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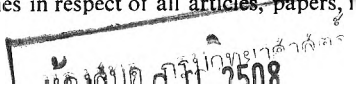

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Research Papers

A preliminary investigation of the pharmacology of longitudinal muscle strips from human isolated jejunum

B. WHITNEY

The effects of drugs on longitudinal muscle strips of human jejunum were studied *in vitro*. The muscarinic site of action of acetylcholine was demonstrated. The sympathomimetic amines phenylephrine, noradrenaline and isoprenaline each produced only a relaxation by an action on adrenergic α- and β-receptors. The presence of both types of receptor was demonstrated by selective adrenergic blockade with pronethalol or Hydergine. The ganglion stimulating agent dimethylphenylpiperazinium produced a contraction by an action on intramural cholinergic nervous tissue. When the contractile response was blocked by hyoscine, a relaxation occurred, due to the stimulation of an adrenergic mechanism which could be either the sympathetic nerves of the intrinsic nerve plexus or adrenergic stores in the bowel wall. The contraction produced by histamine was not inhibited by hyoscine or hexamethonium but was blocked by mepyramine, thus indicating a direct effect of the drug on the smooth muscle. 5-Hydroxytryptamine produced a contraction by a direct action on the muscle, since the response was not inhibited by hyoscine or hexamethonium but was blocked by methysergide (UML 491).

THE effects of drugs on human oesophageal muscle have been studied by Ellis, Kauntze, Nightingale & Trounce (1960), and Trounce & Nightingale (1960) made similar experiments on normal and diseased colonic muscle as part of an investigation of Hirschsprung's disease. The pharmacology of the circular muscle of the human colon has been recently reported by Fishlock & Parks (1963) and the human isolated taenia coli preparation has also been examined by Bucknell & Whitney (1964). The present paper reports the results of a preliminary investigation of the pharmacology of longitudinal strips of human isolated jejunum.

Experimental

Methods

Longitudinal strips were cut from the jejunum at operations in which gastro-jejunostomy was being performed for duodenal ulcer or carcinoma of the stomach. The specimen was immediately placed in cooled Krebs solution containing half the usual concentration of calcium, and brought to the laboratory. The composition of the modified Krebs solution expressed in g/litre was NaCl 6·9 ,KCl 0·35, $CaCl_2.6H_2O$ 0·27, KH_2PO_4 0·16, $MgSO_4.7H_2O$ 0·29, (+)-glucose 1·0 and $NaHCO_3$ 2·1.

In most experiments the mucosa was removed and the longitudinal muscle strip set up in a 15 ml organ-bath containing the modified Krebs solution at 37°, and gassed with 5% carbon dioxide in oxygen. In a few experiments the strip was set up without first removing the mucosa; no differences in response were observed between tissues with and without

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mucosa. The muscle strip was usually about 30 mm long by 1 mm wide. Recordings were made on a smoked drum using a frontal writing isotonic lever. The load on the tissue was 1 g. The responses were magnified eight times. Tissue was often stored overnight in modified Krebs solution at 4°, when only minimal changes in sensitivity were seen, or left set up in the organ bath at 37°. Often the "tone" was not high enough to show a relaxation, and in these experiments the inhibitory effect of the sympathomimetic amines was demonstrated as a reduction in standard responses to acetylcholine or 5-HT.

Drugs. Acetylcholine perchlorate, (−) noradrenaline bitartrate, (±) isoprenaline sulphate, (±)-phenylephrine hydrochloride, 5-hydroxytryptamine creatinine sulphate (5-HT), histamine acid phosphate, dimethylphenylpiperazinium iodide (DMPP), physostigmine sulphate, (−)-hyoscine hydrobromide, mepyramine maleate, procaine hydrochloride, hexamethonium bromide, pronethalol hydrochloride, Hydergine (a mixture of equal parts of dihydroergocornine, dihydroergocryptine, and dihydroergocristine as methanesulphonate) and methysergide hydrogen maleate (UML 491). Drug concentrations are expressed as μg/ml of the final bath concentration of the base, with the exception of DMPP, phenylephrine, procaine, pronethalol, Hydergine and methysergide, which are expressed as the salt.

Results

Spontaneous activity

Most preparations exhibited large, regular spontaneous activity. The lowest point of the wave often corresponded with maximal relaxation of the tissue. Krebs solution containing half the usual amount of calcium reduced the amplitude of the spontaneous activity, but not the responses of the tissue to the various drugs.

Actions of acetylcholine

The tissue contracted to acetylcholine in a concentration range of 0·01–0·2 μg/ml. Physostigmine, 0·3 μg/ml, left in contact with the tissue for 10 min, potentiated the effect of acetylcholine. The contractile response to acetylcholine was not modified by hexamethonium, 30 μg/ml, which completely blocked a response to DMPP at 10 μg/ml. Exposure of the tissue to hyoscine, 0·01 μg/ml, for 3 min completely inhibited the effect of the largest dose of acetylcholine in the range of 0·01–0·2 μg/ml used to establish the dose response curve.

Actions of phenylephrine, noradrenaline and isoprenaline

The sympathomimetic amines phenylephrine, noradrenaline and isoprenaline relaxed the tissue and inhibited spontaneous activity. The range of potency of the sympathomimetic amines sometimes changed when the tissue was kept overnight at 37°. In one experiment noradrenaline 0·02 μg/ml, was equivalent to isoprenaline 0·2 μg/ml when the

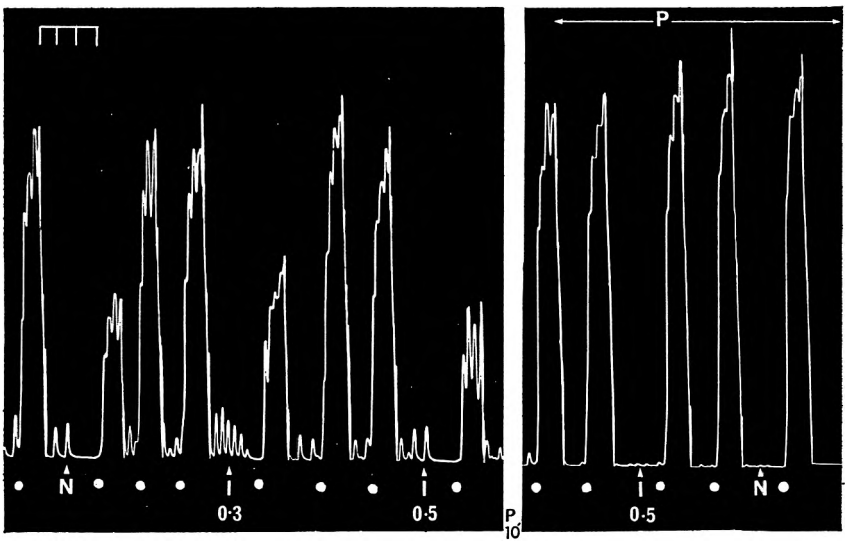

FIG. 1. The effect of pronethalol (P) on the response of the longitudinal strip of jejunum to noradrenaline (N,0·03 μg) or isoprenaline (I, μg). l.h. panel shows the reduction in the standard response to acetylcholine (0·015 μg at white dots) when noradrenaline or isoprenaline was added to the bath 30 sec before the dose of agonist. After incubation of the tissue with pronethalol (2 μg/ml for 10 min), r.h. panel the inhibitory effect of noradrenaline or isoprenaline, on the response to acetylcholine (0·045 μg), no longer occurred. Time signal 30 sec.

