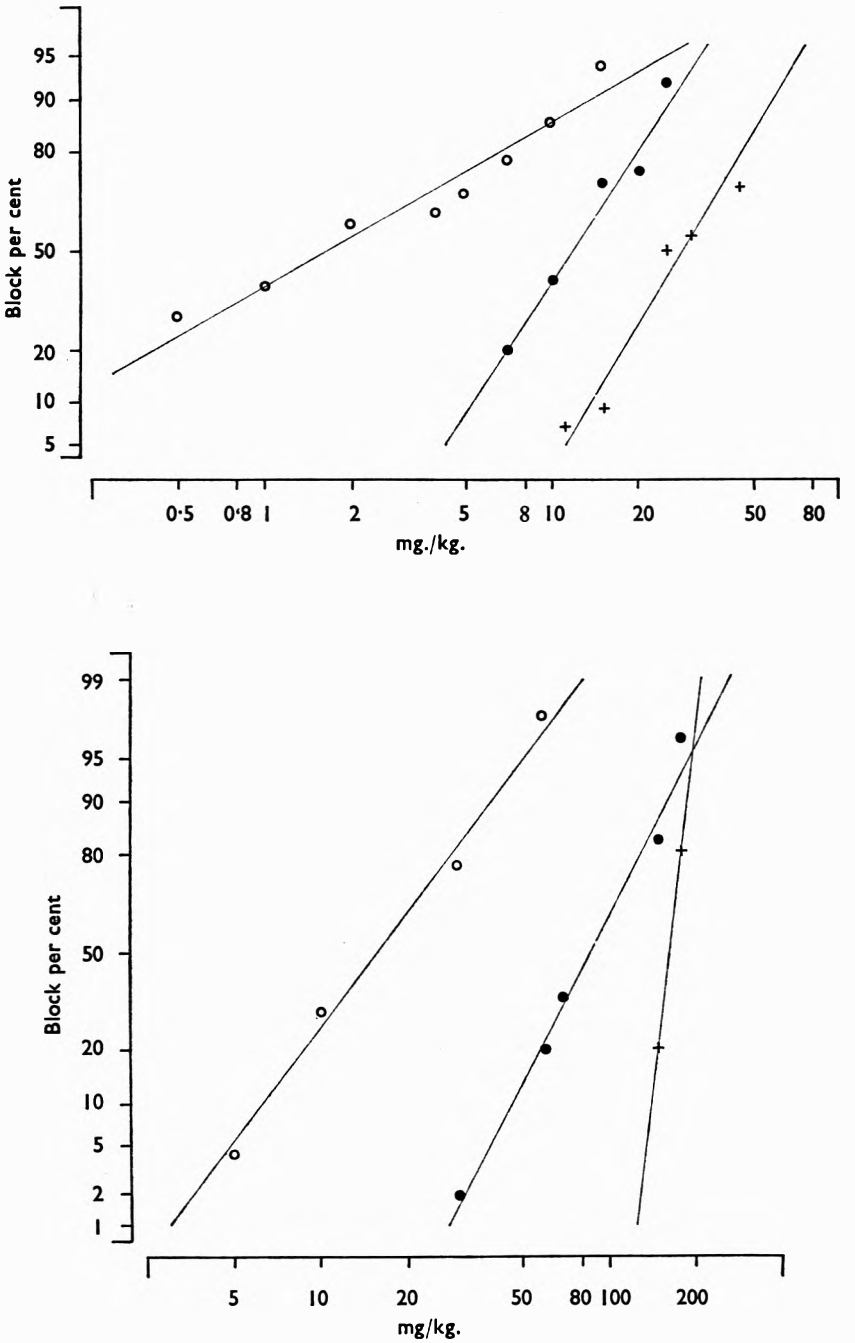

FIG 1. Block of secondary conditioned response (○), conditioned response (●) and unconditioned response (+) by different compounds. Upper graph—Chlorpromazine by oral route. Lower graph—Promazine by oral route

The duration of the effectiveness of both substances is shown in Table V.

Reserpine was given by intraperitoneal injection in doses from 0·3 to 2·5 mg./kg. A large variability was found particularly in the abolition of the U.R. Nevertheless, the results enable us to affirm that this substance specifically blocks both S.C.R. and C.R. (Table II). The ED50 for blocking the S.C.R. was 0·46 mg./kg. and 1·5 mg./kg. for the C.R. The U.R. was affected at doses higher than 2 mg./kg. As shown in Table V, the onset of reserpine activity has been observed 120 minutes after administration and lasted for more than 10 hours.

Hydroxyzine, like reserpine, was given intraperitoneally and was found to specifically suppress the S.C.R. From Table II, the ED50 for blocking the S.C.R. and particularly the C.R. can be seen to be close to the ED50 for blocking the U.R. as with chlorpromazine.

Azacyclonol, intraperitoneally, produces a specific block of S.C.R., but not of C.R. This means that the doses affecting the C.R. (Table II) also affect the motor function and block the U.R. in some animals. Such doses therefore approach the lethal dose.

Benactyzine, by the oral or intraperitoneal route, gave inconsistant results. The S.C.R. appears to be blocked by between 20 and 40 mg./kg. The C.R. was concurrently affected. However, a linear relationship of doses to effects was not found because of the great variability in the responses of different animals and even in the same animal on different days.

Meprobamate, both orally and intraperitoneally, failed to produce any specific block of C.R. Rats began to lose the C.R. at dose-levels that also block the U.R. because of ataxia and incapacitation of motor function. However, meprobamate was shown to have a specific blocking action of the S.C.R. The oral and intraperitoneal ED50 estimates are given in Table II.

Mephenesin, a drug similar in action to meprobamate, failed to produce any specific block of the S.C.R. as well as of the C.R.

Phenaglycodol, tested only by oral route, produces like meprobamate, a specific block of S.C.R. The C.R. was blocked only with doses that also affect the U.R. in different degrees.

Among the barbiturates, *barbitone sodium* does not produce any specific block either of S.C.R., or of the C.R. Also, *pentobarbitone*-induced inhibition of S.C.R. is caused by doses near the neurotoxic ones. On the contrary, *phenobarbitone* produces a moderate but significant degree of specific block of S.C.R. In this respect phenobarbitone was found to differ not only from other barbiturate derivatives but also from other hypnotics, such as *glutethimide*, which behaves like barbitone (Table II).

Morphine sulphate, by subcutaneous injection, produces minimal inhibitory effects on the S.C.R. after 0·5 mg./kg. The calculated ED50 for blocking of S.C.R. was 0·94 mg./kg. Morphine also produces a specific block of C.R. in doses much lower than those which suppress the U.R. Its action on conditioned behaviour of rats, very much resembles that of chlorpromazine.

2-*Thienyl*-5-*amino*-1 : 3 : 4-*thiadiazole* (L1458), a muscle relaxant recently described[5], and in some aspects of its pharmacological action resembling benzimidazole[6], blocks in a moderate degree only the S.C.R. and fails to affect the normal C.R.

A specific block of S.C.R. is also produced by *iproniazid* but only in very high doses, the estimated intraperitoneal ED50 being 84 mg./kg. It was found that 200 mg./kg. produced a block of the C.R. only in 10 per cent of the tested rats (1/10).

Mescaline behaves very similarly to iproniazid, although at a lower dosage. The intraperitoneal ED50 for blocking the S.C.R_t was found to be 33 mg./kg. Following 50 mg./kg. only 10 per cent of the tested rats showed a loss of C.R.

Observations on Dose : Activity Curves

By analysing log-dose : per cent blocking-activity curves it was found that the slopes of the curves for blocking the S.C.R. were almost always higher than those for blocking the C.R. but only in a few instances was this difference significant ($P = 0{\cdot}05$). Of the 14 substances considered in this respect, only three—chlorpromazine, meprobamate by i.p. route and phenobarbitone—show dose : activity curves for S.C.R. blocking, significantly different from those for C.R. blocking. The slopes of the curves for C.R. and U.R. block were always statistically equivalent for every product and route of administration. With few exceptions, the slopes obtained with different products are not significantly different.

Onset and Duration of Action

Remarkable differences were observed in the time of onset and in the duration of action of the tested drugs. As reported, the ED50 estimates have been calculated on the basis of the maximum effect, irrespectively of the time. In Table V the times of onset of the blocking-activity and its duration are given for each of the tested drugs. The effects of the respective ED50 doses on the three responses are shown.

With the exception of reserpine the onset of the S.C.R. block is very rapid for the drugs. The suppression of this response in 10 per cent of the animals is produced usually within 30 or 40 minutes after administration. The duration of maximal and minimal action is more variable.

By comparing the onset of S.C.R. block and the C.R. block produced by approximately equi-active doses of each drug, it appears that an equal or a more prolonged interval after administration is necessary for the suppression of C.R.

The maximal blocking effect on S.C.R. is more prolonged than the maximal blocking effect on C.R. with equi-active doses of chlorpromazine, reserpine, hydroxyzine, oral meprobamate and morphine. An approximately equal duration of inhibition on S.C.R. and C.R. has been observed following azacyclonol, i.p. meprobamate, phenaglycodol and phenobarbitone.

Of the drugs tested reserpine shows a unique behaviour, its action beginning two hours after administration and lasting for more than 10 hours.

Discussion

The results show that the S.C.R. developed by rats in a simple experimental situation, may be made stable in a high percentage of animals. Examples of secondary avoidance conditioning may be found in literature[7–9]. However, we are unaware of any pharmacological application or systematic study of the effects of drugs on these S.C.R. responses.

Our results show that the avoidance S.C.R. may be suppressed by some drugs and that with these a linear relationship exists between log-doses and percentage blocking effect.

It first appeared that many depressive agents in suitable doses may suppress all three responses. To define the "specificity" of the effect on the conditioned behaviour the ED50 ratios have been chosen (Table IV). By analysing the specific activity of each drug, different degrees of potency were found.

TABLE IV

Specificity of drugs activity on conditioned behaviour
ED50 ratios and 19/20 confidence limits*

Substance	Route	ED50 ratio for	
		block of U.R./ block of S.C.R.	block of U.R./ block of C.R.
Chlorpromazine	oral	18·85 (14·28–24·88)	2·84 (2·15–3·74)
Promazine	oral	5·25 (9·13–13·01)	1·94 (1·43–2·61)
Reserpine	i.p.	†4·70	†1·87
Hydroxyzine	i.p.	5·08	1·60 (N.S.)
Azacyclonol	i.p.	4·77	1·20 (N.S.)
Meprobamate	oral	2·91 (1·94–4·36)	1·00 (N.S.)
	i.p.	5·23 (2·17–12·55)	1·00 (N.S.)
Mephenesin	i.p.	1·54 (N.S.)	1·00 (N.S.)
Phenaglycodol	oral	3·54 (2·29–5·45)	1·20 (N.S.)
Barbitone Na	oral	1·57 (N.S.)	1·12 (N.S.)
Pentobarbitone Na	oral	2·03 (1·84–2·23)	1·06 (N.S.)
Phenobarbitone Na	oral	3·38 (2·77–5·47)	1·21 (N.S.)
Glutethimide	oral	1·56 (N.S.)	1·00 (N.S.)
L1458	i.p.	2·30 (1·52–3·47)	1·34 (N.S.)
Morphine	s.c.	16·17 (10·78–24·25)	3·53 (2·37–5·26)
Iproniazid	i.p.	>2·38	—
Mescaline	i.p.	>1·54	—

* When the differences in ED50 estimates are not statistically significant (P = 0·05), the confidence limits are omitted and N.S. (non-significant) added.

† Approximately.

In the works of others on rats in conditioned avoidance situations, only the specific effects on C.R. of the drugs have been considered. We have found that the S.C.R. also is of considerable importance as a test for the activity of drugs.

We consider the tested drugs may be divided in three main groups, according to their activity on conditioned behaviour of rats.

(i) Group reducing a specific block of both the S.C.R. and C.R. This comprises chlorpromazine, promazine, reserpine and morphine. Some of these substances have been differentiated by Cook and Weidley[3] for their blocking activity on C.R. According to Berger[10], they are defined as "autonomic suppressants" because of their antagonism to acetylcholine, histamine and serotonin, which regulate certain functions of the autonomic nervous system. Some of these drugs are also called by Pfeiffer and colleagues[11] "ataractics" as they cause adrenergic blockade,

lower the electrical threshold for convulsions and inhibit conditioned avoidance responses of rat and monkey.

Chlorpromazine, reserpine and promazine also have been called "tranquillisers" by Alexander[12], on the basis of clinical implications, and by Delay and Deniker[13] "neuroleptiques".

Despite the autonomic "side effects" of these substances, it seems evident that they really possess a behavioural specificity in their action. The activity on S.C.R. is, in our opinion, a further confirmation of their specificity.

The high blocking activity of morphine on C.R. has been confirmed by our experiments and the specific behavioural effect of this substance is further shown by its great effectiveness in suppressing the S.C.R. Apparently the changes in conditioned avoidance behaviour of rats do not allow the differentiation of this drug from the tranquillisers, but other pharmacological tests[1,14] may be used to reveal the differences in the activity of morphine and that of other behaviour-affecting drugs.

(ii) A second group of substances were those which specifically block the S.C.R. with little, if any, effect on the C.R.

Different degrees of specificity were found in activity upon S.C.R. In order of decreasing specific potency the drugs are: meprobamate (i.p.), hydroxyzine (i.p.), azacyclonol (i.p.), phenobarbitone (oral), phenaglycodol (oral), meprobamate (oral).

Meprobamate is perhaps one of the most largely used agents for its tranquillising action, but according to others does not affect conditioned and avoidance reflexes[1,10,11,15]. This fact may be explained by a possible inadequacy of the usual techniques. The same may be said for other substances classified in this group such as *hydroxyzine*, *azacyclonal* and *phenaglycodol*. The major differences among the substances of this group have been found in the onset and duration of their activity, as can be seen from Table V.

The experimental results compel us to insert phenobarbitone in this group of substances. The specific block of S.C.R. produced by this drug clearly differentiates it from other barbiturates and hypnotics, an effect for which no ready explanation is available.

(iii) The third group of agents include barbitone, pentobarbitone, mephenesin, L1458 and glutethimide, i.e., hypnotics and centrally acting paralysing drugs. All these substances failed to produce any significant specific block of either S.C.R. or C.R.

We therefore conclude that the effect of drugs upon the secondary conditioned avoidance response permits the selection of substances with high behavioural specific activity. Two different classes of drugs among the agents depressing the behaviour of organisms are apparent. They are the "general deconditioning agents" (chlorpromazine, promazine, reserpine and morphine) and the "secondary deconditioning agents" (meprobamate, hydroxyzine, azacyclonol, phenaglycodol and phenobarbitone).

General deconditioning agents can suppress both conditioned avoidance responses in doses that are not neurotoxic. Some have been found to

suppress also experimental "anxiety"[15,16]. The action of these drugs is characterised by high specificity in blocking the S.C.R. of rats, and usually a marked but minor specificity in blocking the C.R.

Secondary deconditioning agents do not suppress the C.R. of rats, but specifically affect the S.C.R. of rats.

TABLE V

DURATION OF EFFECTIVENESS OF DRUGS IN PRODUCING BLOCK OF THE THREE RESPONSES

Substance	Route	Dose mg./kg.	Response blocked	Time (minutes) after administration and blocking effect			
				Onset 10 per cent	Maximal from	Maximal to	End 10 per cent
Chlorpromazine ..	oral	2	S.C.R.	30	210	270	420
	"	10	C.R.	75	120	210	345
	"	30	U.R.	300	390	—	>390
Promazine ..	oral	10	S.C.R.	40	60	210	345
	"	80	C.R.	30	90	390	>390
	"	180	U.R.	60	300	360	>390
Reserpine	i.p.	0·5	S.C.R.	120	240	560	600
	"	1	C.R.	240	360	440	520
	"	2·5	U.R.	120	240	>360	—
Hydroxyzine ..	i.p.	20	S.C.R.	30	60	210	300
	"	60	C.R.	30	45	90	150
	"	100	U.R.	30	30	45	150
Azacyclonol ..	i.p.	25	S.C.R.	30	120	180	300
	"	150	C.R.	75	120	180	240
	"	200	U.R.	75	150	>390	—
Meprobamate ..	oral	200	S.C.R.	30	60	180	280
	"	500	C.R. and U.R.	<30	30	150	>420
	i.p.	50	S.C.R.	30	60	90	150
	"	200	C.R. and U.R.	<30	30	90	150
Mephenesin ..	i.p.	75	S.C.R.	<30	30	—	90
	"	100	C.R. and U.R.	<30	<30	—	<60
Phenaglycodol ..	oral	100	S.C.R.	<30	90	210	340
	"	200	C.R.	40	150	240	>340
	"	300	U.R.	60	210	>420	>420
Barbitone Na ..	oral	100	S.C.R.	40	180	360	>390
	"	100	C.R.	90	180	240	330
	"	150	U.R.	75	150	300	>390
Pentobarbitone Na	oral	10	S.C.R.	<30	60	100	180
	"	20	C.R.	<30	30	100	180
	"	20	U.R.	<30	30	100	170
Phenobarbitone Na	oral	20	S.C.R.	<30	60	210	300
	"	75	C.R.	45	90	240	360
	"	100	U.R.	30	150	270	>360
Glutethimide ..	oral	200	S.C.R.	45	60	150	360
	"	250	C.R. and U.R.	50	210	360	>360
L1458	i.p.	30	S.C.R.	30	60	150	180
	"	60	C.R.	<30	30	60	120
	"	60	U.R.	<30	30	60	120
Morphine	s.c.	1	S.C.R.	<30	60	120	190
	"	4	C.R.	30	60	90	120
	"	20	U.R.	60	90	150	180
Iproniazid	i.p.	100	S.C.R.	30	60	150	220
Mescaline	i.p.	25	S.C.R.	30	60	150	180

It is not the purpose of this paper to interpret, on the basis of these findings, the mechanism of action of the different drugs. However, one might suggest that although the secondary deconditioning agents, unlike the general deconditioning agents, do not block the behaviour depending upon a conditioned emotional disturbance, they nevertheless influence a process depending in some manner on emotional arousal.

We believe that the study of the S.C.R. will provide an easy experimental approach to the problem of revealing, testing, and classifying new tranquillising drugs.

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PHOLCODINE TARTRATE AND RELATED SALTS

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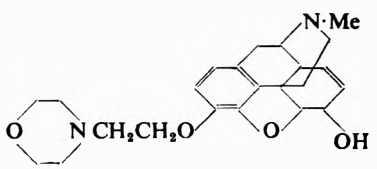
Pholcodine gives stable hydrated salts, crystallisable from water, with a series of dibasic hydroxy acids, the most important salt being the tartrate. The preparation and analysis of these salts is described.

Pholcodine, 3:2′-morpholinoethylmorphine (I), is a cough suppressant[1], and is described in the 1957 Supplement of the British Pharmaceutical Codex. Though soluble in dilute acids it is sparingly soluble in water. It would often be convenient in the manufacture of formulations of pholcodine to have it available as a stable crystalline salt.

