RESEARCH PAPERS

PIRINITRAMIDE (R 3365), A POTENT ANALGESIC WITH UNUSUAL CHEMICAL STRUCTURE

BY PAUL A. J. JANSSEN

From the Research Laboratorium Dr. C. Janssen, Beerse, Belgium

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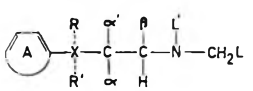
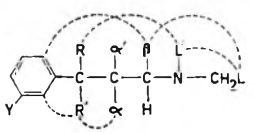
The novelty of the unusual chemical features of the pirinitramide (R 3365) molecule is outlined. It is shown that the compound possesses many morphine-like properties.
In most tests it is more active than morphine by the subcutaneous route, but many times more active orally. As a respiratory depressant agent pirinitramide is possibly less potent in rats than morphine and as an emetic in dogs it is much less active.

MANY synthetic and semi-synthetic organic compounds with pronounced morphine-like activity have been described. All powerful narcotic analgesics have the basic chemical structure (I), in which the nitrogen is usually present as a tertiary, more rarely, secondary or quaternary amine. The basic nitrogen is linked to a flat aromatic ring (phenyl, 2-thienyl) by a chain of three atoms, two of which are carbons and the third X either

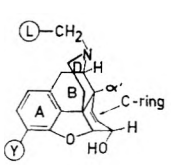


Amines of general structure I.
X = carbon, nitrogen or oxygen.

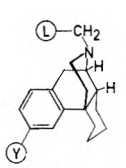


Amines of general structure II.
Y = H, OH, OAc or Oalk;
L′ = H or CH_2L'';
β = H or $CH_2\beta''$;
α = H or $CH_2\alpha''$;
α' = H or OH.

carbon, nitrogen or oxygen. The substituent β on the first carbon atom of this chain is either hydrogen or alkyl. The substituents α and α' on the second carbon atom are two hydrogen atoms, hydrogen and alkyl, or hydroxy and alkyl. The "central atom" X, when carbon or nitrogen, is fully substituted. The substituents L, L′, α, α', β, R and R′ may be



III



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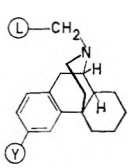
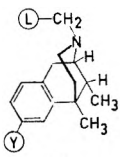


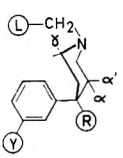
V

III. (−)-Morphine (Y = OH; L = α' = H; absolute configuration).
IV. Morphinans (levorphanol: Y = OH; L = H; absolute configuration).
V. Isomorphinans.

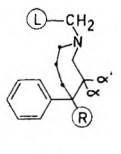
linked with each other and also with the *ortho*-position of ring A. Most of the known morphine-like analgesics are derivatives of general structure II (I: X = carbon). The 5-ring structures related to morphine (structure III shows the absolute configuration of the analgesically active *laevo* isomer, (Beckett and Anderson, 1960), the 4-ring structures of the morphinan type (IV), the B/C-*trans*-isomorphinans (V), the benzomorphans (VI) and their CH_3/CH_3-*cis*-isomers, the 2-ring-compounds related to pethidine (VII), the hexamethyleneimine homologues of VII (VIII) as well as a few less important similar compounds with morphine-like properties, are all derived from II by ring closure between R and L′ to form a piperidine or a hexamethyleneimine-nucleus. The analgesic potency of these drugs depends largely on the nature of substituents L, Y and R (Beckett and



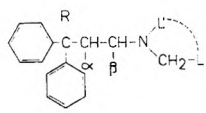
VI



VII



VIII



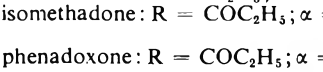
IX

VI. Benzomorphans (−)-phenazocine: Y = OH; L = $CH_2C_6H_5$; absolute configuration.

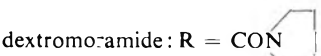
VII. Compounds related to pethidine.
e.g. pethidine: Y = L = α = α′ = γ = H; R = $COOC_2H_5$.
β-prodine: Y = L = α′ = H; α = CH_3; R = $OCOC_2H_5$.
ketobemidone: Y = OH; L = α = α′ = γ = H; R = COC_2H_5.

VIII. Hexamethyleneimine analogues of VII.

IX. 3,3-Diphenylpropylamines
e.g. methadone: R = COC_2H_5; α = H; β = CH_3; NL′CH_2L = $N(CH_3)_2$
isomethadone: R = COC_2H_5; α = CH_3; β = H; NL′CH_2L = $N(CH_3)_2$



phenadoxone: R = COC_2H_5; α = H; β = CH_3; NL′CH_2L =



dextromoramide: R =

; α = CH_3; β = H; NL′CH_2L =

(dextroisomer)

Anderson, 1960; Beckett and Casy, 1954; Eddy, 1959; Eddy, Bezendorf and Pellmont, 1958; Eddy, Halbach and Braenden, 1956; Janssen and Eddy, 1960; May and Eddy, 1959; May and Fry, 1957).

For optimal potency, L must be $CH_2C_6H_5$ or CH_2-2-furyl in III, IV and VI, and $CH_2CHOHC_6H_5$ in VII. In III to VI, Y = OH is optimal, but not necessarily in VII or VIII. In VII or VIII morphine-like activity is associated with R = $COOC_2H_5$, $OCOC_2H_5$, COC_2H_5 or n-C_3H_7 and some lower alkyl analogues of these esters and ketones.

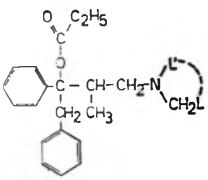
The 3,3-diphenylpropylamines related to methadone (IX) are also derivatives of general structure II (R′ = C_6H_5). High potency is associated with the presence of a methyl-group in α or in β, depending on the nature of substituent R, which may be a ketone (COC_2H_5), a secondary alcohol or its ester ($CHOR'C_2H_5$), a tertiary amide (CON

or $CON(CH_3)_2$)

or a sulphone ($SO_2C_2H_5$). When R is $CONH_2$, CN or OH however, the effects are atropine-like and no analgesic properties are detectable. The potent analgesics of type IX are derivatives of dimethylamine, morpholine, piperidine or hexamethyleneimine. Higher dialkylamines are generally inactive (Janssen, 1959).

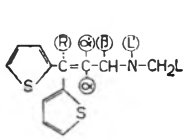
The weakly active propoxyphene-derivatives (X) are chemically closely related to IX.

Isosteric replacement in structure II of phenyl by 2-thienyl leads to the closely related general structure XI, from which the thiambutenes XII are derived. Note the close relationship between IX and XII.

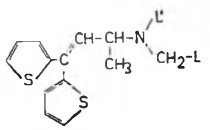


X

Propoxyphene: $NL'CH_2L = NMe_2$



XI



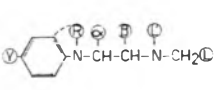
XII

Thiambutenes

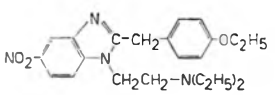
Replacement of carbon in II by nitrogen leads to the derivatives of *N*-phenyl-*N'*-methylethylenediamine of general structure XIII from which the highly potent analgesic 1-diethylaminoethyl-2-(4-ethoxy)-benzyl-5-nitrobenzimidazol (XIV) is derived. In XIV the 5-nitro group as well as the unbranched diethylaminoethyl side chain seem to be important features.

The much weaker phenampromide-like analgesics XV are also derived from structure XIII.

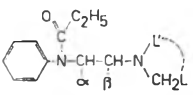
The analgesic activity of a few benzodioxanes of structure XVII which are morphine-like in some respects is evidence for the possibility of



XIII



XIV

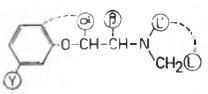


XV

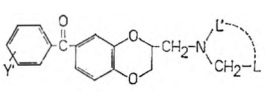
Phenampromide-like analgesics

replacing carbon in II by oxygen to obtain general structure XVI, which is also compatible with morphine-like activity.

A few compounds of structure XVIII were reported to be morphine-like. Their potency is weak and more information is required on their mechanism of action. Whatever the answer to this problem however, there is as yet no exception to the statement that potent morphine-like drugs

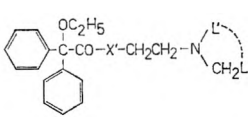


XVI



XVII

Benzodioxanes



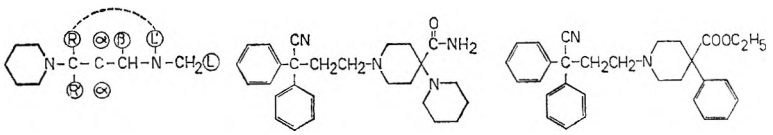
XVIII

X = O or NCH_3

proved in man have a common chemical feature, that is, general structure I in which A is phenyl or 2-thienyl, and X is carbon, nitrogen or oxygen.

In a search for new and potent analgesics, devoid of the classical disadvantages of morphine we have synthesised and screened an extensive series of tertiary amines related to general structure I, but different in at least one respect from the known compounds.

We have found that the chemical variant of I, with the general structure XIX, is compatible with high morphine-like activity. The fact that the piperidino-ring in XIX probably has a chair-conformation shows that the A-ring in I must not necessarily be a flat aromatic structure, as was previously accepted. The presence of this piperidino-ring therefore is



XIX XX XXI

Pirinitramide* (R 3365) Diphenoxylate (R 1132)

regarded as the most important novel chemical feature in the analgesically active compounds of type XIX. In this paper the pharmacology of 2,2-diphenyl-4-(4-piperidino-4-carbamoylpiperidino)butyronitrile, pirinitramide* (R 3365: XX) a typical representative of this new series, will be outlined. The closest known analogues of XX are the analgesically inactive synthetic antidiarrhoeal agents related to diphenoxylate or R 1132 (XXI). (Janssen, Jageneau and Huygens, 1959).

Experimental Methods and Results

We have compared in mice, rats, cats and dogs, the influence of a geometric series of subcutaneous or oral doses (40, 20, 10 . . . mg./kg.) of pirinitramide and of morphine on the characteristic motor activity to various types of stimuli, summarised in Table I.

In tests 1 to 4 of Table I noxious stimuli, that is, stimuli evoking a sensation of pain in man, were used to evaluate appropriate responses in mice, rats and dogs. Contact temperatures of 45° to 60° to the feet not only increase overall motor activity in mice and rats, but also produce other qualitative behavioural changes. In the hot plate test in mice the plate temperature of 55° causes nearly all control mice to lick their paws within 10 sec. after being dropped on the dry plate (Janssen and Jageneau, 1957). In similar conditions Wistar rats react similarly to a dry plate at 55°, but will immediately try to jump out of the restraining glass cylinder when dropped on a plate covered with a thin layer of water. The actual responses depend to a large extent on the plate temperature. On a wet plate at 50° a rat will show considerably increased motor activity without licking or jumping. Pinching a toe of a rat with a forceps results in typical struggle and escape reactions. Tests in which the effects of drugs on the phenomena caused by noxious stimuli are studied, are usually

* Proposed generic name.

referred to as tests for measuring "analgesic" activity. This statement however is valid only for man and not for animals. Pain is a word used for describing a universal subjective experience of mankind. Strictly speaking therefore, pain can be studied only in man. What we are able

TABLE I

SUMMARY OF TESTS FOR STUDYING THE EFFECTS OF DRUGS ON MOTOR ACTIVITY ELICITED BY A VARIETY OF STIMULI, IN MICE, RATS AND DOGS

Species	Test	Situation (unfamiliar unless otherwise noted)	Most important stimuli	Motor activity measured
Mice ..	1. Hot plate test	Dropped on hot plate	Contact heat (55°)	Reaction time of licking reflex (sec.)
Rats ..	2. Cold plate test	Dropped into glass cylinder	New environment	General motor activity (nominal ranking scale)
Rats ..	3. Hot plate test	Dropped on hot plate	Contact heat (50°)	General motor activity (nominal ranking scale)
Rats ..	4. Toe pinching test	Held in hands of observer	Pinching toe of hind paw	Struggling and escape reactions (nominal ranking scale)
Rats ..	5. Open field test	Black arena (open field)	New environment and central illumination	Ambulation, rearing, preening, emotional defecation (ratio scale)
Rats (trained)	6. Weight gain test	Food deprivation schedule	Presentation of food	Food consumption and weight gain in grams per 2 hr.
Rats, dogs (trained)	7. Jumping box test	Familiar avoidance-escape jumping box situation	Buzzer, shock or silence	Pattern of reaction times of avoidance and escape responses (sec.)
Mice (selected)	8. Rotarod test	Balance on rotating rod	Disturbance of balance	Duration of induced coordinating running (sec.)
Mice, rats	9. Righting reflex test	Supine on undulated metal surface (30°)	Release from abnormal position	Reaction time of righting reflex (sec.)
Mice (selected)	10. Fighting test in mice	Isolated aggressive male in familiar cage	Presence of another male	Fighting behaviour (all or none)
Rats ..	11. Palpebral test	Observation cage	Environmental stimuli	Tendency to close or open the eyes (nominal ranking scale)
Rats ..	12. Cornea reflex test	Held in hand of observer	Touching the cornea	Corneal reflex (all or none)
Rats ..	13. Pinna reflex test	Held in hand of observer	Touching the meatus	Pinna reflex (all or none)
Dogs ..	14. Apomorphine test	Special cage	Apomorphine-injection (0·31 mg./kg. s.c.)	Vomiting (all or none)
Rats ..	15. Apomorphine test	Special cage	Apomorphine-injection (1·25 mg./kg. i.v.)	Chewing movements (all or none)
Rats ..	16. Amphetamine test	Special cage	Amphetamine-injection (10 mg./kg. i.v.)	Chewing movements (all or none)
Rats ..	17. Tryptamine test	Special cage	Tryptamine injection (40 mg./kg. i.v.)	Bilateral clonic rythmic movements of front paws (all or none)

to study in animals is not pain itself, but the behaviour of the animal after a stimulus which provokes pain in man (referred to as a "noxious" stimulus in this paper) and also, of course, the influence of a given factor,

such as the administration of an analgesic drug on the reactions of the animal to the noxious stimulus (Janssen, 1959).

Hot Plate Test in Mice (Janssen and Jageneau, 1958)

After subcutaneous injection both pirinitramide and morphine hydrochloride significantly prolong the reaction times of mice. Pirinitramide is faster acting than morphine, while the duration of action of both drugs is similar.

	Pirinitramide	Morphine
mg./kg. s.c.	5·0	10
Onset of action (min.)	8	16
Peak effect (min.)	30	47
Duration of action (min.)	95	92

The slopes of the dose-effect curves do not significantly differ from parallelism and pirinitramide may therefore be said to be almost exactly twice as potent as morphine in this test. A similar investigation of other analgesics gave the following estimated potency ratios, where pirinitramide is 1.

Phenoperidine	= 14	Morphine hydrochloride	= 0·5
Dextromoramide	= 7	Pethidine HCl	= 0·2
Phenazocine	= 4·5	Codeine phosphate	= 0·1
Levorphanol	= 1·5	(+)-Propoxyphene HCl	= 0·07
(±)-Methadone HCl	= 1		

After the injection of ED50-doses, these drugs caused morphine-like excitement, the Straub reaction and mydriasis in nearly all mice.

Cold Plate and Hot Plate Tests in Rats

Method. For each experiment a series of 24 similar pairs of male Wistar rats were selected. The back of one rat of each pair was painted red. Random selection determined the treatment. One rat of each pair was given subcutaneously one of the three doses investigated in each experiment, the other rat being similarly and simultaneously injected with 10 ml. of water per kg. weight. There were eight pairs for each dose and successive pairs were treated at constant intervals of 2 min. The experiment was made by two trained observers, who were unaware of the contents of the solutions as well as of the identity of the treated animal of each pair. Care was taken to rule out conscious or unconscious communication between the observers during the experiment.

One hr. after injection, one of each pair of rats was taken by each of the observers and simultaneously dropped into a restraining glass cylinder onto a copper plate at room temperature and covered with a thin layer of water.

Both rats were observed for 30 sec. The pairs were then dropped in the same order into an identical glass cylinder on a "hot plate" of 50° which was also covered with water, and again observed for 30 sec. At the end of each period both observers independently stated a "preference" for one of the rats of each pair and used a nominal scale to rank the degree of confidence in selection.

This was done according to the following instructions.

Select the animal of each pair that showed less general motor activity than its partner.

Score 1 for failure to observe any difference between both rats which can be considered significant.

Score 2 for a clear difference causing no hesitation in select of the preferred animal.

Score 3 if the observed difference could be due to drug action. The significance of the results is statistically evaluated by binominal analysis, the details of which will be published elsewhere (Janssen, 1961).

Results. The results obtained with a geometric series of subcutaneous doses are graphically summarised in Fig. 1. In these experiments, agreement between the observers was excellent. The conclusion is that

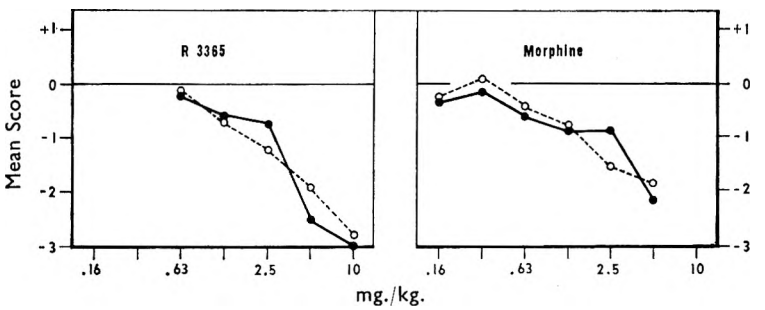


FIG. 1. Hot plate test in rats. ●—● plate at 20° (first trial). o---o plate at 50° (second trial).

pirinitramide and morphine are qualitatively indistinguishable in this test. Both compounds significantly reduce general motor activity as shown by the significantly low frequency of positive scores allocated by the observers. These effects become increasingly pronounced with increasing doses.

We were rather surprised to find that the exploratory motor activity of rats on a cold plate (20°) was inhibited by both compounds to approximately the same extent as the more pronounced motor activity of the same animals on a heated plate of 50°.

The slopes of the "mean score versus dose"—curves of pirinitramide are steeper than the corresponding slopes of morphine Fig. 1, a dose of 0·63 mg./kg. of morphine being slightly but significantly active, whereas the same dose of pirinitramide is inactive. A dose of 5 mg./kg. of pirinitramide however is more active than the same dose of morphine.

The high sensitivity of this simple test procedure is noteworthy as very few pharmacological tests are capable of demonstrating any effect after 0·63 mg./kg. of morphine s.c.

Pinching Test in Rats

Method. The design of the experiment is similar to that used for the hot plate test in rats. Two trained observers select a series of pairs of Wistar rats. They treat one rat of each pair and the other rat as control.

One hour after dosage, they take each pair from a plastic container (one observer) and pinch the toes of the hindpaws with a forceps (the other observer). Repeat as often as desirable.

Without knowing which rat received the drug, they select the animal from each pair which shows less "reactivity" (such as escape or struggling) than its partner. Scores of 1, 2 or 3 are allocated to each selected rat, using the system described for the "cold and hot plate" test. The significance of the results is assessed as before, using binomial statistics.

The "ideal" number of animals per dose may be determined by sequential analysis.

Results. The results obtained after subcutaneous or oral administration of pirinitramide or morphine are graphically summarised in Fig. 2.

After subcutaneous injection pirinitramide is about as active or possibly slightly more active than morphine. After oral administration however

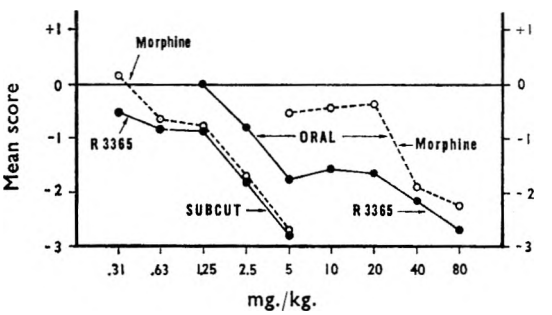


FIG. 2. Mean scores obtained 1 hr. after subcutaneous or oral doses of R 3365 and of morphine in the pinching test in rats (16 pairs per dose).

it is many times more active. A mean score of −2 (clear difference observed in all rats) is obtained after about 2·5 mg./kg. s.c. or 5 mg./kg. orally of pirinitramide and 2·5 mg./kg. s.c. or 40 mg./kg. orally of morphine hydrochloride.

Open Field Test in Rats (Janssen and Jageneau, 1960)

The method consists in assessing ambulation, rearing and emotional defaecation of Wistar rats in an unfamiliar black painted arena (diameter of 1 m.).

Results. The exploratory and emotional behaviour of Wistar rats in an open field situation, measured 1 hr. after subcutaneous injection, is modified in a similar and typical manner by both pirinitramide and morphine (Fig. 3). Motor activity may be either stimulated or depressed, depending on the dose used.

Very high doses (40 mg./kg. s.c. or more) are needed to suppress ambulation, rearing and defaecation completely. At lower doses pirinitramide and morphine cause irregular ambulation-behaviour, periods of increased running activity alternating with periods of complete rest. The mean ambulation scores however are hardly influenced. Increased rearing scores are typical for small or average doses of morphine-like

substances. After injection of 0·63 mg./kg. of pirinitramide and 1·25 mg./kg. of morphine a 100 per cent increase of rearing behaviour was observed. Emotional defaecation decreases with increasing dose levels, morphine being about twice as potent as pirinitramide in this respect.

Qualitatively similar effects are observed with all other morphine-like analgesics in this test, whereas neuroleptics related to chlorpromazine are either ineffective or decrease ambulation, rearing and defaecation, without ever stimulating motor activity.