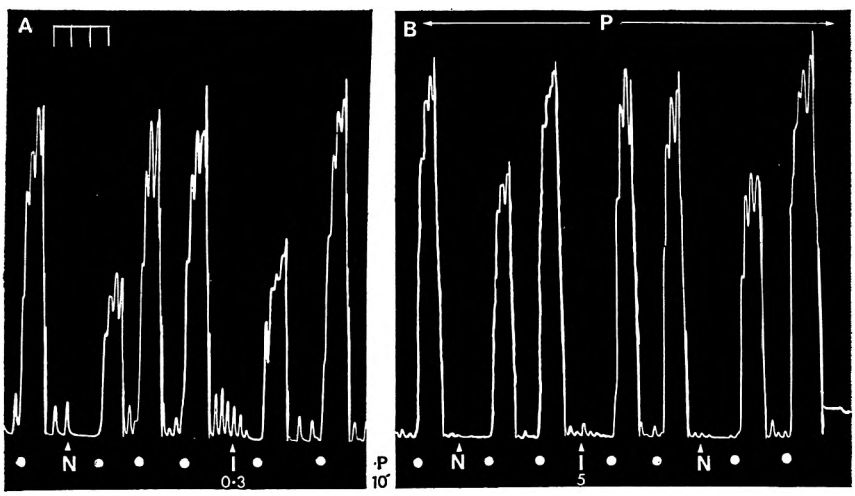

FIG. 2. The effect of pronethalol (P, 0·2 μg) on the responses of the longitudinal strip of human jejunum to noradrenaline (N, 0·03 μg) or isoprenaline (I, μg). Panel A shows the reduction in the standard response to acetylcholine (0·02 μg at white dots) when noradrenaline or isoprenaline was added to the bath 30 sec before the addition of the dose of agonist. Panel B shows the same procedure after incubation of the tissue with pronethalol, 0·2 μg/ml for 10 min. The inhibitory effect of noradrenaline was reduced and that of isoprenaline was abolished. Time signal 30 sec.

tissue was fresh, but the following day the sensitivity of the same piece of tissue was such that isoprenaline 0·7 μg/ml was equivalent to noradrenaline 3 μg/ml. After an initial exposure of the tissue to pronethalol, 2 μg/ml, for 10 min, and subsequently for 3 min before each addition of acetylcholine, the inhibitory effect of noradrenaline and isoprenaline on the contractile response to acetylcholine was abolished (Fig. 1). When 10% of the concentration of pronethalol was used (0·2 μg/ml) the inhibitory effect of isoprenaline was abolished whereas the effect of noradrenaline was only slightly reduced (Fig. 2). Initial exposure of the tissue to Hydergine, 5 μg/ml, for 5 min, and subsequently for 3 min before each addition of acetylcholine, abolished the effect of phenylephrine on the contractile response to acetylcholine, whereas the inhibitory effect of isoprenaline was only slightly reduced.

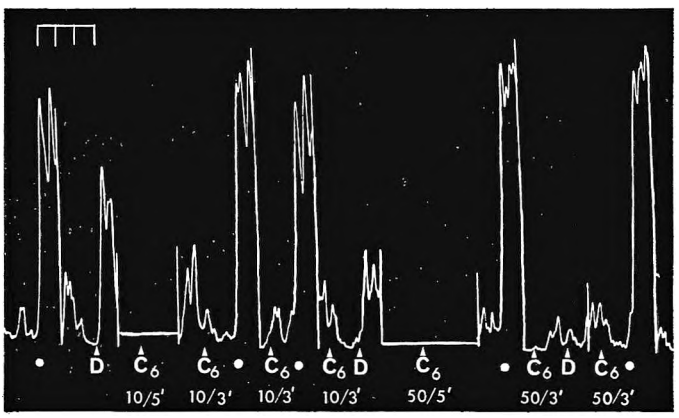

FIG. 3. The effect of hexamethonium (C6, μg/time) on responses of the longitudinal strip of jejunum to DMPP (D, 15 μg). The contractile response to DMPP was reduced by hexamethonium 10 μg/ml and abolished by 50 μg/ml leaving the response to acetylcholine unchanged. Time signal 30 sec.

ACTIONS OF DMPP

DMPP, 10 μg/ml, caused a contraction of the tissue. Minimal responses were obtained with 2 μg/ml and maximal responses with 40 μg/ml. Incubation of the tissue with eserine 0·5 μg/ml, for 5 min, potentiated the response to DMPP by a factor of five. Hexamethonium, 10 μg/ml, reduced the response, and 50 μg/ml caused complete inhibition (Fig. 3). The response to DMPP returned 30 min after hexamethonium had been removed from the bath. Hyoscine, 0·01 μg/ml, blocked the response to DMPP and in some experiments the response to DMPP was converted from a contraction to a relaxation in the presence of hyoscine (Fig. 4A, B). The contractile response was not changed by the removal of the mucosa.

The relaxant response to DMPP, 30 μg/ml, obtained in the presence of hyoscine, 0·05 μg/ml, was abolished after exposure of the tissue to hexamethonium, 50 μg/ml, for 5 min.

ACTIONS OF 5-HT

The tissue responded with a contraction to 5-HT in a concentration range of 0·05 μg–1 μg/ml. The response was not affected by hyoscine, 0·01–1·0 μg/ml (Fig. 4C), mepyramine, 0·01 μg/ml, which inhibited the response to histamine, or by hexamethonium, 50 μg/ml, which blocked the contractile response to DMPP (Fig. 5A, B). Methysergide, 1 μg/ml, completely inhibited the response to 5-HT, leaving the response to acetylcholine unchanged (Fig. 5C).