Pholcodine salts of most acids are so very soluble in water that they cannot be crystallised from aqueous solution; the sulphate, for instance, is soluble in 1·5 parts of water at room temperature[2]. The formation of salts *crystallisable from water*[3], now described, appears to be confined to one particular class of organic acids of general formula (II): of especial



(I)

$$HOOC\cdot(CHOH)_n\cdot COOH$$

(II)

interest in this class are tartronic acid ($n = 1$) and tartaric acid ($n = 2$); the higher members of the class are di-acids derived from sugars, e.g., mucic acid ($n = 4$), and are not so readily available. Oxalic acid ($n = 0$), which might perhaps be considered in this class, also gives a crystalline salt with pholcodine, but this oxalate is much more soluble in water than are the other members of the series; moreover, oxalic acid is a poison and pholcodine oxalate is thus of no practical importance.

The salts may be readily prepared by dissolving pholcodine base in an equivalent amount of a 50 per cent aqueous solution of the appropriate acid at 50–70° and collecting the salt which crystallises on cooling. The salts so prepared contain two molecules of acid per molecule of base and are hydrated. Air-drying to constant weight at 35–40° affords the trihydrate with the tartrate and the tetrahydrate with the tartronate, though indefinite forms containing less water of crystallisation have been obtained on drying at higher temperatures or over a desiccant. These salts on heating dissolve in their own water of crystallisation, thus appearing to melt. They decompose when heated at 100°.

EXPERIMENTAL

Preparative

Pholcodine tartronate was prepared by dissolving pholcodine (21 g.) in aqueous tartronic acid (25 ml. of 50 per cent w/v) at 60°, stirring, and allowing the homogeneous solution to cool. After recrystallisation from water and air-drying at 35–40°, the tartronate (about 25 g.) had an apparent m.p. of 60–65°. (Found: C, 48·3; H, 6·5; N, 3·75, $C_{23}H_{30}O_4N_2$, $2C_3H_4O_5$,$4H_2O$ requires C, 49·0; H, 6·5; N, 3·95 per cent). It lost on drying *in vacuo* 9·9 per cent of its weight ($4H_2O$ requires 10·15 per cent). The tartronate tetrahydrate formed as colourless needles, stable to light, which, in the open, slowly absorbed a small variable amount of water (depending on ambient temperature and humidity).

Pholcodine tartrate similarly prepared apparently melted at about 85°. (Found: C, 49·4; H, 6·4; N, 3·9. $C_{23}H_{30}O_4N_2$,$2C_4H_6O_6$,$3H_2O$ requires C, 49·5; H, 6·4; N, 3·7 per cent). It lost 7·2 per cent of its weight on drying *in vacuo* ($3H_2O$ requires 7·2 per cent). Solubility of the colourless needles in water at 20° is about 1 in 8. It has $[\alpha]_D^{20}$–33° (c = 1 in water) and the pH of a 0·1 M solution in CO_2-free water is 3·1–3·5. The ultraviolet absorption of an aqueous solution shows a maximum at 283 mμ, (ϵ = 1650); this corresponds well with the known value for the base. It may be obtained anhydrous by drying for seven hours over P_2O_5 at 78° and 2 mm. Hg pressure; it then has m.p. 120–122° (sealed capillary) and is hygroscopic.

Pholcodine mucate was less readily obtained. The base (7·5 g.) and mucic acid (7·6 g.) were dissolved in water (11 ml.) by warming to 75°, and the mixture was allowed to cool; the salt crystallised slowly over several days. It was best recrystallised from 50 per cent aqueous ethanol. Drying at 40° over phosphorus pentoxide in a vacuum desiccator gave the monohydrate of m.p. 133–135° (with decomposition). (Found: C, 49·4; H, 7·15; N, 3·25. $C_{23}H_{30}O_4N_2$, $2C_6H_{10}O_8$,H_2O requires C, 50·25; H, 6·15; N, 3·35 per cent).

Analytical

Determination of pholcodine base. The salt (about 0·5 g.) accurately weighed, is dissolved in water (20 ml.) and dilute ammonia solution added until the mixture is distinctly alkaline to litmus. The liberated base is then completely extracted with six 25-ml. portions of chloroform, each extract being washed with the same 5-ml. portion of water. The chloroform is evaporated, the residue dissolved in 95 per cent ethanol (5 ml., previously adjusted to pH 4·8) and the ethanol removed by evaporation. The residue is dried at 105° for 15 minutes and dissolved in 0·1N sulphuric acid (50 ml.). The excess of acid is titrated potentiometrically with 0·1N sodium hydroxide (the end point being near pH 4·8). Each ml. of 0·1N sulphuric acid is equivalent to 0·01993 g. of $C_{23}H_{30}O_4N_2$.

Alternatively a spectrophotometric method of assay may be used. Thus, e.g. anhydrous pholcodine tartrate (about 25 mg. accurately weighed) is dissolved in water and diluted to 100 ml. The extinction of

the solution is determined in a 1-cm. cell at 283 mμ. The E (1 per cent, 1 cm.) of the anhydrous salt at 283 mμ is 24·6.

Determination of moisture content. The salt is dried to constant weight in a "drying pistol" at 78° at a pressure not exceeding 5 mm. The value thus obtained is reproducible; that obtained by drying at 105° is affected by the decomposition of the salts.

Acknowledgements: The authors are indebted to Mr. F. J. Bolton for his encouragement, and to their colleagues Miss A. P. Morrison and Mr. J. H. Smith (who first isolated pholcodine tartrate) for help. Micro-analyses are by Drs. Weiler and Strauss, Oxford.

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THE EFFECT OF ISOPRENALINE ON THE BLOOD FLOW THROUGH INDIVIDUAL SKELETAL MUSCLES IN THE ANAESTHETISED CAT

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The effects of intra-arterially and intravenously administered isoprenaline on the venous outflow from individual skeletal muscles in the hind limbs of cats under chloralose anaesthesia were studied. By intra-arterial injection isoprenaline was more powerful in causing vasodilatation than adrenaline. The dilator response to intravenously administered isoprenaline was shown by cross-circulation techniques and by the use of a blood pressure stabiliser, to be reflexly inhibited by a vasoconstriction initiated by the fall in blood pressure. In the innervated muscle when vasomotor tone was high, intravenously administered adrenaline was more potent than isoprenaline in producing vasodilatation. The reverse was true in the acutely denervated muscle. The dilator response to isoprenaline was shown to be the result of a direct action on the muscle blood vessels; no evidence was obtained of a reflex dilatation, such as has been demonstrated with adrenaline and noradrenaline.

Adrenaline has been shown to cause vasodilatation in the skeletal muscles of the cat by a direct action on the blood vessels and by an action which is mediated through the nerves[1]. The local action of noradrenaline is vasoconstriction, but it, too, can cause a weak vasodilatation in skeletal muscles by a mechanism which involves the nerves[1]. Isoprenaline is known to possess a direct vasodilator action in skeletal muscles as well as in most other tissues[2–5].

The present experiments were designed to compare the vasodilator actions of isoprenaline and adrenaline and to find out whether or not a direct action on the blood vessels is the only mechanism by which isoprenaline produces its effects.

Methods

Cats were anaesthetised with chloralose only (80 mg./kg.) injected into the subcutaneous vein of the fore-limb.

The methods used for recording the venous outflow from the tibialis anterior, the gastrocnemius-plantaris or the soleus muscle and for the preparation of cross-perfusion experiments, in which the gastrocnemius muscle of one cat is supplied entirely by blood from a donor cat, were identical with those previously described[1,6]. The sciatic nerve was exposed high in the thigh to denervate during the experiments. Blood pressure was recorded from the right common carotid artery, a blood pressure stabiliser[7] being connected when required. Drugs were injected intravenously through a cannula in the jugular vein or intra-arterially from a microsyringe into a needle cannula in the cut central end of a branch of the femoral artery. The maximum volume of any solution

administered intra-arterially was 0·01 ml. and control saline injections of the same volume were made throughout each experiment. (For further details see previous paper[1].)

Solutions for injection were made in 0·9 per cent w/v NaCl saline. The drugs used were isoprenaline sulphate and (−)-adrenaline bitartrate. The doses quoted in the text refer to the quantity of amine calculated as base.

Results

Throughout the experiments similar responses were obtained whatever the muscle under study. The results to be described, therefore, apply to all three muscles, the tibialis anterior, the gastrocnemius-plantaris and the soleus.

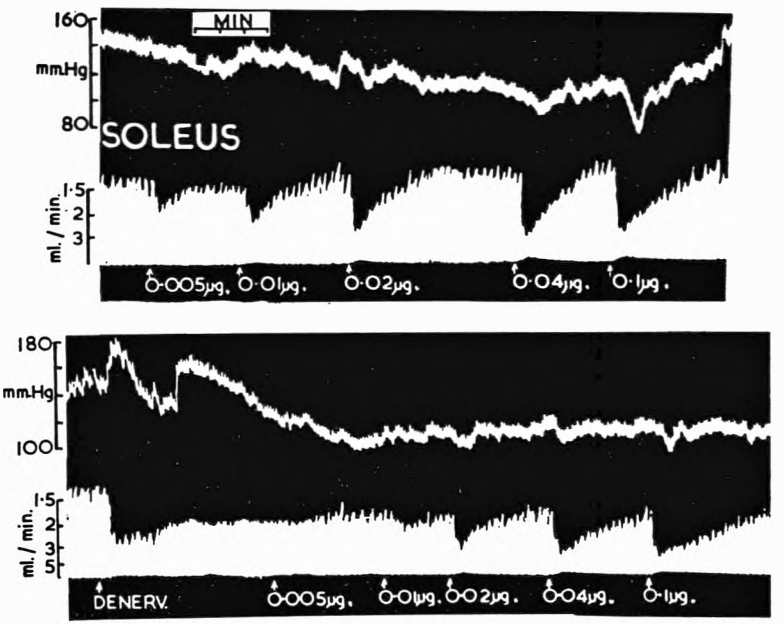

FIG. 1. Cat, 4·2 kg. The effect of intra-arterially administered isoprenaline in the innervated and acutely denervated muscle. Upper record: blood pressure recorded from carotid artery; lower record: venous outflow from soleus muscle.

Intra-arterially Administered Isoprenaline

Minimal effective doses of isoprenaline (0·002 to 0·01 μg.) caused vasodilatation in skeletal muscles and this response increased with increase in the dose up to a maximum with doses of 0·02 to 0·1 μg., after which further increase in the amount merely prolonged the effect. Figure 1 illustrates these results. The minimal effective doses of isoprenaline and adrenaline were similar but with increase in the dosage, the response to adrenaline gradually changed to vasoconstriction[1], whereas vasodilatation was produced by isoprenaline in all effective doses. With doses of equal size, the vasodilatation produced by isoprenaline was always longer lasting than that produced by adrenaline. During the hyperaemia

caused by acute denervation of the muscle, intra-arterially administered adrenaline only rarely caused vasodilatation. Isoprenaline, on the other hand, still caused a marked increase in venous outflow although the minimal effective doses were larger than those required in the innervated muscle (Fig. 1). After denervation, minimal effective doses of isoprenaline were equivalent in size to doses of adrenaline which, in the innervated muscle, produced the onset of vasoconstriction.

When administered intra-arterially, doses of adrenaline up to 3–4 μg. did not affect the general arterial blood pressure[1] but isoprenaline, administered by the same route, often caused a fall in blood pressure in doses as low as 0·05–0·1 μg. (Fig. 1).

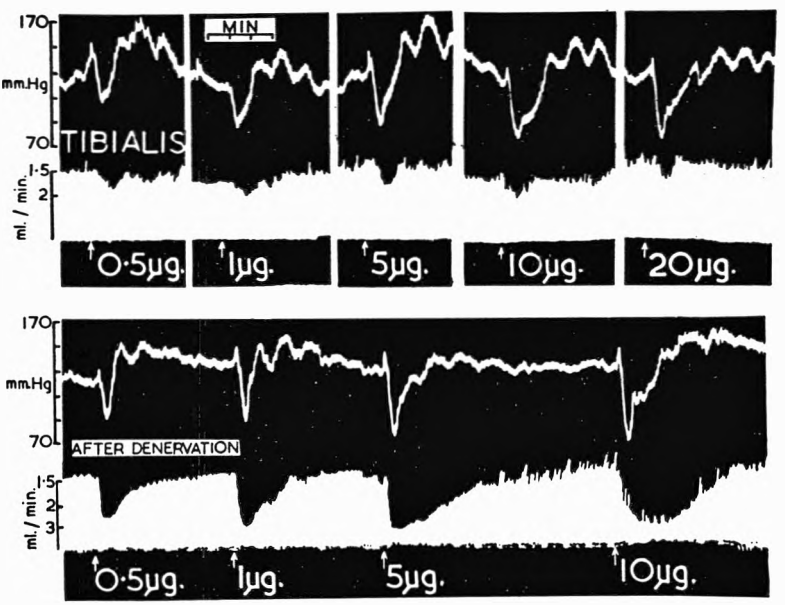

FIG. 2. Cat, 3·6 kg. The effect of acute denervation on the response to intravenously administered isoprenaline.

Intravenously Administered Isoprenaline

Intravenously administered isoprenaline caused a fall in blood pressure and vasodilatation in the skeletal muscles. Minimal effective doses which caused a fall in blood pressure were about 0·025 to 0·05 μg./kg. However, doses approximately four times larger were required to cause vasodilatation in the innervated skeletal muscles. In the acutely denervated skeletal muscles vasodilatation was produced by the smallest dose which caused a fall in blood pressure. In the innervated muscles the vasodilatation was invariably weak and hardly increased with increase in the dosage. It was often preceded by a brief passive reduction in flow as the blood pressure fell. After sectioning the sciatic nerve, intravenously administered isoprenaline produced a much greater increase in venous outflow and here the response increased with increase in dose

up to a maximum with doses of 3–4 μg./kg. after which any further increase merely prolonged the effect. The blood flow response to isoprenaline was increased after acute denervation whatever the original level of vasomotor tone in the muscle. Figure 2 illustrates an experiment in which the vasomotor tone in the innervated muscle was low as shown by the fact that acute denervation, later in the experiment, did not cause an increase in the rate of blood flow. In contrast, in the experiment illustrated by Figure 3, acute denervation caused a pronounced hyperaemia showing that vasomotor tone had originally been high. In both cases the vasodilator responses to isoprenaline were much more pronounced after denervation.

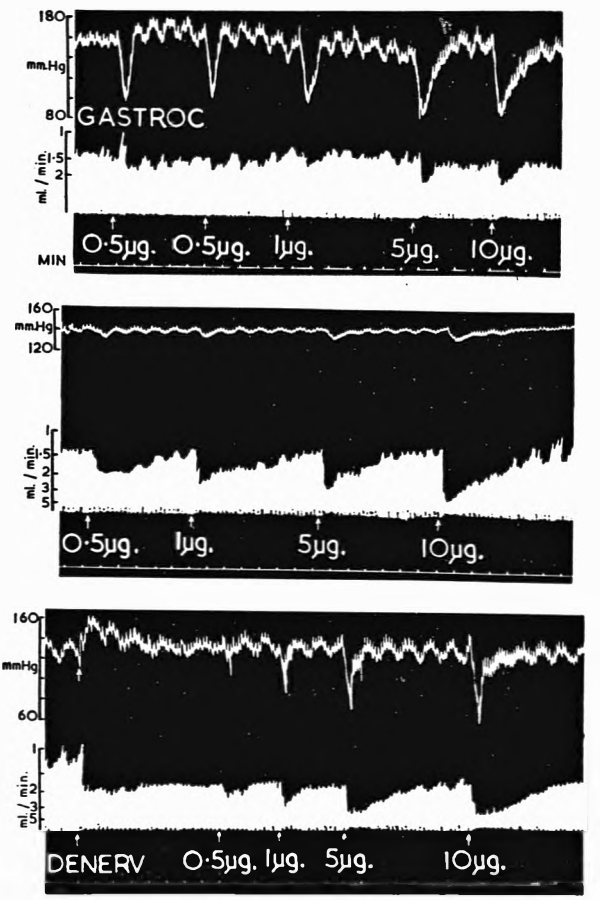

FIG. 3. Cat, 3·0 kg. The effect of stabilising the blood pressure (middle tracing) and of acute denervation (lower tracing) on the response to intravenously administered isoprenaline.

When the blood pressure stabiliser was connected to the carotid artery, the fall in blood pressure produced by intravenously administered isoprenaline was prevented and the vasodilatation produced in the innervated

muscle was very much greater than that which occurred when the blood pressure was allowed to fall (Fig. 3). These results combined indicate that vasodilatation produced by intravenously administered isoprenaline is inhibited in the innervated muscle by reflexes initiated by the fall in blood pressure. This conclusion was confirmed in cross-circulation experiments in which the muscle of one cat was perfused entirely by blood from a donor cat so that the only connection between the recipient cat and the muscle under study was by way of the nerves. In such experiments the intravenous administration of isoprenaline to the recipient cat caused a fall in blood pressure and vasoconstriction in the skeletal muscle. When the fall in blood pressure was prevented by means of the blood pressure stabiliser a similar administration to the recipient cat was without effect on the muscle blood flow. The intravenous administration of isoprenaline to the donor cat caused pronounced vasodilatation in the perfused muscle which was preceded by a short-lasting passive reduction in venous outflow as the blood pressure of the donor cat fell.

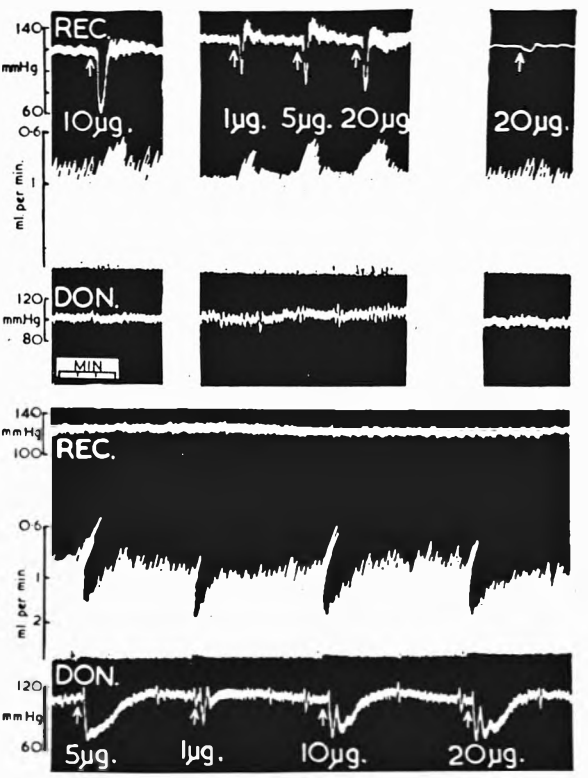

FIG. 4. Recipient cat, 3·5 kg.; donor cat, 2·7 kg. Cross-circulation experiment. REC, blood pressure of recipient cat; middle record, venous outflow from cross-perfused muscle; DON, blood pressure of donor cat. The doses of isoprenaline are marked under the blood pressure record of the cat to which they were intravenously administered. The upper tracing on the extreme right shows the effect of isoprenaline after stabilising the blood pressure of the recipient animal.