Weight Gain Test in Rats

Method. Female Wistar rats of about 250 g. in individual metal cages are put on a 22 hr. food deprivation schedule, standard pellets being offered to them *ad libitum* between 10 and 12 a.m. or between 2 and 4 p.m.

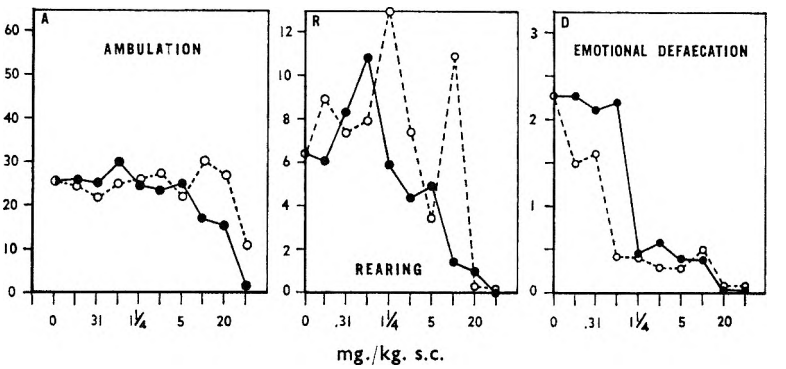


FIG. 3. Influence of R 3365 (●—●) and of morphine (o---o) on ambulation, rearing and defaecation of naive Wistar rats in an unfamiliar open field situation.
Each point represents the average for a group of 8 rats, observed for a period of exactly 3 min. about 1 hr. after dosage.
A = mean ambulation score per rat. R = mean rearing score per rat.
D = mean defaecation score per rat.

Water is available *ad libitum* all the time. Each animal is weighed before and after each eating period to determine weight gain. Food consumption (in g./2 hr.) and faecal excretion (numbers of pellets per 22 hr. after each eating period) are determined as well. After a few weeks of training on this food deprivation schedule the daily values of all three parameters reach surprisingly constant maximal levels, while weight, determined before food consumption, starts to increase gradually and slowly. At this point groups of 5 rats are formed for studying drug effects. After two control days the drug is administered to a group of adequately trained rats by the subcutaneous route, 1 hr. before the usual feeding period of 2 hr. The values are then expressed as percentages of the average values for the two preceding control days (Fig. 4). ED50 values are calculated by probit analysis.

Results. Pirinitramide is about 1·5 times more active than morphine as an inhibitor of food consumption in rats. Its dose-response curve is steeper than the morphine curve. Morphine, on the other hand,

seems to be the more constipating. The following ED50 values (mg./kg.) were calculated by probit analysis.

	Pirinitramide	Morphine
Weight gain	8·7 (6·0–13)	11 (7·1–15)
Food consumed	8·7 (6·3–12)	13 (8·7–20)
Faeces	11 (7·3–15)	11 (6·2–19)

Jumping Box Test in Dogs and in Rats

Method. The avoidance-escape behaviour method for dogs has been described previously by Niemegeers and Janssen (1960). Except for the reduced size of the apparatus, the same method was used for rats.

Results. Pirinitramide, morphine and all other morphine-like drugs we have tested are active only at very high doses in this particular test. Unlike neuroleptic agents they increase the reaction time of avoidance

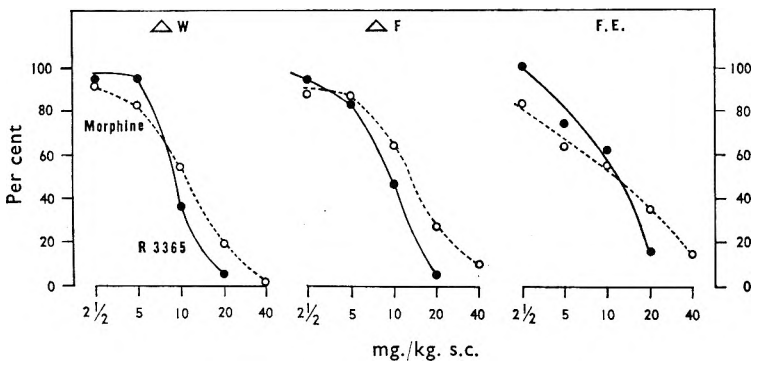


FIG. 4. Influence of R 3365 (●—●) and of morphine (o---o) on weight gain (ΔW), food consumption (ΔF) and faecal excretion (F.E.) of rats trained on a 22 hr. deprivation schedule.

responses only after injection of doses producing overt ataxia and other signs of neurological involvement.

In rats 2·5 mg./kg. s.c. of pirinitramide was inactive. The effects of 5 mg./kg. were very slight. After 10 mg./kg. s.c. some rats failed to avoid shock 1 hr. after injection, but were nearly normal again after 4 hr. Similar effects were obtained with the same doses of morphine.

In dogs 0·31 mg./kg. s.c. of pirinitramide was inactive. A subcutaneous dose of 1·25 mg./kg. had a very slight effect and 5 mg./kg. induced significantly prolonged reaction times in about half of the animals. At this high dose level however an abnormally high frequency of "paradoxical" errors were observed. Maximal effects were again observed 1 hr. after injection. Similar effects were obtained with higher doses of morphine.

Rotating Rod Test

Method. The details of the method have been described previously (Janssen, van de Westeringh, Jageneau, Demoen, Hermans, Van Daele, Schellekens, Van der Eycken and Niemegeers, 1960). Mice are selected

that can maintain equilibrium on a rotating rod (5 revolutions/min.) for at least 3 min. These animals are then injected subcutaneously with a drug under investigation and put again on the rotating rod at various time intervals after dosage.

Results. Like morphine, pirinitramide inhibited the co-ordinated activity required for maintaining equilibrium on a "rotarod" at very high doses only. The ED50 values (mg./kg. s.c., obtained by probit analysis, are pirinitramide, 24(17 — 35) (S = 1·8; fS = 1·4); morphine, 22(15 — 33) (S = 2·2; fS = 1·6). Both drugs were equally active in this test.

Righting Reflex Test in Mice and in Rats

Method. The details of these tests are described by Janssen, van de Westeringh, Jageneau, Demoen, Hermans, Van Daele, Schellekens, Van der Eycken and Niemegeers (1960).

When put on their backs on an undulated metal surface (30°) normal mice and rats show a typical righting reflex immediately after being released. Drugs are given subcutaneously and the animals are put on their backs at fixed time intervals after injection, and the time taken to resume upright posture noted.

Results. Probit analysis gave the following ED50 values (mg./kg.):

	Mice	Rats
Pirinitramide	28 (20–39)	8·9 (6·1–13)
Morphine	45 (30–68)	13 (8·5–19)

These doses are much higher than are required for inducing complete loss of reactivity to noxious stimuli in both species. This is typical for other morphine-like drugs.

Fighting Test in Mice

Method. Aggressive male mice are selected on the basis that they will savagely and immediately attack another male mouse (intruder), after a few days of isolation. (Janssen, Jageneau and Niemegeers, 1960).

Results. Probit analysis shows that pirinitramide and morphine are equiactive at doses lower than the ED50 values (mg./kg.) of the hot plate test: pirinitramide, ED50 = 3·9 (2·0 — 7·6); S = 2·9; fS = 2·2; morphines, ED50 = 4·9 (3·0 — 7·9); S = 5·9; fS = 2·2. Both drugs therefore are relatively selective anti-aggressive agents in mice.

Palpebral Test in Rats

Method. Eight pairs of adult male Wistar rats are selected. One rat of each pair chosen randomly is treated with a subcutaneous dose of drug, the other with solvent. The animals are then put in individual cages provided with a glass window for observation. One hr. after dosage two trained observers independently observe the eyes of each pair of rats without touching the animals. The observers select the rat of each pair having the greatest tendency to close the eyes, *i.e.* the animals having the smaller palpebral aperture. The same score system as described for the

cold and hot plate tests in rats, is then used for expressing the degree of confidence of the observer in his own judgment.

Results. Pirinitramide, morphine as well as many other morphine-like analgesics and CNS-stimulants such as amphetamine, produce exophthalmia in rats. Significant effects were observed in this test with 5 mg./kg. and above of pirinitramide and with 10 mg./kg. and above of morphine. Smaller doses were inactive. Relatively high doses of both drugs are therefore required to produce exophthalmia in the rat and pirinitramide is about twice as active as morphine.

Cornea and Pinna Reflex Tests

Method. Ten adult Wistar rats are subcutaneously treated with a drug and 0·25, 0·5, 1, 2, 4, and 8 hr. thereafter the corneal and pinna reflexes are elicited in the normal way. A rat failing to show a typical reflex at any time after dosage is considered to have temporarily lost that reflex. This never happens in control animals.

Results. Probit analysis shows that the ED50 doses (mg./kg. s.c.) of pirinitramide and of morphine needed to produce temporary loss of the corneal or pinna reflexes in rats are: corneal reflex: pirinitramide, 2·5 (2·1 — 3·0); morphine, 4·0 (2·9 — 5·2); pinna reflex: 4·0 (2·9 — 5·2); morphine, 4·0 (2·9 — 5·2).

Apomorphine Test in Dogs

Method. The method has been described previously (Janssen and Niemegeers, 1959; Janssen, Niemegeers and Schellekens, 1960; Niemegeers, 1960).

A subcutaneous dose of the compound under investigation is followed 30 min. later by a challenging dose of apomorphine 0·31 mg./kg. s.c. This induced emesis in all control experiments.

Results. Most morphine-like drugs produce vomiting in dogs but also block the emetic effects of apomorphine in the same species. As an apomorphine-antagonist in dogs, pirinitramide is about 2·5 times more active than morphine hydrochloride (ED50 values of 0·4 and 1·0 mg./kg. s.c. respectively).

Pirinitramide however is practically devoid of emetic properties at doses of 0·31 to 2·5 mg./kg. s.c., whereas morphine produces vomiting in most dogs at these doses.

Apomorphine-antagonism in Rats

Method. (Janssen, Niemegeers and Jageneau, 1960). Male Wistar rats are isolated in individual cages with shavings on the floor and a glass window for observation. One hr. after a subcutaneous dose of the test drug each rat receives an intravenous injection of 1·25 mg./kg. of apomorphine HCl, and 5, 10 and 20 min. thereafter the animals are observed for about 1 min. by an observer, unaware of the treatment given. The presence or absence of the typical "chewing movements" (compulsory gnawing or "Zwangsnagen") is noted.

Results. The ED50 values (mg./kg. s.c.) obtained by probit analysis were pirinitramide, 23 (16 — 31); morphine, 6·0 (2·9 — 12). Both dose-effect curves differed significantly from parallelism. As an apomorphine-antagonist in rats morphine is about four times more active than pirinitramide.

Amphetamine-antagonism in Rats

Method. Male Wistar rats are isolated in individual cages with shavings on the floor and a glass window for observation. Immediately after a subcutaneous dose of the test drug, each rat receives an intravenous dose of 10 mg./kg. of amphetamine, and 55 and 65 min. thereafter the presence or absence of the typical amphetamine-induced "chewing-movements" ("Zwangsnagen") is noted by an unbiased trained observer, unaware of the treatment given. The probability of occurrence of chewing behaviour in control rats in these conditions is over 95 per cent.

Results. Probit analysis shows that the ED50 in mg./kg. s.c. of pirinitramide was 3·3 (2·3 — 4·7) and of morphine 1·7 (0·78 — 3·7). The dose-effect curves do not significantly deviate from parallelism. The difference between the ED50's is not significant.

Tryptamine-antagonism in Rats

Method. Male Wistar rats are isolated in individual observation cages and subcutaneously treated with the test drug. One hr. thereafter each rat receives an intravenous dose of 40 mg./kg. of tryptamine hydrochloride, which immediately produces, among other effects, typical bilateral clonic convulsions of the forepaws in about 96 per cent of all control rats (Tedeschi, Tedeschi and Fellows, 1959).

Results. At high doses both pirinitramide and morphine are antagonists of the clonic convulsions. The following ED50 values (mg./kg. s.c.) were obtained by probit analysis: pirinitramide, 7·5 (5·4 — 11); morphine, 10 (6·3 — 16). The difference between both values is not significant. Both compounds however are devoid of anticonvulsant activity against leptazol or strychnine in mice.

Nalorphine-antagonism

The well-known morphine-antagonist nalorphine is also a potent antagonist of pirinitramide. An easy way to demonstrate this effect is to give mice, rats or dogs a toxic dose of pirinitramide, followed by an intravenous injection of 0·5 to 10 mg./kg. of nalorphine as soon as the peak effects are observed. Loss of righting reflex, corneal reflex and pinna reflex as well as the bradypnoea, observed after 80 mg./kg. of pirinitramide s.c. in rats, is dramatically antagonised within one min. by 1·25 mg./kg. of nalorphine i.v. Similar results are obtained in dogs treated with 2·5 or 5 mg./kg. of pirinitramide s.c., bradypnoea, ataxia, prostration and insensitivity to noxious stimuli being rapidly abolished by nalorphine.

Respiration

High doses of both pirinitramide and morphine regularly produce striking bradypnoea in mice, rats, dogs and squirrel monkeys. We have studied some of the quantitative aspects of this problem in rats.

Method. A battery of 12 triangular stainless steel cages (7·5 × 7·5 × 18 cm.) in which rats may be immobilised are fixed at the top to a metal bar. The front is made of wire gauze and the back can be closed with a plastic shutter. A special "feeling element," connected to a direct writing four-channel-electrocardiograph, may be fixed in constant contact with the lower part of the thorax of each rat through an aperture on the bottom of the cage. The respiratory movements are inscribed directly on paper (5 mm./sec.).

Results. A randomised 8 × 8 latin square design was used for studying the effects of 2·5, 5·0, 10 and 20 mg./kg. s.c. of pirinitramide and of

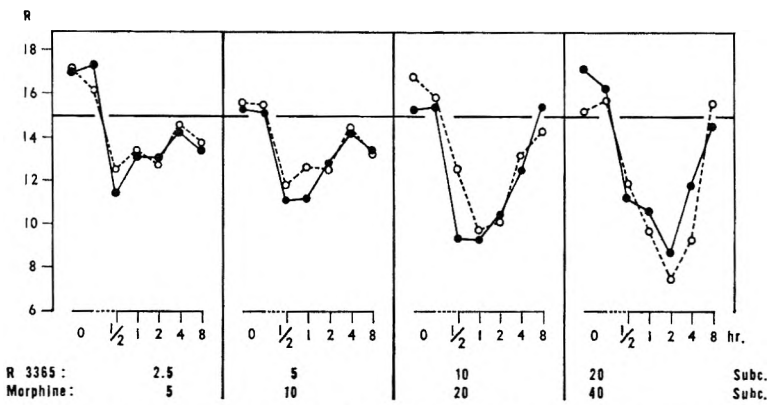


FIG. 5. Influence of R 3365 (●—●) and of morphine (o---o) in mg./kg. on respiratory rate in non-anaesthetised Wistar rats. R = respiratory rate 10^{-3} (frequency/sec./8 rats).

morphine, 5·0, 10, 20 and 40 mg./kg. s.c., in eight adult male Wistar rats. Each animal was treated once a week. After two control readings, the respiratory rate of each rat was recorded over about 30 sec. to 1 min. at ½, 1, 2, 4 and 8 hr. after each dose. There was no evidence of tolerance developing over the experimental period of eight weeks, but much individual variation occurred. As shown in Fig. 5, the time-effect curves of both drugs are similar. Maximal respiratory depression was observed some 30 min. after injection of the smallest doses and 2 hr. after injection of the largest doses. Recovery occurred with comparable speed at all dose levels.

The slopes of both curves are very flat and seem to deviate from parallelism. At the highest dose levels morphine is somewhat more active than pirinitramide as a respiratory depressant in rats, whereas the opposite order of activity is found at low doses. The differences between both drugs are small. Apnoea was not observed in any of these tests. High subtoxic dose levels of both drugs are required to produce a dangerous degree of respiratory depression in the rat.

Mydriatic Activity in Mice

Method. Using a previously described method (Janssen and Jageneau, 1957; Janssen and Jageneau, 1958) pirinitramide was found to be more active than morphine as a mydriatic agent in mice.

Results. Probit analysis gave the following ED50 in mg./kg. s.c.: pirinitramide, 11·6 (8·7–15·5); morphine, 14·9 (14·3–15·5). The ED50 values of most morphine-like analgesics in this test are not significantly different from their respective ED50 values in the hot plate test in mice (Janssen and Jageneau, 1956). For pirinitramide we found a high ratio of about 2.

Blood Pressure and Pressor Effects of Adrenaline and Noradrenaline

Intravenous doses of 0·02 to 0·63 mg./kg. of pirinitramide had no detectable influence on arterial blood pressure in rats and dogs anaesthetised with chloralose. We were unable to prove conclusively that the pressor effects of adrenaline and of noradrenaline (0·0025 mg./kg. i.v. of each) were significantly influenced in the anaesthetised rat by 0·02 to 0·63 mg./kg. of pirinitramide.

There was a suggestion of slight potentiation, particularly of noradrenaline, after administration of the highest doses. This point merits further investigation.

Influence on Adrenaline-induced Mydriasis in Rats

After intravenous injection of 0·04 mg./kg. of adrenaline in normal Wistar rats, a short lasting mydriatic effect is seen in all control rats. The peak effect (about 2 mm.) is obtained within 30 sec. and the total duration of action is about 8 min. Peak effect and duration of this adrenaline induced mydriasis is much enhanced after subcutaneous injection of 10 mg./kg. of both pirinitramide and of morphine.

Acute Toxicity

Method. Groups of 10 adult mice, Wistar rats, cats, dogs and squirrel monkeys were treated with various intravenous, subcutaneous or oral doses of pirinitramide and put in their usual cages. Mortality was recorded 72 hr. after treatment.

Results. Probit analysis gave the following LD50 values (mg./kg.): mice i.v., 34 (23–51); s.c., 280 (230–340); oral, >320; rats i.v., 13 (10–17); s.c., 160 (100–260); oral, 320 (160–640); cats s.c., >10; dogs s.c., >40; monkeys s.c., >10.

After intravenous injection the animals either die within the first hr. or survive, whereas delayed deaths occur as a rule after subcutaneous or oral dosage. Respiratory depression was the main cause of death in all species.

Subacute and Chronic Toxicity in Rats

In one experiment, 12 male and 12 female Wistar rats were selected and divided in 2 × 2 groups of 6 animals. For 15 consecutive days half the animals were given a subcutaneous injection of 1·25 mg./kg. of

pirinitramide daily, the remaining rats being similarly treated with solvent. Weight before treatment and food consumption (standard pellets) was recorded daily. After 15 days all animals were killed and subjected to haematological, pathological and histological examination. In these conditions pirinitramide had no significant effect on growth, food intake: (Fig. 6), haemoglobin content of blood, microhaematocrit values, white blood

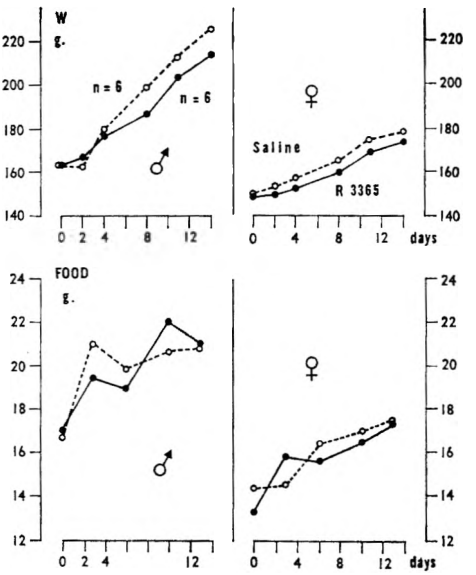


FIG. 6. 15 day subcutaneous rat toxicity of R 3365 (●—●; 1·25 mg./kg. daily) in two groups of 6 male and 6 female young Wistar rats (o—o: 2 × 6 control rats).
W: average daily body-weight per rat in g. FOOD: average daily food consumption (standard pellets) in g./rat.

cells, weight and histology of heart, liver, kidneys, adrenals, pituitary, testes and sex organs. In a second experiment three groups of 10 young male Wistar rats were daily treated with progressively increasing doses of pirinitramide, morphine hydrochloride or solvent for 7 consecutive weeks. Weight was recorded daily immediately before injection. The average weight values (g.) were as follows.

Week	Pirinitramide	Morphine	Solvent
1	1·25 mg./kg.–148 g.	2·5 mg./kg.–148 g.	150½g.
2	2·5 mg./kg.–180 g.	5 mg./kg.–180 g.	185½g.
3	5 mg./kg.–205 g.	10 mg./kg.–200 g.	215 g.
4	10 mg./kg.–226 g.	20 mg./kg.–215 g.	245 g.
5	20 mg./kg.–240 g.	40 mg./kg.–230 g.	265 g.
6	40 mg./kg.–257 g.	80 mg./kg.–232 g.	286 g.
7	80 mg./kg.–270 g.	160 mg./kg.–235 g.	306 g.

It is concluded that both pirinitramide and morphine have a significant growth depressant effect in these conditions, which is probably related to the reduction of food intake caused by these drugs.

Discussion

Pirinitramide, a novel structure lacking some of the classical chemical features of the known morphine-like compounds, has been shown to have typical morphine-like properties in a variety of pharmacological tests. Both pirinitramide and morphine inhibit the reactivity of laboratory animals to various noxious stimuli at doses that are devoid of activity in most other tests. Both drugs also produce the same typical mixture of CNS-depressant and CNS-excitatory effects: excitement is the prominent feature in mice and in cats, while motor activity is mainly depressed in rats, dogs and monkeys. Both substances produce exophthalmia in rats, mydriasis in mice and respiratory depression, loss of corneal-, pinna- and righting-reflexes in all species; at high doses both drugs antagonise some effects of amphetamine, apomorphine and tryptamine, but not of leptazol or strychnine. They are both effectively antagonised by nalorphine.