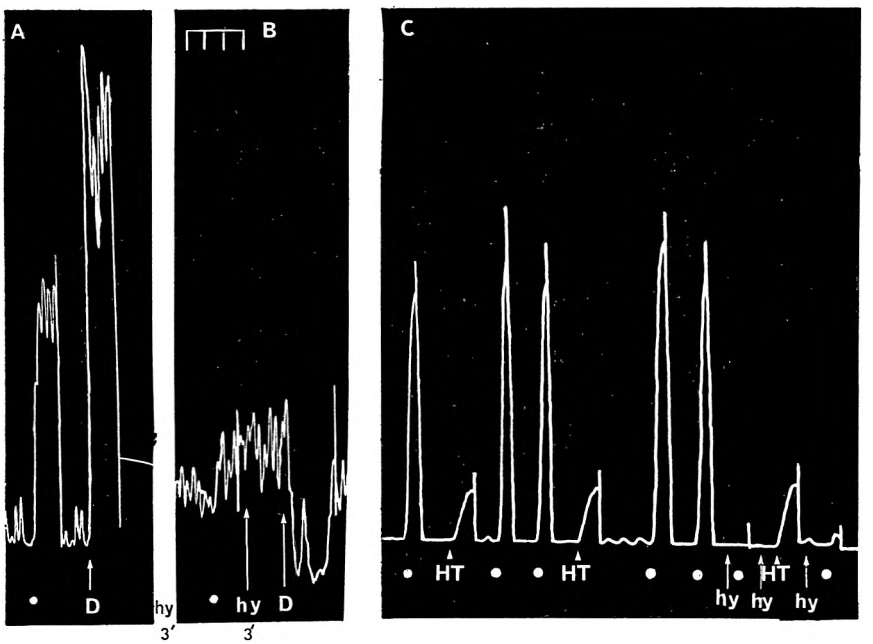

FIG. 4. The effect of hyoscine on the responses of the longitudinal strip of jejunum to DMPP and to 5-HT. Panel A shows the contractile response to acetylcholine (0·03 μg at white dot) and DMPP (D, 15 μg). After incubation with hyoscine (hy, 0·01 μg/ml) for 3 min, panel B, the response to acetylcholine was abolished and the response to DMPP was converted to a relaxation. Panel C shows the contractile response to acetylcholine (0·5 μg at white dots) was inhibited by hyoscine (hy, 0·01 μg/ml for 3 min) whereas the response to 5-HT (2 μg) was unchanged. Time signal 30 sec.

ACTIONS OF HISTAMINE

The tissue responded to histamine with a contraction in a concentration range of 0·5–40 μg/ml. Neither hexamethonium, 33 μg/ml (Fig. 6), nor hyoscine, 0·05 μg/ml, modified the response to histamine. Mepyramine, 0·01 μg/ml for 5 min, completely inhibited the response to histamine, but did not affect the response to acetylcholine or 5-HT (Fig. 7).

Discussion

The preparations of jejunum used were obtained from surgical intervention in man. In no instance was the jejunum itself involved in disease,

and since reproducible responses to drug doses were obtained, the results are considered to represent the pharmacology of longitudinal strips of muscle of normal human isolated jejunum.

The preparation was usually freed from mucosa to eliminate the possibility of a mechanical barrier to the diffusion of drugs; also the isolated mucosa of the human stomach secretes a substance which inhibits the action of drugs (Walder, 1953). Whether the removal of the mucosa and submucosa so damaged most of the intramural nervous plexus that the responses to drugs acting on receptors sited in the nervous tissue were lost, was challenged in experiments in which the jejunum was prepared with the mucosa intact. In these experiments no differences were seen in the responses to either acetylcholine or DMPP when the mucosa was subsequently removed.

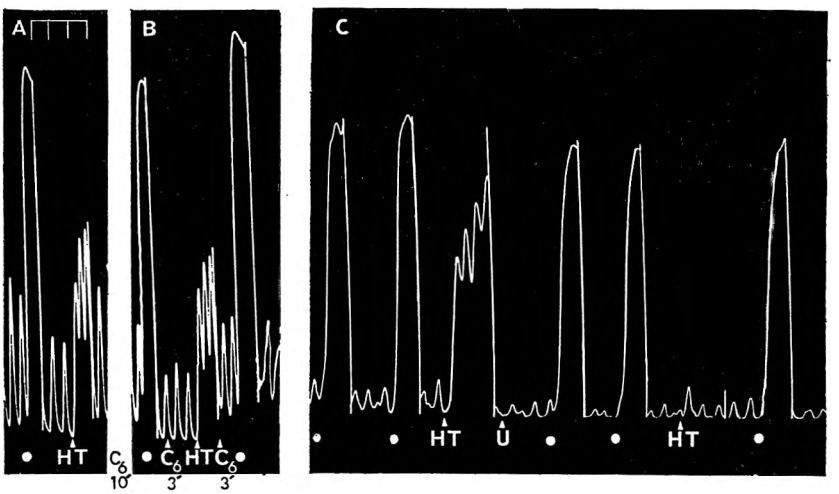

FIG. 5. The effect of hexamethonium and methysergide on the responses of the longitudinal strip of jejunum to 5-HT. Panels A and B show the contractile responses to acetylcholine (0·4 μg at white dots) and 5-HT (1 μg) were not modified by incubation of the tissue with hexamethonium (C6, 50 μg) for 10 min and for 3 min before the test drug. Panel C shows that incubation of the tissue with methysergide (U, 1 μg/ml) for 18 min did not modify the response to acetylcholine (0·1 μg at white dots) whereas the contractile response to 5-HT (0·5 μg) was abolished. Time signal 30 sec.

Acetylcholine produced typical muscarinic effects on human jejunum, the contractile response being blocked by hyoscine, potentiated by physostigmine and unaffected by hexamethonium.

Phenylephrine, noradrenaline or isoprenaline showed only inhibitory effects on the tissue, demonstrated as a reduction in the contractile response to acetylcholine. The inhibitory effect of isoprenaline was abolished by the β-receptor blocking agent pronethalol (Black & Stephenson, 1962) in a concentration which did not affect the response to noradrenaline. Conversely, the α-receptor blocking agent Hydergine blocked the effect of phenylephrine, whereas the response to isoprenaline was only

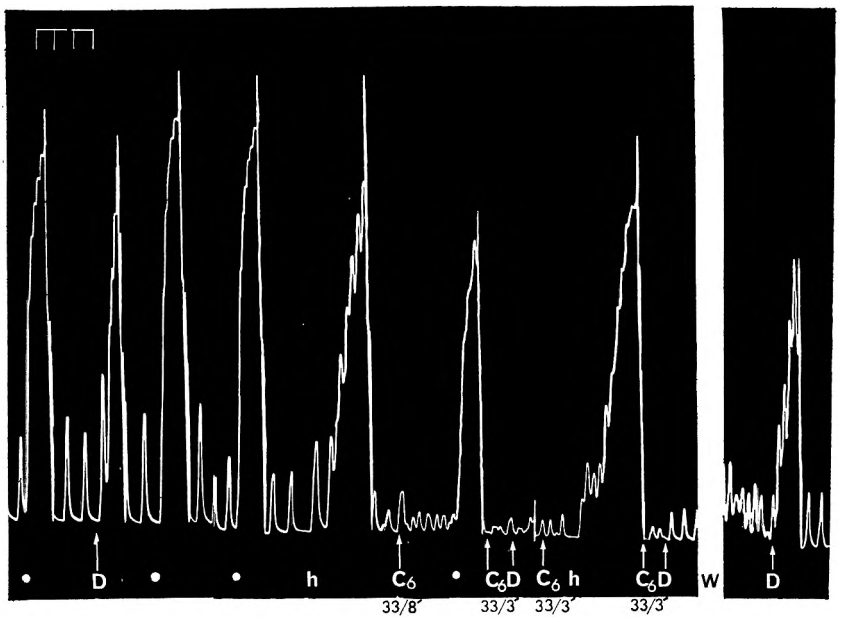

FIG. 6. The effect of hexamethonium on the responses of the longitudinal strip of jejunum to histamine or DMPP. Contractile responses to acetylcholine (0·2 μg at white dots), DMPP (D, 5 μg) and histamine (h, 0·07 μg). After incubation with hexamethonium (C6 μg/time), there was no response to DMPP whereas the responses to acetylcholine or histamine were unchanged. The response to DMPP returned 30 min after hexamethonium had been removed from the bath. Time signal 30 sec.