After acute denervation of the perfused muscle, the administration of isoprenaline to the recipient cat was without effect on the blood flow, while its administration to the donor cat caused effects similar to those produced before denervation. Figure 4 illustrates the results of a typical cross-perfusion experiment.

In the innervated muscle, particularly when vasomotor tone was high, intravenously administered adrenaline was much more powerful in producing vasodilatation than similar doses of isoprenaline. The reverse was true, however, in the acutely denervated muscle.

Discussion

The results obtained on intra-arterial administration confirm the finding by other workers[2–5] that isoprenaline causes vasodilatation in skeletal muscles by direct action on the blood vessels. When vasomotor tone is high, the degree of vasodilatation produced by small intra-arterial doses of isoprenaline is roughly equal to that produced by similar doses of adrenaline, although the effect with isoprenaline is slightly longer lasting. With larger doses, the response to adrenaline is converted to a reduction in venous outflow[1], while isoprenaline continues to cause pure vasodilatation in all doses. When vasomotor tone is low, either naturally or as a result of acute denervation, isoprenaline still causes vasodilatation whereas, in such circumstances, adrenaline frequently causes vasoconstriction[1]. On the whole, intra-arterially administered isoprenaline can, therefore, be said to be more potent than adrenaline in producing vasodilatation in the skeletal muscles of the cat. Working on human subjects, Barcroft and Konzett[4] found that intra-arterially infused isoprenaline was slightly less potent in producing vasodilatation than similar infusions of adrenaline.

The weak vasodilator response to intravenously administered isoprenaline is converted to a much more pronounced effect by sectioning the sciatic nerve. By the use of a blood pressure stabiliser and cross-circulation techniques, the dilator response to intravenously administered isoprenaline in the innervated muscle was shown to be reflexly inhibited by a vasoconstriction initiated by the fall in blood pressure.

Adrenaline and, to a smaller extent, noradrenaline have been shown to produce a vasodilatation in the skeletal muscles of the cat and the dog which is not the result of a direct action on the blood vessels but which is mediated through the nerves[1,8–12]. In cross-circulation experiments, the administration of adrenaline or noradrenaline to the recipient animal causes vasodilatation in the perfused muscles[1,8–12]. The present experiments showed, on the other hand, that a similar administration of isoprenaline causes vasoconstriction. When the blood pressure of the recipient cat is stabilised, the vasoconstriction is abolished but there is still no evidence of vasodilatation. It must be concluded, therefore, that vasodilatation produced by isoprenaline in the skeletal muscles of the cat is entirely the result of a direct action on the blood vessels.

Gruhzit, Freyburger and Moe[12] have provided evidence that in the dog vasodilatation produced by intravenously administered adrenaline

and noradrenaline is mainly brought about by a reflex mechanism, the afferent source of which is mechanoreceptors along the wall of the thoracic aorta, activated by the inotropic cardiac action of these amines.

The inotropic cardiac action of isoprenaline has been shown to be more powerful than that of adrenaline[2,13,14] and yet no reflex vasodilatation could be demonstrated with this substance in the present experiments on the cat. The results obtained with isoprenaline, therefore, supply evidence that it is not the inotropic cardiac action of adrenaline and noradrenaline which is responsible for the nervously activated vasodilatation produced in the skeletal muscles of this species. Taylor and Page[15] demonstrated a reduction in pressure in the trunk when adrenaline or noradrenaline was administered to the perfused head, the only connection between the head and the trunk being the spinal cord. Since the response was independent of pressure changes they concluded that it was due to chemoreceptors in the cephalic circulation. Such a mechanism might be the explanation of the reflex vasodilatation produced in the skeletal muscles of the cat by adrenaline and noradrenaline.

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A NOTE ON GLYCEROL FORMAL AS A SOLVENT IN TOXICITY TESTING

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Twenty-nine organic solvents and emulsifying agents, selected for possible use as injection solvents in toxicity testing, have been submitted to a "range-finding" toxicity screening test on rats. As a result, glycerol formal was examined in greater detail. Glycerol formal is a good inert solvent for a wide range of organic chemicals. It produced no toxic effects or macroscopic pathology when 1500 mg./kg. was administered intraperitoneally to rats, or 1000 mg./kg. to mice or guinea pigs, or 4000 mg./kg. orally to rats, and at high doses the only specific effect found was narcosis. It was correspondingly innocuous subcutaneously or dermally, and was almost non-irritant to the eye surface. There was no detectable effect on the toxicity of parathion. Glycerol formal appears to be a useful addition to the range of solvents suitable for use in toxicity tests.

In toxicological investigations, the chemical under examination has usually to be dissolved in, or, if liquid, diluted with a solvent. An injection solvent for water-insoluble or water-unstable compounds should satisfy, as far as possible, the following main requirements. (*a*) Low toxicity, so that at least 1000 mg./kg. can be injected intraperitoneally without detectable toxic effect or irritancy. (*b*) Complete miscibility with water, giving a neutral solution. (*c*) Good solvent powers for a wide range of materials. (*d*) Chemical inertness. (*e*) A mode of toxic action or detoxication which would not interfere with the absorption or metabolism of a solute. (*f*) Preferably low viscosity and low volatility. (*g*) Ready availability at reasonable cost, in sufficiently pure grade. The absence of local irritative effects is particularly important for intraperitoneal injection.

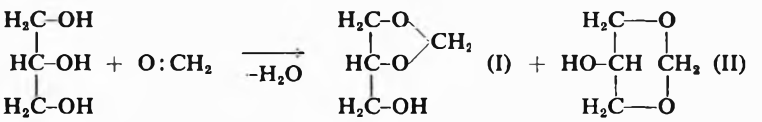
The organic solvent most commonly used in this laboratory is propylene glycol; occasionally-used solvents such as ethanol and acetone cause toxic effects and irritancy at low doses. Propylene glycol cannot be given intraperitoneally to the rat at doses greater than 1000 mg./kg., or toxic effects appear, notably marginal swelling of the liver lobes; also it is not a good solvent, and is at times troublesome to handle owing to its high viscosity.

A number of candidate materials have therefore been submitted to a simple toxicity screening test, to determine whether sufficiently large doses could be injected without detectable effects on the animal. Intraperitoneal injection to the rat was selected as being the most critical route of administration, and that most likely to show up undesirable effects. As this was intended to be merely a preliminary "sorting" test to determine the approximate maximum harmless dose, it was felt permissible to use comparatively small numbers of animals for each material.

In addition to injection, most materials have been examined for ocular irritation in the guinea pig, mainly because many of the possibly anti-cholinesterase compounds submitted to this laboratory for toxicity testing undergo measurement of miotic activity[1].

These tests have shown that glycerol formal was worthy of more detailed investigation, and further tests on this material are reported.

Glycerol formal is a condensation product of glycerol and formaldehyde, and is a mixture of the two materials 4-hydroxymethyl-1 : 3-dioxolane (I) and 5-hydroxy-1 : 3-dioxane (II) obtained by condensation involving respectively the $\alpha\beta$- and the $\alpha\alpha'$- pairs of hydroxyl groups of the glycerol (Hannay, personal communication):—



The proportions of the two compounds in the mixture depend on the reaction conditions, more of the six-membered ring (II) being formed at a lower reaction temperature[2]. The two substances cannot be separated by distillation, but can be benzoylated and the benzoates separated and hydrolysed back to the alcohols in good yield with alkali[3,4].

I and II have very similar boiling point : pressure relationships, with boiling points at 760 mm. Hg respectively 194 and 193°, and approximate vapour pressures at 20° respectively 0·22 and 0·25 mm. Hg[3–6]. Mixtures boil at 192–5° at 760 mm. Hg[7], so there is probably no azeotrope formation. The densities of I and II are respectively 1·2113 and 1·2256 at 20°, and 1·2008 and 1·2200 at 25°[3,4]. There is a difference in refractive indices, n_D^{20} being 1·4477 for I and 1·4533 for II, and n_D^{25} 1·4469 for I and 1·4527 for II[3,4]; in the absence of other gross impurities, it should therefore be possible to determine the approximate composition of a mixture of these components by the refractive index[3]. Both I and II are miscible with water and chemically stable, resisting hydrolysis by hot aqueous potassium hydroxide[3,4,6,7]. No peroxide formation in contact with air has been reported.

No toxicity tests have been reported on glycerol formal or either of its components. However, glycerol formal has been used industrially on the ton scale under the name of Sericosol N as a solvent for cellulose acetate, without special precautions, and has as yet produced no detected hazards (Hannay).

Experimental Methods

In the screening tests, doses of each compound were administered intraperitoneally, undiluted, to groups of semi-adult albino rats (130–200 g.) of either sex, and of Wistar or Glaxo-Wistar strain. Initial tests were on single animals at each dose, which were then made up to groups of 3–4 near the threshold of effects. Animals were observed for mortality and toxic effects for seven days, this being the normal observation period for acute toxicity tests in this laboratory. Survivors were

killed by decapitation and examined macroscopically. Ocular irritancy was tested by placing a 10 μl. standard drop on the corneal surface of one eye of a guinea pig and comparing with the untreated eye. Average lethal dose values were estimated non-statistically, and maximum ineffective doses were the highest at which none of the animals showed any detectable toxic effects. Similar but more extensive methods were used in the detailed tests on glycerol formal.

Most materials screened were of normal laboratory grade, and were checked for gross impurity by measuring the pH change when 10 per cent was added to water, and by comparing the refractive index with values quoted in the literature. Purification was only found necessary with trimethyl and triethyl phosphates; this was done by shaking with anhydrous sodium bicarbonate, drying with anhydrous sodium sulphate, and filtering. The glycerol formal and 1 : 3-dioxolane were experimental samples supplied by Messrs. Brotherton and Co., Leeds. Of the surface active agents, Lissapol NXA was the standard I.C.I. product, the "Sorpol" products are non-ionic emulsifying agents of Japanese manufacture supplied by Internationale Crediet-Handels-Vereeniging, Rotterdam, and the "Tween" products are polyoxyethylene sorbitan esters supplied by Messrs. Honeywill-Atlas Ltd.

Results

Screening tests. The results of the main screening tests are summarised in Table I. In order to indicate the scope and nature of the screening methods used, a typical test, on diethylene glycol monomethyl ether, is given in greater detail in Table II.

As a result of these tests, a more detailed examination was made on glycerol formal.

Glycerol formal. The material tested was a colourless liquid with no appreciable odour, slightly viscous but considerably less so than propylene glycol. It was readily and completely miscible with water, and a 10 per cent aqueous solution had pH between 5·0 and 5·5. The refractive index was 1·4519 at 20°, indicating, on the basis of the literature values[4,5], that it probably contained about 75 per cent II and 25 per cent I; a second sample gave a value of 1·4513, corresponding to about 70 per cent II. The material ignited with difficulty only after warming, and burnt slowly with a non-smoky blue flame. A test for peroxide with ferrous ammonium sulphate and ammonium thiocyanate gave only a very faint positive reaction. A test for free aldehyde with Schiff's reagent was very faintly positive, suggesting that a trace of formaldehyde might have been present.

A series of non-quantitative test-tube solubility experiments were carried out with a range of organic pesticidal chemicals. The results are shown in Table III.

Intraperitoneal injection of undiluted glycerol formal to rats gave the mortality and toxic effects shown in Table IV. At 2000 mg./kg. and above, narcosis occurred. At 2000 mg./kg. this was only moderate and lasted about three hours, while at 4000 mg./kg. weakness persisted until death. No macroscopic post-mortem abnormalities were found in any

TABLE I

SUMMARISED RESULTS OF INTRAPERITONEAL SCREENING TESTS

Material	Rat sex	Estimated approx. rat average lethal dose, mg./kg.	Estimated max. symptomless dose, mg./kg.	Estimated max. dose without macroscopic pathology, mg./kg.	Ocular irritation	Effect*
Methanol	M	1500	500	350		NWUI
Ethanol	M	500	300	300		NWPUI
Acetone	F	500	<500	500		NWPUI
Ethylene glycol monomethyl ether	F	1200	<500	<500	+	NWPUDRLI
Ethylene glycol monoethyl ether	F	1200	500	<500	+	NWUDRLI
Ethylene glycol monomethyl ether mono-acetate	F	1200	700	500	±	WRIX
Diethylene glycol monomethyl ether	M	3000	1500	750	−	NWUDI
Diethylene glycol monoethyl ether	M	3000	1000	750	−	NWURI (? K)
Propylene glycol	M/F	>8000	2000	1000	−	NI
Hexylene glycol	M	1500	500	500	±	NUI
Ethyl glycollate	F	1500	500	500	+	NWRIX
Methyl lactate	F	>2000	500	500	−	NRI
Ethyl lactate	F	1000	750	<500		WRIX
Trimethyl phosphate, tech.	F	1000	750	500		NWUI
Trimethyl phosphate, purified	F	1500	850	750		NI
Triethyl phosphate, tech.	M	500–1000	<500	<500		NWUDI (? L)
Triethyl phosphate, purified	M	800	<500	<500		NI
NN-Dimethylformamide	F	1500	1000	750	±	NWUIL
NN-Dimethylacetamide	F	>2000	1000	<500	±	NPDI
Dioxan	F	1500	<500	<500	++	NWDLKI
Tetrahydrofurfuryl alcohol	M/F	1000	750	750	±	NUR
1:3-Dioxolane	M	500–1000	<500	<500	+	NDU
Glycerol formal	M/F	3000	1500	1500	−	N
2:2-Dimethyl-4-hydroxymethyl-1:3-dioxolane	M	3000	750	750	±	NUI
Emulsifying agents:—						
Lissapol NXA	M	200	100	25		WUI
Sorpol 144	F	700	<500	<500		WRUI
Sorpol 200	F	<500	<500	<500		WCRI
Tween 20	F	3000	1000	<500		WIL
Tween 40	F	>4000	1000	500		WUIL
Tween 60	F	>4000	2000	500		WIL
Tween 80	F	>4000	2000	500		UIL

> indicates highest, and < lowest dose tested.

*Abbreviations: C = convulsions
D = diarrhoea
I = peritoneal adhesions, etc.
K = kidney damage
L = liver damage
N = narcosis
P = pain
R = respiratory distress
U = urinary incontinence
W = weakness
X = congestion, cyanosis, "rubbery" liver, pale contracted gut (acute deaths only).

TABLE II

INTRAPERITONEAL RAT SCREENING TEST ON DIETHYLENE GLYCOL MONOMETHYL ETHER

Dose, mg./kg.	7 day mortality	Survivors affected	Survivors showing macroscopic pathology	Time of onset	Time of death	Effects*
500	0/1	0/1	0/1	—	—	—
750	0/1	0/1	0/1	—	—	—
1000	0/4	0/4	1/4	—	—	I
1500	0/4	0/4	3/4	—	—	I
2000	0/1	1/1	1/1	7 min.	—	NDWI
4000	1/1	—	—	5 min.	3 days	NUW

* Abbreviations as Table I.

TABLE III

SOLUBILITIES OF PESTICIDE ACTIVE INGREDIENTS IN GLYCEROL FORMAL

Solute	Solubility
Parathion	Completely miscible
Malathion	Completely miscible
Hercules 528 ("Delnav")	Completely miscible
Rogor	Very soluble
Dinitrocresol (free acid)	Very soluble
Pentachlorophenol	Moderately soluble, very soluble warm
DDT	Very soluble
Benzene hexachloride	Moderately soluble
Aldrin	Sparingly soluble, very soluble warm
Dieldrin	Sparingly soluble, very soluble warm

TABLE IV

ACUTE INTRAPERITONEAL TOXICITY OF GLYCEROL FORMAL TO RATS

Rat sex	Dose, mg./kg.	7 day mortality	Survivors affected	Time of onset	Time of death
M	500	0/1	0/1	—	—
	750	0/1	0/1	—	—
	1000	0/4	0/4	—	—
	1500	0/4	0/4	—	
	2000	0/1	1/1	1 min.	—
	4000	1/1	—	1 min.	1½ days
F	1000	0/4	0/4	—	—
	1500	0/10	0/10	—	—

TABLE V

INTRAPERITONEAL TOXICITIES TO FEMALE RAT OF SOLUTIONS OF PARATHION

Solvent	Dose of parathion mg./kg.	7 day mortality	Survivors affected	Time of onset	Time of death	Approx. LD50, mg./kg.
Propylene glycol ..	1·5	0/4	4/4	2½ hr.	—	—
	3·0	1/4	3/3	15–20 min.	6–21 hr.	—
	6·0	4/4	—	10–13 min.	¼–21 hr.	4
Glycerol formal ..	1·5	0/4	4/4	2¼–5½ hr.	—	—
	3·0	1/4	3/3	20–30 min.	6–21 hr.	—
	6·0	4/4	—	7–10 min.	¼–21 hr.	4

animals, including two groups of three female rats killed three and twenty hours after 1500 mg./kg., and the rate of weight gain was unaffected in survivors.

Groups of four female rats were given 1000, 2000 and 4000 mg./kg. orally. All were apparently unaffected, gained weight normally, and showed no macroscopic pathology.

A group of four female rats was given 1000 mg./kg. subcutaneously. They were completely unaffected, and showed no evidence of local irritation at the injection site.

A group of four female rats was given 1000 mg./kg. on the clipped skin of the back, which was then covered with waterproof adhesive plaster. This test method is the standard technique of this laboratory for dermal toxicity testing. The solvent was removed after 24 hours by washing with soap and water. The rats were completely unaffected, and showed no evidence of local irritation at the application site.

Intraperitoneal injection of 1000 mg./kg. to two male guinea pigs caused no apparent toxic effects or macroscopic pathology. Similar administration of 1000 mg./kg. to six male albino mice was equally innocuous.

Administration of a single drop (10 μl.) to the eye of a guinea pig caused only slight temporary irritation without pupillary constriction or obscuration.

An experiment was then made to compare the toxic effects of intraperitoneal injection of female rats with 0·6 per cent v/v solutions of the organophosphorus insecticide parathion in glycerol formal and propylene glycol. The results are shown in Table V. There were no detectable differences between the effects of the two solutions, and no macroscopic post-mortem abnormalities.

Discussion

The screening test results summarised in Table I suggested that propylene glycol was a solvent of low toxicity; its most obvious effect at higher non-narcotic intraperitoneal doses, marginal liver lobe swelling probably due to local irritation, can often be allowed for at post-mortem. However, if this swelling be taken into account, glycerol formal could apparently be given in higher doses than propylene glycol without causing macroscopic pathology.

Glycerol formal appeared from Table III to be a useful solvent for a number of classes of compound, and considerably better in this respect than propylene glycol. There are no data on the degree of polarisation of the hydroxyl groups of the components, but they would probably be less polar than in the primary aliphatic alcohols, and therefore less reactive. There was no indication during this work of chemical instability or reactivity.

The toxicity of glycerol formal appeared to be low; average lethal dose values to the rat were estimated from these results as approximately 3000 mg./kg. intraperitoneally, and greater than 4000 mg./kg. orally. The only effect observed was narcosis; there was no evidence of the

mild irritant effects found after injection of propylene glycol. Hence the maximum dose producing no detectable effect of any type, 1500 mg./kg. intraperitoneally in the rat, was greater than with propylene glycol. The absence of more than slight ocular irritation indicated further that the material would be suitable for miosis testing of phosphate esters[1].