In most tests in mice, rats and dogs pirinitramide is about twice as active as morphine by the subcutaneous route. In rats it is many times more active orally. It has a quicker onset of action than morphine, but the duration of action of both compounds is about the same. As a respiratory depressant pirinitramide is not more and possibly less active than morphine. Another significant difference seems to be its low emetic activity in the dog.

The available data indicate that the capacity of pirinitramide to produce physical dependence by chronic administration in dogs or its capacity to suppress morphine abstinence signs in the same species, to be extremely low or possibly nil (J. La Barre, private communication and unpublished results from this laboratory).

Acknowledgment. I wish to thank all collaborators in the departments of pharmacology and chemistry for their valuable contribution in the various phases of this project; also Dr. H. E. Harding of Salisbury (England) for the histological reports on the animal tissues.

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PAPER CHROMATOGRAPHIC SEPARATION OF THE PHLOROGLUCINOL DERIVATIVES FROM *DRYOPTERIS* SPECIES

BY ANERI PENTTILÄ AND JACOBUS SUNDMAN

From The Research Laboratories, Medica Ltd., Helsinki, Finland

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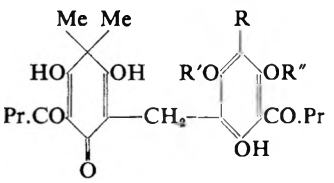
A paper chromatographic technique, using buffered filter papers, is described for separating from *Dryopteris* ferns the phloroglucinol derivatives and some of their decomposition products. The R_F values on papers buffered from pH 4·0 to pH 9·1 are given in the form of diagrams with the aid of which the appropriate buffer can be chosen depending on the mixture to be separated.

THE extracts of the dried rhizomes of different *Dryopteris* species have for long been used as anthelmintic drugs against tape-worm, especially *Diphyllobothrium latum* and *Taenia saginata*. The activity of these extracts is due to the phloroglucinol derivatives of which the following have been isolated in a pure state: aspidin (I), flavaspidic acid (II), desaspidin (III), albaspidin (IV), filixic acid (V), phloropyron (VI),* phloraspin (VII)* and aspidinol (VIII). These substances possess a



I. R = R″ = Me; R′ = H
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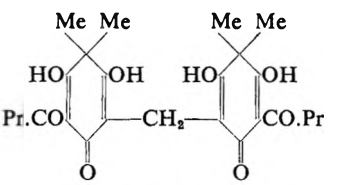
III. R = R″ = H; R′ = Me



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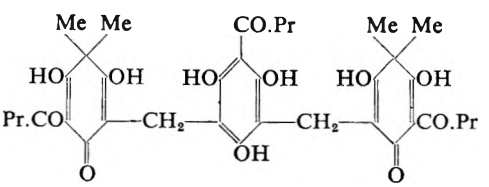

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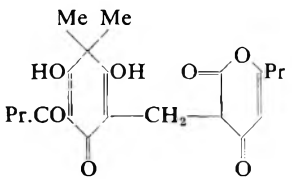
widely different anthelmintic activity as well as general toxicity. The composition of the extracts from different *Dryopteris* species varies considerably, and the same species differ according to habitat and season. The extracts also undergo changes during storage.

Because of the low therapeutic index of the extracts, serious complications have occurred in clinical use and the anthelmintic effect has

* The chemical structure of phloropyron and phloraspin has been resolved by the authors, and the data will be published in *Acta chem. scand.*, 1961.

sometimes proved unsatisfactory. A reliable analytical method is therefore required for the qualitative and quantitative determination of the phloroglucinol derivatives in *Dryopteris* ferns. Such a method would also be useful in taxonomic studies and in separating different *Dryopteris* species which are difficult to identify because of their numerous transition forms.

Chromatographic methods appeared to be the most suitable means of separating the closely related compounds contained in the extracts. Paper chromatography has been used by Klevstrand (1957) for separating some of the phloroglucinol derivatives from *Dryopteris* species. By his



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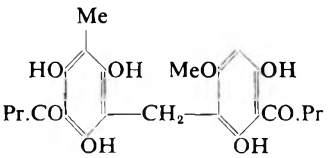
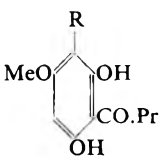


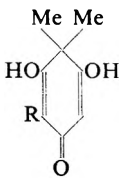
VII

technique favaspidic acid and aspidinol can be separated from albaspidin and filixic acid, but the latter two only partly from each other. Büchi, Aebi and Kapoor (1957) have developed a reversed-phase-method for the separation of the crude aspidin and crude filicin phloroglucinol substances. Godin (1958) applied paper chromatography to the detection of substances isolated from *Dryopteris filix mas*. Klevstrand (1960) has reported a method using two-solvent-systems by which aspidin and phloraspin can be separated and a more satisfactory separation of albaspidin



VIII. R = Me

IX. R = H



X. R = H

XI. R = CO.Pr

and filixic acid obtained. Hegnauer (1961) also describes a two-solvent-system to run parallel to identify the phloroglucinol derivatives from *Dryopteris* species.

We now describe a method which, using buffered filter papers and a single solvent system, has proved successful in separating all known phloroglucinol derivatives from *Dryopteris* species. In addition to the naturally occurring substances of this class, some of their common decomposition products such as filicinic acid (X), 3-butyrylfilicinic acid (XI) and desaspidinol (= phlorobutyrophenone 4-methyl ether) (IX) are included in this investigation.

Experimental Methods and Results

Materials and Solvents

Aspidin (I) m.p. 123–124°, yellow needles from ethanol. Flavaspidic acid (II) m.p. 156°, yellow crystals from benzene. Desaspidin (III) m.p. 152–154°, white crystals from ether. Albaspidin (IV) m.p. 148–150°, white crystals from methanol. Filixic acid (V) m.p. 180–181°, yellow crystals from ethyl acetate. Phloropyron (VI) m.p. 111–112°, white needles from ethanol. Aspidinol (VIII) m.p. 142–143°, yellowish prisms from benzene. Desaspidinol (IX) m.p. 127–128°, colourless needles from 50 per cent ethanol. Filicinic acid (X) m.p. 215–220° (decomp.), white crystals from ethanol. 3-Butyrylfilicinic acid (XI) m.p. 98°, white crystals from xylene.

Filixic acid is isolated from *Dryopteris filix mas* (L.) Schott, all the others from *Dryopteris austriaca* (Jacq.) Woynar with subsp. *dilatata* (Hoffm.) Sch. et Th. and subsp. *eu-spinulosa* (A. et G.) Hyl.

TABLE I

R_F VALUES OF PHLOROGLUCINOL DERIVATIVES ON PAPERS BUFFERED TO pH 5·0 AND 8·8 AND THE COLOURS OBTAINED WITH "FAST BLUE SALT B" MERCK REAGENT

Phloroglucinol compound	R_F values		Colours
	pH 5·0	pH 8·8	
Aspidin	—*	0·73	Yellow
Albaspidin	—*	0·56	Red orange
Desaspidin	—*	0·47	Purplish red
Phloropyron	—*	0·28	Yellow orange
Filixic acid	—*	0·10	Brown
Aspidinol	0·83	0·83	Purple
Desaspidinol	0·74	0·64	Purple
Flavaspidic acid	0·53	0·00	Greyish purple
Butyrylfilicinic acid	0·35	0·00	Carmine
Filicinic acid	0·00	0·00	Blue spot with red centre

* The substance moves with the solvent front.

Chloroform. DAB.6 grade chloroform is extracted with water, dried over Na_2SO_4 and distilled in subdued light. Benzene, pure. Formamide, "Merck" for chromatography. Acetone, pure.

Buffer solutions. pH 4·0–7·4 citrate-phosphate buffer (McIlvaine). pH 7·8–9·1 borate HCl buffer (Sörensen, Clark).

Buffered filter papers. Schleicher and Schüll 2043 b-papers are cut in 20 cm. strips across the machine direction. The papers are dipped in buffer solution, air dried, and stored in air-tight glass containers.

Chromatographic Procedure

The sheets of buffered papers are drawn quickly through the formamide: acetone 2:3 (v/v) solvent and the excess removed. The phloroglucinol substances 50–200 μg. in acetone or chloroform solution are immediately applied on freshly impregnated papers. With crude extracts amounts of up to 500 μg. can be used. The papers are developed by chloroform-benzene solvent 1:1 (v/v). During 2–2½ hr. at 20° the solvent front descends 25–30 cm. After drying, the spots are detected by spraying with tetrazotized di-*o*-anisidine, called "fast blue salt B", in 0·1 per cent

aqueous solution or by drawing them quickly through this solution. Immediately after, the phloroglucinol derivatives give spots of varying colours on a practically colourless background (Table I).

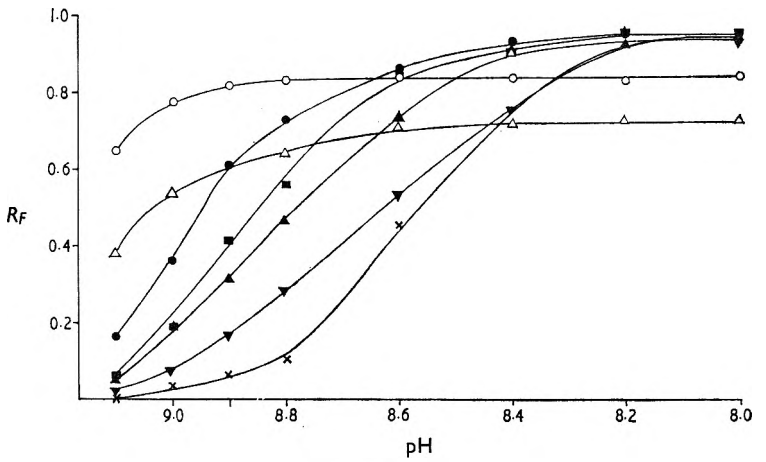


FIG. 1. Variation of R_F values with the pH of the papers.
● Aspidin. ■ Albaspidin. ▲ Desaspidin. ▼ Phloropyron.
X Filixic acid. ○ Aspidinol. △ Desaspidinol.

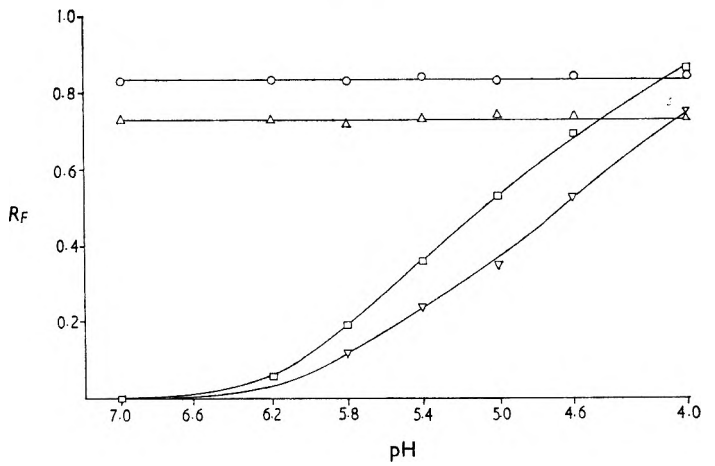


FIG. 2. Variation of the R_F values with the pH of the papers.
○ Aspidinol △ Desaspidinol □ Flavaspidic acid
▽ 3-butyrylfilicinic acid.

Aspidinol, however, first gives a nearly colourless or pale yellow spot which slowly changes to purple. For the detection of the phloroglucinol spots the "fast blue salt B" (Merck) has proved superior to all other reagents. [Ferric chloride - potassium ferricyanide (Klevstrand, 1957); Folin–Denis reagent (U.S.P. XV, 1955); ammonium vanadate - *p*-anisidine (Klinkhammer, 1958); Pauly reagent (Hais and Macek, 1958);

2,4-dinitrophenylhydrazine (Hais and Macek, 1958); vanillin - hydrochloric acid (Hais and Macek, 1958).] The many variations in colour which the different phloroglucinol derivatives give with this reagent, make it possible to identify even quite small quantities of phenolic constituents in raw materials and crude extracts, in which fatty or non-phenolic impurities dominate and may prevent a complete chromatographic separation. Also, with the "fast blue salt B" reagent the colour of the spots remains unchanged for over a year.

Figs. 1 and 2 show that by an appropriate buffering, any mixture of *Dryopteris* fern phloroglucinol derivatives can be separated satisfactorily. For most purposes papers buffered to pH 5·0 and 8·8 are adequate. The R_F values on these papers are listed in Table I. All R_F values were obtained by using 100 μg. amounts, but 2–5 μg. amounts of pure substances can easily be detected. The figures recorded are the average of several determinations giving slightly varying results, but the relative positions of the spots were always the same.

Discussion

On buffering the papers, the purity of the air should be ensured, because the wet papers easily absorb minute amounts of phenolic substances which often occur in laboratory atmosphere. This may later result in a dark coloured background or dark bands along the solvent front.

The paper buffered to pH 8·8 is recommended for the separation of all but the more acidic phloroglucinol substances—flavaspidic acid, filicinic acid and butyrylfilicinic acid. In some instances papers buffered to pH 8·6 or 8·4 give better and more distinct spots with less "tailing" than the paper at pH 8·8. Tailing has in no case badly impaired the results, and it can be avoided by using papers buffered to a lower pH than mentioned above. Aspidinol and desaspidinol always give very distinct spots, regardless of pH.

Separation on non-buffered papers resembles that on papers buffered to about pH 5·6, i.e., flavaspidic acid and butyrylfilicinic acid can be separated from aspidinol and desaspidinol, but all other substances move along the solvent front.

Preliminary attempts to extend the method to quantitative separations have shown promise.

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THE ANALGESIC ACTION OF CHLORMEZANONE

By Mary F. Lockett and U. G. Patel

From the Department of Physiology and Pharmacology, Chelsea College of Science and Technology, London, S.W.3

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Chlormezanone (2-*p*-chlorophenyltetrahydro-3-methyl-1,3-thiazin-4-one 1,1-dioxide) given to mice by intraperitoneal injection has an analgesic action of its own, and greatly augments the analgesia produced by morphine-type drugs in this species whether the latter drugs are given by the intraperitoneal or intracisternal route. The analgesic effect of intraperitoneal chlormezanone also adds with and prolongs that of paracetamol in mice, is only weakly antagonised by nalorphine and is moderately augmented by ethanol. Chlormezanone has however no analgesic action when given to mice by the intracisternal route. The implications of this last finding are briefly examined experimentally.

All the powerful analgesic drugs of the morphine, methadone and pethidine series are drugs of addiction. The weaker antipyretic-analgesic drugs such as acetylsalicylic acid and paracetamol do not suffer from this disadvantage but are ineffective in the relief of severe pain, especially that of visceral origin. Hence any drug capable of augmenting the analgesia caused by these non-addictive drugs appeared to us to merit further study. It was therefore our early observation that chlormezanone could augment the analgesic action of paracetamol in mice that led to this investigation.

Experimental

Detection and Assay of Analgesia

Male white mice, 25–30 g., were distributed at random into groups of either 8 or 10, and separate treatments were assigned to each group. Time-effect curves were constructed for each drug by each route of administration. Individual pain thresholds were recorded in μA needed to elicit a squeak when passed through the body (Lockett and Davis, 1958) before and at intervals after the administration of drugs. Intensity of analgesia was expressed individually as a percentage increase in pain threshold so defined. Assays were made by graded response using 2×2 design and measuring the effect of each drug at the predetermined time of its maximum action.

Drugs. Morphine, methadone and nalorphine were obtained from Burroughs Wellcome and Company Ltd., paracetamol and chlormezanone were gifts from Bayer Products Ltd. These drugs were administered either by intraperitoneal injection in a volume of 0·1 ml., or intracisternally in 0·02 ml. The vehicle used for the intraperitoneal route was either normal saline, in which paracetamol and chlormezanone are ground to fine suspensions, or 1 part of 70 per cent (v/v) ethanol in

9 parts of the saline. All the drugs used were soluble in the latter vehicle which was the only one used for intracisternal injections.

The Metabolism of Chlormezanone by Liver Slices

Slices, 0·7 mm. thick, cut from the livers of freshly killed animals, were weighed and incubated at 38–39° in a nutrient buffered medium containing chlormezanone. The composition of the medium was 0·9 per cent NaCl, 100 parts; 1·15 per cent KCl, 4 parts; 1·2 per cent $CaCl_2$, 3 parts; 3·8 per cent $MgCl_2$, 1 part; 0·1 M phosphate buffer, of pH 7,

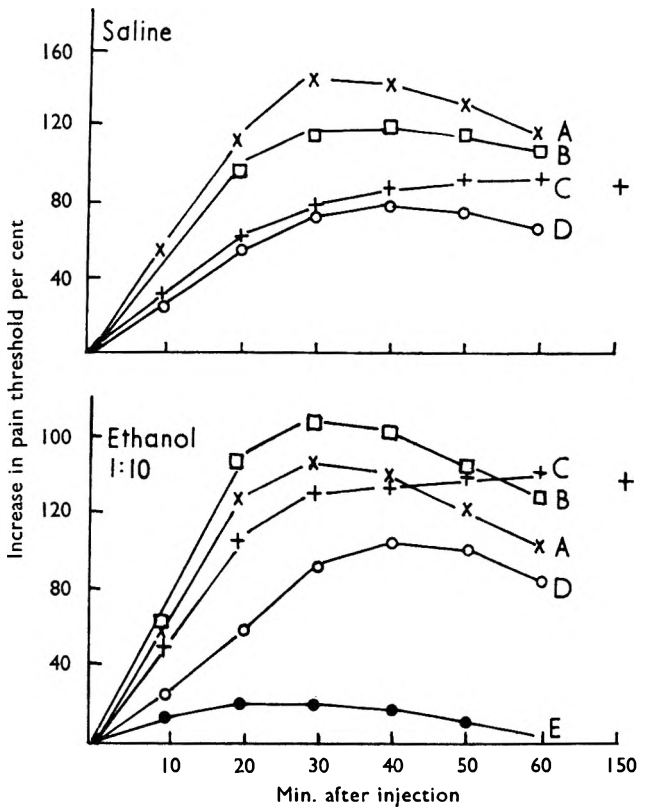

FIG. 1. Comparison of the rates of onset, intensity and duration of analgesia caused by intraperitoneal injections of morphine, pethidine, chlormezanone and paracetamol in mice, ten per group. Vehicles: 0·9 per cent NaCl, and ethanol (70 per cent) 1 in 10 in saline. Drugs: A, morphine HCl, 6 mg./kg.; B, pethidine HCl, 20 mg./kg. (upper), 25 mg./kg. (lower); C, chlormezanone, 50 mg./kg.; D, paracetamol, 300 mg./kg.

20 parts and glucose 1·2 to 2·4 mg./ml., unless otherwise stated. The proteins were coagulated after a 45 min. period of incubation by short immersion of the flasks in a boiling water bath. The flask contents were cooled and centrifuged. Supernatant fluids were examined for analgesic effect by intracisternal injection into mice.

RESULTS

Analgesia Produced by the Intraperitoneal, but not by the Intracisternal, Injection of Chlormezanone in Mice

Comparison was first made of the rate of onset, intensity and duration of analgesia in mice caused by intraperitoneal morphine and pethidine hydrochlorides, chlormezanone and paracetamol, by constructing curves relating the percentage increase in pain threshold to time. Whereas the maximum effect of morphine and pethidine had been reached in 30 min. and of paracetamol in 40–45 min., that of chlormezanone required 60 min. (Fig. 1) and declined little in 2½ hr. The addition of ethanol (70 per cent) (1 in 10) to the saline vehicle slightly increased the rate of onset of analgesia due to all four compounds. It intensified the effects of both paracetamol and chlormezanone, both these drugs being in solution

TABLE I

THE RELATIVE ANALGESIC POTENCIES OF MORPHINE, PARACETAMOL AND CHLORMEZANONE IN MICE. THE FIGURES SHOWN ARE THE MEAN POTENCIES AND STANDARD ERRORS OF THE MEANS, AND THE NUMBER OF ASSAYS WITHIN BRACKETS. THESE ARE RELATED TO PETHIDINE HYDROCHLORIDE (MG./KG. INTRAPERITONEALLY) TO WHICH THE VALUE 1·0 WAS ARBITRARILY ASSIGNED

Drugs	Analgesic potency related to pethidine	
	Drugs suspended in 0·1 ml. aqueous 0·9 per cent solution of NaCl	Drugs dissolved in 0·1 ml. of 1 part 70 per cent ethanol, 9 parts aqueous 0·9 per cent NaCl
Morphine hydrochloride	3·45 ± 0·15 (4)	3·21 ± 0·22 (3)
Paracetamol	0·03 ± 0·002 (3)	0·076 ± 0·002 (3)
Chlormezanone	0·19 ± 0·02 (3)	0·95 ± 0·03 (3)

when injected though they were precipitated in the peritoneal cavity. Next, the relative potencies of morphine and pethidine hydrochlorides, paracetamol and chlormezanone were compared in the presence and absence of this trace of ethanol in a series of 2 × 2 assays in which the maximum effect of each drug was measured and pethidine hydrochloride was the standard of reference. The limits of error ($P = 0{\cdot}95$) for the individual assays varied from ±15·2 to ±31·6 per cent. Direct estimates of mean relative potency, followed by the standard error of the mean and the number of assays are shown in Table I. Whereas the trace of ethanol in the second vehicle had itself insignificant effect on pain threshold (Fig. 1) and did not alter the relative potency of morphine and pethidine hydrochlorides, it doubled the analgesic action of paracetamol and increased that of chlormezanone by approximately five times.

In all experiements, the dose effect curves for chlormezanone have tended to be steeper than those for paracetamol, and slightly less steep than the corresponding curves for morphine and pethidine hydrochlorides. On no occasion have these differences been found to be statistically significant.

