RESEARCH PAPERS

THE INFLUENCE OF THREE PHENOTHIAZINE DERIVATIVES AND OF AMIPHENAZOLE ON THE ACTION OF METHADONE

Studies with Two Algesimetric Methods in Untrained Human Subjects

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The influence of the phenothiazine derivatives chlorpromazine, acepromazine and mepazine and of the phenylthiazole derivative amiphenazole on the pain-threshold-raising action of methadone is described. The tests were made in untrained human subjects by two algesimetric methods, one involving thermal stimulation of the skin, and the other mechanical stimulation of the finger nail-bed. The thermal method was reliable, whereas the mechanical one did not disclose analgesic potency. Chlorpromazine and acepromazine, proved to have analgesic activity but did not significantly increase the analgesic effect of methadone. Mepazine, which lacked analgesic activity, antagonised the methadone analgesia. Amiphenazole was found to exert an analgesic action by itself but it did not decrease the potency of methadone. The side-effects of methadone were increased by chlorpromazine and acepromazine, but were uninfluenced by mepazine and amiphenazole.

Several phenothiazine derivatives are known to produce a potentiation of the effect of analgesics and hypnotics. Chlorpromazine [2-chloro-10-(3′-dimethylaminopropyl)phenothiazine] was shown by Courvoisier, Fournel, Ducrot, Kolsky and Kretschet[1], to potentiate the analgesic effect of morphine in mice. This observation was confirmed by several clinical investigations[2–7]. Acepromazine [2-acetyl-10-(3′-dimethylamino-propyl)phenothiazine], in animal experiments with the radiant heat stimulation method of d'Amour and Smith, potentiated morphine as did chlorpromazine[8]. Mepazine [10-(1′-methyl-3′-piperidylmethyl)phenothiazine] also potentiated the analgesic activity of morphine studied by the hot-plate method in mice[9]. Some clinical observations seemed to confirm this finding. No experimental data were, however, presented[10]. Amiphenazole (2,4-diamino-5-phenylthiazole) is structurally unreleated to the aforementioned phenothiazine derivatives. This substance was introduced clinically to alleviate the respiratory depression, vomiting, drowsiness, depression of the cough reflex and even addiction of morphine[11]. Clinical trials showed that it did not reduce the analgesic action of morphine[12,13].

The present paper deals with the influence of these substances on the analgesic action of methadone. Previously we found that methadone produced a significant analgesic effect in untrained human subjects

studied by a thermal method, radiant-heat stimulation of the skin, and a highly probable analgesic effect tested by an electric method, electric stimulation of the tooth pulp[14].

We now compare the same thermal method with a mechanical method, pressure stimulation of the nail bed.

Material and Methods

Subjects. The experiments were made on 184 untrained, healthy male and female subjects of from 20 to 25 years. Each subject was used for one experiment only and was informed about its nature.

Thermal method. The radiant-heat stimulation method was used. The stimulus, which had a constant intensity of 300 millicalories/sq. cm./sec., was produced by a Hardy-Wolff-Goodell Dolorimeter (Williamson Development Co., West Concord, Mass.) and was applied to the unblackened skin of the forehead. The time required to reach the pain threshold was denoted as the *reaction time.* The analgesic effect is reflected in a prolonged reaction time.

Mechanical method. To obtain a mechanical pain stimulus, the apparatus described by Hardy, Wolff and Goodell[15], manufactured by Williamson Development Co., West Concord, Mass., was used. The apparatus consists of a plunger surrounded by a metal sleeve, within which is mounted a steel spring. The force in grams exerted on the finger nail-bed by the tip of the plunger was read from the scale of the instrument. The force was always applied to the base of the nail, and the subject was instructed to report the first pain sensation. Each measurement of the pain threshold was the mean of 10 readings, one on each finger. An analgesic action is reflected in an increase in the threshold value.

Drugs and dosage. Methadone hydrochloride, 6 mg., chlorpromazine hydrochloride (Hibernal, Leo) 15 mg., acepromazine hydrochloride (Plegicil, Pharmacia) 5 mg., mepazine acetate (Lacumin, Lundbeck) 15 mg., amiphenazole hydrochloride (Fenamizol, ACO) 7·5 mg., or, 1 ml. of saline were given either singly or in combination by intramuscular injection.

Performance of experiments. Threshold determinations were made 15–30 minutes before and 1 and 2 hours after drug administration. All subjects were instructed to rest in an armchair for 30 minutes before and during the experiment. Side-effects occuring during and after the experiment were noted by the subjects. The threshold determinations were carried out as double-blind tests; both methods were used simultaneously.

Statistical analyses. The analgesic effect was computed as the post-medication deviation from the pre-medication threshold value as follows.

In each test subject, X_0 denotes the pre-medication threshold value, and X_1 and X_2 the first and second post-medication threshold value, respectively. A value, Y, of the analgesic effect in each test subject is derived from the following formula: $Y = X_1 + X_2 - 2X_0$. Y represents

the response of each subject. On the basis of the Y values for the test subjects with the *same* medication, the mean, $\overline{Y}$, and the standard error of the mean were calculated.

The significance of the difference between the $\overline{Y}$ values for two medications compared was tested by *t* analysis. The degree of significance was estimated as follows:

Significant:	$P < 0{\cdot}001$: symbol +++
Highly probable:	$0{\cdot}001 < P < 0{\cdot}01$: symbol ++
Probable:	$0{\cdot}01 < P < 0{\cdot}05$: symbol +
Not probable:	$0{\cdot}05 < P$: symbol O

These symbols are used in Figures 1 and 2.

The means of X_0, X_1 and X_2 were computed for each drug treatment and were symbolised by $\overline{X}_0$, $\overline{X}_1$ and $\overline{X}_2$. In Figures 1 and 2, $\overline{X}_0$ was put at the origin. The deviations of $\overline{X}_1$ and of $\overline{X}_2$ from $\overline{X}_0$ were plotted in the Figures to show the variation in analgesic effect of the drugs and drug combination during the post-medication period. The total analgesic potency of each medication must be judged from the estimates of variance. The standard error of the $\overline{Y}$ values varied appreciably.

Each $\overline{Y}$ value and each curve represents 11–13 test subjects, except in the acepromazine experiments (Fig. 1B and 2B), where only 7 test subjects were available for each administration.

Results

Methadone and Chlorpromazine (Fig. 1A and 2A). Both methadone and chlorpromazine exerted an analgesic action tested by the thermal method. Their combined administration had a slightly greater effect, which differed highly probably from that of methadone, but not from that of chlorpromazine. With the mechanical method, only chlorpromazine produced a probable analgesic effect. The action of all other medications was nil.

Methadone and Acepromazine (Fig. 1B and 2B). Both methadone and acepromazine had an analgesic action tested by the thermal method. Their combined administration produced no increase in action. No effect was disclosed by the mechanical method.

Methadone and Mepazine (Fig. 1C and 2C). Only methadone had a significant activity, whereas neither mepazine nor the combination of the two drugs exerted any analgesic action. The antagonistic effect of mepazine on methadone was highly probable. The mechanical method failed to show any effect.

Methadone and Amiphenazole (Fig. 1D and 2D). Methadone and amiphenazole produced a highly probable and a significant degree of analgesia, respectively. There was no difference, however, between the effect of the drug combination and methadone. With the mechanical method, the combination had a probable effect which did not, however, differ from that of methadone. The effect of the other medications was nil.

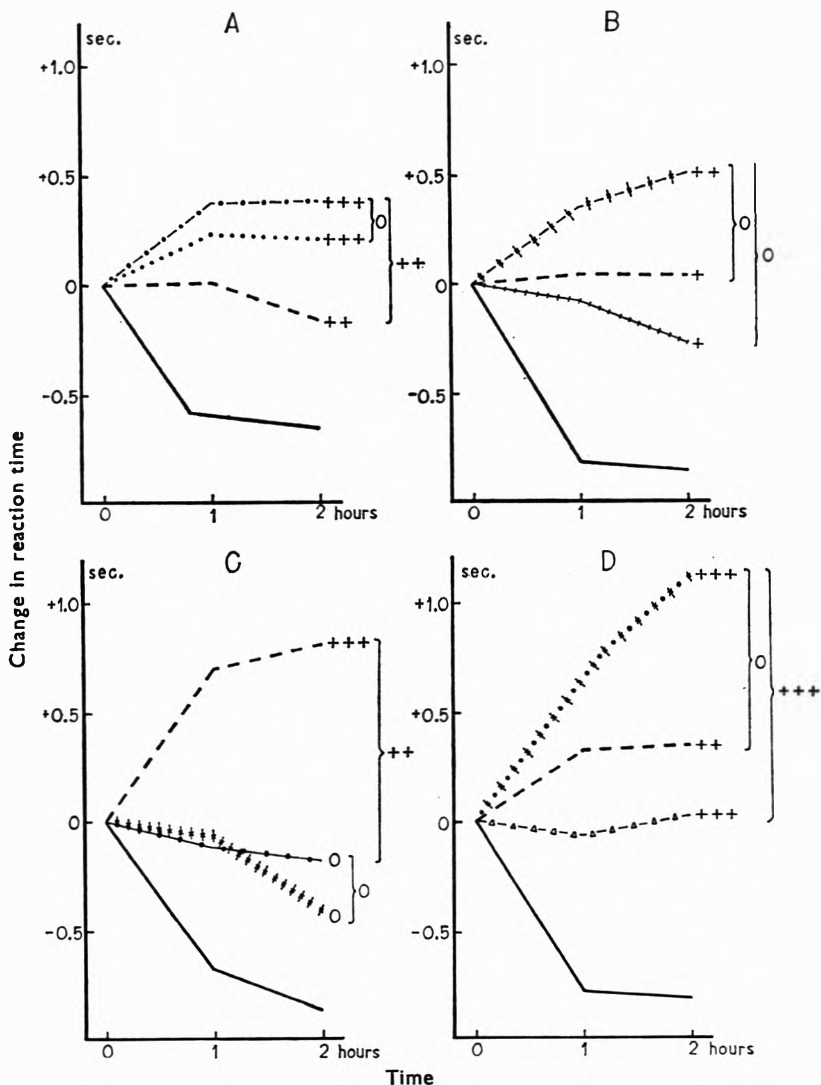

FIG. 1. Average analgesic effect tested by radiant-heat stimulation (see text for further detail).

Symbols by brackets denote statistical significance between curves. +++ = P<0·001; ++ = P 0·001 – 0·01; + = P 0·01 – 0·05; O = P>0·05.

– – – – methadone	mepazine
• • • • • • • chlorpromazine	methadone+mepazine
methadone+chlorpromazine	amiphenazole
acepromazine	methadone+amiphenazole
methadone+acepromazine	placebo

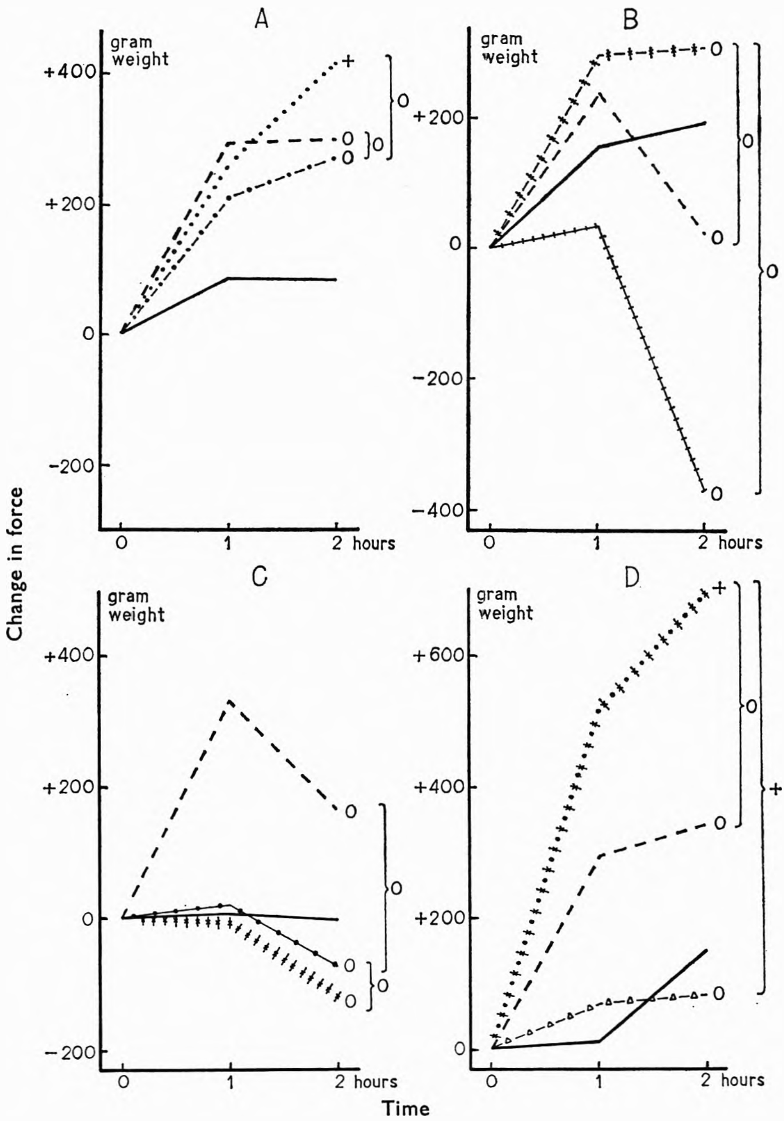

FIG. 2. Average analgesic effect tested by pressure stimulation. Key to symbols is below FIG. 1.

DISCUSSION

Studies on the analgesic action of drugs are associated with considerable methodological difficulties. Thus, the conditions encountered in experimentally induced pain and in clinical pain differ both aetiologically and psychologically. But, study of the synergism and antagonism of different drugs by means of double-blind tests in clinical pain is extremely laborious, in the large number of patients, workers and time involved. For this reason, the present study was made on experimental pain.

When using the experimental pain methods, it is important to bear in mind that different methods may give varying results with the same drug[14]. We therefore considered it reasonable to use two methods with different types of stimulus concurrently; one thermal, the other mechanical.

We showed previously[14] that untrained test subjects could be used, if 10–12 subjects were used for each drug administration. Therefore, except in the acepromazine series, each medication in the present investigation was tested in the same number of subjects.

The thermal method disclosed methadone analgesia in all experiments. The effect of methadone in the acepromazine experiments (Fig. 1B) was only probable, by reason of the small numbers in the experiment.

In the other experiments, highly probable (Fig. 1A and B) and significant (Fig. 1C) effects were obtained.

A constant finding, both in this investigation and in a previous one[14], is the fall in the placebo curves.

Of the three phenothiazine derivatives tested, two exerted an analgesic effect, one, chlorpromazine, was significant, the other, acepromazine being probable. On the other hand, mepazine lacked any analgesic action. As regards the synergistic action of these derivatives on the analgesia produced by methadone, no definite effect could be observed. Mepazine, was, on the contrary, antagonistic.

It is thus interesting to note that mepazine, which had no analgesic potency, antagonised methadone, whereas chlorpromazine and acepromazine, which had analgesic actions of their own, did not antagonise methadone.

The analgesic action of chlorpromazine has been demonstrated earlier in experimental pain, using another kind of thermal method[16], as well as in post-operative pain[7]. The synergism between phenothiazine derivatives and morphine found in previous clinical reports cannot, however, be extended to be valid for phenothiazine derivatives and methadone in experimental pain.

The phenylthiazole derivative amiphenazole has been shown not to reduce the analgesic action of morphine in clinical trials[12,13]. Our results in Figure 1D show that, in experimental pain also, amiphenazole does not antagonise methadone analgesia. This investigation also shows amiphenazole to be analgesic.

The mechanical method did not reveal any analgesic action in any instance, and seems unsuitable for this purpose. It should however, be noted that in the original description of this method[15], the test area was the skin of the forehead. In the present investigation, this area was used for the thermal method and could not be used simultaneously for both methods.

Side-effects. Of the three phenothiazine derivatives mepazine lacked side-effects, whereas chlorpromazine and acepromazine produced drowsiness, nasal congestion, orthostatic hypotension, palpitation and nausea. In combination with methadone, the incidence of hypotension and nausea was still higher. The hypotensive action of acepromazine was greater

than that of chlorpromazine, which caused us to administer smaller doses of the former.

The clinical experience of amiphenazole as an antagonist of the side-effects of morphine[11-13] is interesting, in view of our finding that amiphenazole did not reduce the side-effects of methadone.

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A SPECTROPHOTOMETRIC METHOD FOR THE ESTIMATION OF RESERPINE

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A spectrophotometric method for the estimation of reserpine in crude drugs and pharmaceutical preparations has been described. The method is capable of detecting microquantities of reserpine (0·25 μg./ml.). It is capable of estimating reserpine in crude drugs, alkaloidal mixtures, reserpine and serpentina tablets. The reserpine content in samples of Dehradun and Bengal varieties of *R. serpentina* has been found to be 0·117 and 0·103 per cent respectively.

A NUMBER of methods have been described for the assay of reserpine in pharmaceutical preparations[1–4], but not all are suitable for the estimation of reserpine in crude drug.

Many of the alkaloids present in rauwolfia plants have been grouped as feebly basic and strongly basic. Reserpine can be separated from more polar alkaloids by extraction from acidic solutions with chloroform, and in the present investigation an attempt has been made to estimate reserpine in crude drugs and alkaloidal mixtures by using this property.

As very few methods are available for the estimation of reserpine in natural preparations, it was considered advisable to develop a spectrophotometric method suitable for the micro-determination of this alkaloid. The method is based on the selective solvent extraction of reserpine from other alkaloids and its estimation in the ultra-violet region at 268 mμ. The method has been found to be simple and accurate in the present study.

Material and Method

The present work comprises two parts: the suitability of the spectrophotometric method for the estimation of reserpine in various solutions, and its application to the estimation of reserpine in crude drugs and pharmaceutical preparations.

A Beckman Spectrophotometer Model DU with 1 cm. standard silica cells was used and the absorbance maximum of reserpine in chloroform solvent found to be at 268 mμ. The relation between the concentration of reserpine and light absorption was established according to the following procedure.

Ten ml. of chloroform solution (containing 1 mg. of reserpine) was warmed with 10 ml. of 0·05N sulphuric acid with constant stirring to remove the chloroform. The acid solution was re-extracted with chloroform, the volume made up to 100 ml. and light absorption at various concentrations measured. The observations in Table I show a linear relation.

To remove polar and other interfering alkaloids and basic impurities from alkaloidal mixtures and crude drugs and to prepare a standard curve, the following procedure was adopted.

TABLE I

RELATION BETWEEN CONCENTRATION OF RESERPINE AND LIGHT ABSORPTION AT 268 Mμ WITH SLIT WIDTH AT 0·94 MM.