The fact that glycerol formal is a mixture of possibly variable composition is not necessarily a disadvantage, in view of the low toxicity. Gross changes in composition could be detected by measuring the refractive index. This solvent appears to be a useful addition to the range of solvents suitable for use in acute toxicity tests. It is now in regular use in this laboratory.

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A STUDY OF BACTERIOLOGICAL MEDIA; THE EXAMINATION OF BACTO-CASITONE*

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Three batches of Bacto-Casitone have been examined quantitatively for their free and total amino acids using paper chromatography. Variation occurs in the free amino acids in the three batches. The spots due to the peptides have been found to be a mixture of peptides. When casitone was examined for its strepogenin activity using the medium of Steele, Sauberlich, Reynolds and Baumann[1], and Kodicek and Mistry[2] the stimulatory pattern in the two media appeared to be different. The three batches showed similar strepogenin activity.

Protein hydrolysates by virtue of their content of amino acids and peptides are important ingredients of many microbiological culture media. In previous papers[3,4] the qualitative identification of the constituent amino acids and peptides in "Oxoid" bacteriological peptone was reported. This paper describes the quantitative estimation of the free and total amino acids in three batches of "Difco" Bacto-Casitone, together with a qualitative examination of the constituent peptides. The three batches were examined for their strepogenin activity.

Experimental

Quantitative Estimation of Free Amino Acids

Three batches of casitone designated A, B and C were used. 0·1 g. was dissolved in water and the pH was brought to 9 by 0·5N sodium hydroxide, the volume was adjusted to 15 ml., potassium chloride was added to give an 0·1N solution[5], then 0·5 ml. of fluorodinitrobenzene (FDNB) was added and the reaction was allowed to proceed at 40° for 1½ hours with vigorous stirring, using a magnetic stirrer, the pH was kept at 9 by careful addition of 0·5N sodium hydroxide. After the reaction excess FDNB was removed by extraction with ether. The solution was then acidified and extracted with ether. The combined ethereal extracts were washed with water to which a few drops of 6N hydrochloric acid were added. The ether extract was evaporated under vacuum to dryness and the residue was subjected to the cold finger condenser[6] to remove most of the dinitrophenol. The residue was dissolved in 3 ml. of methanol-methylethyl ketone 1:1 (v/v). The aqueous extract was evaporated to dryness and subjected to column chromatography on talc-celite to remove salts[7]. The column was extruded and the coloured zone

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was eluted with ethanol-6N hydrochloric acid 4:1 (v/v). The eluate was evaporated to dryness and the residue was dissolved in 3 ml. ethanol-6N hydrochloric acid. 0·05 ml. of the ether extract and 0·02 ml. of the aqueous extract were subjected to the quantitative paper chromatography technique of Levy[8], using the ethyl benzene system[9] in the first direction followed after drying the paper by 1·5M phosphate buffer in the second dimension. The excised spots of dinitrophenyl (DNP) amino acids were eluted with a suitable volume of 1 per cent sodium bicarbonate. The optical density reading was converted to the absolute value of the amino acid using the factors previously given[9].

Quantitative Estimation of Total Amino Acid.

0·1 g. of casitone was completely hydrolysed by refluxing with 10 ml. 6N hydrochloric acid for 24 hours after which the solution was evaporated to dryness in a vacuum desiccator over sodium hydroxide. The residue was taken up in water and subjected to the reaction and extraction procedures outlined above. The residue from the ether was dissolved in 2 ml. methanol-methylethyl ketone and that from the aqueous extract in 2 ml. ethanol-6N hydrochloric acid, 0·015 ml. of each was subjected to quantitative paper chromatography. The reaction was carried out in duplicate and paper chromatography in triplicate. The results are given in Table I for the three batches. Figure 1 shows the chromatogram of

TABLE I

THE QUANTITATIVE ESTIMATION OF THE FREE AND TOTAL AMINO ACIDS IN BACTO CASITONE

Amino acid	Free amino acids, g./100 g.			Total amino acid, g./100 g.		
	A	B	C	A	B	C
Gly	0·226	0·197	0·224	1·97	2·03	2·11
Ala	0·513	0·614	0·62	2·97	3·01	3·02
Val	0·802	0·82	0·893	6·25	6·27	5·97
Leu's	4·43	4·57	5·0	13·75	13·3	13·65
Ser	0·635	0·557	0·612	4·25	4·23	4·03
Thr	0·521	0·497	0·574	3·17	3·58	3·57
Tyr	0·272	0·44	0·427	1·56	1·59	1·54
Phe	2·24	2·39	2·66	4·67	4·61	4·75
Met	0·53	0·568	0·72	2·50	2·47	2·36
Arg	1·78	1·62	1·58	2·5	2·55	2·42
His	0·209	0·274	0·293	1·83	2·02	2·33
Orn	0·037	0·082	0·071	0·07	0·09	0·048
CyS	—	—	—	0·288	0·287	0·30
Lys	3·11	1·7	1·92	6·23	6·33	6·22
Pro	0·226	0·202	0·225	8·49	8·60	8·50
Asp and Glu	1·32	0·89	1·22	23·6	24·2	23·9
Try	0·448	0·461	0·488	—	—	—
Total	17·288	15·882	17·528			

DNP amino acids and peptides obtained with batch B. Batch A and C gave similar pictures. It is seen that there exist 3 spots which do not correspond to the position of the amino acids. These disappear on hydrolysis. In addition, there is a trailing spot in the aqueous extract. 0·2 ml. of the ether extract was applied on Whatman 3MM paper and subjected to two dimensional chromatography; 0·2 ml. of the aqueous extract was subjected to chromatography using the ethyl benzene system. Four sheets were thus treated. The spots due to the peptides were eluted with acetone-ammonium hydroxide 4:1 (v/v). The eluate was evaporated

to dryness, the residue was dissolved in 0·2 ml. of 6N hydrochloric acid, and of this 0·1 ml. was hydrolysed in a sealed tube for 8 hours at 105°. After removal of the acid the residue was taken up in a few drops of acetone and an aliquot was subjected to two dimensional chromatography using the ethyl benzene system and 1·5M phosphate buffer to detect the *N*-terminal amino acid of the peptide as the DNP-derivative.

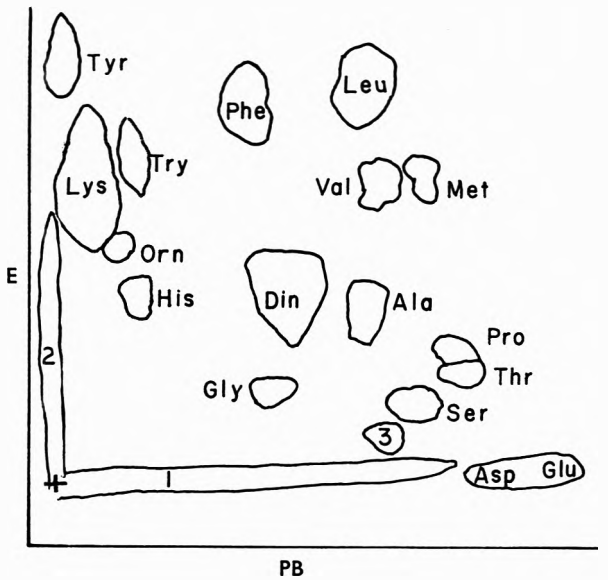

FIG. 1. Two-dimensional chromatogram of DNP-amino acids and peptides in bacto casitone, batch B. + Point of application, *E*, direction of ethyl benzene developer. *PB*, direction of 1·5M phosphate buffer developer. *Din*, dinitrophenol.

The second part of 0·1 ml. was completely hydrolysed for 24 hours at 105° and then after removal of the acid the residue was dissolved in a few drops of 10 per cent *iso*propanol. An aliquot was subjected to two dimensional chromatography using the butanol-acetic acid-water system 4:1:5 in the first direction followed after drying the paper, by spraying the paper with phosphate buffer pH 6·2 and developing in the second direction with phenol saturated with buffer[3]. The quantity of data resulting from this examination does not permit profitable tabulation, for example, in spot No. 1, which is typical, amino acids aspartic, serine*, threonine*, alanine*, glutamic*, valine*, methionine, leucines*, proline, glycine* and possibly histidine were present in the total hydrolysate; while arginine, lysine and those marked with an asterisk were identified as *N*-terminal amino acid residues indicating considerable heterogeneity in the spot.

Bacteriological Examination

Growth stimulating factors have been reported by various workers in enzymatic digests of various proteins. Woolley and others[8,9] found that

casein partially acid hydrolysed and tryptic digests of casein and other purified proteins stimulated the growth of *Lactobacillus casei*. These workers have given the name strepogenin to the growth factor(s) and their studies of the properties of strepogenin led them to suggest it had a peptide character. Merrifield and Woolley[12] have isolated a peptide serylhistidylleucylvalylglutamic acid from acid digested insulin and found that it has 80 units of strepogenin activity. Later Tritsch and Woolley[13] isolated the disulphide of leucylvalylcysteinylglycylglutamylarginine from an enzymic digest of insulin and showed it to have 200 units of strepogenin activity. Ågren[14] found that a commercial tryptic digest of casein stimulated the growth of *L. casei* when grown on the medium of Henderson and Snell[15]. Later[16], he isolated a peptide fraction from calf's plasma and found that it has a stimulating effect both on growth and lactic acid production of *L. casei* after 72 hours incubation when grown on the medium of Steele and others[1]. The three batches of casitone were tested for their stimulatory effect on *L. casei* ATCC (7469). The procedures followed for the culture and inoculum were as described by Ågren[14]. Assays were made in triplicate in 18 × 150 mm. Pyrex culture tubes and in a total volume of 5 ml. The casitone was tested at 0·1, 0·5 and 1·0 mg. concentration. The amount of casitone adjusted to pH 6·8 was added in a volume of 2·5 ml. to 2·5 ml. of the basal medium of Steele and others[15]. Tubes containing 0·1, 0·5 and 1·0 mg. of Wilson's liver fraction L were included for comparison. The tubes were autoclaved at 15 lb. for 5 minutes. A drop of uniform size of a diluted suspension of *L. casei* in saline was added to each tube by a syringe equipped with a needle, the end of which was ground flat. The growth stimulatory effect was given as scale reading on the Klett–Summerson photo-electric colorimeter, this was measured after 24, 48 and 72 hours. The effect on the lactic acid production was measured by titrating with 0·77N sodium hydroxide. The strepogenin activity was also determined on the medium of Kodicek and Mistry[16] in which the glucose content was reduced to 2·5 per cent. Results are given in Table II.

TABLE II

THE EFFECT OF CASITONE ON THE GROWTH OF *L. casei*

Hours	Control	Casitone A, mg.			Casitone B, mg.			Casitone C, mg.			Wilson's liver L, mg.		
		0·1	0·5	1·0	0·1	0·5	1·0	0·1	0·5	1·0	0·1	0·5	1·0
					A. Medium of Steele and others*								
24	2	36	96	109	28	70	90	22	60	95	9	21	30
48	13	96	238	271	92	213	256	93	206	261	28	78	123
72	250	146	271	315	142	253	303	140	247	305	—	143	180
					Acid Production†								
24	0	0·2	0·42	0·57	0·15	0·35	0·50	0·13	0·35	0·45	0·05	0·13	0·15
48	0	2·05	5·15	6·45	1·75	4·6	5·85	1·7	4·7	6·3	0·33	1·45	3·05
72	4·7	4·05	8·9	9·6	3·45	7·95	9·7	3·65	7·7	9·8	—	3·4	4·85
					B. Medium of Kodicek and Mistry*								
24	83	108	145	174	127	147	171	95	152	167	77	118	168
48	267	269	281	285	275	277	282	271	274	280	270	282	295

* Scale reading on the colorimeter. † Ml. of 0·077N alkali.

Discussion

From Table I it is seen that some variation occurs in the free amino acids among the three batches and that the amino acids are liberated to varying degrees. Arginine, phenylalanine, leucines and lysine occur in a higher proportion relative to their total occurrence in casitone than proline, aspartic and glutamic acids which seem to be liberated slowly. This appears to be due to variation in the susceptibility of peptide bonds involving these amino acids. The variation in the free amino acids may be due either to some variation in the purity of pancreatic extract used for the digestion of casein or some variation in the conditions during the digestion. Harding and MacLean[17] subjected casein to the pancreatic enzymes and followed the hydrolysis by the determination of the α-amino acid nitrogen. They found that even at the end of 216 hours the hydrolysis of casein was still proceeding. Ornithine is detected and it seems likely that it occurs only in the free state, it may be produced from arginine during the digestion.

Figure 1 and the results of analysis of the spots, a typical example of which was given in the experimental section, show that the spots due to the peptides consist of a mixture of peptides. This mixture of DNP peptides proved to be difficult to separate either by paper electrophoresis[18] or paper chromatography. There seems to be some variation in the constituent peptides in corresponding spots in the three batches.

It is observed from Table II that casitone has a stimulatory effect on the growth and lactic acid production of *L. casei* when grown on the medium of Steele and others. The stimulatory effect lasts as long as 72 hours. The results are at variance with those recorded by Woolley, Ågren and Kodicek and Mistry for strepogenin where the stimulatory effect is detected after 18 to 24 hours and where after 48 hours all tubes whether supplemented or not showed maximal growth and acid production. That this variation is due to the basal medium used in the assay is evidenced by the results obtained when the basal medium of Kodicek and Mistry is used; the stimulation is seen after a 24 hour incubation while after 48 hours all tubes including the controls showed similar growth. The medium of Steele and others supported little if any growth after 48 hours, this was similar to results obtained by Ågren[16], but his conclusion that the peptide fraction isolated from calf's plasma behaved differently from strepogenin may have been different had he tested for the stimulatory effect on a medium that is used for strepogenin or included in his assay a material with known strepogenin activity for comparison. Tubes containing Wilson's liver fraction L as well as casitone at 0·1 mg. level showed less growth after 72 hours than the controls. The three batches of casitone although they showed some variation in their constituent peptides yet showed similar strepogenin activity. As peptides may have varying strepogenin activity, it seems that strepogenin active peptides are similarly liberated in the three batches.

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STUDIES ON *ACORUS CALAMUS*, PART II

INVESTIGATION OF VOLATILE OIL

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The capacity to potentiate the sedative action of pentobarbitone by the volatile oil of Indian *Acorus calamus* has been used to screen various fractions of the oil for the presence of the principle responsible. The methods used for the removal of the oxygenated components of the oil did not remove the active material. The results show that the active principle resides in the hydrocarbon fraction of the oil or in an oxygenated component not removed by the methods employed. The volatile oil from the European *Acorus calamus* showed activity similar to the oil from the Indian drug.

The sedative potentiating principle of *Acorus calamus* L. of Indian origin was shown to be present in the steam-distillable fraction of the light petroleum extract of the rhizomes[1]. Agarwal, Dandiya, Singh and Arora[2] have described other pharmacological properties for a crude alcoholic extract of the rhizomes of *Acorus calamus* L. of Indian origin. Some of these have been shown to be present in the volatile oil[3], and Chopra, Jamwal and Khajuria[4] have reported carminative and antispasmodic properties, and Chopra, Khajuria and Chopra[5] have reported the acute and chronic toxicities of the oil on guinea pigs as well as its antibacterial properties. More recently an extensive pharmacological study of oil of calamus of Indian origin has been made.[6]

The physical and chemical properties of oil of calamus of Indian, Javanese, European, North American, Japanese and Russian origins have been described. The specific gravities of the various oils have been reported and are listed in Table I. The acid number of the various oils lies between 1 and 3 with the ester values ranging between 4 and 12. Thus the oil of calamus of Indian and Javanese origin differs from the others in having comparatively higher specific gravities, lower optical rotations and higher refractive indices. Kelkar and Rao[7] concluded on the basis of their study of the chemical nature of the volatile oil of calamus of Indian origin that the difference between the Indian and the other commercial varieties of the calamus oil was not due to the presence of any new constituents but due to the predominance of asarone in the Indian oil. The Indian oil has been reported to contain 82 per cent asarone while the other commercial varieties have approximately 7 per cent of this constituent. The physical and chemical properties of the oil have been discussed by Guenther.[8] Rao, Sudborough and Watson[9] have reported the presence of 1·5 per cent of oil in calamus of Indian origin.

* Colombo Plan Fellow.

TABLE I

PHYSICAL CHARACTERISTICS OF THE VARIOUS OILS

Source	Specific gravity at 15°	Optical rotation	Refractive index at 20°
European	0·959 to 0·974	+ 9° 0′ to +31° 0′	1·5028 to 1·5098
Russian	0·952 to 0·974	+ 9° 39′ to +23° 26′	1·5020 to 1·5289
Japanese	0·973 to 1·023	+ 2° 8′ to +26° 30′	1·5051 to 1·528
Indian	1·069 to 1·081	− 1° 30′ to + 6° 12′	1·5030 to 1·5522
Javanese	1·007 to 1·078	+ 0° 51′ to + 0° 53′	1·5504 to 1·5506
American	0·950 to 0·974	+13° 48′ to +15° 0′	1·5013 to 1·5069

EXPERIMENTAL

Volatile oil from Indian drug. (*a*) 500 g. of rhizomes of *Acorus calamus* were cut small (one-half to one inch) and water distilled[10] using a Florentine receiver for oils heavier than water. The yield of oil was 16·1 ml. The oil was separated and dried over calcium chloride in a desiccator. (*b*) Volatile oil distilled from *Acorus calamus* of Indian origin was obtained from a commercial source (Fritzsche Bros. Inc., New York).

Volatile oil from European drug. This was prepared as described for Indian drug.

The physical properties of these three samples are given in Table II.

TABLE II

PHYSICAL PROPERTIES OF THE THREE SAMPLES OF OIL

	Volatile oil distilled by us from Indian drug	Volatile oil distilled by us from European drug	Volatile oil from Indian drug commercial source
Specific Gravity	1·071 at 18°	0·976 at 18°	1·079 at 25°
Optical rotation	+5·7° at 18°	+12° at 18°	−0° 28′ at 18°
Refractive index	1·5522 at 17°	1·5127 at 17°	1·5522 at 20°
Solubility at 20°	1 vol. in 70 per cent alcohol		1 vol. in 70 per cent alcohol
Acid value			2·0
Saponification value			7·0

Chemical Fractionation

Separation of phenolic compounds. 100 g. of oil was shaken with 300 ml. of N KOH and then allowed to separate overnight. The aqueous layer was separated and made acidic with dilute sulphuric acid and was extracted with three successive portions of chloroform (100, 50 and 50 ml.). The combined chloroform extract was washed with 20 ml. of distilled water and the chloroform removed over a water bath. The residual dark-brown liquid was dried in a vacuum descicator at 80°. The dried residue weighed 0·4 g. It gave a green colouration with ferric chloride solution (see Table V).