Chlormezanone failed to influence the pain thresholds of mice when injected intracisternally in doses up to 1 mg./kg. The hydrochlorides of morphine and pethidine had significant action by this route in doses of 4 μg./kg. and 20 μg./kg. respectively.

Augmentation of the Effects of Chlormezanone with Morphine, Pethidine and Paracetamol in Mice

Simultaneous study was made of the effect of a fixed dose of chlormezanone (50 mg./kg.) on the analgesia produced in mice by morphine, pethidine and paracetamol. In this experiment all drug combinations were given in a single intraperitoneal injection (0·1 ml. 0·9 per cent NaCl per mouse). The percentage increases in pain threshold were measured at 10 min. intervals and those found at 30 min. are entered in Table II as means ± their standard errors.

The analgesic actions of chlormezanone and paracetamol little more than added in their intensity (Table II). Synergism was however evident as

TABLE II

AUGMENTATION OF THE ANALGESIC EFFECTS OF MORPHINE, PETHIDINE AND PARACETAMOL IN MICE BY CHLORMEZANONE. ALL COMPOUNDS WERE GIVEN BY THE INTRAPERITONEAL ROUTE; ESTIMATES OF ANALGESIA MADE AFTER 30 MIN. VALUES SHOWN ARE THE MEANS ± STANDARD ERRORS. TEN MICE WERE USED IN EACH DOSE GROUP

Exp. No.	Percentage increases in pain thresholds				Slope of log-dose effect curve for drug I	
	Without chlormezanone		With chlormezanone (50 mg./kg.)		alone	with chlormezanone
	Pethidine hydrochloride		Pethidine hydrochloride			
	20 mg./kg.	40 mg./kg.	20 mg./kg.	40 mg./kg.		
I	55 ± 3·6	81 ± 5·2	87 ± 4·4	162 ± 8·4	87	250
II	62 ± 5·1	98 ± 14·7	108 ± 7·2	202 ± 14·7	120	313
III	64 ± 4·6	106 ± 7·8	124 ± 7·3	208 ± 11·8	140	280
	Morphine hydrochloride		Morphine hydrochloride			
	4 mg./kg.	8 mg./kg.	4 mg./kg.	8 mg./kg.		
I	26 ± 4·1	59 ± 6·3	53 ± 5·6	132 ± 9·4	110	263
II	22 ± 3·6	56 ± 3·8	65 ± 6·2	140 ± 8·7	113	250
III	28 ± 3·2	67 ± 4·4	76 ± 7·4	162 ± 8·8	130	287
	Paracetamol		Paracetamol			
	150 mg./kg.	300 mg./kg.	150 mg./kg.	300 mg./kg.		
I	42 ± 4·4	68 ± 6·4	65 ± 6·6	94 ± 7·5	83	97
II	46 ± 5·2	77 ± 8·3	87 ± 8·2	126 ± 5·8	103	130
III	40 ± 5·5	73 ± 5·8	88 ± 7·0	125 ± 6·6	110	123

Corresponding increases in pain threshold resulting from chlormezanone mg./kg. 50 Exp. I, 21 ± 43. Exp. II, 38 ± 5·1. Exp. III, 44 ± 4·8.

a prolongation of the additive effect (Fig. 2). By contrast, chlormezanone markedly increased the slopes of the log dose per cent effect curves for both morphine and pethidine (Table II), prolonged the duration of their analgesic actions and of observed sedation, but antagonised the respiratory depression which they cause. Intraperitoneal injections of chlormezanone showed similar synergistic action in respect of analgesia and sedation, and again antagonised respiratory depression, when morphine hydrochloride was injected intracisternally. However, intracisternal injection of chlormezanone, even of 1 mg./kg., failed to modify the analgesic, sedative and respiratory effects of morphine given either by the intraperitoneal or by the intracisternal route.

The Effects of Nalorphine on the Analgesic Action of Chlormezanone

The effects of nalorphine on the analgesic and synergistic effects of chlormezanone was now measured in groups of mice. One group received chlormezanone, 50 mg./kg., alone. Other animals received either morphine

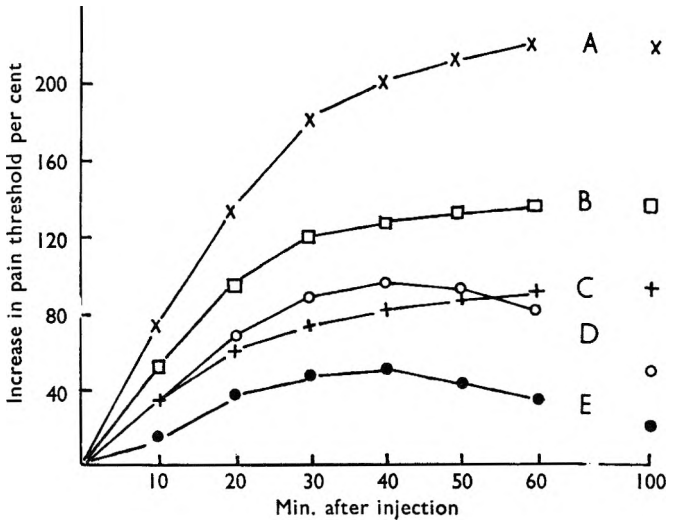

FIG. 2. The additive effects of chlormezanone and paracetamol in mice. A, chlormezanone, 50 mg./kg. and paracetamol 300 mg./kg.; B, chlormezanone, 50 mg./kg. and paracetamol 150 mg./kg.; C, chlormezanone, 50 mg./kg.; D and E, paracetamol 300 and 150 mg./kg. respectively, all given by i.p. injection at zero time.

hydrochloride, 4 mg./kg., or paracetamol, 150 mg./kg. alone, and some were given chlormezanone together with either morphine hydrochloride or paracetamol. Additional groups were treated similarly, but had nalorphine, 4 or 8 mg./kg., as well. All drugs were given in 0·1 ml. 0·9 per cent NaCl by intraperitoneal injection at individual zero times. The percentage increases in pain threshold found 40 min. later have been entered in Table III as means ± their standard errors. The doses of nalorphine used effectively antagonised the analgesic action of morphine and the synergism between morphine and chlormezanone, but caused only

TABLE III

THE ANTAGONISM OF THE ANALGESIC AND SYNERGISTIC ACTIONS OF WIN. 4692 BY NALORPHINE IN MICE. TEN MICE IN EACH DOSE GROUP

Increases per cent in pain threshold 40 min. after intraperitoneal injection						
Without nalorphine			With nalorphine			Dose of nalorphine mg./kg.
Morphine 4 mg./kg.	Chlormezanone 50 mg./kg.	Morphine 4 mg./kg. and chlormezanone 50 mg./kg.	Morphine 4 mg./kg.	Chlormezanone 50mg./kg.	Morphine 4 mg./kg. and chlormezanone 50 mg./kg.	
39 ± 4·8	34 ± 5·6	87 ± 6·2	16 ± 2·2	38 ± 4·6	41 ± 5·5	4
46 ± 5·3	37 ± 4·7	98 ± 7·1	18 ± 3·6	35 ± 3·7	42 ± 5·8	4
52 ± 6·4	37 ± 5·1	101 ± 6·6	11 ± 3·8	21 ± 5·2	33 ± 5·4	8
43 ± 4·4	36 ± 4·9	91 ± 7·8	9 ± 3·1	26 ± 4·0	28 ± 7·2	8
Paracetamol 150 mg./kg. in place of morphine						
33 ± 5·2	31 ± 5·7	71 ± 6·2	—	—	66 ± 8·4	4
38 ± 3·6	36 ± 4·8	78 ± 7·2	—	—	63 ± 9·6	4

slight to moderate reduction in the analgesic action of chlormezanone and failed to influence the combined effects of this latter drug and paracetamol significantly.

The Production from Chlormezanone by Liver Slices of a Compound or Compounds having Analgesic Action on Intracisternal Injection in Mice

The supernatant fluid obtained after incubation of slices of mouse liver in a nutrient medium containing chlormezanone, 0·1 mg./ml., produced analgesia when 0·02 ml. was injected intracisternally into mice. The

TABLE IV

THE FORMATION OF INTRACISTERNALLY ACTIVE ANALGESIC COMPOUNDS FROM CHLORMEZANONE BY THE LIVER SLICES OF VARIOUS SPECIES DURING ANAEROBIC INCUBATION. THE VALUES SHOWN ARE MEAN INCREASES PER CENT IN PAIN THRESHOLD IN THREE EXPERIMENTS CAUSED BY THE INTRACISTERNAL INJECTION OF 0·02 ML. SUPERNATANT OBTAINED AFTER INCUBATION OF 0·5 G. LIVER SLICES PER 2 ML. MEDIUM CONTAINING CHLORMEZANONE 100μG./ML.

Species	Percentage increase in pain threshold			
	pH 6·6	pH 7·0	pH 7·6	pH 8·0
Mouse	14·2 ± 2·8	36·2 ± 4·5	37·3 ± 4·0	27·2 ± 3·8
Guinea-pig	16·1 ± 2·7	22·5 ± 3·1	24·6 ± 3·2	13·8 ± 1·7
Rat	10·9 ± 2·4	24·1 ± 5·1	26·3 ± 3·9	14·8 ± 3·7
Ox	14·2 ± 4·2	23·8 ± 4·4	24·4 ± 4·7	7·4 ± 2·6
Cat	−1·1 ± 2·2	−0·6 ± 1·7	2·5 ± 3·2	4·1 ± 3·1

TABLE V

THE METABOLITES FROM CHLORMEZANONE, FORMED ANAEROBICALLY BY SLICES OF MOUSE LIVER, WHICH ARE ANALGESIC WHEN INJECTED INTRACISTERNALLY INTO MICE, ADD IN THEIR ACTION WITH MORPHINE BUT ARE ONLY PARTIALLY ANTAGONISED IN THEIR ACTION BY NALORPHINE. THE VALUES SHOWN ARE MEAN VALUES FROM THREE EXPERIMENTS ± THEIR STANDARD ERRORS

Injections made		Increases per cent in pain threshold		
		in the absence of nalorphine		in the presence of nalorphine Mg ions present
Intraperitoneal	Intracisternal	Mg ions present	Mg ions absent	
Morphine	Saline	29·6 ± 3·4	28·3 ± 4·1	6·5 ± 3·1
Morphine	Liver and chlormezanone incubated	50·6 ± 4·6	26·3 ± 5·2	20·2 ± 4·1
Morphine	Liver alone incubated	27·7 ± 5·2	25·8 ± 4·4	7·0 ± 3·6
Saline	Liver and chlormezanone incubated	21·4 ± 3·2	4·2 ± 4·7	15·9 ± 2·1
Morphine	Liver boiled then incubated	25·9 ± 6·1	31·2 ± 4·3	6·8 ± 2·3
Morphine	Liver boiled then incubated with chlormezanone	26·2 ± 4·3	27·4 ± 3·7	7·5 ± 2·8

Morphine, 8 mg./kg. nalorphine 8 mg./kg., by intraperitoneal injection throughout.

results are given in Table IV. Neither the fluid incubated with chlormezanone but without the slices, nor the slices incubated in the fluid without the chlormezanone, nor the complete reaction mixture boiled *before* incubation yielded supernatants which caused analgesia in mice by the intracisternal route. Further work showed that magnesium ions were essential for the production of this analgesic activity, and that the highest yields in the supernatant fluid were reached after 45 min. incubation under nitrogen of 0·5 g. slices in 2 ml. reaction mixture buffered with phosphate to pH 7·0 to 7·6, containing concentrations of chlormezanone within the range 80 to 100 μg./ml.

Slices from the livers of mice, rats, guinea-pigs, rabbits and oxen were all able to produce intracisternally active analgesic compounds from

chlormezanone under these conditions; those from cat livers were not. The volume of supernatant fluid injected intracisternally was always 0·02 ml. per mouse of a solution which had originally contained chlormezanone 100 μg./ml. The weight of active compound injected intracisternally is unlikely therefore to have exceeded 2 μg. per mouse and was probably less. The type of analgesia produced by the metabolite or metabolites of chlormezanone differed from that of the parent compound. Whereas intraperitoneal chlormezanone synergised the analgesic effects of morphine in mice, its metabolic products added in effect with morphine when injected intracisternally (Table V), and were only partially antagonised in their action by nalorphine.

Discussion

That analgesia is produced in mice by intraperitoneal injection but not by the intracisternal injection of chlormezanone indicates that this compound owes its analgesic activity in mice to one or more metabolites produced by tissues other than those of the central nervous system. There is also indication that at least two important metabolites are formed in mice. The first of these has an analgesic action which adds with and prolongs that of paracetamol (Fig. 2 and Table II) and is little antagonised by nalorphine (Table III). The analgesic properties of this hypothetical metabolite produced *in vivo* bear some real resemblance to those of the very active metabolite (Table V) of chlormezanone formed during its anaerobic incubation with slices of liver from many but not from all species (Table IV).

It would seem that a second metabolite is also formed *in vivo*, which potentiates the analgesic action of morphine-type drugs and antagonise the respiratory depression that they cause (Tables II and III). Overall, the evidence would seem to indicate that this second metabolite of chlormezanone is formed outside the nervous system, penetrates into the nervous system, has little or no analgesic action itself (Tables II and III), antagonises morphine's action on respiration, and potentiates its analgesic action.

Few of the many methods used for the assay of analgesia in small laboratory animals can adequately measure the effects of the less powerful analgesic drugs such as paracetamol. Indeed, the method used above, and that in which analgesic action is measured as an antagonism of the writhing movements induced in mice by the intraperitoneal injection of benzoquinone or acetic acid, seem the only methods satisfactory for this purpose. In either case the physiological mechanisms by which pain is registered are complex. Interference with this registration would appear as analgesia. We have no evidence to indicate that the increases in pain threshold we have reported were occasioned by any such interference.

Acknowledgement. This work was undertaken whilst U. G. Patel was in receipt of an educational grant for training in research which was provided by Bayer Products Ltd., who also defrayed part of the expense of this work.

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NEUROMUSCULAR BLOCKING AGENTS

PART VIII. LINEAR BIS- AND TRIS-ONIUM ETHERS

BY J. J. LEWIS,* D. E. MCPHAIL,† T. C. MUIR* AND J. B. STENLAKE†

*From *The Division of Experimental Pharmacology, Institute of Physiology, University of Glasgow, and †The Department of Pharmacy, The Royal College of Science and Technology, Glasgow*

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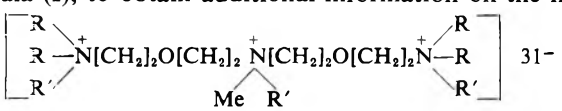
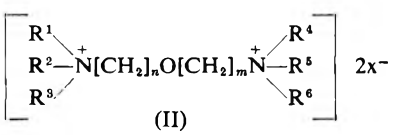
Linear bis- and tris-onium ethers (I and II) have weak tubocurarine-like properties. In both series, potency increased with increase in alkyl group size. No evidence was found for a one point receptor attachment in bis-onium ethers.

IN Part VII (Edwards, Lewis, McPhail, Muir and Stenlake, 1960), we drew attention to changes in neuromuscular blocking potency seen in some linear polyonium ethers in which one of the methylene groups was replaced by an ether oxygen. In the cat, rabbit, chick and mouse, the potency fell sharply, but in the frog it was increased. We have now synthesised and tested a further series of linear poly-onium ethers of the general formula (I), to obtain additional information on the influence of



(I)

the ether oxygen and *N*-alkyl substituents on neuromuscular block in compounds of this type. The influence of the position of the ether-oxygen atom in the chain has not been investigated. Fakstorp and Pedersen (1954, 1957, 1958), however, have studied the ganglion blocking activity of a series of bis-onium ethers of the general formula (II) in which this factor has been considered. Their studies showed that the ether function increased ganglion blocking potency, supported the concept of a one-point receptor attachment at the post synaptic membrane, and indicated that the inter-O-N distance is an important factor in determining potency. They did not, however, investigate the neuromuscular blocking properties of these compounds. We are much indebted to Dr. Pedersen for supplies of the compounds listed in Table II which we have now tested for neuromuscular blocking activity in the cat.



(II)

CHEMICAL

The quaternary compounds (I) were prepared by quaternisation with the appropriate alkyl halides of either *NN*-bis[2-(2-dimethylamino-ethoxy)ethyl]methylamine or *NN*-bis[2-(2-diethylaminoethoxy)ethyl]-methylamine. The latter bases were obtained by the method of Protiva and Pliml (1953) by condensation of di(2-hydroxyethyl)methylamine (*N*-methyldiethanolamine) (Maxwell, 1939) with 2-dimethylaminoethyl chloride and 2-diethylaminoethyl chloride respectively.

EXPERIMENTAL

Di(2-hydroxyethyl)methylamine was prepared as described by Maxwell (1939) and obtained in 81 per cent yield b.p. 130–133°/9 mm. Maxwell gives b.p. 131–133°/9 mm. It was characterised by treatment with ethyl iodide to yield *ethyldi(2-hydroxyethyl)methylammonium iodide*, m.p. 188–190° (from ethanol-ether). Found: N, 5·1; I, 46·0. $C_7H_{18}I\,NO_2$ requires N, 5·1; I, 46·1 per cent.

NN-*Di*[2-(2-*dimethylaminoethoxy*)*ethyl*]*methylamine*. Di(2-hydroxyethyl) methylamine (25·4 g.) in dry toluene (300 ml.) and sodamide (25 g. finely powdered under 50 ml. of toluene) were heated under reflux (oil bath 120–130°) with constant stirring for 2 hr. Heating was interrupted and freshly distilled 2-dimethylaminoethyl chloride (45·67 g.) in dry toluene (50 ml.) was added (20 min.) to the reaction mixture. Refluxing was then continued for a further 6 hr. When cold, the reaction mixture was decomposed by the cautious addition of water. The toluene solution was washed with water (10 ml.), dried (anhydrous K_2CO_3) and removed under reduced pressure. Fractionation of the oily residue gave a forerun of impure starting materials (6·5 g.), b.p. 106–112°/0·07 mm. and then NN-*di*[2-(2-*dimethylaminoethoxy*)*ethyl*]*methylamine* (17·15 g.; 31 per cent) as a pale yellow oil, b.p. 121–123°/0·08 mm. n_D^{23} 1·4520. Found: N, 16·15; equiv. 87·1. $C_{13}H_{31}N_3O_2$ requires N, 16·1 per cent; equiv 87·1.

NN-*Di*[2-(2-*diethylaminoethoxy*)*ethyl*]*methylamine* was prepared from di(2-hydroxyethyl)methylamine (11·9 g.) in dry toluene (150 ml.), sodamide (12 g. finely powdered under 50 ml. of toluene) and freshly distilled 2-diethylaminoethyl chloride (27·1 g.) by the method described for the preparation of *NN*-di[2-(2-dimethylaminoethoxy)ethyl]methylamine, the final reaction mixture being heated for 1 hr. only. Distillation of the crude product gave a forerun of impure starting materials (1·2 g.) b.p. 100–130°/0·05 mm. followed by NN-*di*[2-(2-*diethylaminoethoxy*)*ethyl*]-*methylamine* (9·85 g.; 31 per cent) as a pale yellow oil, b.p. 137–138°/0·06 mm., n_D^{21} 1·4540. Protiva and Pliml (1953) gave b.p. 152°/0·5 mm. Found: N, 13·1; equiv. 105·8. Calc. for $C_{17}H_{39}N_3O_2$, N, 13·2 per cent; equiv. 105·8.

NNN-*Tris-onium Compounds* were prepared by mixing either *NN*-di[2-(2-dimethylaminoethoxy)ethyl]methylamine (1 part) or *NN*-di[2-(2-diethylaminoethoxy)ethyl]methylamine (1 part) with the appropriate alkyl halide (3 parts) and dry ethanol (5 parts), and maintaining the mixture at room temperature. Reaction time, crystallisation solvent and yields are indicated for each compound in that order, in parenthesis.

6 - *Ethyl* - 6 - *methyl* - 3,9 - *dioxa* - 6 - *azoniaundecamethylenebis*(*triethylammonium*) *tri-iodide* (7 days; water-ethanol 4:1; 97 per cent) m.p. 263°. Protiva and Pliml (1953) gave m.p. 263°. Found: N, 5·2; I, 48·4. Calc. for $C_{23}H_{54}I_3N_3O_2$ requires N, 5·35, I, 48·5 per cent.

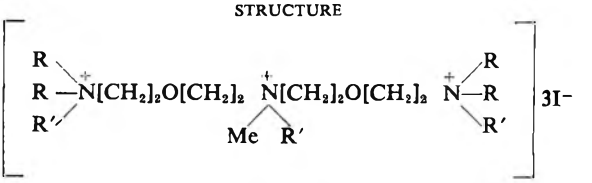
6,6 - *Dimethyl* - 3,9 - *dioxa* - 6 - *azoniaundecamethylenebis*(*trimethylammonium*) *tri-iodide* (3 days; water-ethanol 4:1; 95·0 per cent), m.p. 306–8° (decomp.). Found: N, 6·1; I, 55·1. $C_{16}H_{40}I_3N_3O_2$ requires N, 6·1; I, 55·4 per cent.

6-*Ethyl*-6-*methyl*-3,9-*dioxa*-6-*azoniaundecamethylenebis*(*ethyldimethylammonium*) *tri-iodide* (5 days; water-ethanol 1:9; 97 per cent), m.p. 278° (decomp.). Found: N, 5·7; I, 51·95. $C_{19}H_{46}I_3N_2O_2$ requires N, 5·8; I, 52·2 per cent.