Conc. μg./ml.	0·25	0·50	1·00	2·00	4·00	6·00	8·00	10.00
Optical density	0·006	0·013	0·022	0·051	0·097	0·155	0·194	0·260

Aliquots, containing 1 mg. of reserpine in chloroform, were extracted with 4 × 10 ml. of 0·1N HCl, shaking every time for 2–3 minutes. The combined acid extract was treated with 5 ml. of chloroform, adding the wash to the main reserpine solution. After treating with 10 ml. of 0·05N H_2SO_4, chloroform was removed on a water bath as previously. The acid solution was quantitatively transferred to the separating funnel and extracted thrice with 10 ml. portions of benzene, shaking vigorously every time for 10 minutes. The combined benzene extract was then washed with 5 ml. of 0·05N H_2SO_4 adding the wash to the main acid solution. It was then extracted with 6 × 10 ml. of chloroform. The combined chloroform extract was washed with 5 ml. of 0·1N HCl and evaporated to dryness in a porcelain dish to remove traces of benzene. The residue was redissolved in chloroform and the reserpine content estimated at different concentrations. The findings are shown in Table II from which it may be seen that the relation is again linear.

TABLE II

RELATION BETWEEN CONCENTRATION OF RESERPINE IN μG./ML. AND LIGHT ABSORPTION AT 268 Mμ, WITH SLIT WIDTH 0·94 MM., AFTER TREATMENT WITH HYDROCHLORIC ACID AND BENZENE

Conc. μg./ml.	0·25	0·50	1·00	2·00	4·00	6·00	8·00	10·00
Optical density	0·004	0·010	0·020	0·041	0·088	0·137	0·187	0·252

On an analysis of data presented in Tables I and II, it will be seen that hydrochloric acid extraction did not result in any significant loss of reserpine in as much as only 2·5 per cent deviation in the recovery of reserpine (up to 1 mg.) with 4 × 10 ml. of hydrochloric acid was observed. Also, extraction with benzene did not interfere with the reserpine estimation provided that the solvent was completely removed from the extracts. Neither hydrochloric acid nor benzene thus showed the selective solvent property of chloroform which was found to be the most suitable solvent for the removal of reserpine from acid extracts.

After ensuring the suitability of the above method for the accurate estimation of microquantities of reserpine, it was considered necessary to determine the sensitivity of the test at different concentrations of the alkaloid and establish its optimal range. The estimations were made in graded concentrations of 0·5 to 4 mg. of reserpine and its recovery limits determined spectrophotometrically (Table III).

TABLE III

RECOVERY, PER CENT, OF KNOWN QUANTITIES OF RESERPINE AFTER HYDROCHLORIC ACID, SULPHURIC ACID AND BENZENE TREATMENT

Reserpine content mg.	Reserpine detected mg.	Deviation per cent
0·5	0·500	—
1·0	0·975	− 2·5
2·0	1·900	− 5·0
3·0	1·500	− 50·0
4·0	1·700	− 57·0

A reference to the figures in Table II and III indicates that the method is suitable for estimating reserpine in a range of 0·025 to 1 mg., beyond which the method becomes insensitive.

Estimation of Reserpine from known Alkaloidal Mixtures

After determining the suitability of the method for the estimation of known quantities of pure reserpine, the study was extended to the estimation of this alkaloid in the presence of other alkaloids of *R. serpentina.* Solutions of known quantities of reserpine, rescinnamine, serpentine, ajmaline, yohimbine and methyl reserpate were prepared in chloroform and treated with hydrochloric acid, sulphuric acid and benzene to find out whether the presence of other alkaloids interferred with the estimation of reserpine. The findings are shown in Tables IV and V.

TABLE IV

SHOWING PERCENTAGE RECOVERY OF RESERPINE FROM SOLUTIONS CONTAINING RESCINNAMINE AND RESERPINE

Sample No.	Reserpine µg.	Rescinnamine µg.	Reserpine recovered µg.	Deviation per cent
1	400	100	400	—
2	400	100	410	+ 2·5
3	400	200	410	+ 2·5
4	400	200	415	+ 3·7
5	400	350	414	+ 3·5
6	400	500	450	+ 12·5

TABLE V

SHOWING PERCENTAGE RECOVERY OF RESERPINE FROM ALKALOIDAL MIXTURES CONTAINING RESERPINE, AJMALINE, SERPENTINE, YOHIMBINE AND METHYL RESERPATE

Mixture No.	Reserpine µg.	Other alkaloids µg.	Reserpine recovered µg.	Deviation per cent
1	400	200	410	+2·5
2	400	200	410	+2·5
3	400	400	415	+3·7
4	400	400	410	+2·5

From Tables IV and V it is evident that common alkaloids present in *R. serpentina* in usual quantities, did not interfere in the assay of reserpine to any appreciable extent, excepting rescinnamine which interferes with reserpine estimation beyond a concentration of 350 μg. Since such a high concentration of rescinnamine is not present in the plant and

pharmaceutical preparations, this was not likely to affect the accuracy of the method. It would thus appear that rescinnamine is of limited solubility in benzene and the samples should be very thoroughly shaken to eliminate it completely.

Estimation of Reserpine in Tablets

Commercial samples of ten reserpine tablets were weighed and powdered and known quantities extracted with chloroform and filtered. The aliquots were analysed for reserpine content. The results are given in Table VI.

TABLE VI

RESERPINE CONTENT OF COMMERCIAL TABLETS

Sample No.	Reserpine content as labelled mg.	Reserpine recovered mg.	Deviation per cent
1	0·1	0·98	−2·0
2	0·1	0·096	−4·0
3	0·1	0·103	+3·0
4	0·1	0·102	+2·0
5	0·1	0·105	+5·0

From the above observations it is evident that colouring and other binding materials, present in the tablets did not interfere with the analysis of reserpine and it could be estimated with an accuracy of $\pm$ 4 to 5 per cent.

Estimation of Reserpine in Crude Drug

Though reserpine has now been established as the major alkaloid of *R. serpentina*, the plant contains a large number of other alkaloids which vary from species to species (Chatterjee[5,6]). There is thus considerable variation in the major and minor alkaloidal content in different varieties of *R. serpentina* growing in different regions of India. According to Hoffman[7] reserpine content in *R. serpentina* is about 0·14 per cent.

In the present investigation, the reserpine content in different samples of roots of *R. serpentina* has been analysed spectrophotometrically. Two important varieties of *R. serpentina*, collected from Dehradun and Bengal were selected for the present investigation.

TABLE VII

THE PERCENTAGE OF RESERPINE IN SAMPLES OF DEHRADUN AND BENGAL VARIETIES OF *R. serpentina*. THE RESULTS ARE THE AVERAGES OF FIVE SETS OF EXPERIMENTS IN EACH CASE

Variety of *R. serpentina*	Batch No.	Reserpine content per cent	Mean per cent
Dehradun	I	0·125	0·117
"	II	0·110	
Bengal	I	0·110	0·103
"	II	0·097	
Crude drug tablets	I	0·120	
"	II	0·120	0·126
"	III	0·140	

One gram of powdered root was extracted with chloroform in a micro-soxhlet apparatus for four hours, filtered and the volume made to 50 ml. An aliquot of 10 ml. was analysed for reserpine content as detailed in the section of preparation of standard curve. The findings are shown in Table VII.

As the reserpine content in different varieties of *R. serpentina* has not been definitely estimated, for comparison for the above figures, it was considered advisable to add a known quantity of reserpine to the original chloroform extract of the crude drug to find out whether the results of analysis in these circumstances would compare with the combined figures of reserpine found in the plant and the known quantity added to it. The results are shown in Table VIII.

TABLE VIII

SHOWING PERCENTAGE RECOVERY OF RESERPINE PRESENT IN CRUDE DRUG EXTRACTS TO WHICH ADDITIONAL QUANTITIES OF THE ALKALOID WERE ADDED.

Extract No.	Quantity of reserpine µg.	Quantity of additional reserpine added µg.	Total quantity of reserpine found µg.	Deviation per cent
1	250	200	470·0	+4·4
2	250	400	670·0	+3·1
3	200	200	412·0	+3·0
4	200	400	625·0	+4·1

From the above figures, it will be observed that the method has been found to be sufficiently accurate to measure the correct quantities of reserpine in these experiments with an accuracy of 3 to 4 per cent.

DISCUSSION

In the present investigation, reserpine content in roots of *R. serpentina* and other pharmaceutical preparations, has been analysed spectrophotometrically. In the first stage of this method, hydrochloric acid extraction of the chloroform extract did not cause any loss of reserpine but helped to removing interfering alkaloids, like ajmaline, serpentine, yohimbine and reserpic acid. Reserpine, being a feebly basic alkaloid could be easily taken up in chloroform from sulphuric acid solution in which it has been found to be soluble to a limited extent (1 mg./10 ml.). Extraction with benzene did not affect the content of reserpine in samples though it removed rescinnamine, colouring and other interferring basic impurities.

The method is applicable to samples containing micro-quantities of reserpine (0·25 µg./ml.) and has an added advantage that rescinnamine does not interfere with the assay (up to 350 µg./10 ml.). It has been observed that the method is suitable for analysis of crude drugs in which reserpine is present along with other alkaloids. The average reserpine content in samples of *R. serpentina* (Dehradun and Bengal varieties) has been found to be 0·117 and 0·103 per cent respectively.

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ON THE METABOLISM OF SOME AROMATIC NITRO COMPOUNDS BY DIFFERENT SPECIES OF ANIMAL

PART III. THE TOXICITY OF THE DINITROPHENOLS, WITH A NOTE ON THE EFFECTS OF HIGH ENVIRONMENTAL TEMPERATURES

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An account is given of an investigation of the toxicity of the dinitrophenols to small laboratory animals. A new pattern of toxicity to chemical structure has been discovered and is discussed in relation to other dinitro-compounds. Also the effects of high temperatures on the toxicity of the dinitrophenols have been studied.

THE basic structure of 2:4-dinitrophenol is present in 2:4-dinitro-*o*-cresol (DNC) and a number of similar compounds widely used as herbicides in agriculture. Because all these substances are poisonous to man and to animals it is desirable to seek alternatives that are less toxic. Considerable information exists on some of the substances derived from 2:4-DNP (e.g., Lawford, King and Harvey[1]), but information on its isomers is limited.

Some years ago Magne, Mayer and Plantefol[2] described the main effects of these substances on dogs, but examination of their results showed that further studies would be necessary to supply information on acute toxicities to small laboratory animals at different temperatures, elimination rates from the blood as indications of their possible accumulation in the body, and stimulation or depression of the oxygen consumption. One result of the present study was to reveal a new pattern of chemical structure to toxicity. This bears some resemblance to that proposed by Magne and colleagues, but it also differs from it in two marked respects. This paper describes this relationship, and the results of the other tests mentioned above.

The Preparation, Relationships and Properties of the Dinitrophenols

All six isomers are readily accessible, and three at least are available commercially (2:4-DNP, 2:5-DNP and 2:6-DNP). 2:4-DNP is prepared by the direct nitration of phenol, and no other isomers appear as by-products[3]. 2:4-DNP and 2:6-DNP are prepared by sulphonation and nitration of *o*-nitrophenol, and 2:3-DNP, 2:5-DNP and 3:4-DNP by the nitration of *m*-nitrophenol[4,5]. 3:5-DNP is prepared by replacement of one nitro group by methoxyl in *sym*-trinitrobenzene[6] and demethylation of the dinitroanisole by anhydrous aluminium chloride[7]. The relationship of the isomers and their parent substances is shown in Figure 1 and the main properties in Table I. All the isomers are insoluble in cold water, and some are volatile in steam[5]. They form bright yellow or orange salts soluble in methyl ethyl ketone. This property has been used by Parker[8] for the analysis of 2:4-DNP and DNC in biological fluids.

OH — Conc. H_2SO_4 (3) → OH, HSO_3, HSO_3 — HNO_3 → OH, NO_2, NO_2 2:4-DNP

HNO_3

OH, NO_2 — Conc. H_2SO_4 → OH, NO_2, HSO_3 + OH, SO_3H, NO_2 → HNO_3 → OH, O_2N, NO_2 2:6-DNP

OH, NO_2 — HNO_3 (sp. gr. 1·225) (4, 5) → OH, NO_2, NO_2 2:3-DNP; OH, NO_2, O_2N 2:5-DNP; OH, NO_2, NO_2 3:4-DNP

NO_2, O_2N, NO_2 — NaOMe MeOH (6) → OMe, O_2N, NO_2 — $AlCl_3$ (7) → OH, O_2N, NO_2 3:5-DNP

FIG. 1. Relationships and preparations of the dinitrophenols. Numbers in brackets are references.

Materials and Methods

Preparation of the Isomers

2:4-DNP, 2:5-DNP and 2:6-DNP were obtained commercially, and the remainder prepared by the methods outlined above. Methyl derivatives were also prepared and the melting points of these and of the parent dinitrophenols checked with those given in the literature[9].

Nitrogen Determination and Solubilities

The micro-Kjeldahl technique was used as an alternative method for determining solubilities. Since it is well known that sulphuric acid digestion of aromatic polynitro compounds gives low and variable recoveries of nitrogen, experiments on preliminary reduction were carried out to overcome these difficulties. As a result of these the following method was devised.

To a solution of the dinitrophenol in sodium hydroxide (2–3 mg. in sodium hydroxide, 10 per cent w/v, 1·0 ml.) was added sodium dithionate (50–100 mg.). After 1·5 hours the dark coloured fluid was boiled (1·0 min.), cooled and treated with sulphuric acid (50 per cent v/v, containing selenium dioxide, 2·0 per cent w/v, 2·0 ml.) and digested in the usual way (3·0 hours). The digest was distilled with alkali using a Markham apparatus, the ammonia trapped in boric acid (2 per cent w/v, 5·0 ml.) and titrated with sulphuric acid (0·01N) using a screened indicator. Recoveries of nitrogen from standard solutions of the dinitrophenols in alkali were good. The average recovery from eleven determinations on the six isomers was 100·6 per cent, with a range of 98–106 per cent. The solubilities of the isomers determined by this method are given in Table I.

Chromatographic Separation

During the preparation of the isomers preliminary attempts were made to check their homogeneity by one dimensional paper chromatography. These were partially successful and are shown in Table I. No special precautions were taken to control temperature which ranged from 17–20°.

Estimation of Dinitrophenols

Parker's method (*loc. cit.*) was employed using a Unicam Spectrophotometer and a Hilger "Spekker". Ilford Filter 601 proved to be satisfactory for use with the "Spekker" although recoveries differed for the six isomers. The efficiency of the method for the six compounds in decreasing order was found to be 3:4-DNP; 2:4-DNP; 2:6-DNP; 2:3-DNP; 2:5-DNP; 3:5-DNP.

LD50 Determinations

These were made at 18–20°, 35–37°, 39–41°, representing moderate, warm and hot summer day temperatures, by methods already described for other dinitro-aromatics[10].

Solutions of the dinitrophenols in the requisite quantity of alkali were administered by intraperitoneal injection. Before, during, and after

TABLE I

SOME PROPERTIES OF THE DINITROPHENOLS

General properties						Chromatography (R_F values)			
Dinitrophenol description $-OH = 1$	Crystal form	m.p.	k (25°)	Solubility in in water 35–36° g./100 ml. (*a*)	m.p. cf dinitroanisole	*n*-Butanol, acetic acid, water 4:1:5	*n*-Butanol saturated 5N ammonia	Benzene, acetic acid, water 1:1:2 (*b*)	Colour of alkaline spot (*c*)
						Ascending: 20 hr.	Descending: 5 hr.	Ascending: 3·5 hr.	—
2:3-	Yellow monoclinic prisms	144°	$1{\cdot}3 \times 10^{-5}$	0·22	119°	0·95	0·54	0·83	Red-orange
2:4-	Yellow rhombs or needles	114–5°	$1{\cdot}0 \times 10^{-4}$	0·079	94° (89°)	0·90	0·55	0·95	Yellow
2:5-	Yellow needles	104°	$0{\cdot}7 \times 10^{-6}$	0·068	97°	0·92	0·57	0·95	Red-orange
2:6-	Yellow rhombs	63·5°	$2{\cdot}7 \times 10^{-4}$	0·042	117·5° (118°)	0·86	0·49	0·95	Deep yellow
3:4-	Pale brown or colourless prisms	134°	$4{\cdot}3 \times 10^{-8}$	0·23	70°	0·93	0·59	0·47	Pale yellow
3:5-	Colourless monoclinic prisms	122–3°	$2{\cdot}1 \times 10^{-4}$	0·16	105–6°	0·92	0·60	0·69	Lemon yellow

Notes:

(*a*) The determination was made on a solution of the dinitrophenol prepared by shaking up an excess with water and allowing to stand in warm room for 48 hr.
(*b*) This must be freshly prepared otherwise very streaky runs obtained.
(*c*) Air dried papers held in ammonia vapour.

administration the animals were maintained on normal diets and water *ad lib.*

Elimination rates from the blood. These were determined on mice, rats and rabbits by methods previously described[11].

Oxygen consumption. Following general procedures recommended in a previous paper[12] the effects of sub-acute and low doses were assessed on groups of rats and guinea pigs. Six animals were used for each dose level, and estimations were made 1·0–1–1·25 hours after administration of the dinitrophenol. Measurements were made on a "group" and not on an individual basis. One four point assay of DNC was made on guinea pigs (Fig. 2).

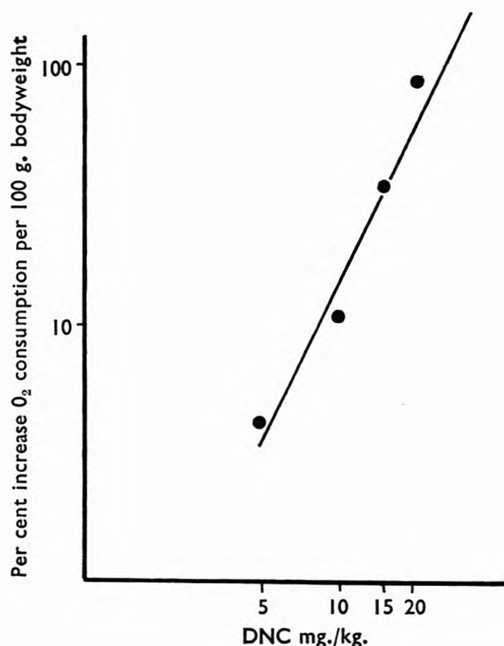

FIG. 2. Oxygen consumption of guinea pigs—average weight of group 1500 g., in response to varied doses of DNC given intraperitoneally. Measurements taken 1·0–1·25 hours after injection. Line by observation.

Body temperatures. These were determined rectally on rats after single sub-lethal doses administered intraperitoneally after three normal readings had been taken at approximately half hour intervals (Fig. 3).