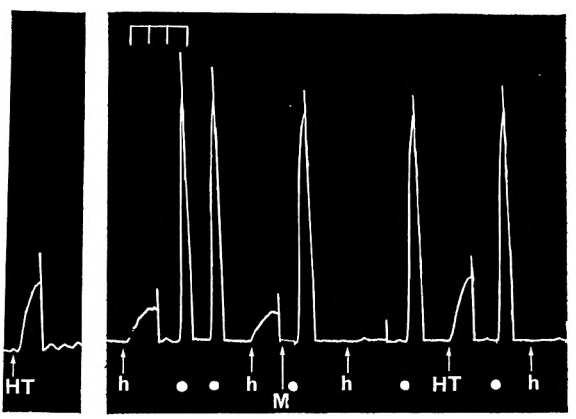

FIG. 7. The effect of mepyramine on the responses of the longitudinal strip of jejunum to 5-HT or histamine. Contractile responses to 5-HT (2 μg) histamine (h, 4 μg) and acetylcholine (0·5 μg at white dots) are shown on the left of the tracing. After incubation of the tissue with mepyramine (M, 0·01 μg/ml) for 5 min, there was no response to histamine whereas the responses to acetylcholine or 5-HT were not reduced. Time signal 30 sec.

reduced. Since phenylephrine acts mainly on the α-receptors, noradrenaline on α- and β-receptors, and isoprenaline on the β-receptors (Ahlquist & Levy, 1959; Levy & Ahlquist, 1961; Kosterlitz & Lees, 1964), the selective blockade of the responses to these drugs by pronethalol or Hydergine suggests the presence of both α- and β-adrenergic receptors in the human jejunum and that stimulation of either produces a relaxation. The concept of two types of receptor was first put forward by Ahlquist (1948) using the range of potency of six closely related sympathomimetic amines on various tissues, and his experiments showed a fixed range of potency for each particular tissue. However, in human jejunum the range of potency for noradrenaline and isoprenaline was not constant, and in one experiment the relative potency reversed when the tissue was stored overnight. This reversal involved a 100-fold decrease in sensitivity to noradrenaline as compared with a 3-fold decrease in sensitivity to isoprenaline.

The ganglion stimulating drug, DMPP, produced a contraction of the tissue which was blocked at neuronal sites by hexamethonium and peripherally by hyoscine. When the contraction was blocked by hyoscine, the response to DMPP was converted to a relaxation. The change in response from a contraction to a relaxation was not obtained by varying the concentration of DMPP as was seen when nicotine was used on the rabbit colon (Gillespie & Mackenna, 1960). The relaxation produced by DMPP in the presence of hyoscine was blocked by hexamethonium, suggesting a neuronal site of action. The ability of the tissue to respond to DMPP with either a contraction or a relaxation demonstrated the presence of both cholinergic and adrenergic nervous tissue in the wall of the human jejunum, and that the cholinergic component was dominant since the adrenergic component was only revealed in the presence of hyoscine. These results are similar to those described for rabbit and kitten ileum by Ambache & Edwards (1951), but differ from those of human colon where only an adrenergic response was demonstrated (Bucknell & Whitney, 1964). The relaxant action of DMPP does not necessarily imply the presence of ganglia with postganglionic adrenergic neurones, since the possibility that the relaxation resulted from an action on adrenergic nerve terminals has not been experimentally excluded.

Histamine has been shown to have a direct action on guinea-pig ileum (Day & Vane, 1963), and an action on both muscle and intramural nervous tissue in the rabbit (Ambach & Lessin, 1955). Brownlee & Harry (1963) have shown a difference between longitudinal and circular muscle strips of the guinea-pig ileum, histamine having a direct action on the longitudinal strips and an indirect action on the circular ones. Longitudinal strips of human jejunum responded to histamine with a contraction due to a direct action, since it was not affected by hexamethonium or by hyoscine. Further support for a direct action of histamine was obtained by selective blockade of the contractile response to histamine by mepyramine.

5-HT has been shown to act on receptors sited both on nervous tissue and on muscle in guinea-pig ileum (Gaddum & Picarelli, 1957). Brownlee

& Johnson (1963) and Day & Vane (1963) showed that 5-HT contracted the longitudinal muscle of guinea-pig ileum mainly by an action through a cholinergic nerve pathway. However, the present experiments on longitudinal strips of human jejunum showed only a direct action of 5-HT, since the contractile response was not inhibited by hexamethonium or by hyoscine. The direct action was confirmed by blockade of the contractile response by methysergide, a substance which is a potent antagonist of the direct actions of 5-HT on smooth muscle.

Acknowledgements. I would like to thank Professor G. Brownlee of King's College, London, for his kindness in discussing this manuscript. I am grateful to the Wellcome Research Laboratories for a gift of dimethylphenylpiperazinium, and to I.C.I. Ltd., for a gift of pronethalol. This work was supported by a research grant from the Medical Research Council.

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Occurrence of anhydrovitamin A and *retro*-vitamin A in pharmaceuticals; their biological potency and effect on the assay of vitamin A

T. N. R. VARMA*, P. ERDODY AND T. K. MURRAY

Analyses of commercial samples of liquid multivitamin preparations revealed that many contained anhydrovitamin A, *retro*-vitamin A ether, vitamin A ether, and *retro*-vitamin A alcohol in varying proportions. Anhydrovitamin A and *retro*-vitamin A ether were present in greater amounts than the other vitamin A-derivatives. Anhydrovitamin A and *retro*-vitamin A ether had no measurable potency in the liver storage and vaginal smear assays, whereas *retro*-vitamin A acetate was 12% as potent as vitamin A. These compounds interfered with the assay of vitamin A by the United States Pharmacopeia XVI and British Pharmacopoeia 1963 methods but the interference could be eliminated by chromatography.