6-*Methyl*-6-*propyl*-3,9-*dioxa*-6-*azoniaundecamethylenebis* (*dimethylpropylammonium*) *tri-iodide* (16 days; ethanol-acetone; 93 per cent),

TABLE I

NEUROMUSCULAR BLOCKING ACTIVITY IN A SERIES OF LINEAR TRIS-ONIUM ETHERS OF STRUCTURE



Compound	Substituents R	Substituents R'	Relative molar potencies (TC = 100) Cat	Hen	Frog	Rabbit	Type of activity
IA	Me	Me	†	•	1·0	•	TC-like
IB	Me	Et	0·4	5·0	2·0	•	TC-like
IC	Me	Pr	1·0	5·2	2·5	•	TC-like
ID	Et	Me	0·7	15·0	4·0	•	TC-like
IE	Et	Et	3·0	20·0	7·0	0·36	TC-like
IF	Et	Pr	14·5	46·0	10·5	•	TC-like

• Insufficient material. † No block at 30 mg./kg. TC = Tubocurarine.

m.p. 196–198° (decomp., after softening at 169–170°). Found, after drying for 5 hr. over P_2O_5 at 56° under high vacuum, N, 5·4; I, 49·1. $C_{22}H_{52}I_3N_3O_2$ requires N, 5·45; I, 49·4 per cent.

6,6-*Dimethyl*-3,9-*dioxa*-6-*azoniaundecamethylenebis*(*diethyl-methylammonium*) *tri-iodide* (7 days; water-ethanol, 4:1; 98 per cent), m.p. 275° (decomp.). Found: N, 5·7; I, 50·95. $C_{20}H_{48}I_3N_3O_2$ requires N, 5·65, I; 51·2 per cent.

6-*Methyl*-6-*propyl*-3,9-*dioxa*-6-*azoniaundecamethylenebis*(*diethylpropylammonium*) *tri-iodide* (70 days; propanol; 79 per cent), m.p. 194° (decomp. after softening at 174°). Found, after drying for 5 hr. over P_2O_5 at 56° under high vacuum: N, 5·0; I, 45·95. $C_{26}H_{60}I_3N_3O_2$ requires N, 5·1; I, 46·0 per cent.

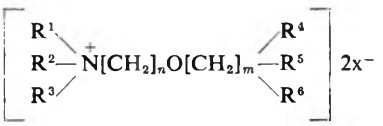
Pharmacological Methods and Results

The methods have been described elsewhere (Edwards and others, 1957, 1958, 1961) and Tables I and II show the comparative molar potencies of the compounds. All possessed muscle-relaxant properties, qualitatively similar to tubocurarine and without depolarising activity. This was true even of the methonium compound IA which caused no contracture of the hen gastrocnemius muscle at doses of 20 mg./kg. which caused an approximately 50 per cent neuromuscular block. In addition, this compound caused no contracture of the isolated frog rectus abdominis muscle. No evidence of sympathetic ganglion-blocking activity was

found in compounds IA to IF when these were tested on the nictitating membrane of the cat and when injected intravenously into the cat anaesthetised with pentobarbitone, doses sufficient to produce an approximately 50 per cent neuromuscular block, caused no significant alteration in the arterial blood pressure level. Compounds IIA to IIH are all ganglion blocking agents (Fakstorp and Pedersen, 1954, 1957, 1958).

TABLE II

NEUROMUSCULAR BLOCKING ACTIVITY IN A SERIES OF LINEAR BIS-ONIUM ETHERS OF STRUCTURE



Compound	Structure					Relative molar potencies Cat (TC = 100)	Type of activity
	$R^1R^2R^3$	n	m	$R^4R^5R^6$	X^-		
IIA (F. & P. 16575) ..	Me_3	2	3	Me_3	I	0·08	TC-like
IIB (F. & P. 16677) ..	$MeEt_2$	2	3	Me_3	I	0·16	TC-like
IIC (F. & P. 8302) ..	$MeEt_2$	2	3	$MeEt_2$	I	0·13	TC-like
IID (F. & P. 16678) ..	Et_3	2	3	Me_2Et	Br	0·30	TC-like
IIE (F. & P. 8303) ..	Et_3	2	3	Et_3	Br	0·45	TC-like
IIF (F. & P. 16701) ..	Me_3	3	3	Me_3	I	No block at 25 mg./kg.	—
IIG (F. & P. 17843) ..	Me_2Et	3	3	Et_3	Br	0·17	TC-like
IIH (F. & P. 8212) ..	Et_3	3	3	Et_3	Br	2·0	TC-like
IIJ	Et_2Pr	3	3	Et_2Pr	I	1·86*	?
Hexamethonium	Me_3			Me_3	I	0·44†	TC-like

* Calculated from the figures of Pradhan and his colleagues (1954).
† Calculated from the figures of Paton and Zaimis (1949).

DISCUSSION

The results confirm those of our earlier work which showed that linear poly-onium ethers of general formula (I) have weak tubocurarine-like neuromuscular blocking activity. In particular, they also confirm that compound IE, which was tested by Vaněček and Protiva (1955) on the rat masseter muscle and phrenic-nerve diaphragm preparation, has only a low potency, although it was one of the more potent of the compounds examined. Our results show this compound to be less potent in the cat than the analogous compound XIIIA $\{[(Et_3\overset{+}{N}[CH_2]_2O[CH_2]_2)_2\overset{+}{N}\ Et_2]3I^-\}$ described in Part VII (Edwards, Lewis and others, 1960), and that it is significantly less potent than the latter in the rabbit. These two substances differ only in the alkyl substituents on the central nitrogen atom, and the observed decline in potency on replacement of an ethyl by a methyl substituent is in agreement with our previous observation on related compounds.

The neuromuscular blocking potency of Fakstorp and Pedersen's compounds is also extremely low and again demonstrates, when IIA is

considered in comparison with hexamethonium, the sharp reduction in potency which is apparently due to replacement of a methylene group by an ether oxygen. As with most of the other groups of linear poly-onium compounds examined, the methonium compounds are weaker than the corresponding ethonium analogues with potency increasing roughly in parallel with the bulk of the onium group. The position of the ether-oxygen atom in the chain appears not to influence neuromuscular block significantly. Thus, although the range of compounds is not fully representative of the series, the observed potencies do not show the marked structural relations which are a feature of the activity of these same compounds as ganglion-blocking agents. This in itself is perhaps not surprising, but it none the less clearly demonstrates that the theory of a one-point receptor attachment at the ganglion postulated by Fakstorp and Pedersen for these and similar compounds does not hold for neuromuscular blockade.

The lowering of potency which is observed with the substitution of an ether-linked oxygen atom in the polymethylene chain of bis- and poly-onium compounds is also in marked contrast to the influence of aromatic ether links on the potency of the tubocurarine and isoquinoline derivatives discussed in Part VII. The potentiating effects of aromatic ether links in these and similar compounds suggests that the real significance of the ether functions may lie in their ability to donate electrons into the aromatic rings. The redistribution of these electrons over the ring would yield a larger charge-bearing structure potentially capable of receptor interaction and hence of reinforcing van der Waal's bonding in a way which is not possible in aliphatic ether derivatives. The reason for the fall in potency when ether links are substituted into aliphatic onium compounds is still not clear, and is being examined further.

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PHARMACOLOGICAL AND BIOCHEMICAL EFFECTS OF SOME RESERPINE DERIVATIVES

By S. Garattini, L. Lamesta, A. Mortari and L. Valzelli

From the Department of Pharmacology, Medical School, University of Milan, Italy

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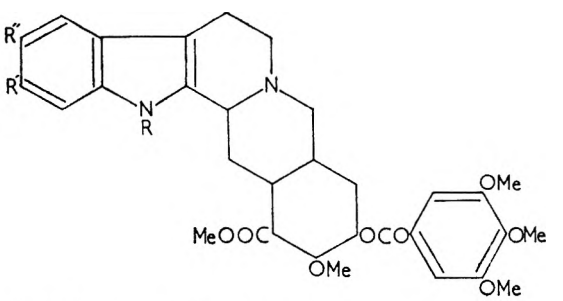
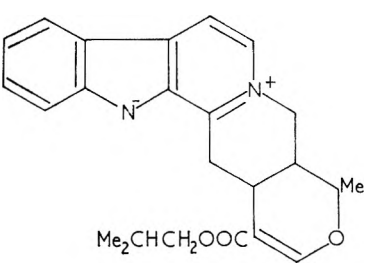
A relation exists between the hypotensive properties of three reserpine derivatives (β-diethylaminoethyl reserpine, isobutylserpentinate, 10-methoxydeserpidine) and their ability to release cardiac catecholamines. These derivatives do not show sedative properties and do not release 5-hydroxytryptamine or catecholamines from the brain.

At the same time as the discovery of carbethoxy-siringoylmethyl reserpate, a reserpine derivative (Plummer, Barrett, Maxwell, Finocchio, Lucas and Earl, 1955) with specific hypotensive effect (Darvill, 1958; Garattini, Mortari, Valsecchi, Valzelli, 1959; Hughes Orlans, Finger and Brodie, 1960; Plummer and others, 1959) was reported, some other compounds,



I. Reserpine, R = H; R′ = OMe; R″ = H.
II. 10-Methoxydeserpidine, R = H; R′ = H; R″ = OMe.
III. DL152, R = $CH_2CH_2N(Et)_2$; R′ = OMe; R″ = H.



IV. Isobutylserpentinate (Ph.458).

with similar properties, were described. Among them the most promising appeared to be β-diethylaminoethyl reserpine (DL 152) (Buzas and Régnier, 1960); isobutyl serpentinate (IBS) (van Proosdij Hartsema, Akkerman and de Jongh, 1959) and 10-methoxydeserpidine (10 MD) (Merlene and Gérard, 1960; Peterfalvi and Jequier, 1960; Velluz, Peterfalvi and Jequier, 1958) (see formulae) since their effect on blood pressure is not

accompanied by depressant activity in the central nervous system. The aim of this investigation was to compare the effect of reserpine and these three new compounds in a number of pharmacological tests and on the 5-hydroxytryptamine and catecholamine content of various organs. It was thought that these experiments might contribute to the elucidation of the mechanism of action of reserpine, particularly to the relation between its pharmacological effects and the release of biogenic amines.

Material and Methods

Sprague-Dawley rats fed a balanced diet have been used throughout. Compounds were dissolved in a mixture of benzyl alcohol (2 g.), propyleneglycol "300" (10 g.), citric acid (0·25 g.) and distilled water up to 100 ml. and injected intravenously or intraperitoneally. Control rats received only the solvent.

For blood pressure experiments, animals were anaesthetised with ethylcarbamate (1 g./kg., s.c.). The carotid artery was isolated and cannulated for recording pressure by means of an electromanometer connected to a No. 150 Sanborn recorder. Iproniazid (100 mg./kg.) was given intraperitoneally 6 hr. before the intravenous injection of reserpine or its derivatives. Tyramine and noradrenaline were injected intravenously at the concentrations shown in Table I.

5-Hydroxytryptamine (5-HT) in extracts of the spleen and intestine was assayed by the method of Bogdanski, Pletscher, Brodie and Udenfriend (1956); noradrenaline assayed in extracts of heart by the method of Shore and Olin (1958), and 5-HT and noradrenaline assayed in extracts of brain by the method of Shore (1959) using a spectrofluorimetric technique.

Isobutylserpentinate was obtained from Prof. D. K. de Jongh (Pharmaco-Therapeutisch Instituut Nedchem, Amsterdam), β-diethylaminoethylreserpine (DL 152) from Dr. A. Buzas (Société Dautreville et Lebas, Paris), 10-methoxydeserpidine from Dr. P. Poirier (Roussel-Uclaf, Paris) and reserpine (Serpasil) from Dr. V. Ghetti (Ciba, S.p.A., Milano).

Results

Reserpine Derivatives on Blood Pressure

Table I summarises the effects of reserpine and its derivatives on the blood pressure of anaesthetised rats. Reserpine is more hypotensive than the other compounds; for example, 4 hr. after intraperitoneal administration, 1·25 mg./kg. of reserpine produces a fall in blood pressure which is comparable with that produced by 2·5 mg./kg. of DL 152 or 5 mg./kg. of IBS or 10 MD.

The doses producing hypotensive effects also prevent the tyramine hypertension. Furthermore, reserpine and its derivatives slightly increase the pressor effect of noradrenaline. In animals pretreated with a monoamine oxidase inhibitor (for example, iproniazid), reserpine elicits a hypertension which may be related to a release of peripheral catecholamines (Garrattini, Fresia, Mortari and Palma, 1960). Unlike reserpine, IBS does not induce hypertension in iproniazid-treated rats, while DL 152 and 10 DM are only slightly active. The increased effect of tyramine in

TABLE I

EFFECT OF RESERPINE AND THREE OF ITS DERIVATIVES ON BLOOD PRESSURE OF RATS IN VARIOUS EXPERIMENTAL CONDITIONS

Compound	Dose (mg./kg.)	Blood pressure after 4 hr.		Pressor effect of compounds in iproniazid-treated rats	Pressor effect of tyramine injected 4 hr. after the compounds				Pressor effect of noradrenaline (10 μg./kg.) injected 4 hr. after the compounds
					Intact rats			Iproniazid-treated rats	
		Max.	Min.		250 μg./kg.	500 μg./kg.	1000 μg./kg.	250 μg./kg.	
Solvent ..	--	155 ± 15	90 ± 13 (10)	0 (10)	+20 ± 5 (10)	+40 (5)	+70 (5)	+70 ± 12 (10)	+50 ± 4 (10)
Reserpine ..	0·63	—	—		±20 ± 5 (5)				
	1·25	120 ± 2	90 ± 9 (5)		0 (5)				
	2·5	95 ± 2	55 ± 6 (10)	+60 ± 15 (10)	+5 ± 4 (10)	0 (5)	0 (5)	+45 ± 14 (10)	+70 ± 6 (5)
IBS ..	1·25	145 ± 19	108 ± 10 (5)		+18 ± 3 (5)				
	2·5	150 ± 9	105 ± 9 (10)	0 (5)					
	5	135 ± 7	87 ± 9 (10)	0 (5)				+60 ± 5 (5)	
	10	127 ± 4	90 ± 9 (10)	0 (10)	*(10)	+30 (5)	+60 (5)	+70 ± 8 (5)	+60 ± 6 (5)
DL 152 ..	1·25	145 ± 9	103 ± 7 (5)		+20 ± 3 (5)				
	2·5	115 ± 7	90 ± 6 (10)	+18 ± 3 (5)					
	5	100 ± 5	60 ± 5 (10)	+17 ± 3 (5)	+2 ± 2 (5)			+65 ± 5 (5)	
	10	100 ± 3	58 ± 4 (10)	+17 ± 2 (5)	+1 ± 2 (5)	+25 (5)	+60 (5)	+70 ± 8 (5)	+75 ± 9 (5)
10 MD ..	1·25	155 ± 12	102 ± 11 (5)		+15 ± 5 (5)				
	2·5	135 ± 11	90 ± 10 (10)	+15 ± 5 (5)	+5 ± 3 (5)				
	5	130 ± 11	86 ± 10 (10)	+20 ± 4 (5)	0 (5)			+60 ± 5 (5)	
	10	115 ± 10	75 ± 9 (10)	+18 ± 4 (5)	0 (5)			+60 ± 5 (5)	+65 ± 8 (5)

* In 4 rats pressor effect was antagonised; in 6 rats pressor effect was comparable to controls.

iproniazid-treated animals is partially prevented by reserpine, but not by the other derivatives.

Other Pharmacological Effects of Reserpine Derivatives

Table II shows the effect of reserpine and its analogues on body temperature, sedation, ptosis, barbiturate potentiation and intestinal function. Except for reserpine, the other compounds in doses up to 10 mg./kg. are almost inactive in these tests.

Reserpine Derivatives and Tissue Amines

Table III summarises biochemical findings concerning the effect of reserpine and its derivatives on tissue amines. Both intestine and spleen 5-HT are decreased by reserpine. However, only spleen 5-HT is decreased by a high concentration (20 mg./kg.) of DL 152 or IBS. The values for intestine and spleen after 10 MD treatment are higher than the control values, due possibly to the fact that 10 MD interferes with the fluorescence

TABLE II

PHARMACOLOGICAL ACTIONS OF RESERPINE AND THREE OF ITS DERIVATIVES IN RATS. EACH VALUE REPRESENTS THE MEAN OF AT LEAST 6 DETERMINATIONS

Compound	Intra-peritoneal dose (mg./kg.)	Body temperature (°C ± S.E.)		Sedation	Ptosis	Diarrhoea	Sleeping time pentobarbitone (20 mg/kg) i.p., given 4 hr. after the compounds
		Controls	After 4 hr.				
Solvent	—	36·9 ± 0·2	36·8 ± 0·2	−	−	−	46 ± 10
Reserpine	1·25	37·1 ± 0·2	35·3 ± 0·2	±	±	±	69 ± 9
	2·5	37·1 ± 0·2	34·0 ± 0·2	+	+	+	173 ± 8
IBS ..	10	37·0 ± 0·2	36·1 ± 0·4	−	−	−	53 ± 6
DL 152 ..	10	37·1 ± 0·1	36·6 ± 0·2	−	−	−	79 ± 4
10 MD ..	10	36·9 ± 0·1	36·7 ± 0·2	−	−	−	71 ± 5

of the 5-HT. Brain 5-HT and noradrenaline are not released by these three reserpine derivatives even when analysis is made 4 or 12 hr. after intraperitoneal doses of 10 and 20 mg./kg.

DL 152, 10 DM and IBS, in decreasing order, deplete heart noradrenaline, but this depletion is less marked than that obtained with reserpine.

DISCUSSION

It is interesting to compare the pharmacological effects exerted by the three reserpine derivatives with their action in releasing tissue amines. DL 152, IBS and 10 MD, which are devoid of sedative effects, do not decrease brain 5-HT and noradrenaline even in doses of 10 mg./kg., while reserpine is active at a dose 8 times lower. On the other hand, the three derivatives, like reserpine itself, possess a hypotensive action. Thus, the hypotensive action may occur without the release of brain amines.

The three reserpine derivatives also fail to decrease the peripheral stores of 5-HT and this finding may be linked with the fact that 10 MD, unlike reserpine, does not increase the urinary excretion of 5-hydroxyindoleacetic acid (Burn and Rand, 1958).

A parallelism exists between the hypotensive properties of these compounds and their activity to decrease cardiac catecholamines. Reserpine

TABLE III

EFFECT OF RESERPINE AND THREE OF ITS DERIVATIVES ON TISSUE 5-HT AND NORADRENALINE IN RATS

Compound	Intra-peritoneal dose (mg./kg.)	Time after administration (hr.)	5-HT μg./g. ± S.E.			Noradrenaline μg./g. ± S.E.	
			Intestine	Spleen	Brain	Brain	Heart
Solvent	—	4	2·67 ± 0·52 (8)	2·34 ± 0·26 (8)	0·35 ± 0·06 (16)	0·24 ± 0·02 (8)	0·57 ± 0·07 (16)
Reserpine	1·25	4			0·27 ± 0·02 (4)	0·05 ± 0·01 (12)	0·17 ± 0·01 (8)
	2·5	4	2·95 ± 0·24 (8)	1·09 ± 0·24 (8)	0·17 ± 0·05 (12)	0·03 ± 0·01 (12)	0·12 ± 0·01 (8)
	2·5	12	2·16 ± 0·15 (4)	1·04 ± 0·20 (4)	0·21 ± 0·02 (4)	0·07 ± 0·01 (4)	0·12 ± 0·01 (8)
	5·0	4	2·16 ± 0·25 (4)	0·60 ± 0·06 (4)	0·10 ± 0·01 (4)		
IBS	2·5	4		2·15 ± 0·16 (4)	0·32 ± 0·01 (4)	0·24 ± 0·01 (4)	
	10	4	2·84 ± 0·26 (4)	2·88 ± 0·56 (4)	0·30 ± 0·04 (4)	0·24 ± 0·01 (4)	0·50 ± 0·02 (8)
	10	12			0·38 ± 0·01 (4)	0·25 ± 0·03 (4)	
	20	4	3·50 ± 0·28 (4)	1·92 ± 0·21 (8)	0·32 ± 0·02 (4)	0·22 ± 0·02 (4)	0·62 ± 0·08 (4)
	20	12					0·37 ± 0·09 (4)
DL 152	2·5	4		2·47 ± 0·21 (4)	0·30 ± 0·01 (4)	0·24 ± 0·01 (4)	
	10	4	2·16 ± 0·16 (4)	1·98 ± 0·19 (4)	0·26 ± 0·02 (4)	0·25 ± 0·01 (4)	0·32 ± 0·01 (12)
	10	12			0·38 ± 0·08 (4)	0·29 ± 0·01 (4)	0·21 ± 0·01 (4)
	20	4	2·53 ± 0·26 (8)	1·25 ± 0·06 (8)	0·30 ± 0·02 (4)	0·21 ± 0·01 (4)	0·20 ± 0·01 (4)
	20	12					0·18 ± 0·01 (4)
10 MD	5	4	3·58 ± 0·56 (4)	3·20 ± 0·70 (4)	0·32 ± 0·04 (4)	0·25 ± 0·03 (4)	0·35 ± 0·04 (4)
	10	4			0·35 ± 0·06 (4)	0·23 ± 0·02 (4)	0·35 ± 0·01 (4)
	10	12					0·22 ± 0·01 (4)

Number of determinations shown in brackets.

is, from both parameters, the most active compound, followed in order by DL 152, 10 MD and IBS. Unlike reserpine, DL 152 and 10 MD elicit only weak pressor responses in rats in which monoamine oxidase is inhibited. This result may be explained by the fact that these compounds release only some of the noradrenaline and that they are probably slow acting. In fact, IBS does not release noradrenaline from the heart and is inactive as a pressor agent in iproniazid-treated animals. The inhibition of the pressor effect of tyramine is common to all the compounds although reserpine is much more active than its derivatives. According to the current views (LaBarre and Hans, 1960), tyramine induces pressor effects through a release of noradrenaline and for this reason tyramine is inactive when reserpine has depleted the noradrenaline stores. However, reduction of the tyramine pressor response may not always be related to a depletion of the noradrenaline stores.