Protection Against the Effects of High Environmental Temperatures

Mice and rats were given a wide range of dose levels of DNPs and DNC and placed in hot room at the highest of the three temperature ranges (37–41°). Varying treatments were then given to animals dosed with dinitrophenols having known stimulant effects (2:4-DNP and DNC) to

investigate the possibility of countering or reducing the exacerbating effect of heat on their toxic activity. The treatments included sponging the animals with water and allowing them continuous contact for 5–6 hours with a little water at the bottom of their cages, immediately, and 1 hour after intraperitoneal injection of the dinitrophenol, parallel and delayed treatment with 4-methyl-2-thiouracil, removal of poisoned animals to a cold room (7°) and treatment with three of the less toxic dinitrophenols (2:3-DNP, 2:5-DNP and 3:4-DNP) with known hypothermic properties. With the exception of those animals removed to the cold room the remainder were returned immediately to the hot room after the other treatments.

TABLE II

LD50 VALUES OF DINITROPHENOLS FOR RATS AND MICE AT THREE TEMPERATURE RANGES, WITH MLD VALUES FOR DOGS (MAGNE AND COLLEAGUES *loc cit.*)

	mg./kg.				
	Rats	Mice	Mice	Mice	Dogs
Dinitrophenol	18–21°	18–21°	35–37°	39–41°	Room temp. (?)
2:3-	190	200	$>160<175$	$>160<175$	1000
2:4-	35	36	35	<5 (*a*)	30
2:5-	150	273	$\sim$250	$\sim$200	100
2:6-	38	45	37	<10 (*a*)	50
3:4-	98	112	$\sim$115	$>100<110$	500
3:5-	45	50	47	50	500

Note.—(*a*) At these dose levels the mortalities were 100 per cent.

TABLE III

COMPARISON OF LD50 VALUES OF DINITROPHENOLS (LD50 FOR 2:4-DNP = 1)

Dinitrophenol	Rats	Mice
2:3-	5·4	5·5
2:4-	1·0	1·0
2:5-	4·3	7·6
2:6-	1·1	1·3
3:4-	2·8	3·1
3:5-	1·3	1·4

RESULTS

Acute Toxicity at Three Temperature Ranges

The summarised results showing LD50 values are given in Table II. A comparison is made also with the limited results obtained on dogs by Magne and colleagues. In view of the well-known action of high temperature in increasing the toxicity of DNC[13–15] it was not surprising that 2:4-DNP behaved similarly. Of the remaining isomers only 2:6-DNP showed a definite increase in toxic activity. Even moderately high temperatures (35–37°) have little effect on the toxicity of 2:4-DNP, although a few degrees higher, the response is very much increased. This suggests

TABLE IV

ELIMINATION RATES OF DINITROPHENOLS FROM THE BLOOD OF MICE AND RATS FOLLOWING A SINGLE LARGE DOSE GIVEN INTRAPERITONEALLY

	Mice					Rats				
Dinitrophenol	Dose mg./kg. (*a*)	Sampling times (min.)	µg. Dinitrophenol/g. blood: Range	µg. Dinitrophenol/g. blood: Mean ± SD	Half time of elimination (min.) (*b*)	Dose mg./kg. (*a*)	Sampling times (min.)	µg. Dinitrophenol/g. blood: Range	µg. Dinitrophenol/g. blood: Mean ± SD	Half time of elimination (min.) (*b*)
2:3-	90	7 12	33–74 8–16	47 ± 16 13 ± 3	2·7	90	15 30 60	70–136 31–107 4–13	104 ± 22 68 ± 32 7 ± 3	12·5
2:4-	20	30 60 90	32–37 21–33 9–14	34 ± 2 28 ± 4 12 ± 2	54·0	20	180 600	29–39 7–11	34 ± 3 8 ± 2	225·0
2:5-	180	6 12	33–60 9–15	47 ± 13 13 ± 4	3·3	90	7 12 40	8–23 8–11 2–4	14 ± 6 9 ± 2 3 ± 1	13·0
2:6-	30	15 120 240	49–71 28–51 10–38	62 ± 8 43 ± 10 21 ± 11	238·0	25	15 150 450	64–76 36–58 10–26	71 ± 10 50 ± 9 19 ± 9	210·0
3:4-	60	5 10	38–55 9–23	47 ± 9 18 ± 6	3·5	90	10 40	41–64 1–5	51 ± 11 4 ± 2	11·5
3:5-	30	4 8	44–61 5–22	56 ± 11 16 ± 8	2·7	30	5 8	22–90 10–19	61 ± 30 14 ± 4	2·1

Notes.

(*a*) Dinitrophenol dissolved in the requisite quantity of sodium bicarbonate or sodium hydroxide.
(*b*) Half time of elimination obtained by plotting mean log concentrations against time and reading off time (min.) for the initial (highest) concentration to be halved.

a "critical" temperature point, at least for mice and rats. Temperatures of 37–41° are not unknown in the United Kingdom, and are certainly common in the tropical countries where DNC may be used as an anti-locust measure.

A comparison of the relative toxicities of the dinitrophenols taking 2:4-DNP as unity is given in Table III. This suggests that on an acute toxicity basis the six isomers may be divided into two equal groups: most toxic—2:4-DNP, 2:6-DNP and 3:5-DNP (*meta* relation of both NO_2 groups common to these isomers); least toxic—2:3-DNP, 2:5-DNP and 3:4-DNP (*meta* nitrophenol group common to these isomers).

TABLE V

RATIOS OF LD50 VALUES OF DINITROPHENOLS TO THEIR HALF-TIMES OF ELIMINATION (× 100) FROM THE BLOOD

Dinitrophenol	Rats	Mice
2:3-	6·6	1·5
2:4-	642·8	154·2
2:5-	8·7	1·2
2:6-	552·6	528·8
3:4-	11·7	3·1
3:5-	6·0	4·2

Elimination Rates from the Blood

These are summarised in Table IV and the ratios of LD50 values to the half-times of elimination (× 100) in Table V. The rabbit detoxicates some of the dinitrophenols (2:3-DNP, 2:5-DNP, 3:4-DNP and 3:5-DNP) very rapidly, so much so that none could be detected in the blood 15 minutes after intravenous injection. This great ability to destroy "unnatural" chemicals is not uncommon with this animal which detoxicates and destroys even a proportion of DNC.

TABLE VI

EFFECT OF DINITROPHENOLS ON THE OXYGEN CONSUMPTION OF RATS AND GUINEA PIGS. MEASUREMENTS TAKEN 1·0–1·25 HOURS AFTER INTRAPERITONEAL INJECTION. (GROUP VALUES, SIX ANIMALS PER DOSE)

Dinitrophenols	Per cent increase in oxygen consumption per 100 g. body weight per min.					
	Rats			Guinea pigs		
	10	20 mg./kg.	100	10	20 mg./kg.	100
2:3-	−16	—	+ 3	+ 1	—	+ 4
2:4-	+17	+21	—	+25	+37 (*a*)	—
2:5-	− 7	—	23	− 5	—	+ 4
2:6-	− 2	+ 2	—	0	+13	—
3:4-	− 4	—	−10	< 1	—	+ 8
3:5-	− 5	−20	—	+18	+19	—

Note.—(*a*) Compare with results in a previous paper.[12]

Oxygen Consumption

Summarised results are given in Table VI. From these it is seen that the most active stimulant is 2:4-DNP and that of the others only 2:6-DNP and 3:5-DNP show any positive stimulation. The remaining isomers have the opposite effects, since oxygen consumption appears to

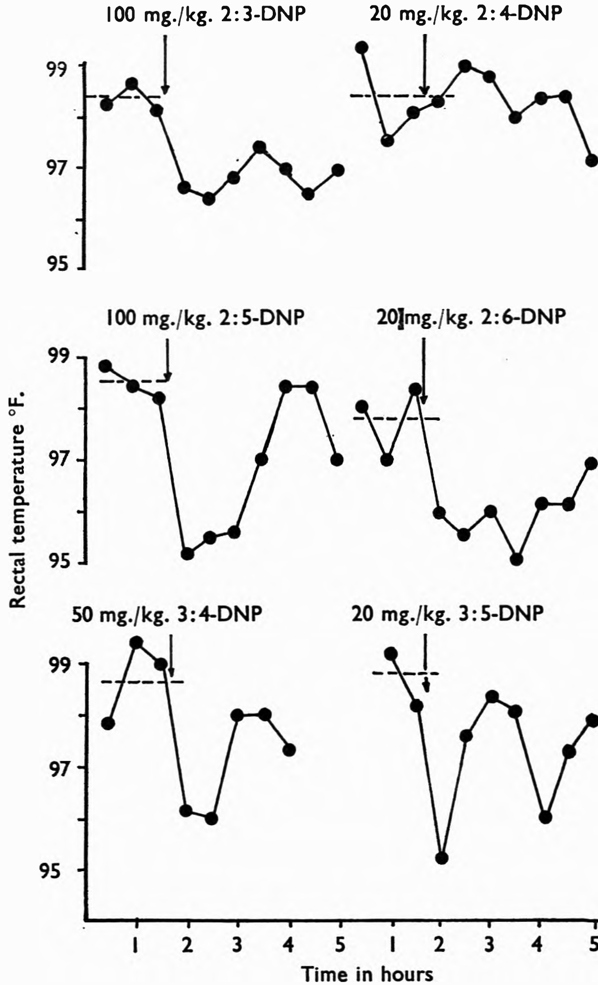

FIG. 3. Rectal temperature of adult rats given single sub-acute doses of the dinitrophenols by intraperitoneal injection after three normal readings.

be retarded, but these differences are insignificant statistically. On the other hand DNC shows a good relation between oxygen consumption and dose, and by simple observation the response appears almost linear. This compared satisfactorily with the results obtained on 2:4-DNP and reported in a previous paper[12]. The statistical analyses of these results will be the subject of a further communication.

Rectal Temperatures

A chronological diagram of the results is given in Figure 3, from which it is seen that only 2:4-DNP is hyperthermic, the remainder all show an initial hypothermia, that diminishes and then increases. This curious double "trough" is a somewhat analogous effect to the reverse and well known phenomenon in which isolated pyrogens (lipopolysaccharides) of bacterial origin cause a double "peak" in an animal's temperature response[16,17].

Protection Against the Effects of High Environmental Temperatures

The protective effect of cooling the animals with water immediately, or 1 hour after administration of 2:4-DNP or DNC is spectacular (Tables VII and VIII). Three of the dinitrophenols and 4-methyl-2-thiouracil were less able to protect, and removal of the animals to a cold room preserved the lives of animals dosed at 10 mg./kg. but not those at 20 mg./kg.

TABLE VII

PROTECTIVE ACTION OF VARIOUS MEASURES AGAINST THE EFFECTS OF HIGH TEMPERATURES ON MICE POISONED WITH DNC AND 2:4-DNP. (39–41° AND 35–37°)

	Mortality per cent over 6 hrs								
		Sponging and contact with water			Hypothermic dinitrophenols immediately after DNC			2:4-DNP	
Dose DNC mg./kg.	Hot room only 39–41°	Immediately after injection of DNC	1·0 hr. after injection of DNC	4-Methyl 2-thio-uracil (*a*)	2:3- at 100 mg./kg.	2:5- at 100 mg./kg.	3:4- at 75 mg./kg.	35–37°	39–41°
2·5	50	0	0	—	—	—	—	0	50
5·0	100	0	0	—	—	—	—	0	100
10·0	100	0	0	60	60	100	50	0	100
20·0	100	10	30	60	100	100	100	0	100
30·0	100	80	70	—	—	—	—	10	100

Note.—(*a*) 10–15 mg./kg. 4-methyl-2-thiouracil intraperitoneally immediately after injection of DNC.

TABLE VIII

PROTECTIVE ACTION OF VARIOUS MEASURES AGAINST THE EFFECTS OF HIGH TEMPERATURES ON RATS POISONED WITH DNC (39–41°)

	Mortality per cent over 6 hr.				
		Sponging and contact with water			
Dose DNC mg./kg.	Hot room only	Immediately after injection of DNC	1·0 hr. after injection of DNC	4-Methyl 2-thiouracil (*a*)	Removed to cold room (7°) 1·0 hr. after injection of DNC
2·5	50	0	0	—	—
5·0	100	0	0	50	—
10·0	100	0	0	100	0
20·0	100	60	100	—	100
30·0	100	100	100	—	—

Note.—(*a*) 10–15 mg./kg. 4-methyl-2-thiouracil intraperitoneally 1·0 hour after injection of DNC.

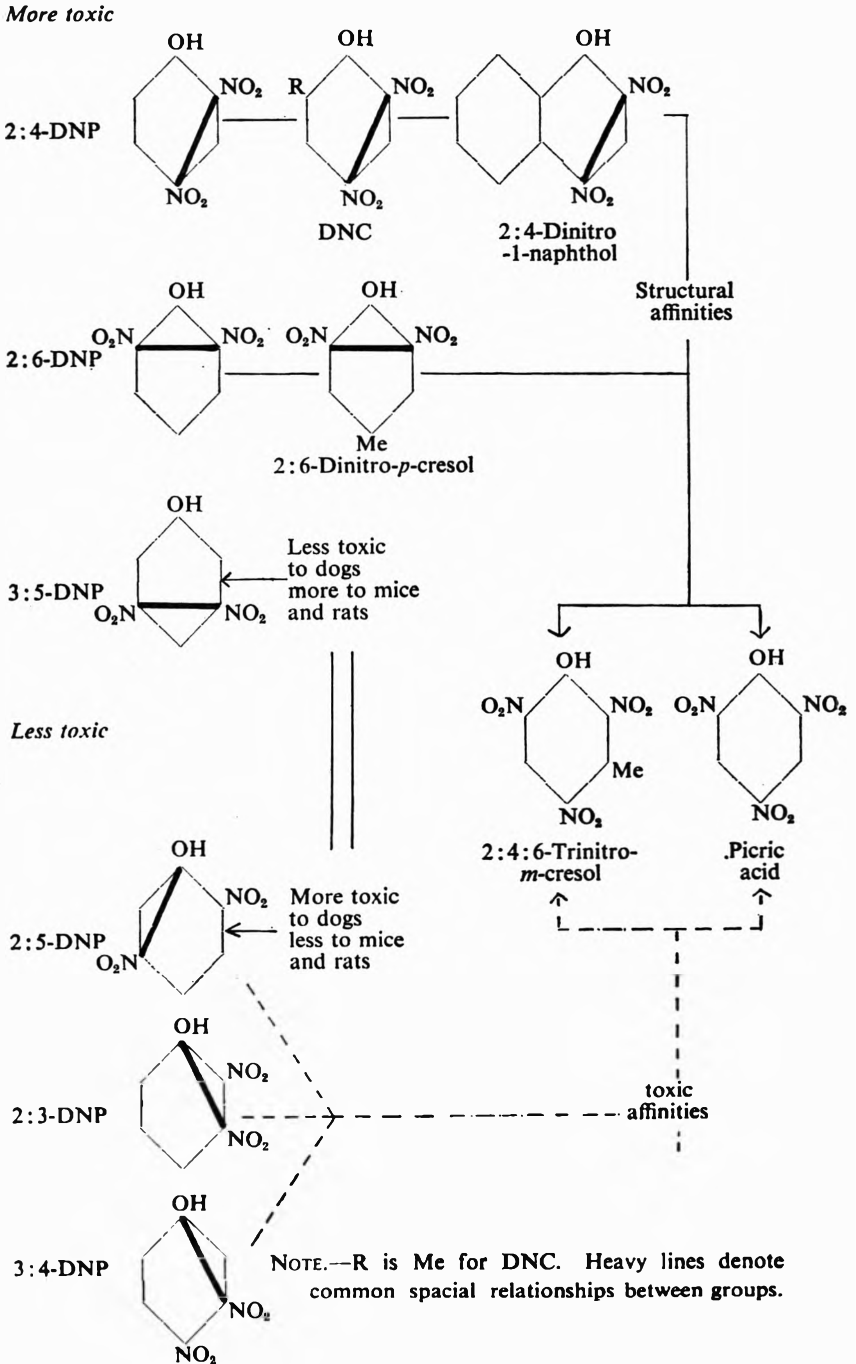

FIG. 4. Toxic relations between the dinitrophenols, 2:4:6-trinitro-*m*-cresol and picric acid.

Discussion

There seem to be alternative patterns of structure to chemical toxicity in the dinitrophenols, and studied in conjunction with the work of Magne and his colleagues there is a hint of an even wider structure-species relation in this interesting group of isomers.

The toxic and structural relationships of the alternatives is shown in Figure 4. The main difference between the two is the reversal in activity between 2:5-DNP and 3:5-DNP. The latter is far more toxic to small laboratory animals than the former, but on the other hand it shares certain common activities with the less toxic groups in both schemes. Thus, it is hypothermic (c.f. Magne and colleagues), it is rapidly eliminated from the blood, and its activity is not increased by high temperatures. The mode of action therefore remains unexplained.

Included in Figure 4 are the cresylic analogues of 2:4-DNP and 2:6-DNP (DNC and 2:6-dinitro-*p*-cresol) and also two closely related trinitro compounds, picric acid and 2:4:6-trinitro-*m*-cresol. From the various experiments reported it can be concluded that addition of an "inactive" group such as methyl, *sec*-butyl or hexyl to the basic 2:4-DNP and 2:6-DNP nucleus will produce relatively minor changes in their quantitative toxicity to man and animals. 2:4-Dinitro-1-naphthol has similar effects to 2:4-DNP. The addition of an "active" nitro-group will alter the degree and nature of the toxicity considerably.

Although the stimulant effects of heat on the action of DNC, 2:4-DNP and 2:6-DNP are severe, the benefits of sponging and contact with water are equally evident. The success of this treatment of severely poisoned rats and mice can not yet readily be explained. A decrease in the environmental temperature alone seems unlikely, because temperatures recorded in the wet cages soon reached the levels of those in the room and in the dry cages. How far skin cooling plays a part remains to be determined, and it is interesting to note that in rats severely poisoned by DNC, the head and shoulders become moist, even though no sweat glands can be identified. It should be remembered that while rats and mice excrete DNC relatively rapidly[15], man excretes it slowly, and therefore sponging and cooling treatment, to be useful, would have to be prolonged and efficient.

The effect of heat in increasing by many times the toxic action of two of the isomers and of DNC, and the very slow excretion by man of the latter, emphasises the need for treating all these substances and related compounds with extreme care, and to apply the proper precautions in handling them.

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2:4-DINITROPHENYLHYDRAZINE, A SUITABLE REAGENT FOR THE COLORIMETRIC DETERMINATION OF CARBONYL COMPOUNDS

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Carbonyl compounds are determined by a method based on the stable orange colour of 2:4-dinitrophenylhydrazones in acidic medium. Under such conditions the blank test is negligible. The method proved useful for the determination of aliphatic, aromatic and steroid compounds.

2:4-DINITROPHENYLHYDRAZONES are highly coloured compounds; however they have but rarely been used for the colorimetric determination of small amounts of aldehydes or ketones, because the interference from excess reagent causes trouble and requires chromatographic separations[1] or extractions with various solvents[2–5]. Addition of alkali to the dinitrophenylhydrazones produces a red colour[6–11] which differs from that of the reagent, but the instability of the resulting colour limits this application.