ANHYDROVITAMIN A (Fig. 1) was first prepared by Edisbury, Gillam, Heilbron & Morton (1932) and later characterised by Shantz, Cawley & Embree (1943) and Meunier, Dulou & Vinet (1943). It showed λ_{max} at 351, 371 and 392 mμ and with antimony trichloride gave a blue colour of the same intensity and wavelength as did vitamin A. Its

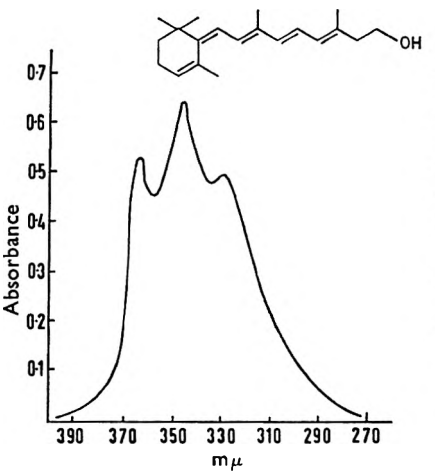

FIG. 1. Absorbance curve of Anhydrovitamin A.

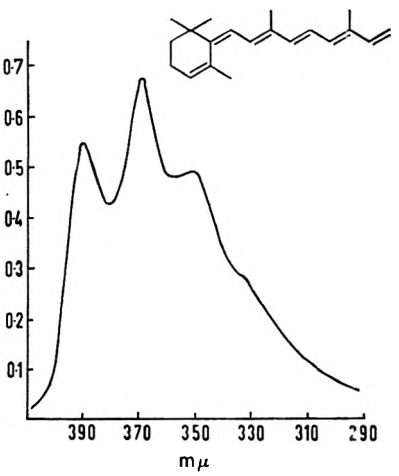

FIG. 2. Absorbance curve of *Retro*-vitamin A.

biological potency, measured by rat growth assay, was about 0·4% that of vitamin A (Shantz & others, 1943). Higuchi & Reinstein (1959) studied the kinetics of formation of anhydrovitamin A from vitamin A alcohol and acetate and noted its possible formation in pharmaceutical preparations.

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Retro-vitamin A, shown in Fig. 2, was prepared by Beutel, Hinkley & Pollak (1955) by reacting vitamin A acetate with aqueous hydrobromic acid. Varma & Murray (1963) fed *retro*-vitamin A to vitamin A-deficient rats and reported that about 10% of the dose was stored in the liver, 8% as vitamin A, 2% as *retro*-vitamin A. With antimony trichloride *retro*-vitamin A produced a blue colour with λ_{max} at 575 mμ.

The presence in pharmaceutical preparations of appreciable amounts of anhydro- and *retro*-vitamin A would pose an analytical problem. Their reported biological activity is negligible but they would contribute to absorbance in the ultraviolet and to the colour produced with antimony trichloride. Murray, Pelletier & Campbell (1963) examined one multivitamin preparation in which the vitamin A was largely replaced by *retro*-vitamin A, possibly in the ether form, but there is no evidence in the literature that this is a problem of any magnitude.

We now report the incidence of anhydro- and *retro*-vitamin A in two types of pharmaceutical preparations, and studies on the biological potencies of these compounds.

Experimental

MATERIALS AND METHODS

Forty-one different liquid* vitamin preparations were obtained from commercial sources. They ranged in complexity from mixtures of vitamins A and D, to multivitamin preparations with minerals and amino-acids. Several samples of some products were examined. In addition, twelve different vitamin tablets were assayed.

The samples were saponified and extracted by the USP XVI method (1960). Vitamin A was separated from interfering compounds by partition chromatography on a celite-polyethylene glycol column (Murray, 1962a) and measured by the USP XVI method. For identification of the vitamin A derivatives it was necessary to separate them on alumina. Woelm alumina (neutral, activity grade 1) was weakened by the addition of 6% water. The sample was introduced onto the column in light petroleum (B.P. 40°–60°) and eluted successively with 2, 4, 8, 12, 20 and 40% (v/v) diethyl ether in light petroleum. The composition of fractions from the chromatographic column and the purity of the preparations used in biological studies was checked by thin-layer chromatography (Varma, Panalaks & Murray, 1964).

The anhydrovitamin A isolated showed λ_{max} at 350, 368 and 390 mμ rather than at 351, 371 and 392 mμ shown by the crystalline material (Shantz, 1950). Estimation of this compound was made on the basis of $E(1\%, 1\text{ cm}) = 3000$ at 390 mμ. *Retro*-vitamin A alcohol and ether were estimated on the basis of $E(1\%, 1\text{ cm}) = 2200$ at 366 mμ, the value for the ether being expressed in terms of the alcohol.

For biological studies, anhydrovitamin A was prepared from crystalline all-*trans* vitamin A alcohol by the reaction with anhydrous methanolic

* These were almost all water dispersions, none were oil solutions.

hydrogen chloride (Shantz, 1950). It was purified by repeated chromatography on alumina until the purest fractions gave $E(1\%, 1\ cm)$ values of 2340, 3130 and 2600 at 350, 368 and 390 mμ respectively. *Retro*-vitamin A acetate was prepared as described by Beutel, Hinkley & Pollak (1955) and purified by chromatography on alumina. It had $E(1\%, 1cm)$ values of 1500, 2030, and 1910 (equivalent to 1730, 2340 and 2200 for the alcohol form) at 332, 348 and 366 mμ respectively. *Retro*-vitamin A ether was purified by chromatography from a liquid multivitamin preparation and had E (1%, 1 cm) values of 587, 653 and 594 at 332, 348 and 366 mμ respectively.

Vaginal smear assays were made according to Pugsley, Wills & Crandall (1944) and liver-storage assays by the method of Ames & Harris (1956), except that liver stores were not estimated by the usual antimony trichloride reaction but spectrophotometrically after saponification and separation of the components on alumina. The relative proportions of vitamin A alcohol and *retro*-vitamin A alcohol were estimated as described by Varma & Murray (1963). For both kinds of biological assays the doses were given orally in corn oil and subcutaneously in water dispersions.

Results and discussion

CHEMICAL

Table 1 contains information on the properties of the various vitamin A derivatives found in the liquid pharmaceutical preparations. Vitamin A ether and *retro*-vitamin A alcohol were found in several products but only in small amounts. No quantitative measurements were made of these derivations.

TABLE 1. PHYSICO-CHEMICAL PROPERTIES OF VITAMIN A ALCOHOL AND RELATED COMPOUNDS ISOLATED FROM THE LIQUID MULTIVITAMIN SAMPLES

Compund	Eluting solvent % ether in light petroleum*	Distribution ratio**	max (mμ)	
			Ultraviolet	$SbCl_3$ colour test
Anhydrovitamin A	0	98 : 2	350,368,390	618
Retro-vitamin A ether ..	0 to 2	95 : 5	332,348,366	575
Vitamin A ether	2 to 4	95 : 5	324	618
Retro-vitamin A alcohol ..	15 to 20	48 : 52	332,348,366	575
Vitamin A alcohol	18 to 22	50 : 50	324	618

*From Alumina.
**Between light petroleum and 83% EtOH–H_2O mixture.