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THE ANTIBACTERIAL ACTIVITY OF NEW DERIVATIVES OF 4-AMINOQUINOLINE AND 4-AMINOQUINALDINE

By D. Caldwell, W. A. Cox, P. F. D'Arcy and L. R. Rowe

From the Research Division, Allen & Hanburys Limited, Ware, Hertfordshire

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Two new series of *N*-alkyl derivatives of 4-aminoquinoline and 4-aminoquinaldine are described. Compounds in both series show antibacterial and protein precipitating properties and it has been possible to correlate bactericidal action and protein precipitation for homologous members of the series. The bacteriostatic activity of both series of compounds is characterised by maxima at C = 12 to 14, with a marked decrease in potency at longer chain lengths. The bactericidal activity differs from this in that activity is not greatly diminished with the higher homologues. The 4-aminoquinaldinium salts are slightly more potent bactericidal agents than the corresponding 4-aminoquinolinium compounds. It is concluded that the mechanism of the antibacterial action of these derivatives may be closely related to the means by which they interact with proteins.

The antimicrobial activity of certain polymethylene heterocyclic bis-quaternary ammonium salts has been described on a number of occasions (Collier, Potter and Taylor, 1953, 1955; Babbs, Collier, Austin, Potter and Taylor, 1956; Austin, Potter and Taylor, 1958; Austin, Lunts, Potter and Taylor, 1959). These compounds possess marked antifungal and antibacterial activity; the former reaching its highest point in the polymethylene bis-isoquinolinium series at a chain length of sixteen methylene groups (hedaquinium, Teoquil), and the latter its maximum activity in the polymethylene bis-4-aminoquinaldinium series at a chain length of ten methylene groups (dequalinium, Dequadin).

Continued interest in this field has led to the present investigation of the allied 1-alkyl derivatives of 4-aminoquinoline and 4-aminoquinaldine. A preliminary note on the activity of the latter series has already appeared (Cox and D'Arcy, 1959). The formation of insoluble complexes between quaternary ammonium compounds and proteins has been long known and the relationship between this property and antibacterial activity has been sought. For example Kuhn and Bielig (1940), using benzyl dimethyl dodecyl ammonium bromide and various proteins, demonstrated that the concentrations of quaternary salt required to precipitate proteins and to kill bacteria were about the same. Larose and Fischer (1952) using wool as the test protein showed that a similar relation existed between the bactericidal activity of some antiseptics and their degree of adsorption to wool.

Since, during our present investigation, it became clear that a rapid screening test would be useful to eliminate those compounds likely to have little or no antibacterial activity, before proceeding with more rigorous bacteriological testing, we have investigated a test based on the ability of certain quaternary compounds to precipitate proteins from very dilute solution. Using this test an attempt has been made to correlate protein

precipitating activity and the antibacterial activity of the 4-aminoquinolinium and the 4-aminoquinaldinium series.

EXPERIMENTAL

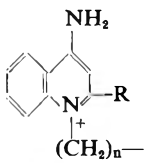
Materials and Methods

Chemical syntheses. 2-Methylcinchoninamide was prepared by a modification of a published method (German Patent 1913) and was converted into 4-aminoquinaldine by the Hofmann reaction using sodium hypochlorite; 4-aminoquinoline was prepared by the method of Royer (1949); all the alkyl halides were obtained using standard procedures.

The quaternary ammonium iodides were prepared by the reaction of a slight excess of the alkyl iodide with the heterocyclic amine in ethyl methyl ketone at 80° for from 6 to 90 hr. After recrystallisation and analysis the

TABLE I

ANALYTICAL RESULTS: 1-ALKYL 4-AMINOQUINOLINIUM AND 4-AMINOQUINALDINIUM IODIDES



R	n	Reaction time hr.	m.p. uncorrected	Crystn solvent*	Found per cent C	H	I	N	Formula	Required per cent C	H	I	N
H	9	6	139·5	E.M.K.	55·3	7·2	31·15	7·0	$C_{19}H_{29}IN_2$	55·3	7·1	30·8	6·8
,,	10	7	143	,,	56·2	7·3	30·2	6·9	$C_{20}H_{31}IN_2$	56·3	7·3	29·8	6·6
,,	11	6	136	,,	57·0	7·6	28·8	6·1	$C_{21}H_{33}IN_2$	57·3	7·6	28·8	6·4
,,	13	6	145·5	,,	58·7	8·1	27·5	6·0	$C_{23}H_{37}IN_2$	59·0	8·0	27·1	6·0
,,	15	16	157·5	,,	60·6	8·25	25·6	5·6	$C_{25}H_{41}IN_2$	60·5	8·3	25·6	5·6
,,	17	16	180·5	,,	61·9	8·6	24·05	5·4	$C_{27}H_{45}IN_2$	61·8	8·6	24·2	5·3
Me	6	68	228	EtOH	53·0	6·4	33·3	7·3	$C_{17}H_{25}IN_2$	53·2	6·6	33·0	7·3
,,	9	80	197	I.P.A.	56·1	7·4	29·7	6·8	$C_{20}H_{33}IN_2$	56·3	7·3	29·75	6·6
,,	10	90	192	E.M.K.	57·4	7·6	28·5	6·6	$C_{21}H_{33}IN_2$	57·25	7·55	28·8	6·4
,,	11	50	182	,,	58·5	7·7	27·6	6·4	$C_{22}H_{35}IN_2$	58·2	7·7	28·0	6·2
,,	13	80	180	,,	59·8	7·9	26·4	5·8	$C_{24}H_{39}IN_2$	59·8	8·1	26·3	5·8
,,	15	80	158	I.P.A./H_2O	61·4	8·35	24·8	5·5	$C_{26}H_{43}IN_2$	61·2	8·5	24·85	5·5
,,	17	100	165	E.M.K.	62·5	8·9	23·25	5·1	$C_{28}H_{47}IN_2$	62·4	8·8	23·6	5·2

*E.M.K. = ethylmethyl ketone, I.P.A. = isopropanol.

iodides were converted by the conventional silver salt method to the corresponding acetates. The latter were more soluble in water than the iodides and were therefore more convenient for the antibacterial testing.

Protein precipitation test. The proteins used for this test were pepsin B.P., casein (soluble and reprecipitated), gelatin (275-Duche), and egg albumin (Judex).

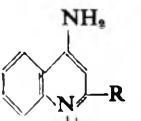
A solution of protein of concentration 0·125 per cent was prepared in 0·05 M sodium acetate and adjusted to pH 8·5 with sodium hydroxide. To 10 ml. portions of the solution specific amounts of a 0·125 per cent solution of a quaternary ammonium salt were added, and the final volume in each tube made up to 20 ml. with distilled water. This gave a protein final concentration of 62·5 mg./100 ml. The tubes were examined in a strong light, and after 1 hr. the weight of the quaternary salt required to cause turbidity was calculated.

Test for bactericidal activity. A bacterial cell suspension was prepared by washing the 20 hr. growth from an agar surface, centrifuging the bacterial cells (R.C.F. 600*g*/15 min.) and suspending them in sterile distilled water to a standard opacity (Wellcome opacity tube No. 7). The quaternary salts under examination were made up to a specified concentration in distilled water with a final volume of 9 ml. A control tube containing 9 ml. of sterile distilled water alone was included in each test.

At zero time, 1 ml. of culture suspension was added with shaking to each quaternary solution and also to the control tube. After time intervals of 2·5 and 15 min. 1 ml. samples of culture-quaternary mixture

TABLE II

ANALYTICAL RESULTS: 1-ALKYL 4-AMINOQUINOLINIUM AND 4-AMINOQUINALDINIUM ACETATES



R	n	Reaction time hr.	m.p. uncorrected	Crystn* solvent	Found per cent C	H	N	Formula	Required per cent C	H	N
H	9	18	120	I.P.A./I.P.AcO.	71·4	9·2	8·0	$C_{21}H_{32}N_2O_2\frac{1}{2}H_2O$	71·4	9·4	8·0
,,	10	18	125	,, ,,	73·7	9·8	7·9	$C_{22}H_{34}N_2O_2$	73·7	9·6	7·8
,,	11	18	122	,, ,,	74·0	9·8	7·8	$C_{23}H_{36}N_2O_2$	74·2	9·7	7·5
,,	13	18	128	,, ,,	75·3	9·8	7·2	$C_{25}H_{40}N_2O_2$	75·0	10·1	7·0
,,	15	17	133·5	,, ,,	75·3	10·45	6·7	$C_{27}H_{44}N_2O_2$	75·7	10·35	6·5
,,	17	17	130·5	,, ,,	76·5	10·6	6·2	$C_{29}H_{48}N_2O_2$	76·3	10·6	6·1
Me	6	16	207	,, ,,	70·4	8·7	8·4	$C_{19}H_{30}N_2O_2\frac{1}{2}H_2O$	70·15	9·0	8·6
,,	9	6	182	,, ,,	70·2	9·6	7·5	$C_{22}H_{34}N_2O_2H_2O$	70·2	9·6	7·4
,,	10	18	178	,, ,,	70·75	9·7	7·5	$C_{23}H_{36}N_2O_2H_2O$	70·7	9·8	7·2
,,	11	3	170	Acetone	71·0	10·0	7·1	$C_{24}H_{38}N_2O_2H_2O$	71·25	10·0	6·9
,,	13	14	161	I.P.A./I.P.AcO.	72·3	10·1	6·3	$C_{26}H_{42}N_2O_2H_2O$	72·2	10·25	6·5
,,	15	18	154	,, ,,	73·3	10·1	6·2	$C_{28}H_{46}N_2O_2H_2O$	73·0	10·5	6·1
,,	17	4	159	,, ,,	76·7	10·9	5·5	$C_{30}H_{50}N_2O_2$	76·6	10·7	5·9

* I.P.AcO. = isopropyl acetate.

were withdrawn and immediately mixed with 9 ml. of 2 per cent Bacto-oxgall to inactivate the excess quaternary ammonium compound in the sample. The inactivated suspensions were then shaken and, from these, 1 ml. quantities were serially diluted tenfold in distilled water and plated out on agar. The number of bacterial colonies formed after incubation for 24 hr. was counted, and the loss of viability determined by reference to the control count.

Test for bacteriostatic activity. The culture medium used was glucose-peptone water (0·5 per cent glucose, 1 per cent peptone (Bacto), 0·5 per cent sodium chloride; at pH 7·2). Five ml. quantities of this medium were distributed into test tubes and sterilised by autoclaving at 115° (10 lb. sq. in./10 min.). The quaternary compounds were dissolved in sterile distilled water to give double-strength solutions, and these were mixed aseptically with double-strength medium in the first tube of each series. Serial twofold dilutions were then made leaving 5 ml. of medium in each tube. The tubes were inoculated with 0·02 ml. of the appropriate

bacterial culture (*Staphylococcus aureus* C.N. 491: *Streptococcus viridans* Eden; *Escherichia coli*, Laboratory strain) containing approximately 1×10^6 organisms/ml. The tubes were incubated at 37° and the minimal inhibitory concentration (M.I.C.) of each quaternary was determined after 24 hr. and 5 days incubation.

TABLE III

KNOWN ANTIBACTERIAL AGENTS: CONCENTRATION OF CATION TO PRECIPITATE PROTEINS

Antibacterial agent	Cation μg./ml. to precipitate protein				Antibacterial activity against *Staph. aureus* C.N. 491 M.I.C. μg./ml.
	Casein	Pepsin	Gelatin	Egg albumin	
Chlorhexidine acetate	9·1	13	254	14	0·25
Benzalkonium chloride	51	27	250	40	0·31
Cetrimide	33	24	180	24	0·63
Dequalinium chloride	12	9	174	18	0·31
Domiphen bromide	33	5·1	76	15	0·31
Hedaquinium chloride	28	21	>625	26	0·31
5-Aminoacridine	37	>625	340	97	6·25
Polymyxin B	6	5	>625	12	0·63

TABLE IV

CONCENTRATION OF QUATERNARY CATION REQUIRED TO PRECIPITATE PROTEINS μg./ml. 4-AMINOQUINOLINIUM ACETATES

No. of carbon atoms in chain	Casein	Pepsin	Gelatin	Egg albumin
10	24	24	230	27
11	19	19	89	20
12	7·5	6·5	35	8
14	7	4·5	20	5
16	5	3	24	4·5
18	5	4·5	24	4

TABLE V

CONCENTRATION OF QUATERNARY CATION REQUIRED TO PRECIPITATE PROTEINS μg./ml. 4-AMINOQUINALDINIUM ACETATES

No. of carbon atoms in chain	Casein	Pepsin	Gelatin	Egg albumin
7	61	410	>525	127
10	23	26	156	21
11	10	13	58	24
12	11	10	32	5
14	11	5·5	19	4
16	11	5·5	19	3
18	8	5·5	25	4

RESULTS

Analytical. The results of analytical tests on the 1-alkyl-4-aminoquinolinium and the 1-alkyl-4-aminoquinaldinium iodides are shown in Table I and those of the corresponding acetates in Table II.

Protein precipitation test. Initial experiments with this technique were made with known quaternary ammonium compounds, and a wide difference in the behaviour of the compounds was noted. For example the following quaternary salts failed to precipitate any of the proteins tested: hexamethonium iodide, decamethonium iodide, decane-1,10-pyridinium bromide, benzyltrimethylammonium hydroxide, phenyltrimethylammonium-*p*-toluenesulphonate, decane-1,10-*N*-methylpyrrolidinium iodide, 3-phenylpropyl-4-aminoquinolinium acetate, and hexyl-3,5,5-trimethyl-4-aminoquinolinium acetate. However, it is known that

these compounds possess little or no antibacterial activity. In contrast to these results protein precipitation was caused by other quaternary ammonium compounds of known antibacterial activity, namely: benzalkonium chloride, cetrimide, dequalinium chloride, domiphen bromide, hedaquinium chloride; the antibacterials chlorhexidine acetate, 5-aminoacridine, and the antibiotic polymyxin B. The results with these latter compounds are summarised in Table III.

Figures for the protein precipitating activity of the acetates of the 4-aminoquinolinium series are shown in Table IV and those of the 4-aminoquinaldinium series in Table V.

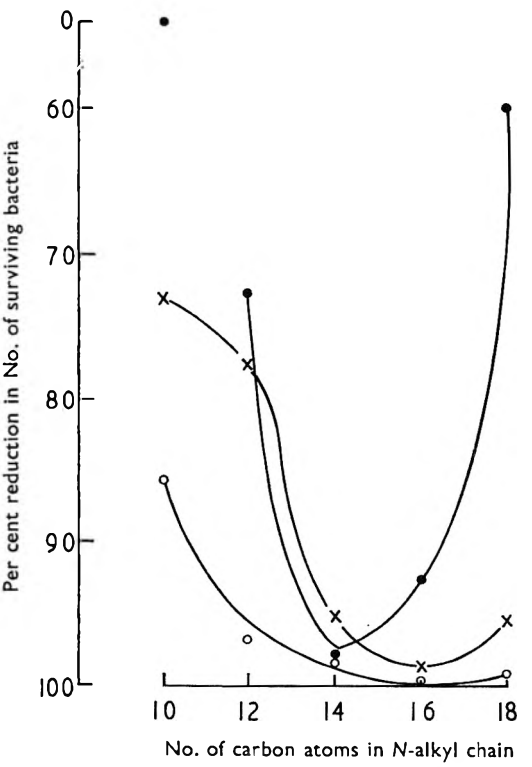


FIG. 1. Bactericidal activity of the 4-aminoquinolinium acetates against *Staph. aureus* (○—○), *Str. viridans* (●—●) and *E. coli* (×—×) at a drug concentration of 10 μg./ml., and a bacteria-drug contact time of 2·5 min.

Bactericidal activity. The results of bactericidal tests on members of the two series of compounds against the three organisms at a drug concentration of 10 μg./ml. and at a bacteria-drug contact time of 2·5 min. are shown graphically in Figs. 1 and 2. In a similar manner, Figs. 3 and 4 summarise their bactericidal activity at a contact time of 15 min. On these graphs the number of carbon atoms in the *N*-alkyl chain has been plotted against the percentage reduction in the survival of the exposed

bacteria. In each experiment the drug-bacteria ratio was 100 μg. drug: 0·7 mg. dry weight of bacteria.

From these results, it is apparent that both series of compounds have bactericidal activity against the organisms tested. In the 4-aminoquinolinium series, the hexadecamethylene member would seem to be the

TABLE VI

BACTERIOSTATIC ACTIVITY 4-AMINOQUINOLINIUM AND 4-AMINOQUINALDINIUM ACETATES

Micro-organism	Chemical series	Bacteriostatic activity (Minimal Inhibitory Concentration μg./ml. at 24 hr.)					
		Homologue: No. of carbon atoms in chain					
		7	10	12	14	16	18
Staphylococcus aureus C.N. 491	4-Aminoquinolinium	—	0·63	0·16	0·44	1·25	10
	4-Aminoquinaldinium	1·90	0·31	0·14	0·24	0·84	3·12
Streptococcus viridans	4-Aminoquinolinium	—	3·12	0·55	0·78	1·10	4·4
	4-Aminoquinaldinium	17·7	1·40	0·35	0·49	0·99	12·5
Escherichia coli	4-Aminoquinolinium	—	25	12·5	50	100	>100
	4-Aminoquinaldinium	100	12·5	8·8	17·7	100	>100

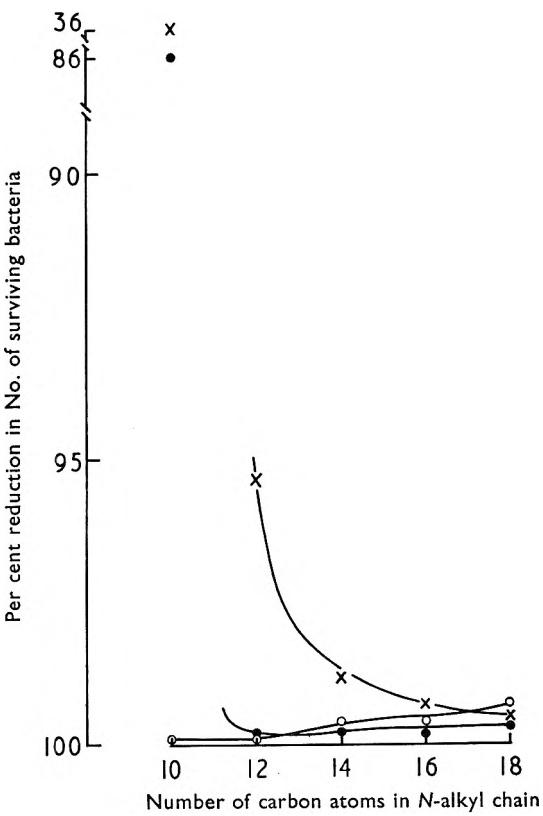


FIG. 2. Bactericidal activity of the 4-aminoquinaldinium acetates against *Staph. aureus* (○—○), *Str. viridans* (●—●), and *E. coli* (×—×), at a drug concentration of 10 μg./ml. and at a bacteria-drug contact time of 2·5 min.

most potent against the three species after 15 min. contact. In the 4-aminoquinaldinium series the hexadecyl and the octadecyl members are equally the most effective against *E. coli*, whilst against *Staph. aureus* and *Str. viridans* good activity is shown by the C_{12} and C_{14} members as well.

Bacteriostatic activity. The results of the bacteriostatic experiments with the 4-aminoquinolinium and the 4-aminoquinaldinium acetates are shown in Table VI; activity is shown against *Staph. aureus*, *Str. viridans* and *E. coli*.

The individual members of both series have high activity against the three bacterial species; in each series the maximal activity against *Staph.*

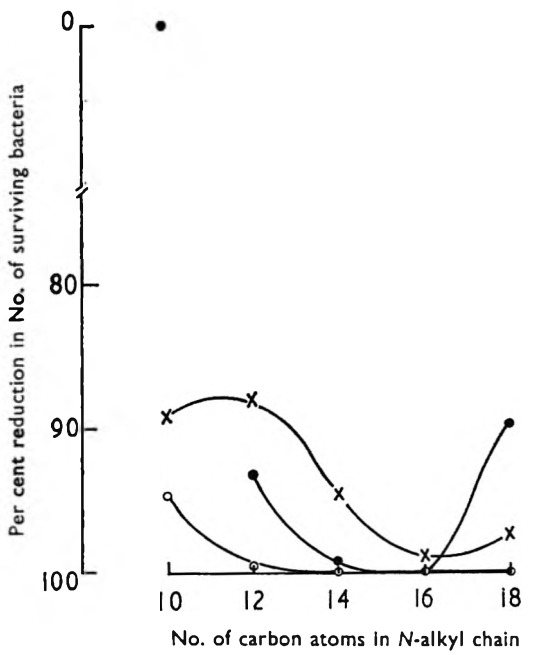


FIG. 3. Bactericidal activity of the 4-aminoquinolinium acetates against *Staph. aueus* (○—○), *Str. viridans* (●—●), and *E. coli* (×—×), at a drug concentration of 10 μg./ml. and at a bacteria-drug contact time of 15 min.

aureus and *Str. viridans* is shown by members with a polymethylene chain length of C = 12 to 14, whilst against *E. coli* activity is maximal at a chain length of C = 10 to 12.

Protein precipitating activity and bactericidal potency. The protein precipitating activities (μg. quaternary cation/ml.) of the two series against casein, pepsin, gelatin and egg albumin (Tables IV and V), have been plotted against chain length to give the results in Figs. 5 and 6, and it is interesting to note the similarities between these graphs and those obtained by plotting bactericidal activity against chain length, particularly in the 4-aminoquinaldine series (Figs. 2 and 4). For example: the chain length range over which the compounds display strong activity of both types

seems to be the same, and in addition both these activities seem to decrease at chain length C = 18 and beyond.