According to our experiments the 2:4-dinitrophenylhydrazones can be obtained at room temperature in acetic-hydrochloric acid medium. Under these conditions the colour of the blank is only light yellow, whereas the condensation of the reagent with the carbonyl compounds yields a stable orange colour with an absorption maximum at 4125 Å. The intensity of the colour follows Beer's law; the determination of aldehydes and ketones can therefore be applied to samples ranging from 10 to 100 μg.

The intensity of the colour varies according to the conjugation of the carbonyl compound involved (Table I); differentiation is thus possible, especially between aliphatic and aromatic compounds. Benzophenone, camphor and sugars react rather slowly at room temperature, and require 15 minutes' heating on the boiling water bath, to ensure condensation, the absorption maximum being slightly shifted to 4325 Å. It should be noted

TABLE I

MOLECULAR ABSORPTION AFTER REACTION OF ALDEHYDES, KETONES OR SUGARS WITH 2:4-DINITROPHENYLHYDRAZINE

Aldehydes	ϵ	Ketones	ϵ
Acetaldehyde	4,225	Acetone	2,320
Benzaldehyde	8,480	Acetophenone	6,240
Cinnamaldehyde	22,970	Acetylacetone	null
Citral	9,120	Benzophenone (*)	765
Diphenylacrolein	27,450	Camphor (*)	80
Formaldehyde	2,790	*cyclo*Hexanone	1,765
Furfural	11,800	*cyclo*Pentanone	2,690
p-Hydroxybenzaldehyde	17,570	Ethylacetoacetate	1,690
Piperonal	18,000	Ethylmethylketone	2,090
Propionaldehyde	4,870		
Salicylaldehyde	14,275	SUGARS	
Vanillin	19,600	Glucose (*)	3,780
Veratraldehyde	19,420	Ribose (*)	4,500

(*) Heating 15 minutes at 100° C.

that acetylacetone does not develop any colour even after heating, because its hydrazone undergoes cyclization to the slightly coloured 1-(2′:4′-dinitrophenyl)3:5-dimethylpyrazole[12].

In the steroid series the condensations are made at 100°. The colour differences can be enhanced (Table II) for the 3-ketonic compounds, by adding an ethanolic solution of potassium acetate. A conjugated double bond in the C4(5) position enhances the molecular absorption whereas an additional C1(2)-double bond decreases it. Of all carbonyl compounds tested, only 11-keto steroids did not react.

TABLE II
CONDENSATION BETWEEN KETOSTEROIDS AND 2:4-DINITROPHENYLHYDRAZINE

Name of compound	Position of carbonyl	Position of double bonds	Molecular absorption	
			without AcOK	with AcOK
3α-Acetoxy 11-keto etiocholane ..	11		null	null
Cortisone	3, 11 and 20	4	2,600	6,000
3:17-Diketo androsta-4-ene	3 and 17	4	3,600	9,600
3:17-Diketo androsta-1:4-diene ..	3 and 17	1 and 4	700	1,700
Dehydro *epi*androsterone	17	5	735	735
3:12-Dihydroxy-7-keto cholanic acid	7		560	560
3:20-Diketo pregnane	3, 20		2,400	3,250
Estrone	17	1, 3, 5	725	725
Hydrocortisone	3, 20	4	2,400	8,000
17β-Hydroxy-3-keto androstane ..	3		450	1,500
3β-Hydroxy-20-keto pregnane ..	20		1,650	1,650
3-Hydroxy-12-keto cholanic acid ..	12		280	280
Prednisone	3, 11 and 20	1 and 4	1,200	2,550
Prednisolone	3, 20	1 and 4	1,400	2,000
Testosterone	3	4	2,250	7,300

The determination with 2:4-dinitrophenylhydrazine has been applied to pharmaceutical preparations after extraction of the carbonyl compound. Aqueous or oily solutions can be directly determined after suitable dilution with pure acetic acid.

EXPERIMENTAL*

Reagent. 0·1 per cent solution of pure 2:4-dinitrophenylhydrazine in acetic acid containing 0·5 per cent of concentrated hydrochloric acid.

General procedure. The sample is dissolved in 1 ml. of acetic acid; 5 ml. of the reagent is added. After mixing the solution is allowed to remain in the dark for 1 hour at room temperature.

For steroid compounds, the reaction is carried out by 15 minutes' heating on a boiling water bath. After cooling to 20°, 0·5 ml. of a molar ethanolic solution of potassium acetate is added.

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* With the co-operation of Miss G. Clement.

SOME PHARMACOLOGICAL PROPERTIES OF A COMMERCIAL HEART EXTRACT

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An investigation was made of the pharmacological properties of a commercial heart extract (Recosen), said to be of value in the treatment of angina pectoris. This extract increased coronary flow but decreased force of beat in isolated perfused mammalian hearts. It potentiated the pressor effects of adrenaline in the spinal cat whilst not affecting those of noradrenaline, and it antagonised the actions of adrenaline and noradrenaline upon intestinal muscle. A mild anticholinesterase activity was demonstrated on striped muscle. The significance of these findings is discussed.

The work described in this paper was performed in an attempt to clarify the pharmacological properties of Recosen, a protein-free water-soluble preparation made from the hearts of freshly killed healthy young pigs, horses, and sheep. This extract is said to increase the coronary blood flow in the hearts of dogs[1], guinea pigs[2], and cats[3], and to increase the mechanical efficiency of the dog's heart by depressing cardiac metabolism[4]. It has been used clinically in the treatment of angina pectoris, and favourable results have been reported from its use in this disease[5,6]. It is also claimed to reduce the toxicity of digitalis alkaloids[7].

In spite of the fact that no specific active principle has as yet been isolated from this extract, it was felt that any substance which was claimed to be of value in the treatment of angina pectoris was worthy of investigation. Experiments were therefore performed which might determine or confirm the cardiac and other general pharmacological actions of this extract. Attempts were also made to identify by biological methods a specific active constituent of this mixture.

Methods

Spinal cats. Cats were spinalised either under intraperitoneal pentobarbitone (30 mg./kg.) or ether anaesthesia. Blood pressure was recorded from a carotid artery by a mercury manometer, and injections of drugs were given into an external jugular vein.

Intestine. Rabbit duodenum was suspended in Locke's solution at 37° through which was bubbled a mixture of 95 per cent O_2 and 5 per cent CO_2. Contractions were recorded with a frontal writing lever on a kymograph.

Perfused hearts. Isolated mammalian hearts were perfused with Locke's solution at 37° using a modified Langendorff apparatus[8]. The constitution of the Locke's solution was NaCl, 9·0, KCl, 0·42, $CaCl_2$, 0·24, dextrose, 1·0, $NaHCO_3$, 0·5 g., distilled water to 1000 ml., and it was

oxygenated with a mixture of 97 per cent O_2 and 3 per cent CO_2. Coronary flow was recorded on the input side of the apparatus using either a phototransistor or a platinum wire drop recorder, feeding a Thorp impulse counter. Drugs were given either by the injection of single doses into the coronary inflow, or by continuous perfusion of fixed concentrations.

Frog rectus muscle. Frog rectus abdominis muscle was suspended in Ringer's solution and aerated at room temperature. Contractures were recorded on a kymograph by a gimbal lever.

RESULTS

Physical Properties

The Recosen used in these experiments was supplied in ampoules, each ampoule containing "heart extract", 0·014 ml., *m*-cresol, 0·003 ml., distilled water to 1 ml. All doses of heart extract stated in this paper refer to amounts of the commercially available ampoules, and not to the pure heart extract. These ampoules contained a brown odourless fluid, pH 6·5, which exhibited a marked fluorescence under ultra-violet light. Full activity was retained after boiling for 3 hours, and after prolonged acid or alkaline hydrolysis.

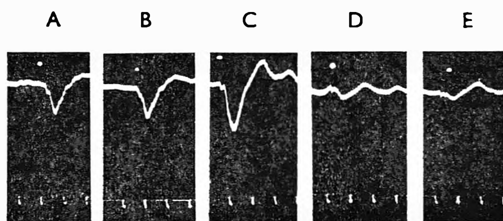

FIG. 1. Spinal cat, 2·7 kg. Upper tracings show blood pressure and lower tracing is time marker (30 sec.). At A and B, 0·5 ml. of heart extract injected. Between A and B, atropine sulphate, 0·5 mg./kg. At C, histamine, 3 μg. Between C and D, mepyramine maleate, 1 mg./kg. At D, histamine, and at E, heart extract, both in the same doses as before.

Spinal Cats

Cardiovascular responses to the heart extract were studied in seven cats. When this extract was given intravenously in doses of 0·1–2·0 ml./kg. there was a fall in blood pressure resembling that caused by histamine. This effect was not affected by the prior administration of atropine, but could be totally abolished by suitable doses of the antihistamines, diphenhydramine and mepyramine (Fig. 1).

Quantitative assay by this blood pressure effect revealed a histamine content of 3 μg./ml. of heart extract. This would be in accord with the results communicated to me by Kocher, who estimated the histamine content of the extract as 2–3 μg./ml. That this effect was not due to histamine release by the extract was shown by the fact that the fall in blood

pressure was immediate in onset, and there was no initial delay as is seen with histamine-releasing compounds.

The most remarkable effect of this extract seen in the spinal cat was a differential potentiation of the pressor actions of adrenaline and noradrenaline. Doses of 0·5–2·0 ml./kg. of heart extract increased the hypertensive response to adrenaline whilst having a negligible effect on the pressor action of noradrenaline.

The potentiated pressor response to adrenaline was accompanied by an increased tachycardia; there was no alteration in the effect of noradrenaline on heart rate. This action of the extract was markedly different from the potentiation of adrenaline and noradrenaline caused by cocaine, in which the pressor effects of the two amines were potentiated to about the same degree (Fig. 2), and were accompanied by a pronounced increase in the response of heart rate to noradrenaline with only a slight increase in this response to adrenaline.

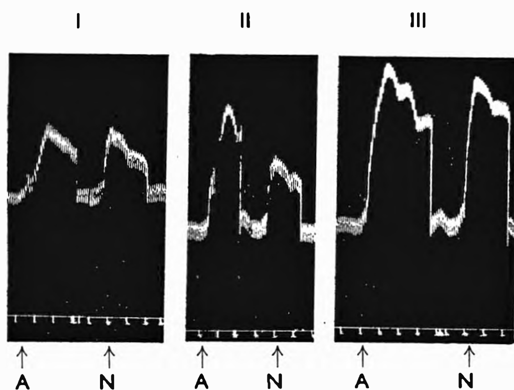

FIG. 2. Spinal cat, 3·9 kg., showing the effects on blood pressure of injection of 10 μg. adrenaline (A) and 1 μg. noradrenaline (N). I is a control; II is after 2 ml. of heart extract; III is after 10 mg. cocaine intramuscularly.

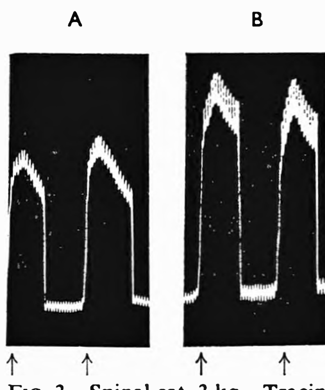

FIG. 3. Spinal cat, 3 kg. Tracing shows blood pressure effects of intravenous injection of 10 μg. adrenaline (indicated by arrows). A is before and B is after the injection of 1·5 ml./kg. of heart extract.

This potentiating action of single doses of the heart extract was prolonged in duration, giving the same quantitative results after 4 hours as it did after 10 minutes.

Differential potentiation of the pressor actions of adrenaline and noradrenaline has not been previously described for this extract. It seemed possible that the mechanism underlying this potentiation could be either an increase in the excitatory cardiovascular actions of adrenaline, or a decrease in the peripheral vasodilator response to adrenaline. Further experiments were therefore made to discover whether either or both of these phenomena could be demonstrated.

Rabbit Duodenum

As adrenaline has an inhibitory effect on the smooth muscle of the gastrointestinal tract similar to its action on the smooth muscle of certain

peripheral vascular beds, eight experiments were performed on rabbit duodenum to see whether the action of adrenaline on this preparation was modified in any way by the heart extract. In these experiments concentrations of the extract of 0·1–1·0 ml. per litre had no action of their own upon intestinal tone or spontaneous contractions, but these concentrations of the extract greatly reduced the inhibition of tone and motility caused by adrenaline. This effect was more pronounced with noradrenaline than with adrenaline. Thus, in the presence of 1·0 ml. of heart extract per litre, the inhibition of tone due to adrenaline was reduced by 32·5 per cent (S.D ± 1·5 per cent) whilst the inhibition caused by noradrenaline was reduced by 58 per cent (S.D. ± 4 per cent).

Isolated Mammalian Hearts

The actions of the heart extract were studied upon rabbit, guinea pig, and kitten hearts, to confirm results previously reported claiming a coronary vasodilator action of this extract[1–3], to assess its actions upon force of beat, and to study any modification that it might produce upon the actions of sympathomimetic amines on isolated hearts.

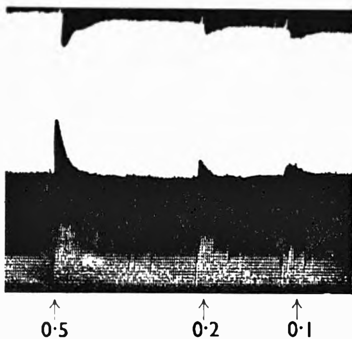

FIG. 4. Isolated perfused rabbit heart, showing responses to 0·5, 0·2 and 0·1 ml. of heart extract. Upper tracing shows myocardial contractions, lower tracing shows coronary flow, the height of each vertical line indicating flow in drops per five seconds.

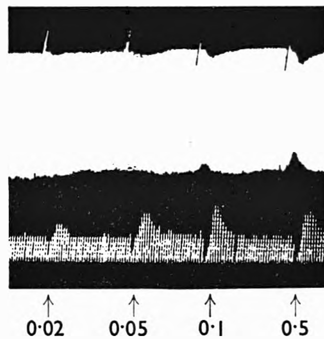

FIG. 5. Isolated perfused guinea pig heart, showing responses to 0·02, 0·05, 0·1 and 0·5 ml. of heart extract. Upper and lower tracings are as for Figure 4.

When single doses of the extract were given, a pronounced species difference was noted between the effects on rabbit hearts and those on the other species studied. In 10 experiments on rabbit hearts, doses of up to 0·1 ml. were usually without effect. This lack of effect was not due to lack of reactivity of the heart, as vasodilatation of the coronary vessels could still be produced with glyceryl trinitrate, and vasoconstriction with adrenaline. Doses of 0·1–0·5 ml. of the extract increased coronary flow, but also caused marked decrease in the force of the heart beat (Fig. 4). Doses of more than 0·5 ml. usually caused transient cardiac arrest, and the beat, when resumed, remained of reduced amplitude.

In 11 experiments on guinea pig hearts, single doses of the extract had a much greater effect on coronary flow than on the force of beat (Fig. 5). Doses of 0·001–0·05 ml. caused a marked increase in the coronary flow without affecting the force of beat. Higher doses caused similar, though much less marked, actions to those seen with rabbit hearts. In neither species were there any consistent alterations in heart rate when the heart extract was given in non-toxic doses. In 3 kitten hearts studied, the effects on coronary flow and force of beat were similar to, though less pronounced than, those seen in the guinea pig heart, but in this species these effects were accompanied by a moderate increase in the heart rate.

In view of the alterations in response to adrenaline and noradrenaline caused by the extract in spinal cats, attempts were made to see whether the heart extract modified in any way the coronary vasodilatation that these amines cause in kitten hearts, and the coronary vasoconstriction that they produce in isolated perfused rabbit and guinea pig hearts. But, when continuous perfusions of Locke's solution containing 0·001–0·1 ml. of extract per litre were used, it was not possible to demonstrate any alteration in the cardiac actions of adrenaline or noradrenaline. Concentrations of more than 0·1 ml./litre caused cardiac irregularities which obscured the effects of the sympathomimetic amines.

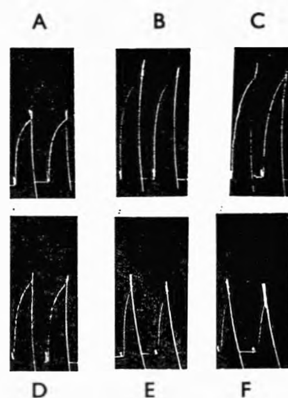

Fig. 6. Frog rectus abdominis muscle, showing contractures produced by A, Acetylcholine (ACh), 0·5 µg./ml. B, ACh, 1·0 µg./ml. C, ACh, 0·5 µg./ml. + heart extract, 0·2 ml./ml. D, ACh, 0·5 µg./ml. + heart extract, 0·1 ml./ml. E and F show contractures in the presence of ethyl pyrophosphate, 5 µg./ml. E, ACh, 0·1 µg./ml. F, ACh, 0·1 µg./ml. + heart extract, 0·2 ml./ml.

Striped Muscle

In view of the deleterious effect that larger doses of the heart extract had on cardiac muscle of certain species, a series of experiments were performed on frog rectus muscle, a simple striped muscle preparation, to see whether similar deleterious effects could be reproduced in skeletal muscle. In 6 experiments on this preparation, the heart extract in doses of 0·02–0·2 ml./ml. of Ringer's solution had no direct action on the muscle, but potentiated contractures caused by acetylcholine. Carbachol-induced contractures were unaffected, and the potentiation of acetylcholine-induced contractures was abolished after treatment with the anticholinesterases, physostigmine and ethyl pyrophosphate (Fig. 6).

Experiments on the Chemical Constituents of the Extract

(*a*) In view of the marked fluorescent properties of the heart extract, solutions of flavine adenine dinucleotide were prepared, ranging in concentration from 0·001 µg./ml. to 10 mg./ml., since this compound is a

riboflavine precursor which exhibits marked fluorescence and which has been isolated from pig heart[9].

(*b*) A number of amines and amino acids have been determined in the heart extract (Kocher, personal communication), and its constitution has provisionally been stated as follows (concentrations are given in μg./ml.):—histamine, 3; acetylcholine, less than 0·1; catechol derivatives, 0; adenosine and derivatives, 2; arginine, 130–150; cystine, 90–120; histidine, 60–70; leucine, 160–190; methionine, 40; tryptophane, 18; tyrosine, 60; potassium, 510; sodium, 340; magnesium, 95; calcium, 40. A solution of this constitution was prepared, using the laevorotatory forms of the amino acids and the upper limits of the concentrations stated. Adenosine triphosphate was used in place of the "adenosine and derivatives" stated above.