Almost all products contained anhydrovitamin A and *retro*-vitamin A ether but only six contained appreciable amounts of these derivatives. The data on these six products are summarised in Table 2.

The data do not indicate the cause or mechanism of formation of the vitamin A derivatives. The products which contained appreciable amounts of these derivatives were complex mixtures but in many similar mixtures the vitamin A had not deteriorated. All samples tested were acidic, but differences in pH were not related to the presence or absence of vitamin A derivatives. Age did not appear to be an important factor

except when the deterioration of a single product was considered. Samples F_1 to F_6 were the same product assayed at various times after manufacture and the results illustrate the rapid changes that may take place in water dispersions of vitamin A. Detectable amounts of anhydrovitamin A and *retro*-vitamin A ether were present within one month of manufacture and within a year the destruction of vitamin A was almost complete. The loss of vitamin A could not fully be accounted for by the appearance of anhydrovitamin A and *retro*-vitamin A ether, and the small amount of vitamin A ether and *retro*-vitamin A alcohol (not shown in Table 2). It is likely that much of the vitamin A was destroyed by oxidative processes.

Measurable amounts of anhydro- or *retro*-vitamin A were not found in the multivitamin tablets examined.

TABLE 2. VITAMIN A, ANHYDROVITAMIN A AND *retro*-VITAMIN A ETHER IN LIQUID MULTIVITAMIN SAMPLES

Product	Age (months)	Vitamin A*	Anhydrovitamin A*	*Retro*-vitamin A ether*
A	17	60	28	3
B_1	?	140	7	4
B_2	18	120	2	2
C_1	?	61	5	4
C_2	?	63	5	3
D	9	30	30	22
E	13	29	33	28
F	6	5	0	78
F_1	1	70	12	3
F_2	2	62	18	9
F_3	3	20	28	14
F_4	8	8	30	16
F_5	10	6	20	22
F_6	13	7	20	21

*Expressed as % total vitamin A claimed on label.

BIOLOGICAL STUDIES

Anhydrovitamin A will support growth if given in sufficient amounts; thus Shantz & others (1943) found the biological potency to be about 0·4% that of vitamin A in the rat-growth assay. An attempt was made to confirm this finding by liver storage and vaginal smear assays. When anhydrovitamin A was administered to vitamin A-deficient rats orally or by subcutaneous injection, no vitamin A was found in the liver. In view of this, the validity of a liver-storage assay of anhydrovitamin A was questionable. The nature of the compounds stored in the liver has been reported by Varma, Erdody & Murray (1965). Furthermore, a potency value for anhydrovitamin A could not be obtained from the vaginal smear assays. The smears were changed from those typical of a vitamin-deficiency to normal by an oral dose of 375 μg anhydrovitamin A but within four days the rats were again deficient. A fourfold increase in the size of the dose did not increase the number of days between dosing and depletion. Similar results were obtained when the doses were administered subcutaneously. This response was reminiscent of that observed by Murray (1962b) when vitamin A acid was fed to vitamin A-deficient rats, except that anhydrovitamin A gave rise to liver storage of related compounds (Varma & others, 1965). Two explanations

seem possible. The compounds stored in the liver after the administration of anhydrovitamin A may not be converted to vitamin A but may be capable of performing some of its functions (growth) although not all (maintenance of a normal vaginal smear). Alternatively, the compounds stored in the liver may be converted to vitamin A (or some "active form") at a very slow rate, sufficient for growth, but not to maintain a normal vaginal smear. The metabolism of vitamin A derivatives is being further examined, but it is safe to conclude that anhydrovitamin A has, at best, very low biological potency.

The potency of orally administered *retro*-vitamin A was 11% ± 10% that of vitamin A by the liver storage assay and 12% ± 10% by the vaginal smear assay. A 94:6 mixture of vitamin A and *retro*-vitamin A was found in the liver in contrast to the 80:20 mixture previously reported by Varma & Murray (1963). The higher ratio of vitamin A to *retro*-vitamin in the present experiment might be due to the longer period (3 instead of 2 days) between dosing and killing the rats.

When the doses for the vaginal smear assay were given subcutaneously in aqueous dispersion, the biological potency of *retro*-vitamin A was only 4% ± 12% that of vitamin A and no vitamin A or *retro*-vitamin A was found in the liver indicating rapid destruction and little or no conversion of the derivative to vitamin A. It is likely, therefore, that the major site of conversion of *retro*-vitamin A to vitamin A is the gastrointestinal tract.

The biological potency of chemically prepared *retro*-vitamin A was similar to that of the "rehydrovitamin A" isolated by Shantz (1950) from the livers of rats fed anhydrovitamin A although the two compounds are not identical. "Rehydrovitamin A" had ultraviolet absorbance maxima very close to those shown by *retro*-vitamin A prepared from vitamin A by the aqueous hydrobromic acid reaction but the former had λ_{max} near 618 mμ and the latter near 575 mμ in the antimony trichloride reaction. The *retro*-vitamin A alcohol and ether isolated from pharmaceutical products reacted with antimony trichloride in the same manner as did the chemically prepared compound.

Retro-vitamin A ether isolated from pharmaceutical products was biologically inactive.

EFFECT ON VITAMIN A ASSAY

Anhydro- and *retro*-vitamin A were mixed in various proportions with pure vitamin A alcohol and the vitamin A of the mixtures estimated by the USP XVI method. By this means it was found that if the absorbance ratio 350 mμ/325 mμ exceeded 0·640, chromatographic purification was necessary.

The USP XVI method makes use of a Morton & Stubbs correction based on absorbance readings at 310, 325 and 334 mμ without regard to the shape of the absorbance curve. The B.P. 1963 states that if, after saponification, maximum absorbance lies outside the wavelengths 323–327 mμ, or if the extinction at 310 mμ relative to that at 325 mμ exceeds 0·730, the sample must be chromatographed.

It is suggested that, in addition to the safeguards of the B.P. method, the absorbance be measured at 350 mμ and the condition added that chromatography is necessary if the absorbance ratio 350 mμ/325 mμ exceeds 0·640.

The antimony trichloride reaction is commonly used for the determination of vitamin A when the ultraviolet absorbance curve is distorted and, in fact, this reaction has been suggested (Ames, Swanson & Lehman, 1960) as a means of estimating the isomer content of water-dispersed vitamin A. This method would result in serious overestimation of potency if anhydrovitamin A were present. *Retro*-vitamin A ether and alcohol found in the non-saponifiable fractions of the pharmaceutical products have λ_{max} near 575 mμ in the antimony trichloride test and interfere only to a limited extent with the colorimetric determination of vitamin A.