Separation of aldehyde. The oil after removal of the phenolic compounds was shaken with 150 ml. of saturated solution of sodium bisulphite. The aqueous layer was separated and treated with a solution of sodium hydroxide until it became alkaline. The liberated oily matter was extracted with successive portions of ether (150, 50 and 50 ml.). The ethereal extract on removal of ether by evaporation, and drying gave a bright golden-yellow viscous residue weighing 0·03 g. (see Table V).

Separation of fractions in succession. (i) *Free acids.* 200 g. of oil was dissolved in ether (500 ml.) and shaken with 50 ml. of 3 per cent sodium carbonate solution. The aqueous layer was separated and acidified with dilute sulphuric acid. An oily layer which separated was extracted with ether. The residue, weighing 0·3 g., after evaporation of ether, melted at 28°.

(ii) *Phenols.* The etheral solution of oil was shaken with 100 ml. of 2 per cent aqueous KOH. The liberated phenol weighing about 0·7 g. gave a blue colouration with solution of ferric chloride. It melted at 26°.

(iii) *Acids present as esters.* The ether was removed and the residual oil was refluxed on the water bath with 5 per cent alcoholic KOH (80 ml.) for 3 hours. The acids which separated on acidifying the alcoholic layer were in the form of a semi-solid residue weighing 0·5 g.

(iv) *Aldehydes.* The oil after treatment with alcoholic KOH was shaken with 80 ml. of saturated solution of potassium metabisulphite. The aqueous layer on acidification yielded an oil which was extracted with ether. The ethereal extract on evaporation yielded a solid residue (weight 0·056 g.) which could be crystallised from absolute ethanol. The crystalline material melted at 190–210°.

(v) *Alcohols.* The residual oil after treatment as described above, was dried over anhydrous sodium sulphate and dissolved in 200 ml. of dry ether. The ethereal solution was shaken with small pieces of metallic sodium. The reaction was allowed to take place for 24 hours, after which the solution was extracted with three portions (100, 50 and 50 ml.) of water. The combined aqueous extract was made acidic with dilute hydrochloric acid and then extracted with chloroform. The chloroform extract was evaporated to dryness and the semi-solid residue weighed 0·4 g.

The following fractions of the oil were collected and reserved for pharmacological testing: (A) free acids, (B) phenols, (C) acids present as esters, (D) aldehydes, (E) alcohols, (F) oil treated to remove free acids, (G) oil treated to remove acids and phenols, (H) oil treated to remove free acids, phenols and acids present as esters, (I) oil treated to remove free acids, phenols, acids present as esters, aldehydes, (J) oil treated to remove total acids, phenols, aldehydes and alcohols (see Table VI).

Pharmacological Observations

Acute toxicity. The LD50 was assessed using doses varying between 0·12 to 0·22 g./kg. of the volatile oil which were injected intraperitoneally into male mice weighing between 20 to 40 g. for acute toxicological studies. The mortality was recorded 24 hours after the injection (see Table III).

Potentiation of sedative activity. Male, white, mice weighing between 20 to 40 g. were used. Each mouse was weighed and a dose of the drug directly proportional to the weight of the mouse was injected intraperitoneally. After 30 minutes from the time of injection of the drug, pentobarbitone sodium, 30 mg./kg. body weight was injected intraperitoneally

using a 0·3 per cent solution. The same dose of barbiturate was given to a group of control animals to which no drug had been administered earlier. The number of mice which lost their righting reflex was recorded.

Oils from different sources were tested according to the method given. In all cases dilutions of 1 to 50 (w/v) in water containing 3 per cent Tween 80 were employed (see Table IV).

The potentiation action of the other fractions was also studied using this procedure (see Table VI).

Results and Discussion

The physical properties determined for the volatile oil from various sources are recorded in Table II. The physical properties of the volatile oil of the Indian *Acorus calamus* distilled in our laboratories and that obtained from a commercial source had similar physical properties. The volatile oil from the European drug differed in its physical properties, having a lower specific gravity, higher optical rotation and a lower refractive index than the other two samples of the oil. However, there appeared to be no significant difference in the potentiation action of these three samples of oil on the sedative property of pentobarbitone sodium as indicated by the results in Table IV.

Table VI indicates that fractions separated by means of chemical fractionation did not show any appreciable potentiating activity on the sedative action of pentobarbitone sodium (A to E). Treatment of the oil to remove phenols and aldehydes and some of the related carbonyl compounds, however, did cause an appreciable increase in this activity of the oil (I and J). The increase was such that only half the dose of the oil was required for the same potentiation action (Table V and Table VI, I and J). Since the volume of these two fractions represents less than 1 per cent of the total volume of the oil this increase could not be explained on the basis of an increase in concentration due to the removal of inert material. The removal of only one of these fractions did not result in an increase or enhancement of activity (Table V, Table VI, compare H, I, J). It appears that the presence of both of these fractions in the oil causes an antagonistic action to the potentiation of the sedative property of the volatile oil of *Acorus calamus*.

The results obtained appear to indicate that the potentiation activity resides in the hydrocarbon fraction of the oil or in an oxygenated component not removed by the methods employed. It seems unlikely that asarone (1:2:5-trimethoxy-4-propenyl benzene) is responsible for the activity evidenced by this oil since it is present to the extent of approximately 80 per cent in the Indian oil and about 7 per cent in the European oil. It has been already stated that the results obtained indicated no appreciable difference in the sedative potentiation action of these oils.

The acute toxicity of the Indian oil is given in Table III. The dose estimated to cause 50 per cent mortality was 0·177 g./kg. It will be observed from Table V that the elimination of the phenolic and aldehydic fractions from the oil resulted in an increase in the toxicity of the oil as well as in the sedative potentiation activity of the remaining oil.

TABLE III

ACUTE TOXICOLOGICAL STUDIES OF VOLATILE OIL OF *Acorus calamus* ON WHITE MICE

Dose of the volatile oil	No. of mice injected	Mortality up till 24 hours	Mortality per cent
0·12 g./kg.	20	nil	0
0·14 g./kg.	20	1	5
0·16 g./kg.	20	4	20
0·18 g./kg.	20	13	52
0·20 g./kg.	20	18	90
0·22 g./kg.	20	20	100

TABLE IV

POTENTIATION ACTION OF VOLATILE OIL OF *Acorus calamus* FROM DIFFERENT SOURCES, ON THE SEDATIVE ACTION OF PENTOBARBITONE SODIUM 30 mg./kg.

Source of oil	Dose g./kg.	No. of mice injected	No. of deaths	No. which lost righting reflex
Volatile oil from Indian drug distilled in our laboratory	0·1	20	nil	19
Volatile oil from Indian drug, commercial source	0·1	20	nil	20
Volatile oil from European drug distilled in our laboratory	0·1	20	nil	18

TABLE V

POTENTIATION ACTION OF PHENOLIC AND ALDEHYDIC FRACTIONS OF VOLATILE OIL OF *Acorus calamus* ON THE SEDATIVE ACTION OF PENTOBARBITONE SODIUM 30 mg./kg.

Fraction	Dose g./kg.	No. of mice injected	No. of deaths	No. which lost righting reflex
Phenolic	0·1	20	nil	3
Aldehydic	0·1	10	nil	4
Oil devoid of phenolic fraction	0·1	20	nil	19
Oil devoid of phenolic and aldehydic fraction	0·1	5	5	
Oil devoid of phenolic and aldehydic fraction	0·05	15	nil	15
Control	nil	10	nil	nil

TABLE VI

POTENTIATION ACTION OF SUCCESSIVE FRACTIONS OF VOLATILE OIL OF *Acorus calamus* ON THE SEDATIVE ACTION OF PENTOBARBITONE SODIUM 30 mg./kg.

Fraction	Dose g./kg.	No. of mice injected	No. of deaths	No. which lost righting reflex
A	0·1	10	nil	nil
B	0·1	10	nil	1
C	0·1	10	nil	nil
D	0·1	10	nil	nil
E	0·1	10	nil	1
F	0·1	10	nil	10
G	0·1	10	nil	9
H	0·1	10	nil	9
I	0·1	10	9 died before pentobarbitone sodium was given	
I	0·05	10	nil	9
J	0·05	10	nil	10

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SOME PHARMACOLOGICAL AND MICROBIOLOGICAL PROPERTIES OF CHLORHYDROXYQUINOLINE AND RELATED COMPOUNDS

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Chlorhydroxyquinoline, prepared by the chlorination of 8-hydroxyquinoline under controlled conditions, has been found to be active *in vitro* against a variety of micro-organisms. Its bacteriostatic activity appears to be greater than that of certain other halogenated derivatives and its oral toxicity in rats is low. After oral administration, chlorhydroxyquinoline is apparently excreted mainly in the faeces of rats and bacteriostatic levels have been observed in the stools of rats and dogs.

5-Chloro-7-iodo-8-hydroxyquinoline has been employed in the treatment of amoebic and bacillary dysenteries and 5:7-di-iodo-8-hydroxyquinoline has been used in intestinal amoebiasis. Also 5:7-dichlor-8-hydroxyquinaldine has been found to be effective clinically and *in vitro* against a variety of organisms encountered in the intestinal tract[1]. It seemed likely to us that chlorhydroxyquinoline, prepared by the chlorination of oxine under controlled conditions, would be active against organisms commonly responsible for intestinal infections.

A number of investigators have observed that oxine and several of its derivatives are effective against Gram-positive and Gram-negative bacteria and pathogenic fungi, while certain halogenated compounds are active against protozoa. There is some uncertainty about the fate of these substances in the body and the mode of their action against organisms in the intestinal tract. Leake[2] found that iodochlorhydroxyquinoline is rapidly excreted by man in the urine and Drill[3] concludes that the drug is absorbed from mucous membranes, excreted in the urine and detoxified in the liver, but Goodman and Gilman[4] suggest that most of an ingested dose probably passes through the intestinal tract without being absorbed. Haskins and his associates[5] studied the distribution of ^{131}I-labelled iodochlorhydroxyquinoline and found the compound seems to be absorbed and eliminated without marked decomposition. The drug may act in the lumen of the gut with an additional effect due to absorption into the intestinal circulation[4]. Di-iodohydroxyquinoline is absorbed to a less extent[6], but the blood-iodine levels[5,7] after oral administration indicate the possibility of some absorption occurring, although part may be iodine dissociated. Grabbe[8] reported that oxyquinoline sulphate is rapidly absorbed from dog intestines and excreted in the urine, mainly conjugated with sulphate, and in the bile.

It was, therefore, decided to compare the activity *in vitro* of chlorhydroxyquinoline with that of oxine, iodinated derivatives and various additional agents employed clinically and to investigate the toxicity and excretion of chlorhydroxyquinoline.

Antibacterial Activity

Many of the drugs tested are almost insoluble in water and the cup-plate technique has limitations owing to poor diffusion in solid media, but this method was considered useful in giving an indication of activity. In a series of preliminary tests, the bacteriostatic effects of various compounds and mixtures were investigated by placing the drugs in 5 mm. cups made with a cork-borer in nutrient agar and measuring the average annular zones of inhibition. The test-organisms were *Salmonella typhi*, *Salm. enteritidis*, *Shigella dysenteriae* (shigae), *Sh. sonnei* and *Sh. flexneri*.

The results indicated that under these conditions of testing, oxine exhibits effects comparable with those of the soluble salts of streptomycin, neomycin and tetracycline. In this series, chlorhydroxyquinoline was less active than oxine but more active than iodochlorhydroxyquinoline, whilst various mixtures of chlorhydroxyquinoline and phthalylsulphathiazole, streptomycin and neomycin appeared to show no additive effects.

Since solubility and diffusion are important factors in the measurement of antibacterial activity, a suitable solvent was sought and it was found that oxine, its halogenated derivatives and other drugs dissolve in dimethylformamide, although they precipitate on dilution with water. Filter paper discs were saturated with 0·4 per cent solutions of the compounds in dimethylformamide and placed while damp on agar plates, the test-organisms being *Salm. typhi*, *Salm. typhimurium*, *Salm. enteritidis*, *Sh. sonnei*, *Sh. flexneri*, *Sh. dysenteriae* (shigae) and *Escherichia coli*. The results, which are shown at Table I indicate that, under these conditions, the activities of oxine and chlorhydroxyquinoline are similar and that these two compounds are more active than iodochlorhydroxyquinoline and di-iodohydroxyquinoline. The solvent alone had no appreciable antibacterial activity.

TABLE I

ACTIVITY OF OXINE AND SOME HALOGENATED DERIVATIVES AGAINST THE TEST ORGANISMS USING FILTER-PAPER DISCS SATURATED WITH SOLUTIONS IN DIMETHYLFORMAMIDE AND PLACED WHILE STILL DAMP ON AGAR PLATES

Compound	Depth of Zone of Inhibition in mm.						
	Salm. typhi	*Salm. typhimurium*	*Salm. enteritidis*	*Sh. sonnei*	*Sh. flexneri*	*Sh. dysenteriae*	*E. coli*
Oxine	7·5	4	7	6	20	6·5	6
Chlor-oxine ..	7	6	5	6	21	8	5·5
Iodochlor-oxine ..	3·5	1·5	1·5	1·5	3·5	3	3
Di-iodo-oxine ..	1	1	2·5	1	3·5	1	3·5

Oxine and its derivatives were also made into pastes with water; porcelain cylinders were filled with these pastes and then placed on plates inoculated with other bacteria which may be associated with intestinal infections. The resulting zones of inhibition are recorded in Table II.

TABLE II

INHIBITION OF TEST ORGANISMS BY AQUEOUS PASTES OF VARIOUS AGENTS USING THE CYLINDER-PLATE METHOD

Organism	Depth of Zone of Inhibition in mm.				
	Oxine	Chlor-hydroxyquinoline	Iodochlor-hydroxyquinoline	Di-iodo hydroxyquinoline	Nystatin
Staph. aureus .. 47	13	13	2	<1	
Staph. aureus		9	2		
Proteus vulgaris	9	8	<1	<1	
Ps. aeruginosa	<1	<1	<1	<1	
Salm. paratyphi A	4	7	1	<1	
Candida albicans	11	10	2		9
Epidermophyton floccosum	17	3	3		1
Trichophyton mentagrophytes	10	6	2		2
Microsporum gypseum ..	12	7	2		2

Fluoro-Derivatives of Oxine

The 5-fluoro and 5:7-difluoro derivatives of 8-hydroxyquinoline were prepared for preliminary screening for bacteriostatic activity *in vitro* against *Staph. aureus*, *E. coli*, *P. vulgaris* end *Sh. sonnei* in comparison with chlorhydroxyquinoline. Results obtained by means of discs impregnated with dimethylformamide solutions indicated that the 5:7-difluoro-compound has a similar order of activity to chlorhydroxyquinoline, while 5-fluorohydroxyquinoline appeared to be less active than the other two substances.

ANTIFUNGAL ACTIVITY

The procelain cylinder method described above was used to give a preliminary estimation of activity of oxine, chlorhydroxyquinoline, iodochlorhydroxyquinoline and the antifungal antibiotic, nystatin, against certain pathogenic fungi. The results, shown in Table II, demonstrate the importance of diffusion because a large number of reports indicate that nystatin, which is insoluble in water, has high antifungal activity *in vivo* and that intense activity is exhibited *in vitro* by solutions in suitable organic solvents and by suspensions or other preparations which enable the antibiotic to be brought into intimate contact with the organisms.

PHARMACOLOGICAL PROPERTIES

Chlorhydroxyquinoline was given by mouth to groups comprising five male and five female rats for a period of 14 days. In one group of 10 animals, 0·1 g. was given per kg. of body weight daily, each member of another group received 0·2 g./kg. daily and the third group were controls. Each rat was weighed every third day, but only insignificant differences in body weight were observed in the three groups during and after the test-period. All animals survived and at the end of the fourteenth day, 2 rats

from each group were killed and examined, particular attention being paid to the livers and kidneys. No pathological changes were found.

The killed animals were also used in a preliminary study of the distribution and excretion of chlorhydroxyquinoline, based mainly on the ultra-violet absorption spectrum. No traces could be detected in the blood, but a compound appearing to have the quinoline nucleus was present in the intestinal contents.

In a second series of tests, groups of 4 adult rats were used. Four animals were given oxine 0·3 g./kg. daily by mouth, four received chlorhydroxyquinoline 0·3 g./kg. daily and four were left untreated as controls. No toxic effects were evident during the test-period of 3 days but it was noted that, although each rat in the control and oxine-treated groups excreted about 26 ml. of urine per day, the average output of animals in the chlorhydroxyquinoline-treated group was only about 3 ml. For this reason, particular attention was paid to the kidneys when the animals were killed at the end of the third day, but no pathological changes were evident.

Only small amounts of oxine or oxine-like substances could be found by the absorption spectrum method in the intestinal contents of the oxine-treated rats. The urine, however, consistently contained a compound resembling oxine in amounts which may represent 60–70 per cent of a daily dose; this substance was not unchanged oxine, but was most probably a conjugated compound[8].

The mass of the intestinal contents in the chlorhydroxyquinoline-treated group was much greater than that of the other groups and although no diarrhoea was evident, the faeces were soft. It is highly probable that the quantity and texture of the stools account for the reduced urinary output. A substantial amount of chlorhydroxyquinoline was present in the contents of the lower intestinal tract and the faeces had a yellow tinge which is likely to be due to break-down products. No unchanged chlorhydroxyquinoline appeared to be present in the urine, but a compound showing a similar, although not identical ultra-violet absorption peak was detected. Each rat received approximately 75 mg. of 8-hydroxyquinoline or chlorhydroxyquinoline daily and it was estimated that the average daily urinary excretion of changed oxine was about 52 mg. and that of changed chlorhydroxyquinoline only about 1·7 mg. Although various methods were used, the content of chlorhydroxyquinoline in the faeces could not be estimated satisfactorily and additional rats were given 0·16 g. of the drug per kg. daily in an endeavour to obtain more precise information. By means of modified methods of extraction and estimation, the average daily content in the urine at this dose-level was found to be approximately 0·5 mg., but the faecal content could not be estimated consistently.

Microbiological Tests on Faeces

Faeces were obtained daily for 3 days from four rats kept in metabolism cages; chlorhydroxyquinoline 0·16 g./kg. was given daily for 3 more days to two of these rats and the faeces were again collected. Faecal pellets of

approximately uniform weight and size were embedded in nutrient agar inoculated with an over-night culture of *Sh. flexneri* and the plates were incubated at 37° for 24 hours. No zones of inhibition were produced by pellets from untreated animals or by those obtained from the treated rats on the first day of medication. The second-day pellets of the treated rats, however, gave zones varying from 3 to 5 mm. in depth and those of the third day gave zones of 5, 6, 5 and 7 mm.

Small porcelain cylinders were filled with stools obtained from two dogs on 2 consecutive days and used to test for bacteriostatic activity against *Sh. flexneri.* No zones of inhibition were evident after incubation for 48 hours. The unused second-day faeces were mixed and a portion was sterilised and retained. The dogs were then given approximately 0·03 g. of chlorhydroxyquinoline per kg. daily as enteric coated tablets of 0·1 g., for 2 days and the faeces were collected for 4 days. This dose was selected because it would probably be similar to that which may be used clinically. Faeces obtained on the first day of medication produced no zones, but those collected on each of the next three days gave zones of 2–4 mm., 4–6 mm. and 0–3 mm. respectively. Chlorhydroxyquinoline was mixed evenly into the retained sterile faeces to give a range of concentrations and the zones of inhibition for *Sh. flexneri* were measured after incubation for 48 hours. Although the organism is highly sensitive to chlorhydroxyquinoline, this method is not satisfactory for estimating the faecal content.