Attempts were made to see whether the effects of the heart extract could be reproduced by solutions (*a*) and (*b*) when they were used in experiments on the spinal cat, perfused hearts, rabbit duodenum, and frog rectus muscle. With neither of these solutions was it possible to reproduce any of the actions of the heart extract, apart from the effects due to the histamine content of solution (*b*) upon the blood pressure of the spinal cat.

Discussion

A most interesting result of the above experiments was the modification of the responses of the spinal cat to adrenaline and noradrenaline, where the effects of adrenaline on blood pressure and heart rate were potentiated but those of noradrenaline were unaffected. It has been shown by Innes[10] that the potentiation of sympathomimetic amines caused by cocaine and by certain antihistamines is accompanied by a rise in the heart rate response to noradrenaline with no alteration in this response to adrenaline. The heart extract appeared to produce the opposite phenomenon to this.

The increased pressor response to adrenaline after injections of this extract may be caused by an increase in the excitatory cardiovascular actions of adrenaline (i.e., an increased cardiac output or increased vasoconstriction), but it may also be due to a modification of the peripheral inhibitory actions of adrenaline. The pressor response to adrenaline is normally accompanied by vasodilatation in certain peripheral vascular beds, such as those in the liver and in skeletal muscle. It is possible that the heart extract antagonises this peripheral vasodilator effect of adrenaline, increasing the total peripheral resistance and so causing an increased rise in blood pressure.

This suggested mode of action of the heart extract in potentiating the pressor response to adrenaline by altering the peripheral response to this drug is supported in part by the actions seen in the rabbit duodenum. In this preparation sympathomimetic amines usually cause a relaxation of tone and an inhibition of spontaneous contractions, a manifestation of the properties of these amines in relaxing smooth muscle. This effect was greatly reduced in the presence of the heart extract, and it is possible that similar effects may occur on the smooth muscle of peripheral blood vessels. This hypothesis is not invalidated by the observation that in the

rabbit duodenum the heart extract had a greater inhibitory effect on the responses to noradrenaline than it did on those to adrenaline. The pressor effect of noradrenaline, unlike that of adrenaline, is not accompanied by significant peripheral vasodilatation. If the heart extract did potentiate the pressor effect of adrenaline by inhibiting the peripheral vasodilatation caused by sympathomimetic amines, it would not by this action affect the pressor response to noradrenaline.

The results elicited in isolated mammalian hearts confirm the results of Haemmerli[11], and of Ryser and Wilbrandt[2], who showed that this extract caused a dilatation of the coronary arteries in guinea pig hearts. Increased force of heart beat was not seen with the heart extract in these experiments; high doses of the extract decreased the force of beat in all species examined, an effect that was much more marked in rabbit hearts than in those of the other species.

The results of the experiments on frog rectus muscle, in which the extract potentiated acetylcholine contractures whilst not affecting those produced by carbachol, and in which this potentiation was abolished by the addition of anticholinesterases, suggest that the heart extract has a mild anticholinesterase activity.

The investigations performed on the constituents of the extract show that its action cannot be attributed to any of the substances so far identified in it. If the fluorescent property of the extract is due to the presence of flavine adenine dinucleotide, this fraction would also appear to be devoid of biological activity. Ryser and Willbrandt[2] in experiments on the coronary resistance of the isolated guinea pig heart demonstrated that this extract had a different dose-response curve to that of adenylic acid and other adenosine derivatives. Whilst it is still possible that the actions of the extract are due to the presence in it of an adenosine derivative, the actions of which are modified by some other constituent, the stability of the extract, and the failure of solutions of adenosine triphosphate to cause similar actions to those of the extract make this possibility unlikely.

Since it is not possible to draw comparisons between the results of acute laboratory experiments performed on healthy experimental animals and the therapeutic results of trials of the drug on patients who suffer from chronic cardiac disorders and who are on a prolonged course of therapy, the results of the experiments described in this paper should not be construed as detracting from the therapeutic properties claimed for this extract. True assessment of the therapeutic worth of this drug will not be possible until there is a careful clinical trial of the double-blind type. It is understood that such a trial is at present in progress. The effects of this drug in dilating the coronary vessels, and its relationship to the actions of sympathomimetic amines would seem to be worthy of further pharmacological study.

Acknowledgements. I am greatly indebted to Dr. J. B. E. Baker for much helpful advice on the planning of these experiments and in the preparation of this paper. The Recosen used in these experiments was made available by Dr. N. Levinson, of Robopharm Ltd., to whom I am also indebted for supplying much useful information about this drug.

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2-HYDROXYIMINOMETHYL-*N*-METHYLPYRIDINIUM METHANESULPHONATE (P2S), AN ANTIDOTE TO ORGANOPHOSPHORUS POISONING. ITS PREPARATION, ESTIMATION AND STABILITY

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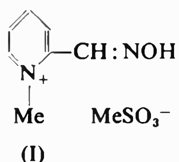
2-Hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate (P2S) is prepared by boiling pyridine-2-aldoxime with methyl methanesulphonate in benzene. It can be estimated both in water and in biological materials by measuring its ultra-violet absorption in alkaline solution. In aqueous solution it is slowly decomposed by heat or prolonged storage with some cyanide ion formation. The stability varies with pH, being optimal at pH 4–5. P2S can be satisfactorily sterilised by filtration through a "millipore" filter.

2-Hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate (P2S) (I) has recently been shown[1] to be very effective when given in conjunction with atropine in the treatment of animals severely poisoned with organophosphate anticholinesterases. It is relatively non-toxic to animals[2] and has been given to man without ill-effects, in doses of a size known to be effective against poisoning in animals[3]. P2S is closely related to the better-known PAM (II) which has been successfully used in parathion poisoning



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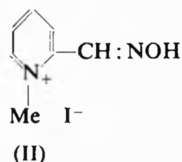

(II)

in man[4], but it has the advantage over PAM in being much more water-soluble so that the relatively large doses necessary may be administered intramuscularly instead of intravenously as with PAM. As P2S may replace PAM we have provided this note on some of its properties of pharmaceutical interest.

Experimental and Results

Preparation and Physical Properties

100 g. pyridine-2-aldoxime (commercial) was boiled under reflux for about 3 hours with 120 ml. of methyl methanesulphonate in 1 l. of benzene. When cool the crude product was filtered off, sucked fairly dry, and then dissolved in 500 ml. boiling ethanol. Ethyl acetate was added to the filtered hot ethanol solution until crystallisation began. P2S is obtained in a yield of about 100 g. of white, very hygroscopic crystals, m.p. 155°. It is very soluble in water (at least 1 g. in 2 ml.) and is a weak acid (pKa in water, 8·0).

Estimation

P2S ionises in dilute alkaline solution to give a yellow anion with a strong ultra-violet absorption maximum at 335 mμ. (see Fig. 1). This absorption provides a satisfactory method of estimation. 0·2 ml. 20 per cent sodium hydroxide is mixed in a 1 cm. silica cell with 3 ml. of an aqueous solution containing about 30 μg. P2S. The optical density is measured at 335 mμ and the P2S content read from a calibration curve (Fig. 2).

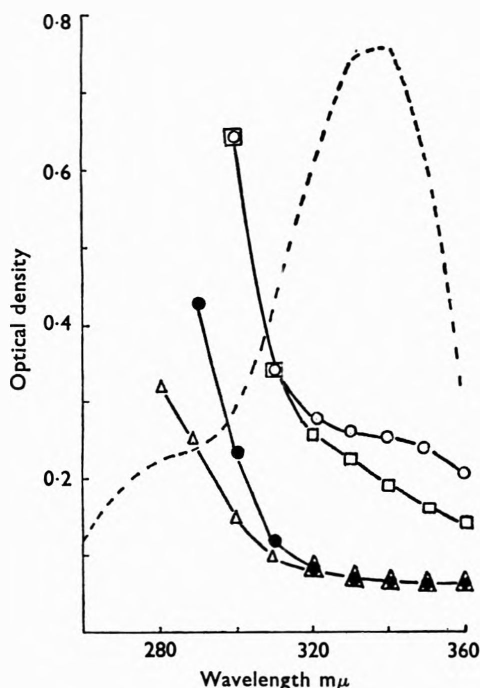

FIG. 1. Ultra-violet spectra of P2S and some tissue extracts in 1·25 per cent NaOH.

- - - 30 μg P2S + 0·2 ml. of 20 per cent NaOH + 3 ml. of water.

—○— Kidney, —□— Liver, —●— Muscle, —△— Whole blood: 3·0 ml. of protein-free solution (equivalent to 0·6 g. tissue or 0·6 ml. blood) + 0·2 ml. of 20 per cent aqueous NaOH.

None of the likely contaminants of reasonably pure samples, such as pyridine-2-aldehyde or pyridine-2-aldoxime, absorb light appreciably at 335 mμ, and do not interfere. All the spectra were measured with a Unicam SP500 spectrophotometer.

Estimation of Cyanide in P2S Solutions

One of the decomposition products of P2S on prolonged storage or on heating the aqueous solution is cyanide ion (see below). This cyanide can be satisfactorily estimated by Aldridge's colorimetric method[5], but as the colour produced may be affected by the presence of P2S it was found desirable to incorporate a suitable control (method A below) or separate the cyanide from the P2S before estimation (method B).

Method A. 1 ml. of 20 per cent aqueous P2S was diluted with 3·9 ml. of water and 0·1 ml. 10N sulphuric acid. The cyanide concentration in 3 ml. of this solution was estimated immediately by Aldridge's procedure. To provide a control, the hydrogen cyanide was removed from the remaining 3 ml. of the solution by the passage of a steady stream of air through the solution for 1 hour. A repeat "cyanide" estimation on 2 ml. of this solution gave an appropriate control and the true cyanide concentration was obtained by difference.

Method B. 1 ml. N sodium hydroxide was placed in the inner well of a Conway dish and in the outer compartment were placed (separately, on opposite sides) 1 ml. 20 per cent aqueous P2S and 0·2 ml. 10N sulphuric acid. The lid was rapidly placed in position before the two solutions in the outer compartment had come into contact. The dish was then shaken gently about 120 times a minute for 1 hour. 0·5 ml. of the sodium hydroxide in the inner compartment was then mixed with 0·1 ml. 10N sulphuric acid and 1·4 ml. water and the cyanide was estimated as above.

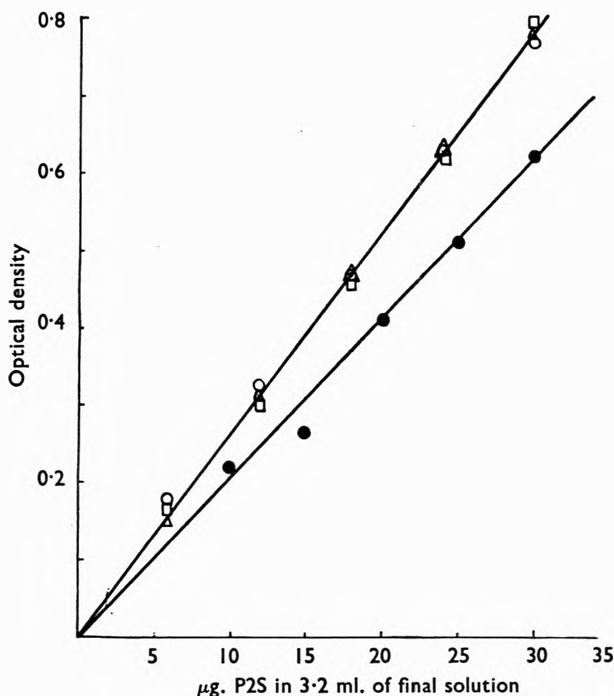

FIG. 2. P2S calibration curve. The tissue curves have been corrected for the absorption due to the tissue itself. —△— Water. —□— Whole blood. —○—Muscle homogenate. —●— Liver homogenate.

Stability of P2S

When P2S was heated in solution in an attempt to sterilise it, it was found that small quantities of cyanide were formed accompanied by a change in colour of the solution from very pale yellow to orange-brown. These changes are dependent on the temperature and pH of the solution. When 20 per cent aqueous P2S was heated at 100° for 30 minutes the following percentages (by weight based on the initial amount of P2S) of cyanide were found: at pH 5, 0·3; at pH 6, 1·1; and at pH 7, 2·8 per cent. In Table I the amounts of cyanide formed and P2S remaining are shown after storage of 20 per cent aqueous P2S in glass at room temperature for 109 days at pH values in the range 3–7.

TABLE I

pH	3	4	5	7
Cyanide formed, per cent	0·04	0·07	0·30	0·50
Unchanged P2S, per cent	93	97	99	61

When a solution of P2S was adjusted to pH 6·0, freeze dried and stored in a sealed glass container for 6 months some cyanide was detected by its smell on opening the container. But, when P2S was dried from solution at pH 4·5 no cyanide was detected after storage either by smell or by chemical analysis.

Sterilisation of P2S

From the above experiments it is clear that any method involving heat would be unsuitable for the sterilisation of P2S. However, filtration through a "millipore" filter has been found to be adequate. If an asbestos filter is used the P2S solution becomes yellow-coloured with a pH about 6 due to the extraction of alkali from the filter. Our practice is to make a 15 per cent solution of P2S at pH 4·5 in pyrogen-free water which is then filtered. 3 ml. portions of the filtrate are freeze-dried into sterile ampoules, which are then sealed. When required for use the ampoule is opened and the P2S is dissolved in 3 ml. sterile pyrogen-free water. Sg. t. G. D. Wedd tells us this solution can be safely injected into the thigh muscle without causing either pain or tissue damage. Samples from each batch are tested for sterility, cyanide and P2S content.

Estimation in Biological Materials

The simple ultra-violet absorption method is applicable to estimating P2S in biological material provided a correction is made for the absorption of the tissues themselves (see Fig. 1), and provided these are first deproteinised by treatment with zinc sulphate and barium hydroxide as in the examples below.

Tissues were ground with an equal weight of sand and twice their weight of 0·9 per cent sodium chloride. The mixture was centrifuged and 3 ml. of the supernatant was mixed with 1 ml. 0·3M barium hydroxide and 1 ml. 0·33M zinc sulphate. The slight excess of zinc sulphate was needed to give a clear supernatant. The precipitated protein was removed by centrifugation and the supernatant used in the P2S estimation. With liver extracts, this supernatant was often opalescent because of the presence of glycogen; this was removed without significantly affecting the P2S by heating the solution at 100° for 3 minutes with 1 per cent by volume of concentrated hydrochloric acid.

Blood. 2·0 ml. of whole blood was mixed with 3·8 ml. water, and 1 ml. 0·3M barium hydroxide; after haemolysis was complete, 1 ml. 0·33M zinc sulphate and 0·2 ml. 20 per cent sodium chloride were added and the mixture centrifuged. The sodium chloride caused the precipitate to shrink, thus giving the maximum volume of supernatant; this was then used in the P2S estimation.

Urine. 0·2 ml. of urine was usually diluted 1 : 10 before treatment with the barium and zinc solutions. Diluted urine 0·2 ml. was mixed with water 2·8 ml., 1 ml., 0·3M barium hydroxide and 1 ml. 0·33M zinc sulphate. The supernatant obtained by centrifuging was used for the estimation of P2S.

Faeces. Dried faeces were ground with 3 times their weight of water and were then centrifuged. 1 ml. of the supernatant was deproteinised by addition of 5 ml. water, 1 ml. 0·3M barium hydroxide and 1 ml. 0·33M zinc sulphate followed by centrifugation. The supernatant was used in the estimation of P2S.

3 ml. of the final protein-free supernatant from any of the above materials was mixed with 0·2 ml. 20 per cent sodium hydroxide and the optical density measured at 335 mμ. The P2S concentration was read from a predetermined calibration curve (Fig. 2) after correction for the absorption of the biological materials themselves at this wavelength (Fig. 1). The minimum sensitivity is about 1 μg. of P2S per ml. of the final solution. Complete recovery of P2S added to various tissue homogenates was obtained except in the case of liver homogenate which slowly decomposed P2S (see Fig. 3).

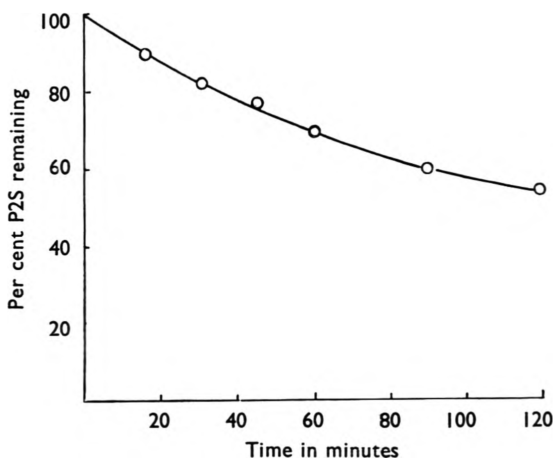

FIG. 3. Breakdown of P2S by liver homogenate at 37°. 300 μg. of P2S added per ml. of 25 per cent. rat liver homogenate in saline.

It was impossible to obtain tissue blanks from the same animals that had been treated with P2S. However, the variation in tissue blanks was not large and a blank value previously determined for a group of animals was used to calculate individual tissue levels within certain limits. The mean value of the blanks for 12 different samples of rat skeletal muscle expressed as μg. of P2S per g. was 5·6 (S.D. 1·1); 5·6 μg./g. was therefore used as a fixed blank to be subtracted from all estimates of muscle P2S levels. The corresponding values used for other biological materials were: whole blood 2·7 μg./ml. (S.D. 0·4), liver 11·4 μg./g. (S.D. 2·4), kidney 21 μg./g. (S.D. 3·7); urine diluted 1 : 10 had a negligible blank.

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SOME BIOLOGICAL PROPERTIES OF DIMETHISTERONE "SECROSTERON" A NEW ORALLY ACTIVE PROGESTATIONAL AGENT

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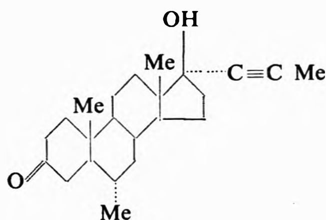
Dimethisterone, 6α:21-dimethylethisterone possesses well-marked oral progestational properties with 11·6 times the activity of ethisterone in the Clauberg assay. It has an oral LD50 in mice of 7·65 g./kg., no apparent anabolic, androgenic properties and no significant effect on sodium, potassium or water excretion in saline loaded rats.

THE preparation and progestational activity of a number of alkylated ethisterones have previously been reported[1] from our laboratories. Dimethisterone, the most active of the series, was submitted for clinical evaluation and its progestational superiority confirmed by Jackson (unpublished) and Matthew[2]. This paper describes its acute toxicity, progestational, anabolic, androgenic and oestrogenic properties and its effect on sodium potassium and water excretion in saline loaded rats.