The correlation of protein precipitating activity and bacteriostatic activity is not so striking. Graphs drawn plotting bacteriostatic activity against chain length for the two series, reach maxima at C = 10 to 14 and then fall away rapidly at higher chain lengths.

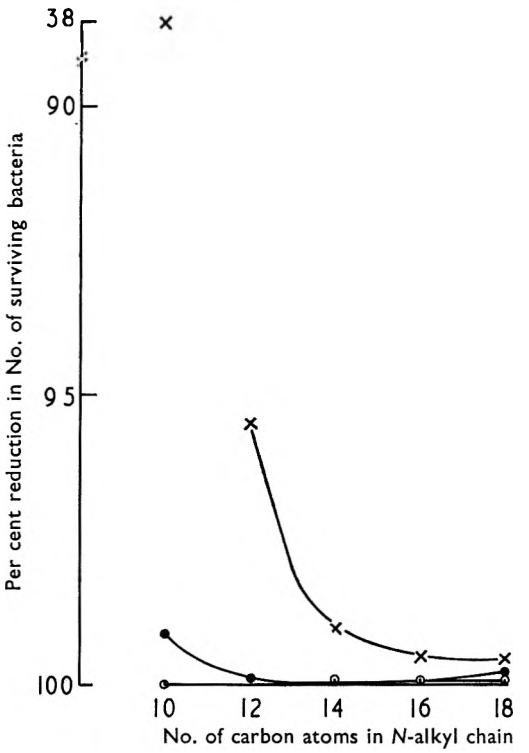


FIG. 4. Bactericidal activity of the 4-aminoquinaldinium acetates against *Staph. aureus* (○—○), *Str. viridans* (●—●), and *E. coli* (×—×), at a drug concentration of 10 μg./ml. and at a bacteria-drug contact time of 15 min.

DISCUSSION

Members of the 4-aminoquinolinium and 4-aminoquinaldinium series possess marked protein precipitating, bactericidal and bacteriostatic activity. Both series of compounds show maximal activity over the range of polymethylene chain length C = 12 to 16.

The results of the bactericidal tests show that members of the 4-aminoquinaldinium series are more potent than the homologous members of the 4-aminoquinolinium series. In addition the length of time that the organism is in contact with the drug has less effect upon bactericidal activity in the quinaldinium series than it has in the quinolinium series. In the latter it is much greater after 15 min. than after 2·5 min. contact time. In contrast, there is little difference in the bactericidal effects of the members of the quinaldinium series at the two times of contact.

Protein precipitation is caused by much the same concentration of each of the quaternary homologues in both series. However, higher concentrations of these compounds are needed to precipitate gelatin than to precipitate casein, pepsin and egg albumin. This is especially evident with the C_{10} homologues of both series, and there is an interesting analogy between the relatively poor activity of these members as bactericidal

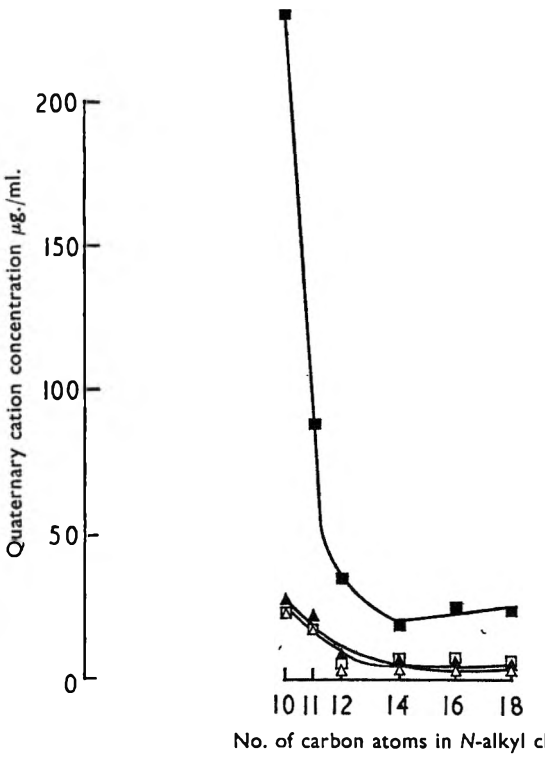


FIG. 5. Protein precipitating activity of the 4-aminoquinolinium acetates against casein (□—□), pepsin (△—△), gelatin (■—■), and egg albumin (▲—▲).

agents and their low activity as gelatin precipitants. The relation between protein precipitating and bactericidal activity can be put on a semi-quantitative basis by comparing the quaternary compound: protein ratio with the quaternary compound: bacteria ratio at the critical concentration of quaternary compound causing both protein precipitation and bactericidal effects.

A comparison of the relative amounts of protein and bacterial cells used in the experiments may be obtained from the formula, concentration of protein, divided by concentration of bacteria, both as mg. of dry weight per cent. This equals $62{\cdot}5 \div 7 \simeq 9$.

In this formula 62·5 is derived from the final concentration (mg. per cent) of protein in the quaternary compound-protein mixture used in the

protein precipitating experiments (0·625 mg./ml.), and where 7 is derived from the final strength (mg. per cent) of bacteria in the quaternary compound-bacteria mixture used in the bactericidal experiments (0·07 mg./ml.).

Therefore, since the concentration of quaternary compound used in all bactericidal experiments was 10 μg./ml., the equivalent concentration of quaternary compound required, in protein precipitating experiments, to give an equivalent quaternary compound: protein ratio to that in the

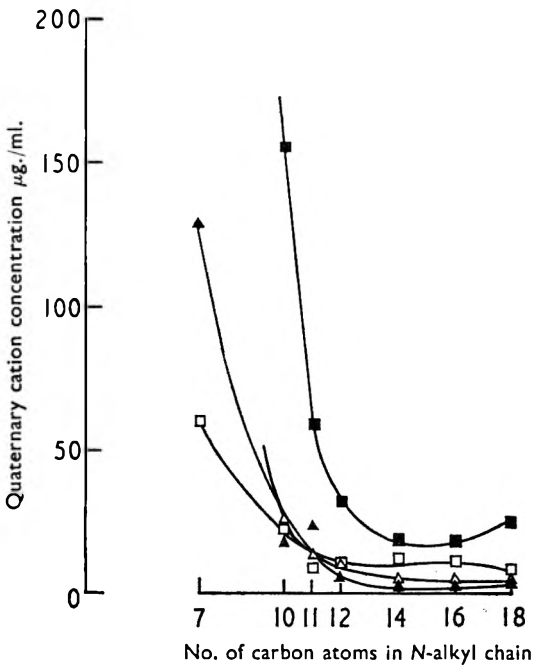


FIG. 6. Protein precipitating activity of the 4-aminoquinaldinium acetates against casein (□—□), pepsin (△—△), gelatin (■—■), and egg albumin (▲—▲).

bactericidal experiments should be 90 μg./ml. In the protein precipitation studies, it is found as expected that at a concentration of 90 μg./ml. the homologous salts of 4-aminoquinoline and 4-aminoquinaldine, in the range C = 10 to 18, precipitate albumin, casein and pepsin and, with the sole exception of the C_{10} quinolinium member, they also precipitate gelatin.

The results of the bacteriostatic experiments show maxima of activity in both series of compounds in the region of the C_{12}—C_{14} members, a range which also includes those compounds having good bactericidal activity. However, with polymethylene chain lengths greater than this range it is difficult to determine any correlation of bacteriostatic and bactericidal activity, since the bacteriostatic results are confused by precipitation of the salts in the culture media, and these are then not available to exert their antibacterial effect. There does not appear to be any close relation between the protein precipitating activity of the salts and

their bacteriostatic activity, but there is a good correlation of their protein precipitating and bactericidal activities which indicates that the mechanism by which these agents kill bacteria may well be related to their effects on protein.

The dodecyl acetate member of the 4-aminoquinaldine series (Laurodin) has been selected for fuller investigation.

Acknowledgements. We would like to thank Miss J. A. Moore and Miss C. A. Bedford for their assistance with the antimicrobial experiments, and Mr. R. Dellar for his assistance in the synthesis of the compounds examined.

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THE OXYTOCIC ACTIVITY OF DL-5-HYDROXYTRYPTOPHAN

By Ž. Lovašen, Z. Supek and M. Randić

From the Biology Division, Institute "Rudjer Bošković," Zagreb, Yugoslavia

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Incubation of the isolated rat uterus with α-methyldopa or bromolysergic acid diethylamide produces a complete inhibition of oxytocic activity of 5-hydroxytryptophan.

Studying the formation of 5-hydroxytryptamine (5-HT) in X-irradiated aqueous solution of 5-hydroxytryptophan (5-HTP) we observed that 5-HTP possesses a slight oxytocic activity, the sensitivity threshold being 5×10^{-5}. It seemed to us of interest to find out if this activity can be attributed to 5-HTP itself or to its decarboxylation product 5-HT. For that reason the influence of α-methyl-β-(3,4-dihydroxyphenyl)alanine (α-methyldopa) which is an inhibitor of 5-hydroxy-L-tryptophan decarboxylase, and bromolysergic acid diethylamide (bromLSD) which is an antagonist of 5-HT, was investigated on the oxytocic activity of 5-HTP.

Methods

The isolated oestrous rat uterus suspended in de Jalon solution (Gaddum, Peart and Vogt, 1949) was used to test the oxytocic activity. The

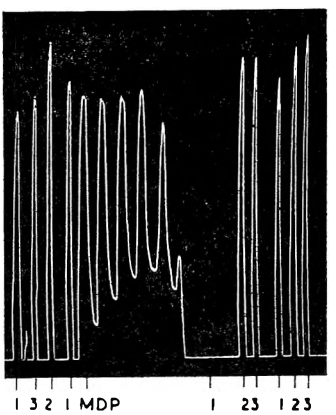

Fig. 1. Isolated oestrous rat uterus, 1, 0·1 mg./ml. 5-hydroxytryptophan. 2, 0·003 μg./ml. 5-hydroxytryptamine. 3, 0·15 μg./ml. acetylcholine. MDP, 1·5 mg./ml. of α-methyldopa. 10 min.

temperature of the organ bath was 28–29°. The following substances were used, DL-5-hydroxytryptophan monohydrate (Roche, England); 5-hydroxytryptamine creatinine sulphate monohydrate (Fluka, Switzerland); α-methyl-β-(3,4-dihydroxyphenyl)alanine (M.D.); bromolysergic acid diethylamide (Sandoz, Switzerland); acetylcholine hydrochloride (Roche, Switzerland).

Results

As shown in Fig. 1 the oxytocic activity of 5-HTP was completely inhibited by α-methyldopa, while this activity of 5-HT and acetylcholine remained unaltered. In few experiments, besides the complete inhibition of 5-HTP, a partial and transient inhibition of 5-HT was observed (Fig. 2). 5-HT and 5-HTP were both completely antagonised by bromLSD (Fig. 3).

Discussion

It is well known that α-methyldopa is an inhibitor of 5-hydroxytryptophan-L-decarboxylase (Westermann, Balzer and Knell, 1958). Since 5-HTP is inhibited by this agent in our experimental work, it is very likely that its oxytocic activity can be ascribed to 5-HT formed by decarboxylation in the uterus tissue. The experiments with bromLSD, an antagonist

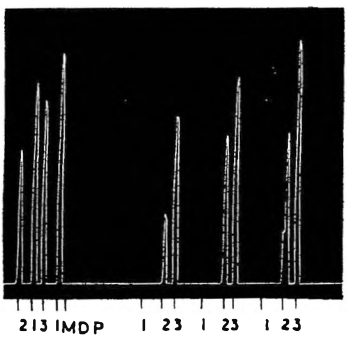

FIG. 2. Isolated oestrous rat uterus. 1, 0·3 mg./ml. 5-hydroxytryptophan. 2, 0·004 μg./ml. 5-hydroxytryptamine. 3, 0·1 μg./ml. acetylcholine. MDP, 2·5 mg./ml. α-methyldopa. 10 min.

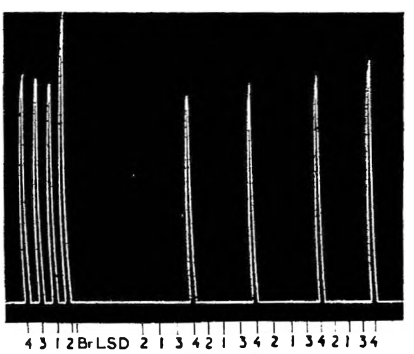

FIG. 3. Isolated oestrous rat uterus. 1, 0·05 mg./ml. 5-hydroxytryptophan. 2, 0·1 mg./ml. 5-hydroxytryptophan. 3, 0·004 μg./ml. 5-hydroxytryptamine. 4, 0·1 μg./ml. acetylcholine. BrLSD, 0·1 μg./ml. bromLSD. 5 min.

of 5-HT (Cerletti and Konsett, 1956), also support this presumption. Reported observations might suggest the presence of 5-HTP decarboxylase in the tissue of uterus. This possibility should be taken into account while using the uterus as test organ for studies on the biosynthesis of 5-HT.

Acknowledgements. The authors wish to thank Prof. V. Erspamer, Parma, Italy, for his advice and helpful discussion, Dr. C. N. Mushett of Merck Sharp & Dohme Laboratories, Rahway, New Jersey, U.S.A., for a supply of α-methyldopa and Messrs. Sandoz Ltd., Basle, Switzerland, for a sample of bromLSD.

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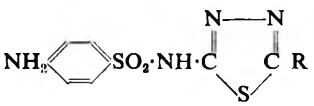
HYPOGLYCAEMIC AGENTS. PART I

By B. G. Boggiano, V. Petrow, O. Stephenson and A. M. Wild
From The British Drug Houses Ltd., Graham Street, London, N.1

Received April 20, 1961

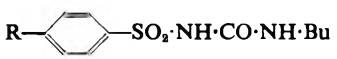
Some structural analogues of the active compounds (I), (II) and (III) are described. Their biological study failed to reveal significant hypoglycaemic activity.

We began a search for new hypoglycaemic agents in 1956 when the main structural types exciting attention were: (*a*) derivatives of 5-alkyl-2-*p*-aminobenzenesulphonamido-1,3,4-thiadiazole (cf. I), which had been



(I)

discovered by Loubatières in 1944 and (*b*) analogues of 1-butyl-3-benzenesulphonylurea (II) [Compare Franke and Fuchs (1955) and Miller and Dulin (1956) who reported on the *p*-aminophenyl (carbutamide; II;



(II)

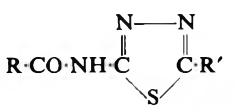
R = NH_2) and *p*-tolyl (tolbutamide; II; R = Me) analogues respectively.] A year later Ungar, Freedman and Shapiro (1957) and Pomeranze, Fujiy and Mouratoff (1957), described phenformin (N^1-phenethylbiguanide; *N*-β-phenethylformamidinyliminourea) (III), the first example of a

(III)

non-sulphamoyl compound to survive clinical studies for hypoglycaemic activity.

Initially we prepared a few compounds related to structures (I) and (II).

Analogues of (I) were synthesised in which the *p*-aminophenyl group was replaced by a *p*-carboxyphenyl, by a 2-pyridyl and by a 4-acetamido-2-pyridyl group. These compounds were readily obtained by reaction of the appropriate sulphonyl chlorides with 2-amino-5-t-butyl-1,3,4-thiadiazole in pyridine solution. Related types (IV) in which the sulphonyl group



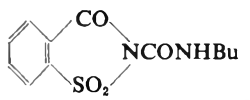
(IV)

was replaced by a carbonyl group were also synthesised. The R = *p*-tolyl, 3-pyridyl and 2-furyl analogues (IV; R′ = CMe_3) were prepared from the

appropriate acid chlorides and the aminothiadiazole. The *p*-aminophenyl derivative (IV; R = *p*-$NH_2.C_6H_4$, R′ = CMe_3) was obtained directly using thionylaminobenzoyl chloride (cf. Graf and Langer, 1937) or by reduction of the corresponding nitro-compound (IV; R = *p*-$NO_2 \cdot C_6H_4$; R′ = CMe_3). In addition, the 5-carbamoyl (IV; R′ = $CONH_2$) and 5-carbamoylmethyl (IV; R′ = CH_2CONH_2) derivatives of 2-amino- and 2-acetamido-1,3,4-thiadiazole (IV; R = Me, R′ = H) were prepared.

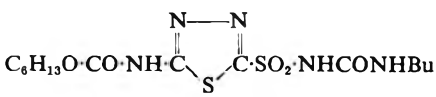
Attention was then directed to analogues of 1-butyl-3-sulphonylurea (II). These included the higher homologues (II; R = $AcNHCH_2$· and $AcNH(CH_2)_2$) of carbutamide, as well as the 1-butyl-3-(2-thienyl), 1-butyl-3-(2-pyridyl) and 1-butyl-3-(3-pyridyl) ureas. 1-Butyl-3-(3-nicotinoyl)-urea (V) and the corresponding isonicotinoyl urea were also synthesised. By condensing saccharin with butyl isocyanate, the carboxybutylamide (VI) was obtained which contained both the 1-butyl-3-carbonylurea and

(V)



(VI)

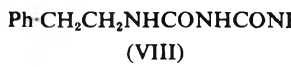
1-butyl-3-benzenesulphonylurea moieties. 1-Butyl-3-(2-hexyloxycarbonamido-1,3,4-thiadiazole-5-sulphonyl)urea (VII), a mixed type resembling



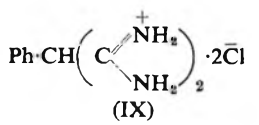
(VII)

both (I) and (II) was similarly obtained from 2-hexyloxycarbonamido-1,3,4-thiadiazole-5-sulphonamide (Petrow, Stephenson, Thomas and Wild, 1956).

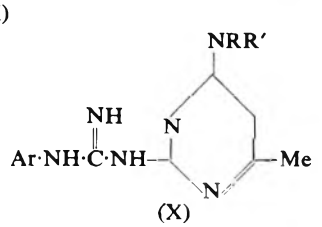
Later, when reports on phenformin (III) became available, the related 1-phenethylbiuret (VIII) and β-phenylmalondiamidine (IX) were synthesised. In addition, the guanidinotetrahydropyrimidines (X) were



(VIII)



(IX)



(X)

obtained by condensing the 2-arylguanidino-4-chloro-6-methylpyrimidines with phenethylamine or with 1,2,3,6-tetrahydropyridine. Some derivatives of thiosemicarbazide, which cannot be classified with the previous groups of compounds are reported in the Experimental.

Biological study of the above compounds by Dr. A. David and his colleagues failed to reveal hypoglycaemic activity superior to that of the parent structures.

EXPERIMENTAL

2-(2-*Pyridinesulphonamido*)-5-*t-butyl*-1,3,4-*thiadiazole*

(*a*) *Pyridine*-2-*sulphonyl chloride*. A solution of 2-mercaptopyridine (58 g.) (Thirtle, 1946) in concentrated hydrochloric acid (649 ml.) and ice water (142 ml.) was cooled below 10° and chlorine passed through the solution until absorption of the gas was complete (*ca* 90 min.). Ice-water (1740 ml.) was then stirred into the mixture, which was cooled to 0°. The crystalline *product* which separated was collected, washed with cold water and drained as far as possible. It was used without delay for the next stage of the reaction.

(*b*) The foregoing crude sulphonyl chloride was added in portions to a solution of 2-amino-5-t-butyl-1,3,4-thiadiazole (42 g.) in pyridine (160 ml.) at below 40°. Reaction was completed by heating at 60° for 35 min. when the mixture was cooled, added to ice and acidified. The *product* (66 g.) had m.p. 167° (decomp.) after crystallisation from aqueous ethanol. Found: C, 44·5; H, 4·6; N, 18·7; S, 21·7. $C_{11}H_{14}N_4O_2S_2$ requires C, 44·3; H, 4·7; N, 18·8; 21·5 per cent.

2-(5-*Acetamido*-2-*pyridinesulphonamido*)-5-*t-butyl*-1,3,4-*thiadiazole*, was prepared by reaction of 5-acetamidopyridine-2-sulphonyl chloride (Caldwell and Kornfeld, 1942) with 2-amino-5-*t*-butyl-1,3,4-thiadiazole in pyridine. It had m.p. 210° (decomp.) after crystallisation from aqueous ethanol. Found: N, 19·8; S, 18·2. $C_{13}H_{17}N_5O_3S_2$ requires N, 19·7; S, 18·0 per cent.

2-(4-*Carboxybenzenesulphonamido*)-5-*t-butyl*-1,3,4-*thiadiazole*, had m.p. 370° (decomp.) after crystallisation from pyridine or a large volume of acetic acid. Found: N, 12·0. $C_{13}H_{15}N_3O_4S_2$ requires N, 12·3 per cent.

2-(*p-Aminobenzamido*)-5-*t-butyl*-1,3,4-*thiadiazole*

(*a*) 2-(*p-Nitrobenzamido*)-5-*t-butyl*-1,3,4-*thiadiazole*, prepared by reaction of 2-amino-5-t-butyl-1,3,4-thiadiazole (6·3 g.) with *p*-nitrobenzoyl chloride (7·4 g.) in pyridine (30 ml.) had m.p. 296-297° after crystallisation from glacial acetic acid. Found: C, 50·9; H, 4·8; S, 10·5. $C_{13}H_{14}N_4O_3S$ requires C, 51·0; H, 4·5; S, 10·6 per cent.

A suspension of the foregoing nitro-compound (1·53 g.) in boiling ethanol (100 ml.) was treated with hydrazine hydrate (1·0 g.) and Raney nickel (*ca* 2 g.) and the mixture heated under reflux for 5 hr. It was then filtered, concentrated and diluted with water. The product was dissolved in hydrochloric acid, reprecipitated by neutralisation and recrystallised from acetic acid. It had m.p. 323–325° (some decomp.). Found: C, 56·1; H, 5·9; N, 20·2; S, 11·6. $C_{13}H_{16}N_4OS$ requires C, 56·5; H, 5·8; N, 20·3; S, 11·6 per cent.