The faeces of the last group of rats and of the dogs were dispersed in sterile Ringer's solution to give dilutions ranging from 1/100 to 1/10,000 and 1 ml. of each dilution was transferred to an agar plate and incubated aerobically for 72 hours. It was observed that the administration of chlorhydroxyquinoline modified the normal flora and reduced or even eliminated Gram-negative bacteria. Streptococci were not markedly affected.

Discussion

The results of these tests suggest that chlorhydroxyquinoline may be useful in bacillary infections of the intestinal tract. Under the conditions described this substance inhibited the multiplication of a variety of micro-organisms; it has a low oral toxicity in the rat and a substantial portion of an oral dose appears to be excreted in the faeces.

Methods of testing for bacteriostatic activity are limited by insolubility, and diffusion through solid media must be considered in interpreting the results obtained with the cup-plate techniques. Dimethylformamide has been found to be suitable for use by the filter paper disc method, but the oxine derivatives are precipitated from this and other solvents on adding water and so serial dilutions cannot easily be prepared in liquid media. We have employed suspensions in broth and found that, in a concentration of less than 0·1 per cent, chlorhydroxyquinoline is bacteriostatic for *E. coli*, but the minimal inhibitory concentration is influenced by several factors, notably size of particles.

Rats given chlorhydroxyquinoline by mouth in doses of 0·2 g./kg. daily for 14 days and 0·3 g./kg. daily for 3 days did not exhibit any toxic effects, while four rats given a single oral dose of 0·48 g./kg. showed normal gains in weight and no toxic symptoms over 14 days. By analogy with iodochlorhydroxyquinoline, di-iodohydroxyquinoline and chlorhydroxyquinaldine, it might be expected that chlorhydroxyquinoline would be largely excreted in the faeces, although the observations quoted in our introductory paragraphs suggest that there is some disagreement on the fate of certain of the halogenated derivatives of oxine in the body. Tests in this series have indicated that only a small part of an oral dose of chlorhydroxyquinoline is absorbed and excreted in the urine of rats and that the major portion remains in the intestinal tract, but the faecal content has not yet been estimated satisfactorily. In both rats and dogs treated with the substance, the levels in the stools are adequate to suppress the multiplication of *Sh. flexneri*. The term "chlorhydroxyquinoline" has been employed because spectrophotometry, chromatography, determination of melting points of fractions and other procedures have shown that the substance under consideration is not a single compound. An exhaustive study of individual chloro-derivatives of oxine has not yet been undertaken but tests so far conducted indicate that more highly chlorinated products and various fractions obtained by chromatographic separation are less active *in vitro* than chlorhydroxyquinoline.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

Reserpine, Rescinnamine and Deserpidine, Content of, in Rauwolfia Roots. D. Banes, A. E. H. Houk and J. Wolff. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 625.) Alkaloids of the reserpine-rescinnamine group were extracted from a sample of rauwolfia root by the method previously described (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 708). The chloroform extract was mixed with ethanol and evaporated to dryness at 70° *in vacuo*. The residue was incorporated in a silica column and fractionated chromatographically by the method of A. L. Hayden and colleagues (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 157). The fractions containing deserpidine, reserpine and rescinnamine were mixed and diluted as necessary, and the content of the alkaloids determined spectrophotometrically. The results of analyses of powdered root of *Rauwolfia serpentina* by this method were generally in agreement with those obtained by the nitrite colorimetric method, but discrepancies were observed with some samples. The proposed method is believed to be more reliable than the colorimetric method, since the active alkaloids are isolated individually and determined by direct measurement. G. B.

ANALYTICAL

Antibiotics, Paper Chromatographic Method for the Determination of Suitable pH Values for the Extraction of. V. Betina. (*Nature, Lond.*, 1958, **182**, 796.) pH Chromatograms are determined only when cultures of antibiotically active micro-organisms are available, before even crude substances have been isolated. The solubility of the antibiotic in ten solvents (distilled water, 3 per cent w/v ammonium chloride, methanol, acetone, ethyl acetate saturated with water, *n*-butanol saturated with water, chloroform, benzene, light petroleum, and ether saturated with water) is determined as follows. Agar plugs cut from colonies of the micro-organism are placed in contact with filter paper strips for 15–50 minutes. The latter are dried, and chromatographed by assending technique with each of the above solvents, and the spots detected bioautographically. pH Chromatograms are then run with the most suitable solvent on a series of paper strips buffered with McIlvain citrate-phosphate buffers of pH 2·2, 3, 4, 5, 6, 7 and 8, and with phosphate buffers of pH 9 and 10 respectively, using the same technique, and allowing the solvent front to advance 15 cm. in each case. The pattern of spots obtained is a characteristic of each antibiotic. Acidic substances advance further with acid buffers, whilst basic substances advance further with alkaline buffers. The movement of neutral antibiotics is unaffected by pH. R_F values are dependent on the partition coefficient of the antibiotic between the organic and aqueous phases, and extraction with an organic solvent will be most successful at the pH value which gives the highest R_F value. Conversely re-extraction into aqueous media will be most effective at that pH at which the R_F has a low value. J. B. S.

Glycosidal Alkaloids of the Solanine Complex, separation of, by Paper Electrophoresis and Chromatography and their Colorimetric Determination. J. Šerák and M. Kutáček. (*Českoslov. Farm.*, 1958, **7**, 322.) A sample of the mixture (80 to 500 μg.) is subjected to electrophoresis on a sheet (30 × 45 cm.) of Whatman No. 1 paper for 18 hours at a voltage of 140 V., 2·5 M acetic acid being used as electrolyte. The paper is dried at 60 to 80° and then used for paper chromatography in a direction perpendicular to that for the electrophoresis; a mixture of ethyl acetate, glacial acetic acid and water (11 : 1 : 2) is used as solvent system. The spots are located by a satured solution of antimony trichloride in chloroform. For the quantitative determination of the glycosidal alkaloids a standard chromatogram is run at the same time as the test and the spots on both are eluted with a 1 per cent solution of acetic acid. To the eluate (3 ml.) 5 ml. of conc. sulphuric acid and 2·5 ml. of a 1 per cent solution of formaldehyde are added, the sample being cooled during the addition. The maximum colour develops in 70 minutes and remains stable for 3 hours. The average recovery is 94·3 per cent; the loss can be allowed for by the use of a calibration curve constructed from results obtained on standards. E. H.

Meprobamate, Identification of, by Adsorption Chromatography on Chromatoplates. A. Fiori and M. Marigo. (*Nature, Lond.*, 1958, **182**, 943.) Chromatoplates are prepared by pouring a paste, prepared from silicic acid, rice starch and water onto glass plates, and spreading to form a homogeneous layer. After drying at room temperature the plates are activated by heating in an oven for 30 minutes at 100–105°. Meprobamate is isolated from urine by making alkaline, extracting with ether, and evaporating the latter solution; it is applied (in ethanol) to the starting line of the chromatoplate in a stream of hot air. Plates are developed in *cyclo*hexane and ethanol for 2 hours, when the solvent front moves about 10–11 cm., dried at room temperature and sprayed with concentrated sulphuric acid. Yellow spots appear after the plates are heated at 110–115° for 2–3 minutes, sprayed lightly with distilled water and re-heated. Meprobamate has R_F 0·30, and unidentified impurities migrate with the solvent front, and become violet under sulphuric acid treatment. Quantitative determination can be made by cutting out the spots, adding to water, treating with hydroxyquinone in sulphuric acid, heating at 100° for 20 minutes, and measuring the yellow colour at 420 mμ. J. B. S.

Morphine, New Method for the Determination of Small Quantities of. G. Nadeau and G. Sobolewski. (*Canad. J. Biochem. Physiol.*, 1958, **36**, 625.) The method depends upon the development of a blue fluorescence when morphine is warmed with concentrated sulphuric acid, and ammonia added. The solution containing morphine is evaporated to dryness, the last traces of moisture being removed under reduced pressure, and the residue is heated with 0·5 ml. of sulphuric acid in a water bath at 50° for 8 minutes, after which 5 ml. of water and 6 ml. of strong solution of ammonia are added. The liquid is allowed to stand at 50° for 2 hours, and then cooled. The fluorescent product is extracted by shaking with 10 ml. of *iso*butanol, and the fluorescence of the extract is determined in a fluorimeter. The solution may be centrifuged if necessary to remove turbidity. The intensity of fluorescence is proportional to the concentration of morphine for samples containing 0·02–20μg. Quenching occurs with higher concentrations, but the method can be used without serious error up to 100μg. The method is sensitive, and as the fluorescence is not given by other alkaloids of opium, morphine can be estimated in opium preparations without a preliminary separation. Diamorphine gives a fluorescence about one-third the intensity of that given by morphine. G. B.

Piperitone, Colorimetric Estimation of, with 3 : 5-Dinitrobenzoic Acid. D. H. E. Tattje. (*Pharm. Weekbl.*, 1958, **93**, 694.) To 4 ml. of an ethanolic solution of (—)-piperitone containing up to 1·6 mg. is added 5 ml. of a 4 per cent solution of 3 : 5-dinitrobenzoic acid in ethanol and 2 ml. of 3N sodium hydroxide. The colour intensity at 5375Å is measured in a 0·5 cm. cell 40 minutes after the addition of the sodium hydroxide. The blank consists of a solution identical in all respects except for the omission of the (—)-piperitone, and the temperature of measurement is 20°. A calibration curve is essential since the relationship of intensity of colour to concentration deviates appreciably from linearity. Cineole and 1-α-phellandrene do not interfere. Results obtained on mixtures of piperitone and oil of *Eucalyptus globulus* were in fair agreement with the theoretical values, whereas two oils of the *Eucalyptus dives* type gave results about 10 per cent lower than those obtained by the neutral sulphite method. This may be due to the greater specificity of the colorimetric method D. B. C.

Salicylic Acid, An Inverse Isotope Dilution Analysis of. H. A. Swartz and J. E. Christian. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 701.) Samples of salicylic acid are dissolved in glacial acetic acid and allowed to react with the appropriate quantity of iodine monochloride reagent labelled with iodine-131. The reaction is completed by heating in a water bath at 70°–80° for 20 minutes, shaking repeatedly. 3 : 5-Di-iodosalicylic acid in hot glacial acetic acid solution is added as carrier, and the solution set aside to cool and crystallise. After purification, the specific activity of the 3 : 5-di-iodosalicylic acid is determined, and from the activity of the labelled iodine monochloride reagent, the quantity of salicylic acid is calculated. The method of standardising the reagent is described. The method was verified using quantities of 0·2 to 20 mg. of salicylic acid, and should be applicable to smaller quantities. Acetylsalicylic acid may be determined by the same method, after quantitative hydrolysis to salicylic acid. G. B.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Adrenaline and Noradrenaline, Association of, with Blood Platelets. H. Weil-Malherbe and A. D. Bone. (*Biochem. J.*, 1958, **70**, 14.) Blood platelets, in addition to their role in blood coagulation, act as carriers of pharmacologically active amines. In this paper the distribution of adrenaline and noradrenaline between plasma and blood platelets is studied by a fluorimetric method. It was found that in human plasma the platelets contained approximately 50 per cent of the total catechol amines present in platelet-rich plasma. The mean amounts contained in 10^9 platelets were 2 ng. of adrenaline and 7 ng. of noradrenaline. The concentration of catechol amines is about 125 times higher in platelets than in plasma. No amines passed into the serum from the platelets during clotting, whether spontaneous or induced. Lysis of platelets by freezing and thawing or by treatment with a surface-active agent resulted in a partial release. No uptake of adrenaline by platelets was observed in heparinized platelet-rich plasma at an adrenaline concentration of about 10 μg./l. An uptake, resulting in a final concentration of platelet-bound adrenaline of about three times the initial concentration, was found in citrated platelet-rich plasma at an adrenaline concentration of 80–200 μg./l. An increase of adrenaline was found in platelet-rich plasma after electroconvulsive treatment in mental patients. M. B.

Intrinsic Factor and Pernicious Anaemia, Studies on. H. Berlin, R. Berlin, G. Brante and S-G. Sjoberg. (*Scand. J. clin. and Lab. Invest.*, 1958, **10**, 278.) A study has been made of the relationship between intrinsic factor (IF) activity and vitamin B_{12}-binding capacity for hog pylorus concentrates. The investigations were carried out in well established pernicious anaemia (p.a.) patients. The B_{12}-binding capacity was determined by the method of Hoff-Jørgensen and others (*Nord. Med.*, 1952, **48**, 1754) and 1,000 C.U. defined as the amount of the IF preparation capable of making 1 μg. vitamin B_{12} unavailable to *E. coli*. The IF activity was determined by measurement of the urinary radioactive B_{12}–^{60}Co excretion according to Schilling. The results showed that a significant increase in B_{12} uptake was obtained by IF doses higher than 500 C.U. A dose of 1,000 C.U., which was "equivalent" to the B_{12} dose given in terms of binding capacity, was not sufficient for maximal response. The response increased most sharply in the range of approximately 1,000 to 3,000 C.U. Maximal response occurred when an IF dose of 10,000 C.U. was given. The individual response to the same IF dose was highly significant between patients, which may have been due to cases being included at different stages of development of the p.a. Some patients appeared to become refractory to oral treatment with B_{12} and IF.

G. F. S.

Radiovitamin B_{12} Bound in Pig Liver, Intestinal Absorption of. W. Nyberg and P. Reizenstein. (*Lancet*, 1958, **2**, 832.) Support is given to the theory of Heathcote and Mooney (this Journal, 1958, **10**, 593) that the absorption of vitamin B_{12} is enhanced by binding with peptides. Liver containing native radiovitamin B_{12} was obtained by injecting a pig with radiovitamin B_{12} daily for one month. The liver contained approximately 6 mg. vitamin B_{12}–^{60}Co and approximately 560 mg. non-labelled B_{12} per g. of wet tissue. Of the vitamin in the liver approximately 88 $\pm$ 3 per cent was non dialysable. Samples of this liver were fed to five healthy volunteers and to four patients with untreated pernicious anaemia. Faeces were collected for seven days and the excreted radioactivity measured. Eight of the patients then received comparable, though somewhat larger doses of crystalline radioactive B_{12} as a control test. Absorption of liver B_{12} was not significantly higher in the controls than in the cases of pernicious anaemia, and more of the bound B_{12} than of the crystalline vitamin appeared to be absorbed. This may indicate that B_{12} in the form in which it occurs in the liver is more easily absorbed than crystalline B_{12}.

G. F. S.

Sorbitol, Failure of, to Replace Intrinsic Factor in the Gastrectomised Rat. B. A. Cooper. (*Nature, Lond.*, 1958, **182**, 647.) In a series of controlled experiments on gastrectomised rats, the oral administration of a preparation containing D-sorbitol was shown to be without effect on the absorption of a small dose (0·015 μg.) of labelled vitamin B_{12}. On the other hand the administration of rat gastric juice or of homogenised rat stomach promoted absorption to an extent comparable with that in the normal rat. These results showed that D-sorbitol did not take the place of rat intrinsic factor. It has been demonstrated that in pernicious anaemia two mechanisms seem to exist for the absorption of vitamin B_{12}. Rapid absorption, independent of, or even inhibited by, intrinsic factor, follows the administration of a large dose of the vitamin. On the other hand a slower physiological mechanism involving the intrinsic factor, is required for the absorption of small amounts. From these results and those of other workers, the authors conclude that D-sorbitol affects only the absorption of large doses of vitamin B_{12}, that is, the mechanism which is independent of intrinsic factor activity.

W. C. B.

BIOCHEMICAL ANALYSIS

Ethanol, Optimum Conditions for Determination of, in Body Fluids; using the Acid Dichromate Method. L. Wilkinson. (*Analyst*, 1958, **83**, 390.) The rate of reduction of potassium dichromate at room temperature by ethanol was studied in concentrations of sulphuric acid ranging from 5–37N, the optimum lying between 18·5 and 23N and the rate also rising with increase in dichromate concentration. Loss by volatilisation and the effect of light were found to be negligible. In the method described, ether, acetone, ethyl chloride, chloroform and methylamine do not interfere and formaldehyde can be polymerised by treatment with potassium carbonate. Conway diffusion can be used in the routine determination. D. B. C.

Glucocorticoids, A Simple and Rapid Method for the Biological Assay of. B. P. Block and P. F. D'Arcy. (*Nature, Lond.*, 1958, **182**, 181.) It is well known that adrenocortical steroids have a pronounced effect on carbohydrate metabolism and in particular on the liver glycogen level. Various methods for the assay of adrenocorticoids, based on the deposition of liver glycogen in adrenalectomised rats and mice, have been reported but in all instances they are too complex and tedious for rapid screening procedures. The authors then give a detailed description of their simple and rapid, yet highly sensitive, method of assaying glucocorticoids, based on the liver glycogen method of Venning, Kazmin and Bell. The results are expressed in terms of mg. of liver glycogen equivalent per 100 g. of mouse. Using this method it was found that doses of either 10 or 20 mg. of glucose per mouse, incorporated into the injection solution, greatly increased the response to a given dose of cortisone acetate, without themselves having any pronounced effect on the liver glycogen level. Doses greater than 20 mg. per mouse caused glycogen deposition. The only disadvantage of the method is that, because the livers are bulked for assay, no estimate of the scatter of the individual responses within each group can be obtained. However, the method is most suitable for the routine screening of new compounds for glucocortical activity. M. B.

Tranquillisers and Sedatives, Bioassay of Potential, Against Audiogenic Seizures in Mice. N. P. Plotnikoff. (*Arch. int. pharmacodyn.*, 1958, **116**, 130.) The bioassay consists of selecting mice which are sensitive to audiogenic seizures and placing them in groups of five. The responses of mice susceptible to the auditory stress (ringing of two door-bells for 90 sec.) consist of running, jumping, circling, convulsing and passing into catalepsy. Each type of response is graded one point if present so that any one mouse might have a maximum of 5 points. Each group acts as a control one day and is drug tested the next, all agents being administered orally one hour prior to testing. Percentage protection is calculated on the basis of the control level and is plotted against log dose. Chlorpromazine, reserpine and meprobamate inhibited both running and convulsive seizures in small doses. High doses of chlorpromazine (200 mg./kg.) and reserpine (300 to 400 mg./kg.) potentiated convulsive seizures. Sodium phenobarbitone, methylpentynol and chloral hydrate inhibited convulsive seizures but not running seizures even at high doses. It is suggested that new agents may be classified as potential tranquillisers if they inhibit running seizures. W. C. B.