Dimethisterone is an odourless and tasteless white crystalline compound with a molecular weight of 340·4. It is almost insoluble in water, slightly soluble in acetone and chloroform and soluble in ethanol. Its preparation and properties are described by Barton, Burns, Cooley, Ellis and Petrow[3]. It melts at about 102°, is dextrorotatory with a specific rotation in chloroform $+12°$ and has E(1 per cent, 1 cm.) in *iso*propanol 450 at 240 mμ.

It has the structural formula



(I)

METHODS

Acute Oral Toxicity

Male albino mice, weighing approximately 20 g. each, were fasted overnight. Three groups of 10 animals were given a suspension of dimethisterone, by stomach tube, in the following aqueous suspending medium.

Sodium carboxymethylcellulose ..	1·2 g.
Tween 80	1·5 g.
Methyl-*p*-hydroxybenzoate	0·06 g.
Propyl-*p*-hydroxybenzoate	0·03 g.
Distilled water to 100 ml.	

All volumes were adjusted to 0·5 ml./20 g. body weight. The LD50 was estimated, using Karber's[4] formula from the seven days mortalities.

Progestational Activity

McPhail's[5] modification of the Clauberg test was used. Thirty immature rabbits weighing between 800 and 1,200 g. were sensitised with three doses of 5 μg./kg. of oestrone, intramuscularly in 0·2 ml. of 20 per cent ethyl oleate in arachis oil on days one, three and five of the experiment.

Dimethisterone and ethisterone, the latter being used as the reference compound, were administered by stomach tube as suspensions in 5 per cent mucilage of acacia on days seven, eight, nine and ten of the experiment. Twenty-four hours after the last injection the animals were killed, the uteri removed and frozen sections 20 μ thick prepared and stained with haematoxylin and eosin. The progestational response was estimated by measuring the fraction of endometrium occupied by glandular tissue and also by McPhail's grading system. Relative potencies were estimated from the data obtained by the first method.

Anabolic and Androgenic Activity

A modification of Eisenberg and Gordon's[6] method, using testosterone propionate as the reference compound, was used. Both compounds were administered subcutaneously in 20 per cent ethyl oleate in arachis oil, volumes being adjusted to 0·5 ml./100 g. body weight. Thirty rats, castrated 3 weeks previously, with a mean weight of 156 g. were divided into five groups each of six animals. Seven daily injections were given with a 72-hour interval between the third and fourth injections. Three groups were given 4, 16 and 64 mg./kg. of dimethisterone respectively, the fourth group 2 mg./kg. of testosterone propionate and the remaining group, which acted as castrated controls, the vehicle only. Twenty-four hours after the last injection the animals were killed and the mean wet weight of the levator ani muscle and seminal vesicles per 100 g. weight recorded. The mean pre- and post-injection weights were also recorded.

Oestrogenic Activity

The Allen Doisy vaginal cornification method using spayed mice was employed, for reference see Emmens[7]. All compounds were given subcutaneously in 20 per cent ethyl oleate in arachis oil. The ovariectomised mice were primed with oestrone, 0·5 μg./20 g. weight, on two successive days. Three weeks later thirty-five mice were divided into seven groups of five mice. Oestrone, 0·25 μg. and 0·5 μg./20 g. weight, was given subcutaneously in two divided doses to two groups on days

one and two of the experiment; five doses of dimethisterone 2, 4, 8, 16 and 32 μg. respectively were given in a similar manner to the remaining groups. Vaginal smears were examined 36 hours following the last injection and at 12-hourly intervals on days four and five. The responses were treated quantally and a positive scored if any one of the five smears showed nucleated or cornified cells with the absence of leucocytes.

Effect on Salt and Water Excretion in Saline Loaded Rats

Four groups of 16 male albino rats were deprived of food and water overnight. The following morning a 0·9 per cent saline water load, 25 ml./kg. was given by stomach tube to each rat. At the same time dimethisterone, 2, 8 or 32 mg./kg. was administered as a suspension in 5 per cent gum acacia to three of the groups, the fourth acting as a control. The urine was collected and measured over a 24-hour period after administration and recorded as ml./kg. body weight. The Na^+ and K^+ concentrations were estimated by means of a flame photometer and recorded as mEq./kg. weight.

RESULTS

Acute oral toxicity. The acute oral LD50 in mice was 7·65 g./kg. Table I records the mortalities in 7 days after the administration of 4, 6 and 9 g./kg. of dimethisterone.

TABLE I

ORAL TOXICITY OF DIMETHISTERONE IN MALE ALBINO MICE

Compound	Dose g./kg.	Seven day mortalities	LD50 g./kg.
Dimethisterone	4 6 9	1/10 2/10 6/10	7·65

Progestational activity. Dimethisterone had an oral progestational activity in oestrone primed rabbits 11½ times that of ethisterone. Table II records the mean responses to varying amounts of dimethisterone and ethisterone.

TABLE II

THE MEAN PROGESTATIONAL RESPONSE IN FIVE RABBITS AFTER ORAL ADMINISTRATION OF DIMETHISTERONE AND ETHISTERONE

Compound	Total dose mg./kg.	Response		Relative potency
		McPhail	Per cent glandular tissue	
Dimethisterone	0·3	1·6	53·4	
,, ...	0·6	2·4	59·6	11·5
,, ...	1·2	3·2	73·4	
Ethisterone	2·5	1·2	45·8	
,, ...	5·0	1·8	54·8	1·0
,, ...	10·0	2·5	68·4	

Anabolic and androgenic activity. Testosterone propionate at 2 mg./kg. had well-marked androgenic and anabolic properties. Dimethisterone in amounts up to 64 mg./kg. had no apparent androgenic or anabolic properties, the weights of the seminal vesicles and levator ani muscles of treated and castrated controls being similar. Table III records the results.

TABLE III

THE ANABOLIC AND ANDROGENIC ACTIVITY OF DIMETHISTERONE GIVEN SUBCUTANEOUSLY TO GROUPS OF SIX CASTRATED RATS

Compound	Dose mg./kg./day	Body weight g.		Wet weight mg. seminal vesicles	Wet weight mg. levator ani
		Pre-injection	Post-injection		
Dimethisterone	4	157	145	10	22
	16	155	161	9	23
	64	155	166	13	24
Testosterone propionate ..	2	156	163	239	76
Controls		156	167	10	20

Oestrogenic activity. Table IV records the number of ovarectomised mice showing a positive oestrogenic response to various doses of dimethisterone and oestrone. Dimethisterone has no oestrogenic properties in amounts up to 32 μg./20 g.

TABLE IV

THE OESTROGENIC RESPONSES IN OVARIECTOMISED MICE GIVEN DIMETHISTERONE SUBCUTANEOUSLY

Compound	Total dose µg./20 g.	Response
Dimethisterone	2	0/5
	4	0/5
	8	0/5
	16	0/5
	32	0/5
Oestrone	0·25	4/5
	0·50	4/5

Effect on salt and water excretion in saline loaded rats. Table V records the results. At 32 mg./kg. dimethisterone had some diuretic effect. There was no Na^+ retention or undue K^+ excretion in the 24 hours after oral administration.

TABLE V

THE MEAN TWENTY FOUR HOUR RENAL OUTPUT OF URINE, Na^+ AND K^+ IN SALINE LOADED RATS GIVEN DIMETHISTERONE ORALLY

Dimethisterone mg./kg.	Urine output		mEq./kg./24 hr.	
	ml./kg./24 hr.	Relative activity	Na^+	K^+
2	6·9	0·9	2·7	1·5
8	8·7	1·2	3·2	1·6
32	12·0	1·6	3·2	1·9
Controls	7·3	1·0	2·3	1·3

Discussion

Dimethisterone, 6-α:21-dimethylethisterone is a comparatively non-toxic synthetic steroid with an acute oral LD50 in mice of 7·65 g./kg. It has oral progestional activity in oestrone-primed immature rabbits with an activity 11·6 times greater than ethisterone. It has no anabolic or androgenic properties in castrated immature rats in amounts up to 64 mg./kg. and no oestrogenic activity in ovariectomised mice in the Allen Doisy test. Salt and water balance were not markedly affected and it has no antidiuretic properties.

Dimethisterone should be of value in gynaecological conditions where an oral progestational agent is indicated.

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A STUDY OF BACTERIOLOGICAL MEDIA: THE EXAMINATION OF CASAMIN E*

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Three batches of Casamin E have been examined quantitatively for their free and total amino acid content. Variation occurs in the free amino acid content in the three batches examined. The peptides were examined qualitatively. The batches were examined for their strepogenin activity using the media of Steele, Sauberlich, Reynolds and Baumann[1] and Kodicek and Mistry[2]. The stimulatory pattern appeared to be different with the two basal media.

In a previous paper[3] the quantitative examination of the amino acids in Difco Bacto-Casitone was reported. This paper describes the quantitative estimation of the free and total amino acids in three batches of Casamin E which is a pancreatic digest of casein manufactured by Mann Research Laboratories. The qualitative examination of the constituent peptides is given. The three batches were examined for their strepogenin activity.

Experimental

Quantitative estimation of the free amino acids. Three batches of casamin E designated A, B and C were examined. 0·1 g. of casamin was reacted with fluorodinitrobenzene (FDNB) at pH 9 and 40° for 1½ hours in 0·1 N potassium chloride solution with vigorous stirring. Excess FDNB was extracted with ether, after acidification the dinitrophenyl (DNP) amino acids and peptides were extracted with ether, a part remained suspended in the ether layer. This was removed by dissolving in acetone and adding it to the washed ether extract. The ether extract was evaporated to dryness under vacuum then subjected to the cold finger condenser to remove most of the dinitrophenol. The residue, as well as that from the aqueous extract remaining after ether extraction of the DNP-amino acids, was subjected to the quantitative paper chromatography technique of Levy[4] using the ethyl benzene system[5] in the first direction followed by 1·5 M phosphate buffer as previously described[3].

The total amino acids were estimated by hydrolysing 0·1 g. of casamin and reacting the hydrolysate with FDNB and then subjecting the DNP-amino acids to quantitative paper chromatography. The amino acid content of casein was determined by the same method and results were computed for a nitrogen content of 12·5 per cent. The results are given

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in Table I which shows in column 9 the amino acid content of casein obtained from the literature[6–15]. Results obtained by Gordon and others[16] are included in column 10. Results in columns 9 and 10 have been computed for a nitrogen content of 12·5 per cent.

TABLE I

THE QUANTITATIVE ESTIMATION OF THE FREE AND TOTAL AMINO ACIDS IN CASAMIN E

	Free amino acid g./100 g.			Total amino acid g./100 g.					
							Casein		
Amino acid	A	B	C	A	B	C	Determined	Literature*	Gordon and others[16]
Gly ..	0·191	0·16	0·38	1·61	1·64	1·76	1·56	1·52 (6)	2·16
Ala ..	0·643	0·605	1·58	2·56	2·70	2·69	2·59	2·8 (7)	2·40
Val ..	1·63	1·51	3·43	5·9	6·16	6·35	6·0	5·75 (8)	5·75
Leu's ..	6·45	5·90	9·25	12·35	13·23	13·32	12·85	14·3 (8)	12·25
Ser ..	0·98	1·0	1·81	4·92	4·90	5·01	3·92	4·72 (9)	5·03
Thr ..	0·85	0·833	1·78	3·57	3·83	3·71	3·85	3·60 (9)	3·92
Tyr ..	0·642	0·353	0·743	1·29	1·51	1·47	4·75	4·87 (10)	5·03
Phe ..	2·97	2·68	3·47	4·3	4·62	4·62	4·55	4·4 (8)	4·0
Met ..	1·37	1·33	2·36	2·47	2·33	2·47	2·3	2·48 (11)	2·24
Arg ..	1·90	1·99	2·49	2·5	2·85	2·70	3·4	3·2 (12)	3·28
His ..	0·92	0·733	1·23	2·34	2·25	2·14	2·46	2·56 (12)	2·48
Cys ..	—	—	—	0·242	0·242	0·254	0·164	0·272 (11)	0·272
Lys ..	4·2	4·0	4·73	6·1	6·25	6·25	6·97	6·55 (13)	6·55
Pro ..	0·13	0·12	1·1	8·6	8·9	8·5	8·23	9·27 (8)	9·05
Asp ..	1·46	2·35	6·4	23·6	23·4	23·9	23·4	23·4 (14, 15)	23·6
Glu ..	1·26								
Try ..	0·84	0·772	1·08	—	—	—	—	—	—
Total ..	26·436	24·286	41·833						
Nitrogen per cent	12·45	12·33	12·5						

* Number in parentheses refers to reference number.

Figure 1 shows the chromatogram of the DNP-amino acids and peptides of batch A, similar chromatograms were obtained with batches B and C. Three spots correspond to peptides and a fourth trailing spot in the aqueous extract is present. 0·2 ml. of the ether extract was applied on each of four sheets of Whatman 3 MM paper and the papers were subjected to two-dimensional chromatography. 0·2 ml. of the aqueous extract was chromatographed using the ethyl benzene system. The spots corresponding to the peptides were excised and eluted with water. The eluates from corresponding spots in the three batches were pooled together and then concentrated under vacuum. The solution was acidified with 6 N hydrochloric acid and the DNP-peptides were extracted with ethyl acetate to free them from salts and the extract evaporated to dryness under vacuum. The residue was dissolved in 0·3 ml. of 6 N hydrochloric acid and 0·15 ml. of the solution was completely hydrolysed at 105° for 24 hours. An aliquot was subjected to two dimensional chromatography for amino acid analysis[17]. The remainder of the solution was hydrolysed for 8 hours at 105° and an aliquot was examined for the *N*-terminal amino acid as the DNP-derivative by two dimensional chromatography[5]. All the peptides spots gave several DNP-amino acids thus indicating that they consist of a mixture of peptides. For example spot I of batch A gave aspartic* and glutamic* acids, trace of threonine, alanine*, valine*, leucines*,

proline, serine*, glycine, phenylalanine* and a trace of arginine. Lysine and the amino acids marked with an asterisk appeared as *N*-terminal residues.

Bacteriological examination. Three batches were tested for their strepogenin activity on *Lactobacillus casei* ATCC (7469) using the media of Steele and others[1] and Kodicek and Mistry[2]. The procedure followed for the culture and inocula was as that described by Ågren[18]. The

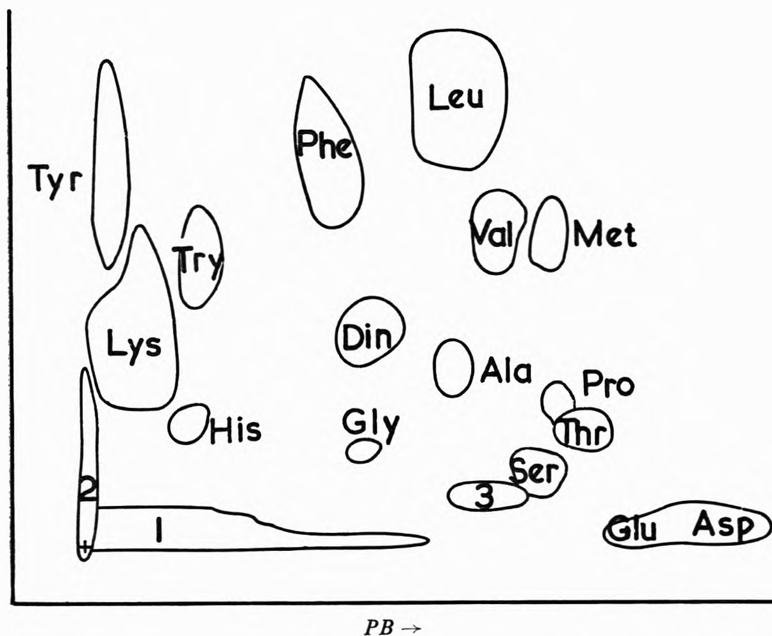

FIG. 1. Two dimensional chromatogram of DNP-amino acids and peptides in Casamin E, batch A. + point of application. *E*=direction of ethyl benzene developer. *PB*=direction of 1·5M phosphate buffer. *Din*=dinitrophenol.

method of assay was as previously reported[3]. The growth stimulatory effect was given as scale reading on the Klett-Summerson photoelectric colorimeter measured after 24, 48 and 72 hours incubation. The effect on lactic acid production was measured by titrating with 0·077 N sodium hydroxide using bromothymol blue as indicator. Results are given in Table II.

DISCUSSION

Table I shows that the content of free amino acids in the three batches of casamin varied from 24 to 42 per cent. While the difference is small between batches A and B, batch C shows more than 60 per cent increase in the total free amino acids over either A or B. The three batches of casamin show a higher content of free amino acids than the three batches of bacto-casitone previously examined[3], in which variation was from 15·9 to 17·5 per cent. This difference probably originates in the conditions of the enzymatic digestion of casein during the manufacturing

process. The free amino acids are not liberated in proportion to their occurance in the protein. The total amino acids were similar in the three batches and with the exception of tyrosine compared favourably with the reported data of casein and also with the values obtained by the quantitative paper chromatography technique. The uncorrected value for serine in column 8 was lower than that reported in the literature. This may result from the greater lability to acid hydrolysis of serine combined as phosphoserine in phosphoproteins compared with that of serine itself. It seems that some destruction of tyrosine takes place during the manufacturing process as evidenced by the low values of tyrosine in the three batches. From Figure 1 and *N*-terminal analysis of the peptide spots it is concluded that they consist of a mixture of peptides. There appears to be some variation in the peptides in corresponding spots in the three batches.

TABLE II

THE EFFECT OF CASAMIN E ON THE GROWTH OF *L. casei*

Time hours	Control	Casamin A mg.			Casamin B mg.			Casamin C mg.			Wilson's Liver L		
		0·1	0·5	1·0	0·1	0·5	1·0	0·1	0·5	1·0	0·1	0·5	1·0
A—Medium of Steele and Others*													
24	7	53	114	145	45	92	117	24	59	84	5	15	23
48	17	131	239	287	119	235	286	75	201	235	20	56	88
72	256	164	255	300	163	260	305	128	237	273	70	118	137
Acid Production†													
48	0	1·7	4·15	5·75	1·45	3·6	4·2	1·0	3·15	4·25	0·15	0·35	1·1
72	5·25	4·15	8·6	9·50	4·15	8·45	9·7	2·95	7·3	8·8	0·95	1·4	3·1
B—Medium of Kodicek and Mistry*													
24	81	85	150	178	87	147	177	75	155	171	66	103	148
48	267	278	297	300	288	296	307	280	298	310	278	286	298

* Scale reading on the Klett-Summerson colorimeter. † ml. 0·077 N sodium hydroxide.

The three batches of casamin showed a stimulatory effect on the growth and the lactic acid production of *L. casei*. The pattern of stimulation differed with the basal medium used. Thus using the medium of Steele and others the stimulatory effect of casamin continued even after 72 hours. The growth stimulatory effect of batch C appears to be about 10 per cent less than that of batch A and B, whether this decrease is significant can not be decided from the data presented. The tubes containing 0·1 mg. casamin as well as those containing Wilson's liver fraction L showed less growth than the control after 72 hours incubation. Using the medium of Kodicek and Mistry[2] it was found that after 48 hours, the tubes containing all levels of casamin showed comparable growth.