Separation of vitamin A from any of the common types of interference can be accomplished by the partition system described by Murray (1962a) or on alumina. The former system is easier to use and has the added advantage that the column may be re-used many times. For separation of the individual vitamin A derivatives alumina is to be preferred. Neither system will separate vitamin A alcohol from *retro*-vitamin A alcohol but in our experience the latter occurs in pharmaceutical products in very small amounts.

It was concluded that anhydrovitamin A and *retro*-vitamin A may occur in pharmaceutical products in amounts that interfere with the assay of vitamin A by the USP XVI and B.P. methods. Analysts should be aware of the errors caused by these compounds and should check for their presence by use of the absorbance ratio 350 mμ/325 mμ.

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Interaction between imipramine-like agents and catecholamine-induced hyperthermia

A. JORI AND S. GARATTINI

Imipramine-like drugs potentiate the hyperthermic response obtained by infusion of noradrenaline, adrenaline and isoprenaline. In addition to this effect, desipramine increases the hyperthermic response induced by L-dopa (in monoamine oxidase blocked rats), reserpine (immediately after intravenous injection) and dexamphetamine. On the contrary, other types of hyperthermia, such as the one induced by phenethylamine in monoamine oxidase treated rats, and that by yeast, were not increased by desipramine. These results are discussed in relation to the mechanism of action of antidepressant drugs.

PREVIOUS work has shown that the hypothermia elicited by reserpine is prevented or counteracted by imipramine (Costa, Garattini & Valzelli, 1960), amitriptyline (Vernier, Hanson & Stone, 1962) and their nor-derivatives (Garattini, Giachetti, Jori, Pieri & Valzelli, 1962; Askew, 1963). The mechanism of such an antagonism is not well understood. It was also established that imipramine-like drugs do not change the level of brain 5-hydroxytryptamine (5-HT) and noradrenaline and do not affect

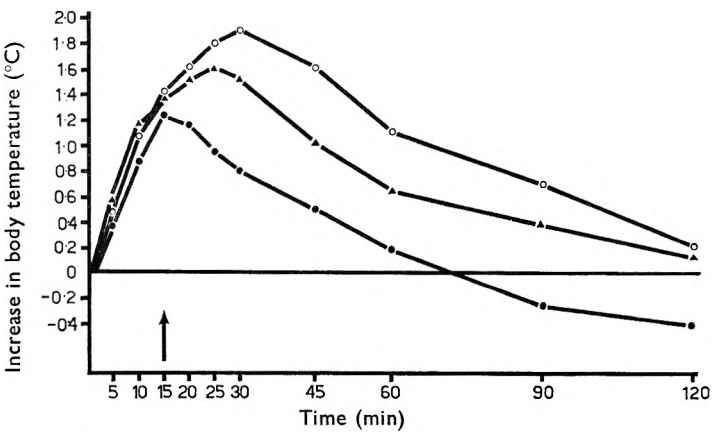

FIG. 1. Increase of body temperature in rats infused with noradrenaline (total dose 60 μg rat in 15 min) at a room temperature of 22°. The infusion was stopped at the arrow. Desipramine and imipramine, 15 mg/kg i.p. were given 1 hr before the infusion. —○— Desipramine + noradrenaline. —▲— Imipramine + noradrenaline. —●—Noradrenaline.

the depletion of these amines induced by a treatment with reserpine (Garattini & others, 1962) or reserpine congeners (Sulser, Watts & Brodie, 1962). Other investigations showed that imipramine and its analogues potentiate the pressor response (Sigg, 1959) and the contraction of the nictitating membrane (Sigg, 1959; Schaeppi, 1960) following the administration of noradrenaline, and the reduction of spleen volume (Thoenen, Huerlimann & Haefely, 1964) following adrenergic nerve stimulation.

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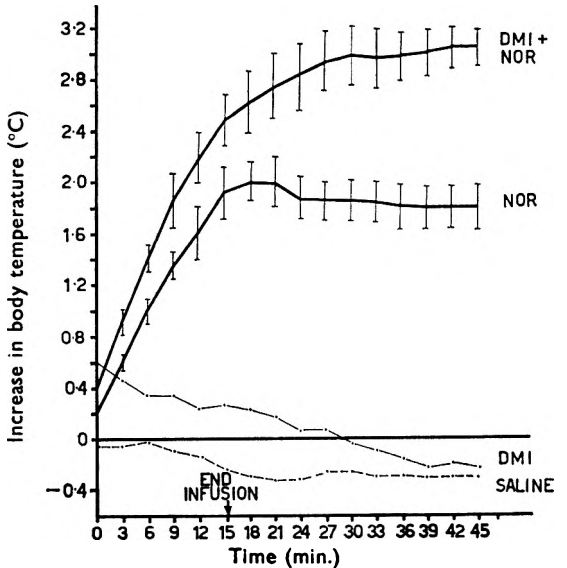

FIG. 2. Increase of body temperature in rats infused with noradrenaline NOR (total dose 60 μg/rat in 15 min) at a room temperature of 30°. The infusion was stopped at the arrow. Desipramine (DMI), 15 mg/kg i.p., was given 1 hr before the infusion.

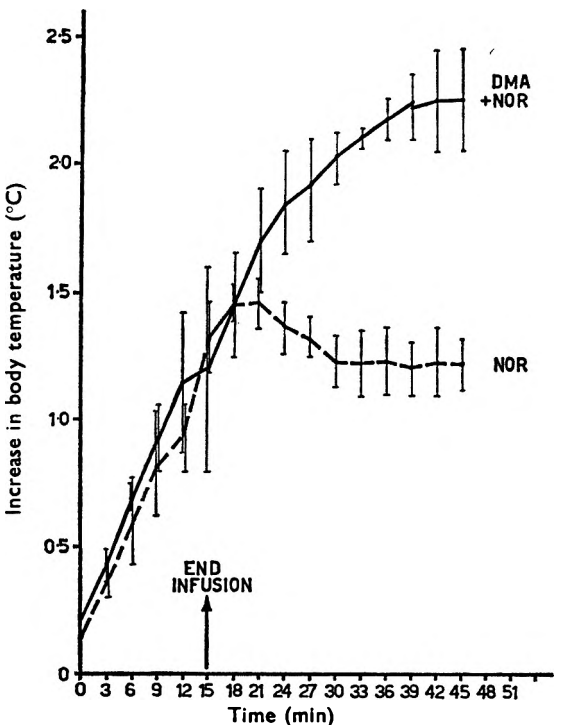

FIG. 3. Increase of body temperature in rats infused with noradrenaline (total dose 60 μg/rat in 15 min) at room temperature of 30°. The infusion was stopped at the arrow. Nortriptyline (DMA), 15 mg/kg i.p., was given 1 hr before the infusion.

Biochemical investigations have demonstrated that imipramine derivatives inhibit the uptake of labelled noradrenaline so that a larger concentration of the amine would probably be available at receptor sites (Hertting, Axelrod, Whitby & Patrick, 1961; Axelrod, Hertting & Potter, 1962; Titus & Spiegel, 1962). These data suggest that imipramine could antagonise the hypothermia induced by reserpine, through a potentiation of the activity of the endogenously formed catecholamines. The results reported in this paper are not in disagreement with this hypothesis.