PHARMACY

Ammonium Alginate Wool as a Filter for Collecting Micro-organisms from large Volumes of Air. E. C. Hammond. (*J. gen. Microbiol.*, 1958, **19**, 267.) Filters containing ammonium alginate wool were sterilised by autoclaving at 10 lb. wt./sq. in. for 30 minutes. Air containing the test organism (*Bacillus subtilis*), either in the form of an aerosol or of a dry dust prepared from sifted soil was blown through the filters at speeds varying from 1 to 5 cu. ft./min., any organisms which had passed through the alginate wool being collected on membrane filters. The alginate wool from each filter was dissolved in a sterile 0·5 per cent solution of dipotassium hydrogen phosphate and samples plated out with glucose nutrient agar and incubated at 30° for 3 days. The membrane filters were shaken with 0·5 per cent sodium chloride solution and glass beads and the solution plated out with nutrient agar. In these experiments, ammonium alginate wool retained the dry spores of *B. subtilis* with an efficiency of 99·13 to 99·96 per cent. It appeared to be 100 per cent efficient for micro-organisms of diameter greater than 2μ, including the majority of yeasts and moulds. G. B.

Enteric Coated Tablets, Correlation of *In Vivo* with *In Vitro* Disintegration Times. J. G. Wagner, W. Veldkamp and S. Long. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 681.) Tablets of barium sulphate (3 grains) were undercoated, and then enteric-coated, using five different enteric-coating preparations. The 5 batches of enteric-coated tablets were submitted to the action of simulated gastric fluid (U.S.P.) for 2 hours at 37°. The tablets, which were unaffected by this treatment, were placed in artificial gastric fluid at 37° in the U.S.P. disintegration test apparatus. The tablets with undercoating only were also tested in artificial intestinal fluid, and by subtracting the disintegration time for these from that for the enteric-coated tablets, an estimate of the disintegration time of the enteric coating was obtained. Dogs were given 4 enteric-coated tablets and 50 ml. of 0·1N hydrochloric acid after an overnight fast. X-ray photographs were taken after 45 minutes, and subsequently at intervals of 15 minutes. In some experiments to determine the stability of the enteric coating in the stomach, the dogs were fed 30 minutes before receiving the 4 tablets. Graphs are presented showing the relationship between the *in vitro* and *in vivo* disintegration times. The methods appear to be useful for investigating enteric coatings and the effect of storage conditions on them, but it is suggested that similar experiments, together with blood level determinations, should be carried out in human subjects before attempting to lay down standards for the disintegration of enteric-coated tablets. G. B.

6-Fluorothymol, The Synthesis and Antifungal Properties of. C. A. Discher, J. M. Cross, P. F. Smith and M. Iannarone. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 689.) 6-Aminothymol fluoroborate was diazotised and, after purification, the diazonium salt was distilled with benzene and toluene to remove the gaseous products of decomposition. 6-Fluorothymol was extracted from the reaction mixture with sodium hydroxide solution and subsequently purified. The antifungal activity of this compound was compared with that of other 6-halothymols and thymol, using the serum-agar cup-plate method, with 8 test organisms (*Candida*, *Microsporon*, *Trichophyton* spp.). At a concentration of 0·05 per cent in propylene glycol solution, 6-iodothymol was the most effective antifungal agent, followed by 6-bromothymol, 6-chlorothymol, 6-fluorothymol and thymol. G. B.

Mould-inhibiting Compounds, The Effect of pH on the Efficiency of. F. J. Bandelin. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 691.) A modified Sabouraud agar, enriched with yeast extract was buffered to pH 3, 5, 7 and 9 with the aid of citrate-phosphate buffer solutions. The compounds under test were incorporated in various concentrations in the warm fluid medium, and sterilised slopes prepared. These were inoculated with standard suspensions of spores of the test organisms, *Aspergillus niger*, *Penicillium citrinum*, *Alternaria solani* and *Chaetomium globosum*. The slopes were incubated at 30° for 14 days, the criterion of inhibition being lack of growth at the end of the incubation period. Benzoic, salicylic, propionic and sorbic acids were effective at pH 3–5, but lost their activity at higher pH values. Dehydroacetic acid (3-acetyl-6-methyl-1:2-pyran-2:4(3H)-dione) was effective at concentrations of 0·001–0·005 per cent in acid media, but less effective in alkaline media. Esters of *p*-hydroxybenzoic acid were most effective in acid media, but less affected by pH changes than the other compounds. Vanillin and ethylvanillin were effective in concentrations up to 0·2 per cent, and relatively little affected by pH changes. Kojic acid, an antifungal substance isolated from *Aspergillus* sp., was the least effective substance examined. G. B.

Pyrogens, Bacterial, Determination of the Components of, by Chromatography. K. Macek and J. Hacaperková. (*Československ. Farm.*, 1958, **7**, 300.) A sample of pyrogen (200 mg.) is hydrolysed by heating with dilute sulphuric acid and the purines are precipitated by silver sulphate. Sugars are detected by chromatography on Whatman No. 1 or No. 3 paper with *n*-butanol:pyridine:water (6:4:3) as solvent system; the spots are located by spraying the paper with a reagent comprising 4 per cent ethanolic aniline, 4 per cent ethanolic diphenylamine and 85 per cent phosphoric acid (5:5:1). For the detection of glucose, mannose and galactose the chromatogram is moistened with yeast suspension, incubated for 2 hours at 38°, and then treated with diphenylamine reagent. In the separation of purines, water-saturated *n*-butanol containing 5 per cent of formamide is used as solvent. For amino acids the solvent system *n*-butanol:acetic acid:water (4:1:5) is used. Results are given for pyrogens produced by *S. typhimurium*, *E. typhi*, *P. vulgaris* (Westphal), *P. vulgaris* (Dare) and *S. abortus equi*. E. H.

PHARMACOGNOSY

Digitalis Glycosides, Enzymatic Decomposition of. O. Gisvol. (*J. Amer. Pharm. Ass.*, 1958, **47**, 594.) Glycosides were extracted from the leaves of several species of *Digitalis* using two different procedures. (i) Fresh leaves were disintegrated in the presence of water and dilute methanol, heated for a short time and then filtered. The filtrate was extracted successively with a mixture of ether and methylene chloride (3:1) and with ethyl acetate and the extracts examined chromatographically. By this procedure the terminal glucose of the primary glycoside was removed and certain of the resulting desglucoglycosides could be obtained crystalline. (ii) Fresh leaves were immersed in boiling phosphate buffer (pH 7) and then disintegrated in the medium, filtered, and the filtrate treated by suitable solvent solvent extraction and examined chromatographically. This second procedure resulted in the production of primary glycosides as the preliminary heat treatment evidently inactivated the carbohydrases responsible for splitting off the terminal glucose. It is claimed that this simple procedure is more satisfactory than that originally used by Stoll.

Fresh leaves which had been dried in an incubator at 60° were examined in a similar manner: it was shown that considerable decomposition of the primary glycosides had taken place. Two solvent systems were used in the paper-chromatographic work; the first consisted of methyl *iso*butyl ketone and *iso*-propyl ether (100:25) saturated with formamide. The stationary phase, formamide, was applied to the paper as a 30 per cent solution in acetone. The second system was prepared from methyl*iso*butyl ketone, *iso*propyl ether, tetrahydrofuran and formamide (40:10:15:15). The stationary phase was the same as in the first solvent system. J. W. F.

Digitalis Glycosides, Enzymatic Decomposition of. O. Gisvol. (*J. Amer. pharm. Ass.*, 1958, **47**, 600.) Fresh leaves of *D. purpurea* were carefully dried below 60° and examined by the methods already described. The results showed that only a small proportion of the primary glycosides had been decomposed. Similar results were obtained with a commercial sample of dried leaf and with U.S.P. Digitalis Reference Standard 1942. In contrast, fresh leaves dried at 100° showed almost complete decomposition of the primary glycosides into desglucoglycosides. Extraction of carefully dried leaf with 66 per cent methanol (or stronger) inhibited enzyme action. Since the primary extract of Digitalis Reference Standard used in assay work is made with strong ethanol the primary glycosides will be the chief reference standard. With unknown samples of leaf, the amount of primary glycosides present will depend on the care with which the leaf has been dried and stored. J. W. F.

PHARMACOLOGY AND THERAPEUTICS

Adrenaline and Noradrenaline, Replacement of, in the Innervated and Denervated Adrenal Gland of the Rat, following Depletion with Reserpine. B. A. Callingham and M. Mann. (*Nature, Lond.*, 1958, **182**, 1020.) It has already been shown that reserpine causes a similar percentage loss of adrenaline and noradrenaline, followed by slow recovery, in the normal rat adrenal gland. Experiments have now been performed to show whether this action is central or peripheral. The left adrenal gland was denervated by cutting the splanchnic fibres. Subsequently the rats were given reserpine and then killed at suitable time intervals in order to study both the depletion and the replacement of adrenaline and noradrenaline in the innervated and denervated glands. It was found that at three days there was a similar degree of depletion of both amines in the innervated and the denervated glands. The adrenaline then slowly recovered in both groups, while the noradrenaline content rose well above its normal level of 10 per cent before returning to normal. Complete recovery of the two amines had taken place in both the groups by three weeks. Thus no appreciable difference, in either the depletion or the replacement of adrenaline and noradrenaline could be found between innervated and denervated adrenal glands of the rat. It may thus be concluded that this action of reserpine is peripheral. M. B.

Bis-Quaternary Ammonium Compounds, the Pharmacology of Some. Z. P. Horovitz, E. C. Reif and J. P. Buckley. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 718.) Tests were carried out to compare the pharmacological effects of 4 experimental hypotensive agents with chlorisondamine dimethochloride. The compounds examined were 1:6-bis-*NN*-dimethylamino-2-hexyne dimethobromide (JB-520), 1:6-bis-*NN*-morpholino-2-hexyne dimethobromide (JB-540),

β-(3-dimethylamino)propylamino-*N*-methylpiperidine dimethobromide (JB-549) and β-dimethylaminoethyl-*N*-methylpipecolinate dimethobromide (JB-591). All these compounds blocked the transmission in the superior cervical ganglion of the cat, blocked the hypertensive activity of 1:1-dimethyl-4-phenylpiperazinium iodide and dilated the mesenteric blood vessels in the rat. JB-520 and chlorisondamine depressed the auricular musculature of the perfused turtle heart, and JB-520 produced a negative inotropic effect on the isolated rabbit heart. All of the compounds except JB-591 increased the activity of the isolated rabbit ileum, and none of the compounds produced alterations in the blood pressure of the pithed rat. G. B.

Dihydrocodeine and Morphine in Man, Comparison of the Analgesic and Respiratory Effects of. J. C. Seed, S. L. Wallenstein, R. W. Houde and J. W. Bellville. (*Arch. int. pharmacodyn.*, 1958, **116**, 293.) In a double-blind, random-sequence, cross over study inpatients suffering from chronic pain due to cancer, 68 mg. of dihydrocodeine was found to be equivalent in analgesic potency to 10 mg. of morphine. The respiratory effects of the two drugs were compared by determining the displacement of the alveolar ventilation-alveolar partial pressure of carbon dioxide gas response curves in normal volunteers and in patients. When given in doses which were equi-analgesic with those of morphine, dihydrocodeine was found to produce an equal amount of respiratory depression. It appeared that the effects of the two drugs on the response curve were primarily due to an effect on the respiratory control mechanism and not to changes in cerebral circulation, cerebral metabolism, airway resistance, or physiological dead space. W. C. B.

Dioscorine and Dioscine, Pharmacological Properties of. J. L. Broadbent and H. Schnieden. (*Brit. J. Pharmacol.*, 1958, **13**, 213.) These alkaloids are present in certain varieties of "yams", ingestion of which may cause convulsions. In both rats and mice these drugs caused clonic followed by tonic convulsions. The convulsions resemble those produced by picrotoxin and were antagonised by sodium pentobarbitone. The LD50 values in mice were 60 mg./kg. for dioscorine and 100 mg./kg. for dioscine. The toxicity of dioscine solutions rapidly decreased on storage even when stored in the refrigerator. Both drugs had an analeptic action in anaesthetised rats but the effective dose was close to the convulsant dose. Solutions of both drugs showed a local anaesthetic action when injected intradermally into the guinea pig but were inactive on the cornea. In the anaesthetised cat neither drug affected the blood pressure in doses up to 20 mg./kg. but the hypotensive action of acetylcholine was reduced and the hypertensive action of adrenaline was increased. On the guinea pig isolated ileum the responses to acetylcholine and histamine were reduced. On the isolated heart doses of 2 mg. had no effect but the response to acetylcholine was reduced. Both drugs had an antidiuretic action. G. F. S.

Ganglion-blocking Agent. (189c56) an Orally Effective. S. Locket. (*Brit. med. J.*, 1958, **2**, 74.) This ganglion-blocking agent is chemically related to pentacynium methylsulphate. Compared with pentacynium, about twice the dose of the active cation of 189c56 by subcutaneous or intravenous injection produces the same fall in blood pressure. It produces hydrodynamic changes and effects on renal function comparable to those of pentacynium. Its valuable features are (1) that it is invariably effective when taken by mouth, (2) that when given daily before breakfast it consistently produces the expected degree

of hypotension, (3) that its duration of action is such that a single daily dose treatment of hypertension becomes possible in some patients, (4) that dosage is highly critical (a small alteration in the controlling dose of 5–12 per cent causes a marked effect on the extent and duration of the hypotension), (5) constipation is absent or minimal, even with large doses, and ileus does not occur, and (6) disturbances of bladder emptying do not occur. The initial dose employed is usually 200 mg. This is increased by 50 mg. each day until an appreciable hypotensive effect is obtained. This dose is then given daily. In a few days the patient becomes accommodated to the hypotension and the dose is then increased by 25–50 mg. every few days. The degree and duration of hypotension achieved show little daily variation once a satisfactory drug level is obtained. After several weeks of daily administration of this dose it may be necessary to reduce it because of increased severity and duration of the fall in blood pressure. Dryness of the mouth occurs with the larger doses, and disturbance of vision occurs as with other ganglion-blocking agents. This treatment was given to 11 patients. S. L. W.

Glycyrrhizin-induced Inhibition of the Pituitary Adrenal Response. S. D. Kraus. (*J. exp. Med.*, 1958, **108**, 325.) Glycyrrhizin has been shown to have an action like deoxycortone, reducing the resistance of mice to a cold stress and decreasing the ability of rats deprived of food to mobilize glucose. The average survival time of normal mice, weighing from 8 to 10 g., at 5° was 6·5 hours. Mice which had received 0·4 per cent ammoniated glycyrrhizin, in place of drinking water, for four days previously only survived for 2·5 hours. Rats fasted for 24 hours showed hypoglycaemia, but after 48 hours mobilized sufficient glucose to bring the blood sugar level up to the normal value. Rats pretreated for seven days with ammoniated glycyrrhizin in their drinking water showed much less mobilization. It is concluded that glycyrrhizin, like deoxycortone, depressed the output of ACTH by the pituitary gland. G. F. S.

5-Hydroxytryptamine and Cooling, Effect of, on the Peristaltic Reflex. D. Beleslin and V. Varagić. (*Brit. J. Pharmacol.*, 1958, **13**, 266.) The effect of 5-HT and cooling has been studied on the peristaltic reflex of the guinea pig isolated intestine to obtain more information about the physiological role of 5-HT in peristalsis. A modification of the method of Trendelenburg was used so that the drug could be introduced directly into the lumen. The results showed that lowering the temperature to 19–26° temporarily abolished the emptying phase of the reflex, while cooling to 5° for 3 to 8 hours caused this to be permanent. While 5-HT added to the bath at 37° depressed or abolished the reflex, in the cooled preparation at 19° introduction of 10 to 400 ng. into the lumen easily restored the reflex. This effect may be due to sensitizing receptors in the mucosa which trigger the reflex, the facilitation of transmission at the synapse or to both actions, according to the site of application. The effects of 5-HT were prevented by 2-bromolysergic acid diethylamide. G. F. S.

Isoetharine, the Pharmacology of. A. M. Lands, F. P. Luduena, J. O. Hoppe and I. H. Oyen. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 744.) Isoetharine (Dilabron, Win3046), a bronchodilator drug, is the hydrochloride of 1-(3:4-dihydroxyphenyl)-2-*iso*propylaminobutan-1-ol, the *N-iso*propyl derivative of ethylnoradrenaline. Using intact anaesthetised guinea pigs it was shown that isoetharine is an effective antagonist to bronchostriction induced by histamine. The drugs were given in the form of an aerosol and the inclusion of thenyldiamine hydrochloride in the aerosol enhanced the action of isoetharine. Subcutaneous injection of isoetharine with phenylephrine was found to increase

the protective action of thenyldiamine against toxic doses of histamine administered intravenously to dogs. The increase in pulse-rate following subcutaneous administration of isoetharine to dogs was decreased by the addition of 25 per cent of phenylephrine and 10 per cent of thenyldiamine to the injection. Isoetharine is rapidly absorbed following oral administration, and usually gives rise to tachycardia; experiments in dogs showed that the absorption of the drug may be suitably delayed by giving it in the form of enteric-coated tablets. Isoetharine, alone or mixed with phenylephrine and thenyldiamine has a low acute toxicity for mice when given by intravenous injection. G. B.

Laminarin Sulphate (LM 46), Effect of, on Bone Growth. S. S. Adams, H. M. Thorpe and L. E. Glynn. (*Lancet*, 1958, **2**, 618.) LM 46 was administered subcutaneously or intravenously to rabbits (15, 30, and 60 mg./kg.), rats (15 and 30 mg./kg.) and guinea pigs (15 mg./kg.) on five days of each week for 2–9 weeks. Control animals received a dose of heparin equivalent in anticoagulant activity to the laminarin sulphate. In all animals there was a pronounced loss of weight after prolonged dosing and the rabbits and guinea pigs had a white discharge in the eyes. Bone lesions developed which appeared to depend primarily on disturbance of the endochondrial bone formation that normally takes place on the diaphyseal side of the epiphyseal cartilage. In rats and guinea pigs the normal sequence of maturation of the cartilage cells was impaired and this was associated with virtually complete absence of the vascular invasion that normally precedes the actual deposition of bone on the newly formed tongues of calcified cartilage. In rabbits it was apparently this deposition of bone which was impaired. The structural similarity of laminarin sulphate to chondroitin sulphate suggested that it might be acting by interfering with the normal metabolism of chondroitin sulphate. In addition to the bone lesions all the animals showed accumulation of metachromatic material in the reticuloendothelial system and in the proximal convoluted tubules of the kidneys. W. C. B.

Metamidium: a New Trypanocidal Drug. W. R. Wragg, K. Washbourn, K. N. Brown and J. Hill. (*Nature, Lond.*, 1958, **182**, 1005.) This paper describes a new trypanocide which possesses considerable potentiality, both as a curative and as a prophylactic drug. Metamidium chloride hydrochloride (M & B 4404) was examined for activity against three strains of *Trypanosoma congolense* which gave an acute infection in mice. A single subcutaneous injection was given to mice with an infection in the peripheral blood stream of from 1–10 trypanosomes per high power field. Ten mice were used for each dose and five untreated mice which died within 5–7 days were used as controls in each experiment. Homidium chloride was used as the standard. Wet blood smears were examined three times a week for four weeks and the number of animals cleared of trypanosomes for that period was noted. The subcutaneous LD50 for mice was also determined. The results show that metamidium was more active than homidium against all three strains. Prophylactic experiments in mice were also carried out and it was found that metamidium completely protected mice for up to sixteen weeks at about one-ninth of the LD50. This is of importance because neither the parent compound homidium chloride (2:7-diamino-10-ethyl-9-phenylphenanthridinium chloride) nor Berenil, with which metamidium shares some structural similarities, has any appreciable prophylactic activity at one-third of the LD50. Of the two isomers of metamidium, the red was more active than the purple, both therapeutically and prophylactically, but there was little difference in their toxicities. M. B.