The three batches of casamin showed comparable strepogenin activity with the three batches of Bacto-Casitone previously examined[3], although the free amino acid content differed. It seems that the strepogenin activity is exhibited by peptides of widely different structure.

Acknowledgements. I wish to express my sincere thanks to Professor Harold G. Cassidy for his invaluable suggestions and advice throughout the work. This work was supported by research grants RG 3207 (C3, C381, C4, C5) from the Division of Research Grants, National Institutes of Health, Public Health Service.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Hexachlorophene and Bithionol, Assay of, in Solid and Liquid Soaps, Emulsions and Dusting Powders. H. G. van der Pol. (*Pharm. Weekbl.*, 1958, **20**, 881.) This paper describes a spectrophotometric method which enables hexachlorophene (2:2′-dihydroxy-3:3′:5:5′:6:6′-hexachlorodiphenylmethane) and bithionol (2:2′-dihydroxy-3:3′:5:5′-diphenyl sulphide) to be determined separately or in the presence of one another. Two equal portions of a methanolic solution or extract are separately diluted to an equal extent by a methanolic acid and alkaline buffer respectively. The two solutions, which are of equal strength and only differ in pH, are subjected to measurement as follows. The extinction of the alkaline solution is measured at 312 mμ using the acid solution as a blank, giving a measure of hexachlorophene, and at 328 mμ for bithionol using 1 cm. cuvettes in each case. Any absorption due to irrelevant ingredients is thus compensated for. Formulae are given for the calculation of the concentrations of the medicaments, and the method is specific, quick and has an accuracy of $\pm$ 2 per cent. D. B. C.

Nicotinic Acid, Quantitative Determination in Pharmaceutical Preparations. K. Howorka. (*Pharm. Zentralh.*, 1958, **97**, 521.) Whereas the cation exchanger Wofatit KPS 200 binds nicotinic acid quantitatively if used in the H-form, it cannot do so in the pyridine-form. If mineral salts are also present, an aliquot part of the solution being assayed is put through a column of the exchanger in the H-form. The difference in the amount of acid liberated can be used to find the amount of nicotinic acid present. In the case of esters and amides of nicotinic acid, saponification using 0·5 N ethanolic KOH is carried out, the solution acidified with sulphuric acid, the sulphate precipitated with excess barium hydroxide solution, and the solution made up to a definite volume. For preparations containing nicotinic acid esters and other saponifiable material, saponification and acidification is carried out as above, and the free fatty acids are allowed to rise to the surface on cooling. Hard paraffin may be used to assist the formation of a solid layer. The aqueous layer is treated as above.
D. B. C.

(+)-Pulegone, Colorimetric Estimation of, with 3:5-Dinitrobenzoic Acid. D. H. E. Tattje. (*Pharm. Weekbl.*, 1958, **93**, 1048.) In this method, 4 ml. of a solution of (−)-pulegone containing up to 0·65 mg./ml. is mixed with 5 ml. of a 4 per cent ethanolic solution of 3:5-dinitrobenzoic acid and 2 ml. of 3 N aqueous sodium hydroxide solution is added. The extinction coefficient is measured at 5375 Å against a blank containing 4 ml. of ethanol, 5 ml. of reagent and 2 ml. of 3 N sodium hydroxide about 40 minutes after the addition of the alkali. The colour remains constant for a further 8 minutes. The temperature must be controlled to within narrow limits, preferably around 20°. For (+)-pulegone, E (1 per cent, 1 cm.) = 82·3 (standard deviation, 1·599).

For (−)-piperitone and piperitenone, which may occur in pennyroyal oil, E (1 per cent, 1 cm.) values are respectively 128 and 235. Menthone does not react with 3 : 5-dinitrobenzoic acid. It was shown that this colorimetric method agreed with the neutral sulphite method, and was probably more specific.

D. B. C.

Piperazine, Quantitative Determination of, in Pharmaceutical Preparations. M. Hädicke. (*Pharm. Zentralh.*, 1958, **97**, 365.) Two methods are suggested involving the precipitation of piperazine with sodium tetraphenylboron or Reinecke's salt, both of which react with each nitrogen atom of the piperazine. After washing, the precipitate in each case is dissolved in acetone and then estimated titrimetrically. In the case of the tetraphenylboron complex, an argentimetric method is used with eosin as an absorption indicator, whereas with the Reineckate complex, excess standard silver nitrate solution is added followed by back-titration with standard ammonium thiocyanate solution using ferric alum as indicator. The results of both methods are in good agreement and the error in each is about $\pm$ 2 per cent.

D. B. C.

Reserpine, Determination of, in Tablets by Infra-red Spectrophotometry. W. R. Maynard, Jr. (*J. Assoc. off. agric. Chem., Wash.*, 1958, **41**, 676.) This method is based on the intensity of a carbonyl band at 5·78 μ in reserpine in chloroform. Although reserpine breaks down slowly in chloroform, no other solvent could be found without encountering absorption difficulties, and the rate of decomposition is not sufficient to affect the accuracy of the assay. The concentration required is about 2 mg./ml. and a standard of this concentration is used for comparison. The most commonly occurring impurities are rescinnamine and deserpidine and these can be determined admixed with reserpine. Rescinnamine is determined by measuring the absorbance at 7·58 μ and comparing with a standard. Reserpine is found by measuring the total absorbance at 6·1 μ and subtracting that due to rescinnamine at this wavelength. Deserpidine is then determined by measuring the carbonyl absorption at 5·78 μ and subtracting the absorbance of reserpine and rescinnamine at this wavelength. Experimental results indicate an accuracy of about $\pm$ 1 per cent for reserpine.

D. B. C.

Strychnine in Nux Vomica, Determination of, by Paper Chromatography, G. P. Briner. (*Nature, Lond.*, 1958, **182**, 742.) A simplified paper chromatographic procedure is described in which a chloroform extract of the alkaloids, containing 60–20 μg. of strychnine is spotted on to Whatman No. 1 paper, and developed overnight with n-butanol: n-propanol: 0·05N hydrochloric acid (1 : 2 : 1) at 20° by an ascending technique. The paper is dried, photographed in the ultra-violet to locate the spots, which are cut out and eluted with water. The strychnine is determined spectrophotometrically in the aqueous solution at λ max 255 mμ.

J. B. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTY

Penicillin, Synthesis of: 6-Aminopenicillanic Acid in Penicillin Fermentations. F. R. Batchelor, F. P. Doyle, J. H. C. Nayler and G. N. Rolinson. (*Nature, Lond.*, 1959, **183**, 257.) The isolation of 6-aminopenicillanic acid

(I, R = H) from penicillin fermentations of *Penicillium chrysogenum* W.51.20 carried out in the absence of side-chain precursor, is reported. The product was detected, after removal of natural penicillins by solvent extraction at low pH, by treating with phenylacetyl chloride in the presence of sodium bicarbonate. The resulting antibiotic substance was indistinguishable from penicillin G (I, R = $CO{\cdot}CH_2C_6H_5$) on paper chromatograms; treatment similarly with phenoxyacetyl chloride gave a product indistinguishable from penicillin V (I, R = $CO{\cdot}CH_2O{\cdot}C_6H_5$).

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Me2C — CH·CO2H
 |      |
 S      N
  \   /  \
   CH    CO
     \  /
      CH·NHR

      (I)
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Crystalline 6-aminopenicillanic acid has been isolated and converted into both benzyl- and phenoxymethylpenicillin, and the *N*-carbethoxy derivative (I, R = EtO) has been degraded by known methods to *N*-carbethoxyaminoacetaldehyde (isolated as the 2:4-dinitrophenylhydrazone). The antibacterial activity of 6-aminopenicillanic acid is less than that of the derived penicillins and the spectrum is different. It is destroyed by penicillinase but more slowly than benzylpenicillin, is unstable to alkali but stable to acid. The isolation of 6-aminopenicillanic acid is consistent with the view that introduction of the side chain is the last step in the biosynthetic process. J. B. S.

PHARMACY

Drug Release from Gradual Release Preparations, Determination of. D. J. Campbell and J. G. Theivagt. (*Drug Standards*, 1958, **26**, 73.) Apparatus similar to that specified in the U.S. Pharmacopoeia for tablet disintegration tests was used, the conditions being arranged to simulate *in vivo* conditions of motion, pH and temperature. The preparation was placed in the basket of the apparatus and treated with simulated gastric juice, followed by solutions containing increasing amounts of simulated intestinal fluid. The solutions were analysed, the percentage of medicament released during each hour calculated, and the general pattern of drug release determined. This procedure was applied to preparations consisting of drug embedded in inert plastic material, coated granules enclosed in capsules, and multilayered tablets, and can be used for evaluation of release patterns of most solid slow release preparations for oral use. G. B.

Pyrogens, Investigations Concerning. G. van der Reijden. (*Pharm. Weekbl.*, 1958, **93**, 657.) In order to ascertain whether an asbestos pad could be used to make solutions pyrogen-free, a 1:50,000 solution of methylene blue was used: 10 sq. cm. of pad should decolorise not less than 300 ml. of this solution. A direct relationship existed between the area of pad and the volume of solution which could be freed from solute. When a test solution containing 4×10^6 cells of *Ps. aeruginosa* per ml. is used, 10 sq. cm. of pad should free 200 ml. of this solution from pyrogen. This contains more pyrogen than would normally occur in practice. It was found that 0·3 per cent activated charcoal was sufficient to adsorb the pyrogen from such a solution, and this was also achieved by 0·6 per cent of asbestos fibre. A rest period of a week or more between the tests was desirable for rabbits, and the test recommended which was based upon the fever reaction is described in detail. Attempts were made to design a seriological pyrogen test, but these failed because the by-products of only a few kinds of bacteria were adsorbed by erythrocytes, and even then the test was far less sensitive than the fever reaction produced in rabbits. Also haemolysis and other unwanted reactions sometimes occurred. D. B. C.

Pyrogens, their Properties and Destruction. K. D. Rudat. (*Pharmazie*, 1958, **11**, 677.) This paper includes a historical survey of work related to the initial recognition, properties and composition of pyrogens, with a discussion on their origin in solutions and their introduction via apparatus. This is followed by recommendations on the removal of pyrogens from solutions by Seitz filtration and destruction in apparatus by hot-air sterilisation at 160° to 200° for long periods. Mention is made of the preference of single-use plastic tubing over rubber tubing. B. R.

Reserpine, Oxidation of, During the Preparation as Tablets. O. Weis-Fogh. (*Arch. Pharm. Chem.*, 1958, **65**, 859.) Butylated hydroxanisole, hydroquinone, nordihydroguaiaretic acid, and propyl gallate were shown to prevent oxidation of reserpine during the preparation of tablets. The most effective antoxidant was nordihydroguaiaretic acid, a quantity of 20 μg. per 0·25 mg. tablet, dissolved in the reserpine solution before adding to the granulate, being sufficient to reduce the loss of reserpine below 1 per cent. The antoxidants did not prevent the deterioration of reserpine tablets stored in daylight. G. B.

***Salmonella abortus equi*, Chemical Analysis of Lipopolysaccharides in.** I. Fromme, O. Lüderitz, A. Nowotny and O. Westphal. (*Pharm. Acta Helvet.*, 1958, **33**, 391.) Lipopolysaccharides isolated from bacteria are extremely specific in immunisation for the particular bacteria, and the lipoid component is essential for the development of endotoxic activity. The highly purified lipopolysaccharide derived from *Salmonella abortus equi* is one of the most active of such agents for higher animals and man. This has been degraded into a phosphorylated polysaccharide and a lipid. The polysaccharide was resolved into glucosamine, galactose, heptose, glucose, mannose, rhamnose and 3-desoxy-D-fucose, and the percentage of each was determined. Stepwise hydrolysis of the lipopolysaccharide with N mineral acid for 3–5 minutes gave 45 per cent of a lipid designated A I and a phosphorylated polysaccharide fraction which was resolved into the above monosaccharides and phosphoric acid after prolonged acid hydrolysis. Lipid A I, after 30 to 60 minutes' further refluxing yielded 26 per cent of Lipid A which was soluble in chloroform and pyridine. Lipid A required 10 to 20 hours' refluxing with 6 N hydrochloric acid for complete hydrolysis into a fatty acid and water-soluble amino acids and phosphoric acid. Among the amino acids were glutamic acid, aspartic acid, α,ϵ-diaminopimelic acid, lysine, alanine and serine. Ethanolamine and other unidentified fractions were also isolated. D. B. C.

PHARMACOGNOSY

***Datura tatula*, Investigation of Mutations in.** E. Steinegger. (*Pharm. Acta Helvet.*, 1958, **33**, 357.) Mutations produced by X-rays in *Datura tatula* L. var. *inermis* Timm. are described. Small packets of seeds from one source were exposed to an equal intensity of X-rays from a copper source of known intensity. The batches were then grown for four generations with further irradiations. The following types of mutation were noticed, firstly, changes in appearance such as dwarfishness, curled leaves, small leaves, fruits and seeds and thorny fruits; secondly, plants with the tetraploid number of chromosomes and increased alkaloidal content and often correspondingly large pollen grains and seeds; thirdly, plants with pollen grains four times the normal size, but with the diploid number of chromosomes; and fourthly, diploid plants with increased alkaloidal content. D. B. C.

***Digitalis lanata* Ehrh., Changes in the Glycosidal Content of, during Development.** L. Fauconnet and D. Kutter. (*Pharm. Acta Helvet.*, 1958, **33**, 369.) A method is described for the paper chromatographic separation and quantitative estimation of up to 15 active principles including some newly found glycosides in *D. lanata* Ehrh. From four to nine of these are found in the seed, three in the young plant, and twelve in the fully developed plant during the first year, and up to fifteen during the second year. Analyses on the leaves, stem and seeds showed that the distribution of glycosides was irregular, and that the concentrations varied with the time of day, the age of the plant and prevailing weather conditions. D. B. C.

PHARMACOLOGY AND THERAPEUTICS

Aspirin: Occult Blood in Faeces following Administration. L. Th. F. L. Stubbé. (*Brit. med. J.*, 1958, **2**, 1062.) Occult blood in the faeces occurred in about 70 per cent of 180 persons (mostly between 40 and 60 years of age) to whom aspirin in the form of tablets was given in doses of from 750 to 3000 mg. daily for periods of 14 days or more. Results obtained with 140 patients in the rheumatology wards were entirely comparable to those found in a control group of 40 healthy volunteers. The quantity of aspirin administered did not greatly influence the occurrence or seriousness of the result. Identical results were obtained whether the aspirin was given in powder form suspended in water or whether it was given as tablets. The value of coated tablets is doubtful, as 15 out of 20 patients gave a positive benzidine reaction following administration of such tablets. On the basis of the strength of the benzidine reaction in a number of cases it was shown that the quantity of blood lost following aspirin administration may not always be ignored, especially if the patient is already anaemic. Sodium salicylate tablets, whether coated or uncoated, did not give rise to a positive benzidine reaction. S. L. W.

Batyl Alcohol, Erythropoietic Stimulatory Activity of. J. W. Linman, F. H. Bethell and M. J. Long. (*J. Lab. clin. Med.*, 1958, **52**, 596.) In controlled experiments, racemic batyl alcohol, the monoglycerol ether of *n*-octadecyl alcohol, was administered subcutaneously to normal rats in doses of 12·5 and 25 mg. daily for 4 weeks. Batyl alcohol stimulated erythropoiesis apparently through accelerating erythroblastic cellular division but without associated augmentation in haemoglobin synthesis. This response was manifested by erythrocytosis due to microcytes with decreased osmotic resistance, reticulocytosis and myeloid erythrocytic hyperplasia. The substance also stimulated thrombopoiesis and probably granulopoiesis. The chemical, physical and physiological characteristics of batyl alcohol are similar to those of the thermostable, ether-soluble plasma erythropoietic factor, indicating that they may be the same or related compounds. The authors suggest that all aspects of myelopoiesis may be under the influence of humoral regulatory mechanisms and that batyl alcohol, produced in yellow bone marrow, may be of primary importance in such a system. W. C. B.

Chloroquine in the Treatment of Rheumatoid Arthritis. H. Fuld and L. Horwich. (*Brit. med. J.*, 1958, **2**, 1199.) Thirty-nine patients with rheumatoid arthritis largely resistant to other forms of treatment were treated with chloroquine and the results followed up for periods of up to 4 years. Complete remission was obtained in 12 patients, a major improvement in 19, and minor or no improvement in 8. The regimen adopted was as follows: (1) chloroquine,

600 mg. daily initially, reduced only when gastric irritation made it necessary (maintenance dose, 200 to 400 mg. daily); as no benefit from the chloroquine can be expected for some 3 weeks from the start of treatment, calcium aspirin was given during this period for symptomatic relief: (2) bed rest for several weeks or until the E.S.R. had returned to normal: (3) induced pyrexia (T.A.B. vaccine intravenously): (4) weekly transfusions of 1 pint of fresh blood for 3 or 4 weeks (in 11 patients): (5) injection of hydrocortisone acetate into single joints: (6) physiotherapy after acute stage. The drug was well tolerated by the majority of patients. Relapses while on chloroquine were not observed in this series. S. L. W.

Cyanocobalamin: Oral Administration in Pernicious Anaemia. J. N. M. Chalmers and N. K. Shinton. (*Lancet*, 1958, **2**, 1298.) From a study of 22 cases of pernicious anaemia in relapse further evidence is presented that oral cyanocobalamin can be absorbed and utilised by these patients. Daily oral doses of from 20 to 500 μg. of cyanocobalamin were given for periods varying from 15 days to 50 months. The drug was taken in a single dose, usually first thing in the morning. It was given as a syrup containing the cyanocobalamin in a 2-drachm dose. The syrup contained 20 per cent w/v sucrose and a small amount of sodium carboxymethylcellulose, with colouring and flavouring substances. The larger the daily oral dose, the more prompt is the response and the return of the serum-vitamin-B_{12} level to the normal range. Long-term studies show that daily oral doses of 100 μg. or more of cyanocobalamin can be effective in maintenance therapy, but there can be haematological and clinical remission without a return of serum-vitamin-B_{12} level to normal. Parenteral therapy with cyanocobalamin is still the most reliable method of ensuring freedom from relapse in pernicious anaemia but oral treatment taken daily may be effective. S. L. W.