(*b*) *p*-Thionylaminobenzoyl chloride (1·56 g.) and 2-amino-5-t-butyl-1,3,4-thiadiazole (1·21 g.) were dissolved in pyridine (5 ml.) and the mixture heated on the steam bath for 30 min. The solution was poured into cold

water to yield the *product* m.p. 323–325° (some decomp.) after purification as described above.

2-*Nicotinamido*-5-*t*-*butyl*-1,3,4-*thiadiazole*

Nicotinic acid (17·2 g.) was dissolved in dry pyridine (70 ml.), heated on the steam bath, and thionyl chloride (14 ml.) added with stirring. After 30 min., a solution of 2-amino-5-*t*-butyl-1,3,4-thiadiazole (22 g.) dissolved in the minimum volume of pyridine was added and the mixture heated for a further hr. It was then cooled and diluted with ice-water. The *product* was collected and washed with cold water. It (26 g.) had m.p. 206–207° after crystallisation from ethanol. Found: C, 55·4; H, 5·2. $C_{12}H_{14}N_4OS$ requires C, 54·9; H, 5·4 per cent.

2-(*p*-*Methylbenzamido*)-5-*t*-*butyl*-1,3,4-*thiadiazole*, had m.p. 217–218° after crystallisation from ethanol. Found: C, 60·8; H, 5·9; N, 15·3; S, 11·7. $C_{14}H_{17}N_3OS$ requires C, 61·1; H, 6·2; N, 15·3; S, 11·6 per cent.

2-(2-*Furoylamido*)-5-*t*-*butyl*-1,3,4-*thiadiazole*, had m.p. 174–175° after crystallisation from ethanol. Found: C, 52·5; H, 4·8; N, 16·8. $C_{11}H_{13}N_3O_2S$ requires C, 52·6; H, 5·2; N, 16·7 per cent.

2-*Amino*-5-*carbamoyl*-1,3,4-*thiadiazole*. Thiosemicarbazide (18·2 g.) and phosphorus oxychloride (35·4 g.) were mixed carefully with cooling. Ethoxalyl chloride (27·4 g.) was then added carefully with cooling and reaction completed by heating at 60° for 1 hr. The cooled mixture was poured into ice water and neutralised with sodium bicarbonate.

2-*Amino*-5-*ethoxycarbonyl*-1,3,4-*thiadiazole* (11 g.), m.p. 165–167° (decomp.), was obtained by crystallisation from water. The compound is unstable in solution. It (2 g.) was added to aqueous ammonia (10 ml., d = 0·880) and water (5 ml.) and the mixture left at room temperature for 2 days. The *product* which separated was crystallised from acetic acid and had m.p. 307° (decomp.). Found: C, 25·2; H, 2·7. $C_3H_4N_4OS$ requires C, 25·0; H, 2·8 per cent.

2-*Acetamido*-5-*ethoxycarbonyl*-1,3,4-*thiadiazole* was obtained when the amine (2 g.) was heated with acetic anhydride (10 ml.) and anhydrous sodium acetate (0·5 g.) for 5 min. It (1·6 g.) had m.p. 230° after crystallisation from acetic acid. Found: C, 39·2; H, 4·3; N, 19·8. $C_7H_9N_3O_3S$ requires C, 39·1; H, 4·2; N, 19·5 per cent.

2-*Acetamido*-5-*carbamoyl*-1,3,4-*thiadiazole*. (*a*) The foregoing ester (13 g.) was dissolved in aqueous ammonia (45 ml., d = 0·880) and water (15 ml.). The *product* (8·9 g.) which separated was collected after 1 day and washed with cold water. It had m.p. >350°. Found: C, 31·9; H, 3·2; N, 29·8; S, 17·1. $C_5H_6N_4O_2S$ requires C, 32·3; H, 3·2; N, 30·1; S, 17·2 per cent.

(*b*) 2-Amino-5-carbamoyl-1,3,4-thiadiazole (17·5 g.) was refluxed with acetic anhydride (75 ml.) and anhydrous sodium acetate (5 g.) for 20 min. Water was added to decompose excess anhydride and the *product* was collected and washed with water. It had m.p. >350°. 2-*Acetamido*-5-*ethoxycarbonylmethyl*-1,3,4-*thiadiazole* was obtained by heating the corresponding 2-amino-compound (4 g.) (Ohta, 1952) with acetic anhydride (10 ml.) for 5 min. It (4·3 g.) had m.p. 193–194° after crystallisation from

acetic acid. Found: C, 41·5; H, 5·0; N, 18·2; S, 14·1. $C_8H_{11}N_3O_3S$ requires C, 41·9; H, 4·8; N, 18·3; S, 14·0 per cent.

1-*Butyl*-3-(2-*pyridinesulphonyl*) *urea*

(*a*) 2-*Pyridinesulphonamide*. Moist pyridine-2-sulphonyl chloride (72 g.) was added with stirring at below 10° to aqueous ammonia (400 ml., d = 0·880). When the addition was complete, excess of ammonia was boiled off and the residue was evaporated to dryness at reduced pressure. The residual solid was triturated with water and collected. It had m.p. 140–142° after crystallisation from water. Found: C, 37·8; H, 3·9 N, 17·8. $C_5H_6N_2O_2S$ requires C, 38·0; H, 3·8; N, 17·7 per cent.

(*b*) A solution of the foregoing sulphonamide (14·4 g.) in water (65 ml.) containing sodium hydroxide (4 g.) was treated with acetone (30 ml.) and cooled to 10°. Butyl isocyanate was added dropwise with stirring which was continued for a further hr. The solution was diluted with an equal volume of water, filtered and the filtrate acidified with hydrochloric acid. The solids were collected, washed with water and dissolved in dilute ammonia solution (100 ml.). The filtered solution was acidified with hydrochloric acid yielding the *product* (19 g.), m.p. 164° after crystallisation from ethanol. Found: N, 16·4; S, 12·6. $C_{10}H_{15}N_3O_3S$ requires N, 16·3; S, 12·5 per cent.

1-*Butyl*-3-(3-*pyridinesulphonyl*)*urea*, had m.p. 104–105° after crystallisation from aqueous ethanol. Found: C, 47·0; H, 5·5; N, 15·9. $C_{10}H_{15}N_3O_3S$ requires C, 46·7; H, 5·9; N, 16·3 per cent.

1-*Butyl*-3-(2-*thiophensulphonyl*)*urea*

(*a*) *Thiophen*-2-*sulphonamide* (described by Langer in 1884). Thiophene-2-sulphonyl chloride (10 g.) was added with stirring to aqueous ammonia (50 ml., d = 0·880) at 0°. After addition was complete the mixture was kept at 0° for 6 hr., then allowed to warm slowly to room temperature when reaction was completed by warming to 40° for 30 min. The solid which separated (7·6 g.), had m.p. 143–145° after crystallisation from water. Found: C, 29·1; H, 3·1; N, 8·9; S, 39·3. Calc. for $C_4H_5NO_2S_2$: C, 29·4; H, 3·1; N, 8·6; S, 39·3 per cent.

(*b*) The foregoing sulphonamide (8·15 g.) was dissolved in a solution of sodium hydroxide (2 g.) in water (35 ml.) and acetone (15 ml.). The solution was cooled to 10° and treated slowly with butyl isocyanate (5·5 g.) added dropwise over 75 min. Stirring was continued for a further 90 min. when a small amount of insoluble material was filtered off and the filtrate acidified with dilute hydrochloric acid. The solid (12 g.) was collected and had m.p. 150–152° after crystallisation from aqueous ethanol. Found: C, 41·3; H, 5·3; S, 23·7. $C_9H_{14}N_2O_3S_2$ requires C, 41·2; H, 5·4; S, 24·4 per cent.

1-(*p-Acetamidomethylbenzenesulphonyl*)-3-*ethylurea*, had m.p. 198–199° (decomp.) after crystallisation from aqueous 2-ethoxyethanol. Found: C, 48·3; H, 5·5; N, 14·0; S, 10·9. $C_{12}H_{17}N_3O_4S$ requires C, 48·1; H, 5·7; N, 14·0; S, 10·7 per cent.

1-(*p-Acetamidomethylbenzenesulphonyl*)-3-*propylurea*, m.p. 176–177°, (from ethanol). Found: C, 49·8; H, 5·9; N, 13·0; S, 9·9. $C_{13}H_{19}N_3O_4S$ requires C, 49·8; H, 6·1; N, 13·4; S, 10·2 per cent.

1-(*p-Acetamidomethylbenzenesulphonyl*)-3-*butylurea*, m.p. 158–160°, (from ethanol). Found: C, 51·4; H, 6·2; N, 12·6; S, 9·6. $C_{14}H_{21}N_3O_4S$ requires C, 51·4; H, 6·5; N, 12·8; S, 9·8 per cent.

1-[*p*-2-(*Acetamidoethyl*)*benzenesulphonyl*]-3-*butylurea*, had m.p. 150–152° after crystallisation from ethanol. Found: C, 52·9; H, 6·9; N, 12·2; S, 8·9. $C_{15}H_{23}N_3O_4S$ requires C, 52·8; H, 6·8; N, 12·3; S, 9·4 per cent.

1-*Benzenesulphonyl*-3-*butylguanidine*. Benzenesulphonyl chloride (30 g.) was added with stirring to a solution of butylguanidine hydrochloride in 10 per cent sodium hydroxide solution (300 ml.) at 0° and stirring continued until the odour of sulphonchloride had disappeared. The *product* was collected and washed with water. It had m.p. 120–122° (from ethanol). Found: C, 52·0; H, 6·9; N, 16·2. $C_{11}H_{17}N_3O_2S$ requires C, 51·8; H, 6·7; N, 16·5 per cent.

1-*Acetylsulphanilyl*-3-*butylguanidine*. Butylguanidine sulphate (1·64 g.) was dissolved in warm pyridine (2·0 ml.) and treated, in portions, with acetylsulphanilyl chloride (2·34 g.). The mixture was heated at 60° for 1 hr., cooled and triturated with ice water. The solid (1·6 g.) was collected and washed with water. It had m.p. 157–159° (from ethanol). Found: C, 49·9; H, 6·4; N, 18·0. $C_{13}H_{20}N_4O_3S$ requires C, 50·0; H, 6·5; N, 17·9 per cent.

N-*Butylcarbamoyl-o-benzoicsulphimide*, had m.p. 130–131° (from aqueous acetone). Found: C, 51·3; H, 5·0; N, 9·8; S, 11·3. $C_{12}H_{14}N_2O_4S$ requires C, 51·1; H, 5·0; N, 9·9; S, 11·4 per cent.

1-*Butyl*-3-(2-*hexyloxycarbamido*-1,3,4 *thiadiazole*-5-*sulphonyl*)*urea*. A solution of 2-hexyloxycarbamido-1,3,4-thiadiazole-5-sulphonamide (10·3 g.), butyl isocyanate (5 g.) and triethylamine (5 ml.) in benzene (40 ml.) was heated under reflux for 10 hr. on the steam bath when volatile material was distilled off at reduced pressure. The gummy residue was dissolved in aqueous ethanol and acidified with acetic acid and the *product* (9·3 g.) collected. It had m.p. 172–173° after crystallisation from aqueous ethanol. Found: C, 41·5; H, 5·9; N, 16·9. $C_{14}H_{25}N_5O_5S_2$ requires C, 41·3; H, 6·2; N, 17·2 per cent.

1-*Butyl*-3-*isonicotinoylurea*. A mixture of isonicotinoyl chloride hydrochloride (44·5 g.) and butylurea (29 g.) in pyridine (40 ml.) was heated on the steam bath for 2 hr., cooled and triturated with water. The solids were collected, dissolved in boiling ethanol and filtered to remove about 5 g. of high melting insoluble material. Dilution of the filtrate with water yielded the *product* (28 g.) m.p. 110–111°. Found: C, 59·5; H, 7·0; N, 19·0. $C_{11}H_{15}N_3O_2$ requires C, 59·7; H, 6·8; N, 19·0 per cent.

1-*Butyl*-3-*nicotinoylurea*, m.p. 103–105° (aqueous ethanol). Found: C, 59·5; H, 6·7; N, 18·6 per cent.

1-*p-Toluoylsemicarbazide*, had m.p. 226–227° (decomp.) (from water). Found: C, 56·4; H, 5·5; N, 21·4. $C_9H_{11}N_3O_2$ requires C, 56·0; H, 5·7; N, 21·8 per cent.

1-*Acetylsulphanilyl*-4-o-*tolylthiosemicarbazide*. A solution of acetylsulphanilylhydrazide (17·4 g.) in ethanol (400 ml.) was treated with *o*-tolyl isothiocyanate (11·4 g.) and the mixture heated under reflux for 12 hr. on the steam bath. The solids (25 g.) were collected, washed with ethanol and had m.p. 228–229° (decomp.) (from acetic acid). Found: C, 50·8; H, 4·6; N, 14·7; S, 17·0. $C_{16}H_{18}N_4O_3S_2$ requires C, 50·8; H, 4·8; N, 14·8; S, 16·9 per cent.

4-o-*Tolyl*-1-*tosylthiosemicarbazide*, had m.p. 204° (decomp.) (from ethanol). Found: C, 54·1; H, 4·8; N, 12·0; S, 19·1. $C_{15}H_{17}N_3O_2S_2$ requires C, 53·7; H, 5·1; N, 12·5; S, 19·1 per cent.

4-*Allyl*-1-*tosylthiosemicarbazide*, had m.p. 174–175° (decomp.) (from ethanol). Found: C, 46·6; H, 5·7; N, 14·6; S, 22·1. $C_{11}H_{15}N_3O_3S_2$ requires C, 46·3; H, 5·3; N, 14·7; S, 22·5 per cent.

1-*Phenethylbiuret*. A solution of phenethylamine (17 g.) and nitrobiuret (20 g.) in 50 per cent ethanol (200 ml.) was warmed at 50–60° for 1 hr. then concentrated to remove most of the ethanol. The product which separated on cooling had m.p. 140–141° after crystallisation from water. Found: C, 58·0; H, 6·2; N, 20·1. $C_{10}H_{13}N_3O_2$ requires C, 58·0; H, 6·3; N, 20·3 per cent.

1-*Diguanidino*-1,2,3,6-*tetrahydropyridine*. A mixture of dicyandiamide (8·4 g.), 1,2,3,6-tetrahydropyridine (9·15 g.) and copper sulphate pentahydrate (12·5 g.) in 2-ethoxyethanol (20 ml.) and water (23 ml.) was heated under reflux with stirring for 2 hr. The mixture was diluted with water and the purple-brown copper complex was collected, washed with water and dissolved in N hydrochloric acid (390 ml.). Copper ions were removed with hydrogen sulphide and the filtered solution evaporated to dryness at reduced pressure. The residual gum crystallised from aqueous ethanol to yield the *product* (9·85 g.), m.p. 220–223°. Found: C, 33·5; H, 6·5; N, 28·1; Cl, 28·7. $C_7H_{15}Cl_2N_5 \cdot \frac{1}{2}H_2O$ requires C, 33·7; H, 6·5; Cl. 28·5; N, 28·1 per cent.

β-Phenylmalondiamidine dihydrochloride. A solution of β-phenylmalononitrile (62·5 g.) in ether (500 ml.) and ethanol (51·4 ml.) was saturated with hydrogen chloride at 0° and left at this temperature overnight. The imino-ether which separated was collected and washed with ether. It (100 g.) was dissolved in 12 per cent methanolic ammonia (1 litre) and kept at room temperature for 4 days. The solution was concentrated and mixed with ether. The *product* (62·5 g.) was collected and crystallised from ethanol-ether, from which it separated as a *monohydrate*, m.p. 208° (decomp.). Found: C, 40·9; H, 6·4; Cl, 26·9. $C_9H_{16}Cl_2ON_4$ requires C, 40·5; H, 6·0; Cl, 26·5 per cent.

6-*Methyl*-4-*phenethylamino*-2-*phenylguanidinopyrimidine*. A mixture of 4-chloro-6-methyl-2-phenylguanidinopyrimidine (16·5 g.) (prepared as described by Curd and Rose, 1946), sodium hydroxide (9·1 g.) and phenethylamine (9·2 g.) in chlorobenzene (60 ml.) and water 960 ml.) was heated under reflux with stirring for 30 min. when the chlorobenzene was distilled off in steam. The *product* was collected, washed with water and then with ethanol. It (13·5 g.) had m.p. 194–195° after crystallisation from ethanol.

Found: C, 69·1; H, 6·0; N, 24·7. $C_{20}H_{22}N_6$ requires C, 69·4; H, 6·4; N, 24·3 per cent.

2-p-*Chlorophenylguanidino*-6-*methyl*-4-*phenethylaminopyridine*, obtained in 95 per cent yield, had m.p. 208–211° (from 2-ethoxyethanol). Found: C, 63·2; H, 5·3; Cl, 9·3; N, 22·2. $C_{20}H_{21}ClN_6$ requires C, 63·1; H, 5·6; Cl,9·3; N, 22·1 per cent.

2 - p - *Chlorophenylguanidino* - 6 - *methyl* - 4 - (1,2,3,6 - *tetrahydropyridino*) *pyrimidine*, m.p. 178–180° (from acetone) was obtained in 67 per cent yield. Found: C, 59·10; H, 5·5; Cl, 10·5; N, 23 9. $C_{17}H_{19}ClN_6$ requires C, 59·6; H, 5·6; Cl, 10·3; N, 24·5 per cent.

2 - p - *Bromophenylguanidino* - 6 - *methyl* - 4 - (1,2,3,6 - *tetrahydropyridino*) *pyrimidine*, obtained in 60 per cent yield, had m.p. 172–175° (from acetone). Found: C, 52 9; H, 4·9; N, 21·5; Br, 20·8. $C_{17}H_{19}BrN_6$ requires C, 52·7; H, 4·9; Br, 20·6; N, 21·7 per cent.

2-p-*Bromophenylguanidino*-6-*methyl*-4-*phenethylaminopyrimidine*, had m.p. 210–212° (from ethoxyethanol). Found: C, 56·4; H, 4·9; Br, 18·5; N, 19·7. $C_{20}H_{21}BrN_6$ requires C, 56·5; H, 5·0; Br, 18·8; N, 19·8 per cent.

2-0-*Methoxyphenylguanidino*-6-*methyl*-4-*phenethylaminopyrimidine*, obtained in 60 per cent yield, had m.p. 218° (from 2-ethoxyethanol). Found: C, 67·2; H, 6·3; N, 22·2. $C_{21}H_{24}N_6O$ requires C, 67·0; H, 6·4; N, 22·3 per cent.

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BOOK REVIEW

OXIDATION-REDUCTION POTENTIALS OF ORGANIC SYSTEMS. By W. Mansfield Clark. Pp. xi + 584 (including Index). Baillière, Tindall & Cox Ltd., London, 1960. 108s.

In his book Dr. Mansfield Clark critically examines the methods used in oxidation-reduction measurements and discusses the results obtained from different organic oxidation-reduction systems in terms of thermodynamic principles.

The author introduces the electrochemistry of oxidation and reduction historically, clearing up some misconceptions in the interpretation of results of early work. This chapter could have been much reduced in content, the practising chemist being familiar with the points the author mentions at great length. This chapter is followed by the thermodynamic principles of electrochemistry; to many this section will serve as a useful revision of the theoretical treatment pertinent to the subject. It is an essential part of the book and will be helpful to the organic and biological chemist. Mention is then made of definitions, conventions and some special terms used in this field of chemistry. This completes the first quarter of the book; the remainder is devoted in a masterly way to a large number of general and specific systems which have been investigated in organic chemistry.

Complexities arising in organic oxidation-reduction systems, for example, dimerisation of the quinhydrone type, aggregation of molecules of dyes, preferential adsorption of a component, free radicals of the triphenylmethane and also the pyocyanine types necessitate the construction of equations to account for the deviations of these systems from the more simple ones. This is done most comprehensively in chapters 6, 7 and 8, which are devoted to the modifications of basic equations to deal with the special cases, both real and hypothetical.

In a short chapter on liquid junction potentials the author brings a fresh approach to the meaning of electrostatic potential between solutions which are in liquid junction and he discusses the cause of this potential in terms of thermodynamical aspects of diffusion. An important chapter on the standardisation of potentials by the standard hydrogen half-cell and pH values of buffer solutions follows. The many results from recent experimental work justifies a revision of pH values of certain buffer solutions. This the author has attempted and in doing so has suggested the preparation of a few important buffer solutions.

The accumulated experience of Dr. Mansfield Clark has produced a very clear and full account in chapter 11 of the methods which are used in obtaining potentiometric measurements. In chapter 12, criteria by which the reliability of redox potentials may be judged is discussed.

In the remainder of the book the author deals admirably with a large number of results from important organic redox systems; he discusses the chemical reactions taking place and interpretation of the results obtained therefrom. This section is also a valuable source of references. The book ends with a glossary of symbols and a useful bibliography.

Some topics such as polarography have been treated sparsely and irreversible systems, of which there are many, are not dealt with at all. However, the book is costly enough and any extension would have made the price prohibitive to most readers. The reviewer thinks the author could have economised in his

use of words and quotations when introducing topics and producing argument, in this respect he has tried too hard to make the book "readable." Nevertheless, Dr. Mansfield Clark has made a valuable contribution to the chemistry of redox potentials of organic systems. His wide experience and critical faculties have been invaluable in emphasising the important aspects of the technique and the necessity of interpreting the method in terms of sound theoretical relationships.

Although the subject is a specialised one, the book is suitable for organic, physical and biochemists. N. ROBINSON.