Pain in Spasmodic Dysmenorrhoea, Relief of, by Bromelain. C. A. Simmons. (*Lancet*, 1958, **2**, 827.) Bromelain is obtained by extraction from the stem of the pineapple. It contains an unstable proteolytic enzyme and is freeze dried for storage. The intravaginal administration of a fresh solution has been shown to rapidly relieve the cramping pain of spasmodic dysmenorrhoea. Its mode of action is unknown, but is possibly due to relaxation of the smooth muscle of the cervix. Ovulation is not inhibited. G. F. S.

Pempidine, Pharmacological Properties of. S. J. Corne and N. D. Edge. (*Brit. J. Pharmacol.*, 1958, **13**, 339.) Pempidine (1:2:2:6:6:-pentamethylpiperidine) is an orally effective, long acting ganglion-blocking agent. In the anaesthetised cat an intravenous injection of pempidine caused, like mecamylamine, a relaxation of the preganglionically stimulated nictitating membrane. The relaxation was slower than with hexamethonium and recovery very prolonged. Pempidine acted specifically at the ganglion and not on the nictitating membrane itself. Large doses did not reduce the output of acetylcholine from the perfused ganglion. In both the anaesthetised cat and the normal mouse pempidine caused mydriasis through its effect on the ciliary ganglion. Doses of 0·05 to 1·0 mg./kg. caused a fall in the cat blood pressure, which developed more slowly than with hexamethonium. Doses up to 10 mg./kg. did not affect the depressor actions of acetylcholine or histamine, but like other ganglionic blocking agents it potentiated the response to adrenaline. A dose of 0·1 mg./kg. abolished the pressor action of nicotine. There was no evidence of histamine release in the atropinised cat, but high doses caused a pressor action, which was not due to adrenaline release. A dose of 0·4 mg./kg. abolished the effect of peripheral vagal stimulation, but not injected acetylcholine. On the isolated rabbit heart a dose of 16 mg. was required to cause cardiac arrest. Smaller doses caused a slowing and decreased amplitude of the beat. Coronary flow increased. In the perfused hind limb of the dog intra-arterial doses of 8 mg. were without effect. Larger doses caused vasodilatation. On the isolated ileum of the guinea pig 800 μg./ml. had little direct effect in most preparations, in others 8 μg./ml. caused a contraction which was abolished by atropine. After 0·8 μg./ml., responses to histamine, pilocarpine and acetylcholine were normal; but the response to nicotine was abolished. Doses of 80 μg./ml. depressed the responses to histamine, pilocarpine, acetylcholine and 5-HT. Pempidine inhibited the peristaltic reflex in doses of 1 μg./ml. On skeletal muscle doses of 10 to 40 mg./kg. were required to block the response of the indirectly stimulated tibialis muscle of the cat, the block being curare-like. The anticholinesterase activity *in vitro* was 10^6 times less than neostigmine. Nicotine induced convulsions in mice were prevented by 0·09 mg./kg. G. F. S.

Perphenazine, A Potent and Effective Antiemetic. S. C. Wang. (*J. Pharmacol.*, 1958, **123**, 306.) Perphenazine (Trilafon), a phenothiazine compound, has been shown to have a potent antiemetic activity. In dogs treated with 0·5 mg./kg. of apomorphine, 0·1 mg./kg. i.v. prevented vomiting and 0·035 mg./kg. was about the 50 per cent effective dose. It was therefore about forty-eight times as effective as chlorpromazine. Similarly good protection was given against vomiting induced by morphine sulphate, Hydergine, and also against Lanatoside C where chlorpromazine was ineffective. Against oral emetic doses of 40 mg./kg. of copper sulphate, 0·1 mg./kg. of perphenazine gave 27 per cent protection, comparable with 2·0 mg./kg. of chlorpromazine. It is concluded that the increased potency of perphenazine is related to its greater affinity for the receptors in the trigger zone located in the area postrema. G. F. S.

Serotonin Antagonism of Noradrenaline *In Vivo*. P. Gordon, F. J. Haddy and M. A. Lipton. (*Science*, 1958, **128**, 531.) It has been reported previously that pretreatment with serotonin (5-HT) reduces the mortality of mice given bacterial endotoxin. Since the administration of the endotoxin is followed by the secretion of and hypersensitivity to adrenaline and noradrenaline, it is possible that 5-HT has antiadrenergic properties. That 5-HT does antagonise noradrenaline has been shown for the following *in vivo* systems: inhibition of the toxicity of noradrenaline in mice, suppression of the pilomotor response in mice, lysis of small blood vessel tone, and inhibition of noradrenaline-induced bradycardia. 5-HT pretreatment of mice considerably reduced the mortality caused by noradrenaline and it also abolished the concomitant pulmonary oedema. The pilomotor response in mice was elicited either by injection of noradrenaline or by exposure to cold or by the injection of reserpine. Prior injection of 5-HT completely suppressed the piloerection when caused by any one of the above methods. Dibenzyline also blocked this response. The effect of 5-HT on the vascular resistance was studied in the dog forelimb. Pressures were measured simultaneously in the brachial artery, a small vein in the footpad, a small vein in the paw and the cephalic vein. Large artery, small vessel (mainly arteriolar) and large vein resistances were calculated separately. The administration of 5-HT decreased the small vessel resistance at all levels of tone, the decrease being proportional to the initial level of tone. The effect of noradrenaline on these blood vessels was also antagonised. Again in dogs, noradrenaline given after 5-HT caused tachycardia instead of the usual bradycardia. Also the tachycardia induced by decreasing the intraluminal pressure of the carotid sinus was abolished during 5-HT infusion. Since noradrenaline-induced bradycardia is dependent on the carotid sinus reflex and since the denervated heart responds to noradrenaline with tachycardia, these results suggest that 5-HT antagonises the action of noradrenaline on the carotid sinus. These results from the different preparations suggest that the biological role of 5-HT lies in its interaction with noradrenaline. M. B.

APPLIED BACTERIOLOGY

Essential Oils, *In Vitro* Antifungal Activity of. J. C. Maruzzella and L. Liguori. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 250.) Essential oils were tested by the filter paper disk method, in which disks $\frac{1}{4}$ inch in diameter were moistened with the oil and placed on plates of Sabauraud's maltose agar, previously seeded with a test organism. After incubation zones of inhibition were measured, and part of the clear zone was incubated in Sabauraud's maltose broth to obtain an indication whether the oil was fungicidal or fungistatic under the conditions of the test. Of 92 volatile oils examined, 90 showed activity against at least one of the test organisms. The most effective were red origanum, lemon grass, red thyme, sweet birch, savory select, coriander, sassafras, cinnamon, distilled laurel leaf and chenopodium oils. Chenopodium, red origanum and terpeneless dill oils were shown to be very effective against *Ustilago avenae*, chenopodium and red origanum against *Trichophyton mentagrophytes*, and cinnamon against *Epidermophyton interdigitale*. *Streptomyces venezuelae* was the most susceptible of the test organisms, and *Candida krusei* the most resistant. Of 12 terpeneless oils examined, cinnamon, caraway, dill and anise showed the greatest activity. Castor, olive and white mineral oils were shown to be devoid of antifungal action. G. B.

Essential Oils and Oil Combinations, *In Vitro* Antibacterial Activity of. J. C. Maruzzella and P. A. Henry. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 294.) Thirty-five volatile oils were examined for antibacterial activity, using the filter paper disk method with 5 test organisms. The most effective oils, giving the greatest sum of diameters of zones of inhibition of the test organisms, were eucalyptus, cinnamon and red origanum. Volatile oils were found to be more effective against Gram-positive than Gram-negative organisms. Castor, cod-liver, olive and white mineral oils, infused oils of asafoetida, burdock, henbane, lobelia and mullein, vitamin K_1, and volatile oils of cedar wood and myrrh were shown to be devoid of antibacterial activity. Mixtures of two or three of the volatile oils examined were usually less effective than the individual oils, but enhancement of effect was observed with mixtures of equal quantities of eucalyptus, cinnamon and dwarf pine needle oils, eucalyptus, cinnamon and juniper berry oils, and eucalyptus, cinnamon and niaouli oils. The activity of volatile oils was markedly diminished by the addition of fixed or infused oils. G. B.

Novobiocin Sodium in Selected Ointment Bases, Antibacterial Activity and Stability of. E. Stempel, L. Greenberg and A. Urdang. (*Amer. J. Pharm.*, 1958, **130**, 116.) Ointments of novobiocin were prepared in a number of bases of different types, and their antibacterial activity against *Micrococcus pyogenes* var. *aureus* determined by the agar cup-plate method. Hydrophilic ointment U.S.P.XV appeared to be the most suitable of the water-miscible bases, as the antibiotic was found to be unstable in polyethylene glycol ointment U.S.P.XV. If a washable absorption base is preferred, the following formula is recommended: cetyl alcohol 12, stearyl alcohol 16, Ethofat '60/60' 8, propylene glycol 21, white soft paraffin 43. Of the greasy absorption bases examined, Plastibase hydrophilic-water (4:1), Falba-water (2:1) or Hydrosorb-water (2:1) were found to be satisfactory. Greasy bases did not release the antibiotic so readily as other types. Ointments prepared with petrolatum rose water ointment U.S.P.XV showed the greatest antibacterial activity after storage for one week, but ointments prepared with Plastibase were more stable.

G. B.

Ophthalmic Ointments, Bacteriological Study of. R. W. V. Wyk and A. E. Granston. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 193.) Commercial opthalmic ointments were examined by the following method. The content of an unused tube of ointment was transferred aseptically to a flask containing 25 ml. of sterile water and a few glass beads. The flask was warmed to 45° and the melted ointment dispersed by placing the flask on a shaker for an hour. 1 ml. of the liquid was mixed with blood agar and a plate poured. Counts were carried out after incubation at 37° for 24 hours. Of the ointments examined 14·5 per cent were sterile, and most had counts of less than 50 organisms per g. For experimental purposes an ophthalmic ointment base consisting of white wax 1, cetyl alcohol 1 and *iso*propyl myristate (Deltyl extra) 4 was inoculated with *Micrococcus pyogenes* var. *aureus*. Various substances were examined for their efficiency in killing or inhibiting growth of the organism, and it was shown that benzyl alcohol (0·5 per cent) is more effective than benzalkonium chloride (0·02 per cent) or chlorbutol (0·25 per cent). A series of ointments prepared extemporaneously with the addition of 0·5 per cent of benzyl alcohol showed a reduction of 97 per cent in their content of bacteria, as compared with similar commercial ointments prepared without benzyl alcohol. G. B.

BOOK REVIEW

THE ANALYTICAL USES OF ETHYLENEDIAMINETETRAACETIC ACID. By F. J. Welcher. Pp. xvii + 366 (including Bibliographical References and Index). D. Van Nostrand Company Ltd., London, 1958. 64s.

The number of published papers which have been devoted to the use of ethylenediaminetetra-acetic acid (EDTA) as an analytical reagent is large and ever-increasing. In this volume Professor Welcher has aimed at providing a comprehensive review of the work and he is to be congratulated on having achieved so great a measure of success. In his preface the author makes it clear that no attempt has been made to give a critical evaluation of each method and that the volume is not to be regarded as a handbook of recommended methods. To have compared and contrasted the many published methods would surely have been a task beyond the power of any one man to perform; even in the limited field of pharmaceutical applications it has already become difficult to examine every procedure from a critical standpoint. It is with this limitation in mind that the book should be considered.

The subject matter has been divided into 18 chapters, the first 4 of which deal with general theoretical and practical considerations. The fifth chapter is devoted to the determination of water hardness and this is followed by a number of sections, forming the major part of the work. These describe the application of EDTA titration methods to the determination of many metal ions. Miscellaneous chapters at the end of the book deal with the determination of anions, the aplication of polarographic, amperometric and colorimetric methods, and the use of EDTA in qualitative analysis and as a masking agent to prevent interference in other methods of analysis. The work is concluded by a list of nearly 1000 references which represents a careful selection of the more important papers up to 1957. So rapid is the development of the technique, however, that new fields of investigation such as the determination of alkaloids through their metal compounds are not mentioned.

To demonstrate the immense value of the book and to draw attention to the possible pitfalls, reference might be made to the section on the determination of aluminium. The newcomer to complexometric analysis will find, in Chapter IX, no less than 10 possible methods set out in detail, together with references to several others. The worker with plenty of time to compare and assess these procedures will find the information of considerable value, but the analyst seeking a reliable method for immediate application may well find that the choice before him tends to bewilder rather than to assist. Usually, published applications are by no means of uniform excellence and all too often in the past authors have failed to make a scientific comparison of their work with other published methods. This is a short-coming which cannot be laid at Professor Welcher's door, and it is to be hoped that his excellent book will stimulate a more critical approach on the part of would-be contributors of papers in the future.

As a practical manual the book is to be very warmly recommended; it has very few typographical errors, is well bound and will remain open at any page on the laboratory bench.

C. A. JOHNSON.

LETTERS TO THE EDITOR

Catechol Amine Excretion after Banana Feeding

SIR,—Marshall in a recent letter to this *Journal*[1] reported that the ingestion of bananas (which contain catechol amines in the pulp[2]) does not increase the urinary excretion of catechol amines. We have investigated this problem by feeding bananas to normal subjects with their meals and determining the subsequent excretion of catechol amines fluorimetrically. The free catechol amine excretion measured as noradrenaline equivalents increased slightly after banana feeding, but not above the normal range. After acid hydrolysis (100° for twenty minutes at pH 0–1) of these same urine specimens, however, the total (free plus conjugated) catechol amine excretion was found to be markedly elevated. The findings are shown in the following Table.

Subject	Day	Banana pulp ingested (g.)	Catechol amine excretion µg./day (as noradrenaline equivalents)	
			Free	Total
A	1	0	37	73
	2	205	48	560
	3	475	70	890
	4	0	41	150
B	1	0	43	94
	2	725	73	1660
	3	0	54	140
Normal range			10–80	30–250
Pheochromocytoma			100–2000	400–3000

Extracts of the hydrolysed urine were then chromatographed on Whatman No. 1 paper in a phenol : HCl system, and the appropriate areas on the chromatograms were eluted and assayed. The increase in catechol amine excretion after banana feeding could be accounted for as conjugates of noradrenaline and 3 : 4-dihydroxyphenylethylamine (dopamine); the latter was found in amounts 20–30 times that of noradrenaline. No increase in adrenaline excretion was found. These results are consistent with the findings by Waalkes and others that dopamine is present in banana pulp in large quantity, that noradrenaline is present in smaller amounts, and that adrenaline is not present. It was estimated from the chromatographic data that approximately one-half of the increase in total noradrenaline-equivalents after banana feeding actually represents noradrenaline and that the remainder represents interfering fluorescence from dopamine. The specific fluorescence of dopamine is 3–5 per cent of that of noradrenaline.

Bioassay of these same urine extracts was performed in an anaesthetised dog by a technique which separates qualitatively the effect of noradrenaline from that of dopamine[3]. The results were compatible with the presence of both amines. In spite of its low biologic activity relative to noradrenaline, dopamine contributed significantly to the total pressor activity of the extracts because of the large amount present. In view of this it seems reasonable to suspect that the biologic activity in banana extracts attributed to adrenaline by Marshall[1] represents in fact the effect of large amounts of dopamine.

The results show that free catechol amine excretion after banana feeding is not increased enough to produce a false positive test for pheochromocytoma.

This finding is in essential agreement with that of Marshall. If the practice of many laboratories of hydrolysing the urine to determine total catechol amines is followed, however, the ingestion of only a few bananas may easily produce levels compatible with pheochromocytoma. This source of diagnostic error may be avoided by collecting specimens on a banana-free diet or by determining free rather than total catechol amines. A full report of these findings will appear elsewhere.

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The Assay of Strychnine in Pharmaceutical Preparations of Nux Vomica

SIR,—The chromatographic separation of the alkaloids from nux vomica in its preparations prior to their spectrophotometric estimation has been described by El Ridi and Khalifa[1] using alumina and 86 per cent ethanol, and by Elvidge and Proctor[2] using oxidised cellulose.

We have found that a clean separation can be obtained using absolute ethanol on a column of highly activated alumina.

Method. Heat "Alumina for Chromatography" (B.D.H.) to 800° for six hours, cool and store in airtight containers. Pack a 1-cm. chromatographic tube "wet" with 10 g. of active alumina in absolute ethanol.

Take an aliquot of the sample containing approximately 0·5 mg. of strychnine, make slightly alkaline with N sodium hydroxide, and evaporate to about 2 ml. Mix this residue with about 1 g. of active alumina, and transfer to the top of the column with the aid of absolute ethanol. Elute at 5 to 10 drops per minute with 50 ml. of absolute ethanol under slight negative pressure. Allow the column to drain.

Evaporate the eluate to dryness and dissolve the residue in 100 ml. N sulphuric acid. Measure the absorption of the solution at 262 mμ (A) and 300 mμ (B) against a blank of N sulphuric acid, in 1-cm. quartz cells.

Then $$\text{per cent strychnine} = \frac{0{\cdot}318A - 0{\cdot}460B.}{100}$$

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Effect of Cutaneous Burn on Histamine and 5-Hydroxytryptamine in Mice

SIR,—Dekanski[1] in 1945 reported that total histamine in the body was approximately doubled in one hour after a superficial skin burn in mice. The increase was mainly due to the rise in skin histamine. In view of the importance of this finding in studies relating to metabolism of histamine, it was decided to repeat and extend this observation. Most of the histamine in the skin is contained in the mast cells[2]. It has also been suggested that mast cells might possibly contain also 5-hydroxytryptamine (5–HT)[3]. Therefore studies were made on the mast cell and 5–HT contents of the skin after a superficial skin burn.

Groups of albino mice were anaesthetised with ether and immersed in hot water at 60° for 10 seconds. After drying, the animals were placed in warm cages. A group of mice, anaesthetised and subsequently killed served as controls. Groups of mice were killed 10 minutes, 2 hours, and 24 hours after being subjected to a superficial skin burn. Mast cell spreads were made from the subcutaneous tissues, and mesentery. The histamine and 5–HT contents of the whole skin, subcutaneous tissue, outer skin, spleen and lungs were separately estimated according to the methods of Parratt and West[4]. The mast cells showed degranulation and rupture within 10 minutes after the superficial burn. The changes were essentially the same in the 2 hour and 24 hour specimens. There was no alteration in histamine or 5–HT values in any of the tissues studied as compared with normal controls. Dr. West writes us he has found similar results in rats. It is difficult to explain this difference between our results and those obtained by Dekanski[1].

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