Dequalinium in the Treatment of Skin Infections. R. B. Coles, C. Grubb, D. Mathuranayagam and D. S. Wilkinson. (*Brit. med. J.*, 1958, **2**, 1014.) Dequalinium chloride (Dequadin), in the form both of paint and cream, was used in 241 patients suffering from various skin conditions. Cure, or considerable improvement, resulted in 43 out of 51 staphylococcal skin infections, usually within a week; good results were also obtained (27 improved out of 35) in conditions of mixed aetiology in which staphylococcal or monilial infections played an important part. The results were especially gratifying in cases of secondarily infected eczema and infective eczematoid dermatitis round the ears and in the body folds. On the hairy areas, the beard and scalp, the paint was particularly useful. Eight out of 13 cases of ringworm were cleared or improved. Non-infective conditions did not respond. It appeared to be as effective as antibiotic preparations and dyes, and is colourless and pleasant to use. Apart from 3 cases of sensitisation, no irritant or toxic effects were observed, and it can be used on the mucous membranes and on the skin of infants. In 37 cases of pruritic skin conditions a dequalinium cream containing 0·5 per cent prednisolone showed a superior effect to the plain dequalinium cream and was successful in all cases. S. L. W.

Methyprylone and Quinalbarbitone Compared as Hypnotics. T. J. Thomson. (*Brit. med. J.*, 1958, **2**, 1140.) The hypnotic effect of methyprylone (Noludar) was compared with that of quinalbarbitone, using the double-blind technique, in 128 patients ranging from 24 to 74 years of age, all of whom required hypnotic drugs. Powders of ascorbic acid were used as a control. The results were assessed by sequential analysis. No important difference could be detected

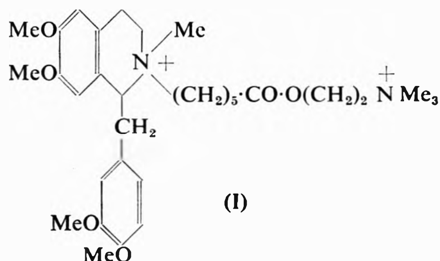
between the apparent hypnotic action of 200 mg. of methyprylone and the control powder, and no important differences were discovered between the hypnotic effects of 400 mg. of methyprylone and 100 mg. of quinalbarbitone.

S. L. W.

Morphine Derivatives, New, Acute Pharmacological Studies of Some. R. Okun and H. W. Elliott. (*J. Pharmacol.*, 1958, **124**, 255.) The analgesic properties, acute toxicity and effects on gastrointestinal motility of morphinone, 6-methylmorphine, 6-methyldihydrodesoxymorphine, 6-methylenedihydrodesoxymorphine and 6-methyl-7-hydroxydihydrodesoxymorphine were studied in mice. Morphinone was less potent in its analgesic effect and had a lower therapeutic index than morphine itself, but the remaining compounds, although their effects were briefer, were considerably more potent than morphine and their therapeutic indices were higher. All five compounds had weaker inhibitory effects than morphine on gastrointestinal motility. 6-methylenedihydrodesoxymorphine appeared to be the most promising compound, being, on a weight basis, 82 times as powerful as morphine in its analgesic action and only twice as toxic. Further tests on this compound were carried out on dogs in which it was again shown to be many times more potent than morphine in its analgesic effect although shorter in its duration of action. The respiratory depression produced by large doses of the compound was antagonised by nalorphine. Side effects were minimal suggesting that the compound may be clinically useful as a short-acting analgesic.

W. C. B.

Neuromuscular Blocking Agents, Some New. H. O. J. Collier, J. M. Z. Gladych, B. Macauley and E. P. Taylor. (*Nature, Lond.*, 1958, **182**, 1424.) The advantage of suxamethonium in producing muscular relaxation during anaesthesia is its brevity of action but its disadvantage is that it has no satisfactory antagonist and that it sometimes causes post-operative muscle pains and stiffness. Laudexium (laudolissin) is antagonised by neostigmine and does not cause muscle pain but it is of longer duration of action than suxamethonium. Compounds have therefore been made, incorporating some of the chemical features of each into one molecule, in an attempt to obtain a substance which has the valuable properties of both suxamethonium and laudexium. Three series of compounds were investigated; the symmetrical bis-esters, the unsymmetrical mono-esters, and the symmetrical bis-amides. All the compounds were examined by intravenous administration to cats, recording blood pressure and tibialis muscle twitch. The potencies and durations of the neuromuscular blocking actions of the new compounds were compared with those of suxamethonium chloride or sometimes with those of tubocurarine chloride or laudexium methosulphate. Neostigmine methosulphate was used in antagonism tests. It was found that the only compound more active than suxamethonium was the unsymmetrical mono-ester, No. 43 (I), which was about



(I)

seven times as active as the reference compound, and appears to be one of the most potent synthetic neuromuscular blocking agents known. It was of long duration of action and did not show antagonism by neostigmine. Cats maintained by artificial respiration tolerated very large doses. The only compound briefer in action than suxamethonium was the symmetrical bis-ester No. 41 (structure given), which was of relatively low potency, and antagonised by neostigmine. M. B.

Nicotinamide, Tranquillising and Antiserotonin Activity of. D. W. Woolley. (*Science*, 1958, **128**, 1277.) Nicotinamide in a concentration of 0·01M completely inhibited the response of the isolated uterus from oestrogenised rats to 5-hydroxytryptamine (5-HT). The concentration of 5-HT used was just sufficient to elicit a maximal contraction of the untreated muscle. The antagonism did not appear to be strictly competitive. The same concentration of nicotinamide was only slightly effective in reducing acetylcholine-induced contractions of the uterus. Sodium nicotinate in a concentration of 0·01M was without antagonistic action to 5-HT in this tissue. In mice, large doses of nicotinamide protected the animals against the effects of hydroxytryptophan. The large doses of nicotinamide used also caused marked tranquillisation of the animals which was more severe than that produced in mice by reserpine.
W. C. B.

Normorphine, Analgesic Properties of, in Patients with Postoperative Pain. L. Lasagna and T. J. De Kornfeld. (*J. Pharmacol.*, 1958, **124**, 260.) The analgesic effects of subcutaneously administered morphine and normorphine were compared in controlled tests on 60 patients suffering from post operative pain. Normorphine in doses of 40 mg. was found to have approximately the same analgesic effect as 10 mg. of morphine. These results supply evidence against the theory that *N*-demethylation of morphine is necessary before its analgesic effects are produced in man. The authors conclude that it is unlikely that normorphine will offer any advantage over morphine in the treatment of acute pain. W. C. B.

Podophyllotoxin, Pharmacology of. G. Valette, M.-L. Hureau and J. Cariou. (*Ann. pharm. franç.*, 1958, **16**, 169.) Podophyllotoxin was shown to stimulate peristalsis in the small intestine and colon of the cat and rat (*in situ*). In experiments on the isolated organs, podophyllotoxin was found to have a depressant action on the rat duodenum, but it stimulated contraction of the rat colon and guinea pig ileum. Podophyllotoxin antagonised the action of acetylcholine on the rat duodenum, and potentiated the effect of histamine on the guinea pig ileum. Podophyllotoxin dissolves in blood serum in which it combines with serum albumin. A similar effect has been observed with anthraquinones and anthranols and may be the basis of their action on the intestine.
G. B.

Pyridine-2-aldoxime Methiodide and Diacetyl Monoxime against Organophosphate Poisoning. H. Edery and G. Schatzberg-Porath. (*Science*, 1958, **128**, 1137.) The protective actions of pyridine-2-aldoxime methiodide (PAM) and diacetyl monoxime (DAM), separately and in combination, were studied in white male mice against poisoning by tetraethylpyrophosphate, dyflos, bis(dimethyl amido) fluorophosphate and diethyl-*p*-nitrophenyl phosphate. Statistical analysis of the results showed that DAM potentiated the protective action of PAM against tetraethylpyrophosphate and bis(dimethylamido)

fluorophosphate but not against dyflos or diethyl-*p*-nitrophenyl phosphate. DAM administered alone showed a weak protective action against tetraethylpyrophosphate but was ineffective against bis(dimethylamido) fluorophosphate and diethyl-*p*-nitrophenyl phosphate. Residual cholinesterase in the brain and blood of animals which had either died from, or had survived, the poisoning was determined and practically no difference was observed in the two cases. The authors conclude that the results do not support the assumption that the protective action of the antidotes can be attributed mainly to their ability to reactivate cholinesterase. W. C. B.

Salicylate Anaemia. W. H. J. Summerskill and A. H. Alvarez. (*Lancet*, 1958, **2**, 925.) Severe 'iron deficiency' anaemia of uncertain cause had required continuous treatment with iron for 5 and 10 years respectively in a man of 47 and a woman of 40; total hysterectomy had failed to influence the anaemia in the woman. Habitually heavy consumption of salicylate compounds for headaches had coincided with the onset and subsequent course of the anaemia in both patients. Occult bleeding from the gastrointestinal tract was demonstrated during controlled periods of salicylate medication (10 grains of soluble aspirin twice or four times daily). After salicylate consumption had been greatly reduced anaemia did not recur during follow-up periods of 10 and 7 months without iron therapy. Salicylate consumption is put forward as a possible cause of anaemia, and where this is suspected as a causal factor the effect of withdrawing the salicylate should be tried before treatment with iron or alternative procedures such as total hysterectomy or laparotomy (which was contemplated in both these patients). S. L. W.

Triamcinolone: Comparison with Prednisolone in Rheumatoid Arthritis. F. D. Hart, J. R. Golding and D. Burley. (*Lancet*, 1958, **2**, 495.) The treatment of 24 patients with rheumatoid arthritis and of 1 with psoriatic arthopathy was changed from prednisolone to triamcinolone and, after periods varying from 5 to 70 days, back to prednisolone. The dose ratio of triamcinolone to prednisolone was 4/5. Of the 25 patients, 11 preferred triamcinolone, 3 preferred prednisolone, and 11 were indifferent. Measurement of sedimentation rate and digital tenderness remained unchanged; finger swelling and grip strength altered in only 5 patients, each of whom improved on triamcinolone. There were no acute rebound symptoms on changing to either drug. In only 5 cases was subjective improvement more than slight, in each case in favour of triamcinolone. Of 7 patients who complained of dyspepsia on prednisolone, 3 improved on triamcinolone and 3 deteriorated. In 3 patients abdominal striae appeared within 3 weeks of changing to triamcinolone, and 3 patients noticed post-prandial flushing on triamcinolone but not on prednisolone. Although triamcinolone does not differ greatly in its therapeutic action or side-effects from prednisolone it is a useful alternative steroid. S. L. W.

Triamcinolone in Lupus Erythematosus. E. L. Dubois. (*J. Amer. med. Ass.*, 1958, **167**, 1590.) Twenty-nine patients with systemic lupus erythematosus were treated with triamcinolone for periods of up to 14 months. The average initial dosage in mild cases was 20·6 mg. daily, and the average maintenance dose used to control mild exacerbations was 26 mg. daily. The pattern of clinical improvement closely paralleled that obtained by previous treatment with the older steroids. The fever abated in 24 hours, joint pains disappeared in several days, and pleural effusions and cutaneous lesions subsided in one to two weeks. Retinal changes, anaemia, adenopathy, and cachexia, gradually improved, and

renal abnormalities often returned to normal during steroid-induced remissions, though long-standing renal lesions were unaffected. The cutaneous side-effects, particularly Cushingoid appearance, hirsutism and striae were more marked than with the older steroids. The most serious side-effect was muscle weakness, most marked in the quadriceps groups, which appeared in 6 patients; the weakness gradually cleared on changing to another steroid. No cases occurred among 7 males in the series, and no male patients showed Cushingoid features. A major difference between triamcinolone and other steroids was a tendency, in 18 patients, towards progressive gradual weight loss, some of which was fluid loss. There was no evidence of sodium retention or potassium loss. There was evidence of peptic ulceration in only one patient, who received 96 mg. daily. Fourteen of the patients had received previous steroid therapy, and 7 of them were better controlled and felt better with triamcinolone than with other steroids. S. L. W.

Trimeglamide, A New Sedative and Soporific Drug. G. Cronheim, J. T. Gourzis and I. M. Toekes. (*Science*, 1958, **128**, 1570.) Trimeglamide (trimethoxybenzoyl-glycine diethylamide) has in animals a somnifacient action which is not preceded or followed by excitement or ataxia. In dogs the oral soporific dose was 50 mg./kg. and sleep lasted 2 to 6 hours. Sleeping animals could be easily aroused; when they responded normally to external stimuli. There were no effects on blood pressure, heart rate and respiration. Larger doses (100 mg./kg.) prolonged the sleep in cats. In dogs side effects appeared, emesis, muscle twitching and slight ataxia. Raising the dose to 500 mg./kg. had a stimulant effect, superimposed upon the soporific action, characterised by restlessness, purposeful locomotion, slight ataxia and some disorientation. There was an absence of hypnosis or anaesthesia. In man single or repeated oral doses of 500 to 1500 mg. caused sedation and drowsiness. There were no changes in electroencephalographic records and there was no hypnosis or anaesthesia. The acute toxicity was very low. Dogs, cats and mice have tolerated single oral doses of 500, 770 and 2000 mg./kg. respectively. In mice and dogs trimeglamide significantly prolonged the sleeping time and some tolerance developed to this effect following repeated doses of trimeglamide. Trimeglamide had an anticonvulsant effect in mice against electro-shocks.

G. F. S.

Vitamin B_{12} Absorption in Pernicious Anaemia. M. Schwartz, P. Lous and E. Meulengracht. (*Lancet*, 1958, 2, 1200.) A study of the absorption of radioactive vitamin B_{12}, by the Schilling's technique, has shown that a blockage of absorption is common with patients with pernicious anaemia who have been treated orally for long periods with vitamin B_{12} and a purified preparation of hog pyloric mucosa. This blockage rarely occurs with unpurified preparations of whole pylorus. The blockage appears to be related to the hog intrinsic factor, a resistance having developed preventing absorption of B_{12}. Such patients absorb B_{12} bound to human intrinsic factor obtained from fundus mucous membrane. Absorption also occurs when vitamin B_{12} is given with rat gastric juice. It is suggested that blockage is due to an antibody being present directed against the activity of the intrinsic factor in hog pyloric mucosa.

G. F. S.

Yeast Extract and Ribonucleic Acid, Radiation Protective Effects of. K. D. Detre and S. C. Finch. (*Science*, 1958, **128**, 656.) In controlled experiments, the intravenous or intraperitoneal injection of a crude autolised extract of yeast was shown to provide a moderate degree of radiation protection in rats and mice

subsequently irradiated with a lethal dose of X-rays. In a further series of similar experiments, the protective effect of a commercial yeast ribonucleic acid preparation was studied and found to be considerably more effective than the yeast extract. These results indicate that ribonucleic acid, or a substance associated with it in the yeast autolysate, may be the principal radiation protective factor. W. C. B.

APPLIED BACTERIOLOGY

Mercurials, Microbiological Assay of in Pharmaceutical Products. D. V. Carter and G. Sykes. (*Analyst*, 1958, **83**, 536.) In order to compensate for the effects of other ingredients, a control preparation is made containing all the basic ingredients of the test preparation but no mercurial. A known amount of the mercurial under test is then incorporated in the control preparation in the same concentration as is assumed to be present in the test preparation, or in graded amounts if desired. Both test and control preparations are then treated in one of three ways. Firstly, serial dilutions are made in a broth of recommended composition and innoculated with a recommended strain of *E. coli* using a diluted culture and incubated at 37° for 48 to 72 hours. The end point is the greatest dilution at which no growth occurs. Secondly, one standard drop of undiluted culture is added to each tube and the result is read after 4 hours at 37°. The broth is weakly buffered to balance any acidity or alkalinity in the test material and contains bromocresol purple. The acid produced by the growth of the organism is sufficient to cause a colour change, which greatly assists with preparations such as creams which give turbid dilutions. Thirdly, a plate-diffusion method may be used following the same procedure as in the assay of penicillin in the B.P. using a recommended formula for nutrient agar and a culture of *Staph. aureus*. This method is more precise but is sensitive only to one part per million, whereas the first method is sensitive to 0·2 and the second to 0·05 parts per million. A table of comparative results is given. D. B. C.

Ristocetin and Framycetin: *In Vitro* Activity. R. W. Fairbrother and B. L. Williams. (*Lancet*, 1958, **2**, 1353.) Sensitivity tests by the serial dilution technique were carried out to assess the *in vitro* activity of ristocetin and framycetin. Ristocetin was shown to be particularly effective against Gram-positive but has little effect against Gram-negative organisms. The pneumococcus was the most sensitive organism; *Str. pyogenes* and *Str. faecalis* were also sensitive. *Staph. aureus* was relatively resistant. Three strains developed a two-fold increase in resistance after 12 passages. Framycetin was shown to have a wide range of activity against both Gram-positive and Gram-negative organisms. The pneumococcus was the most sensitive organism. Strains of *Staph. aureus* showed wide difference in sensitivity and 25 out of 50 were relatively resistant. *Str. pyogens* and *Str. faecalis* show a similar pattern and are both moderately resistant. Activity against Gram-negative organisms was very variable; only 5 out of 23 strains of *Ps. pyocyanea* could be classified as sensitive. Resistant strains of *Staph. aureus* develop rapidly—2 strains showed an eight-fold increase in resistance by the twelfth passage; *Ps. pyocyanea* showed an even more rapid development of resistance. The clinical application of ristocetin is likely to be limited but it may prove useful in infections due to antibiotic-resistant *Staph. aureus* and of enterococcal endocarditis. S. L. W.

LETTER TO THE EDITOR

Histamine and 5-Hydroxytryptamine after Cutaneons Burn in Mice and Rats.

SIR,—We have reportedï[1] that we were unable to confirm the rise in skin histamine after superficial skin burn in mice within 24 hours as noted by Dekanski[2]. The experiments have now been repeated in albino mice and rats and the observations continued for three weeks after superficial skin burns.

Groups of 6 animals were anaesthetised with ether and immersed in hot water at 60° for 10 seconds. The animals were killed at 10 minutes, 2 hours, 24 hours, 7 days, 14 days and 21 days thereafter. Control groups of animals anaesthetised and allowed to recover were killed at similar time-periods for comparison of skin histamine and 5-hydroxytryptamine content. The extraction and assay procedures were similar to those described by Parratt and West[3].

The earlier observation that the skin histamine is not raised within 24 hours after superficial skin burn was confirmed in both mice and rats. On the other hand there was a moderate lowering of the histamine content of the whole skin along with mast cell degranulation and rupture. Values were not very much altered in 7 day samples. Samples examined 14 days and 21 days after burning showed a remarkable change. It was found that both in mice and rats there was a 50 per cent rise in the histamine content of the whole skin after 14 days whereas the corresponding rise was above 150 per cent in 21 days. The experiments were repeated thrice at these times with similar results. During this period, 5-HT values did not alter. Also there was no significant proliferation of mast cells in the subcutaneous tissues of either species.

Most of the animals died after 3 weeks and later changes could not be studied.

The mechanism of the delayed rise in the skin histamine is being studied and will be reported later. Eosinophils are usually associated with histamine metabolism and it has been noted in clinical practice that a delayed eosinophilia often develops 4–6 weeks after a burn and lasts for weeks[4]. It is not improbable that the delayed eosinophilia may be associated with similar alterations in histamine metabolism as has been found in experimental animals.

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