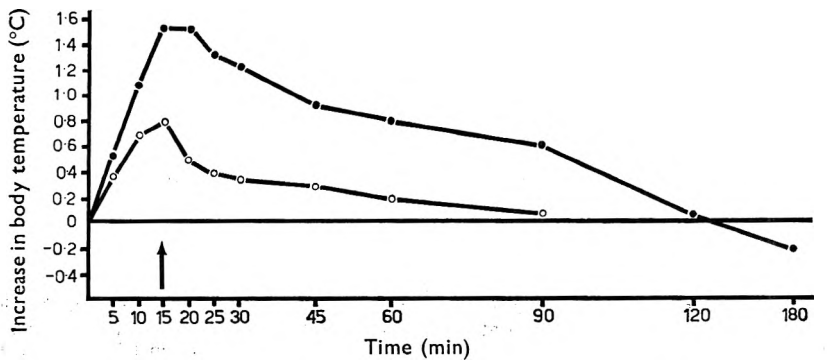

FIG. 4. Increase of body temperature in rats infused with adrenaline (total dose 6 μg/rat in 15 min) at a room temperature of 22°. The infusion was stopped at the arrow. Desipramine, 15 mg/kg i.p., was given 1 hr before the infusion. —●—Desipramine + adrenaline. —○—Adrenaline.

Experimental

MATERIALS AND METHODS

Sprague Dawley rats and Swiss mice were used. Animals were housed in Makrolon cages at a room temperature of 20° and at a relative humidity of 60%. The relatively low temperature was chosen to facilitate the onset of hypothermia in reserpinised animals. During the infusion of noradrenaline into the rat tail vein, with a Braun apparatus at a speed of 0·1 ml/min, the room temperature was elevated to 30° to obtain a significant hyperthermia although the same results were qualitatively obtained at a temperature of 22°. Body temperature was determined by inserting an electrical thermometer into the rectal cavity.

The drugs used and their sources were as follows: L-3,4-dihydroxyphenylalanine (dopa), phenethylamine, iproniazid phosphate (Hoffman-La Roche), dexamphetamine, noradrenaline bitartrate, adrenaline (Recordati), isoprenaline (Biosintex), pheniprazine (Lakeside), reserpine (Ciba), imipramine and desipramine (Geigy), and nortriptyline (Merck, Sharp and Dohme and Pharmacia).

Results

EFFECT ON HYPERTHERMIA INDUCED BY INFUSIONS OF NORADRENALINE

Noradrenaline infused in rats at a concentration of 4 μg/rat/min induced an elevation of body temperature which returned gradually to

normal on termination of the infusion. Animals pretreated with imipramine or desipramine showed a higher rise and, particularly, a more sustained increase of the body temperature (see Fig. 1). Similar results were obtained with desipramine and nortriptyline at a room temperature of 30° (Figs 2, 3).

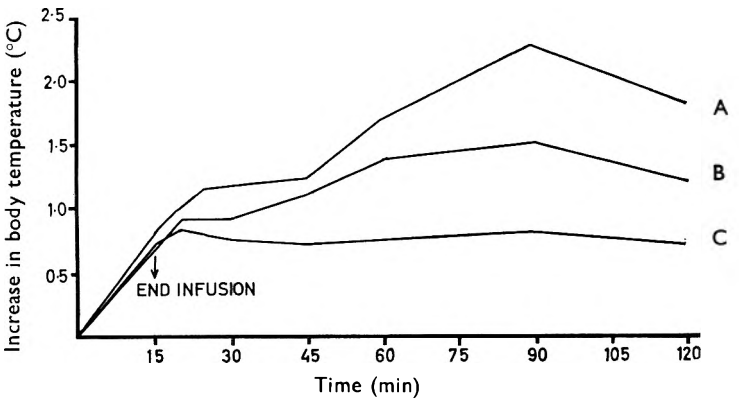

FIG. 5. Increase of body temperature in rats infused with isoprenaline (total dose 600 μg/rat in 15 min) at a room temperature of 22°. The infusion was stopped at the arrow. Desipramine and nortriptyline, 15 mg/kg i.p., were given 1 hr before the infusion. A, desipramine + isoprenaline. B, nortriptyline + isoprenaline. C, saline + isoprenaline.

EFFECT ON HYPERTHERMIA INDUCED BY ADRENALINE

An infusion with adrenaline (0·4 μg/rat/min) produced a hyperthermia which rapidly disappeared after the end of the infusion. Fig. 4 shows that desipramine potentiated and prolonged this effect of adrenaline.

EFFECT ON HYPERTHERMIA INDUCED BY ISOPRENALINE

Isoprenaline infused at the dose of 40 μg/rat/min induced an increase in body temperature lasting for about 2 hr after the end of the infusion. Desipramine and nortriptyline enhanced and prolonged this type of

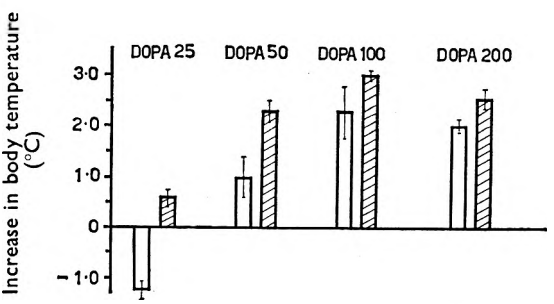

FIG. 6. Increase of body temperature in rats (with a blockade of monoamine oxidase: pheniprazine 10 mg/kg i.p. 16 hr before test) induced by various doses of dopa (25–200 mg/kg i.p.) with (hatched columns) or without (open columns) desipramine 1 hr before at a dose of 7·5 mg/kg. Increase in body temperature was recorded 30 min after administration of dopa.

hyperthermia (see Fig. 5). On the other hand, pretreatment with a monoamine oxidase inhibitor (pheniprazine) or with chlorpromazine (unpublished results from this laboratory) did not potentiate, but rather inhibited, the hyperthermia induced by isoprenaline.

EFFECT ON HYPERTHERMIA INDUCED BY DOPA

In animals with a blockade of monoamine oxidase, dopa induces a hyperthermia which is considered of central origin (Van der Wende & Spoerlein, 1962; Everett, Will & Evans, 1964). In our experiments pheniprazine (10 mg/kg i.p.) was given to mice 16 hr before the test was made. Desipramine was injected intraperitoneally 1 hr before dopa. Under these conditions, there was a clear enhancement of the increase in body temperature over that observed in animals treated only with the monoamine oxidase inhibitor followed by dopa.

The enhancement was evident with doses of dopa which did not induce hyperthermia (see Fig. 6). The effects of larger doses of dopa which in itself induced a hyperthermic action were only prolonged by a pretreatment with desipramine (see Fig. 7).

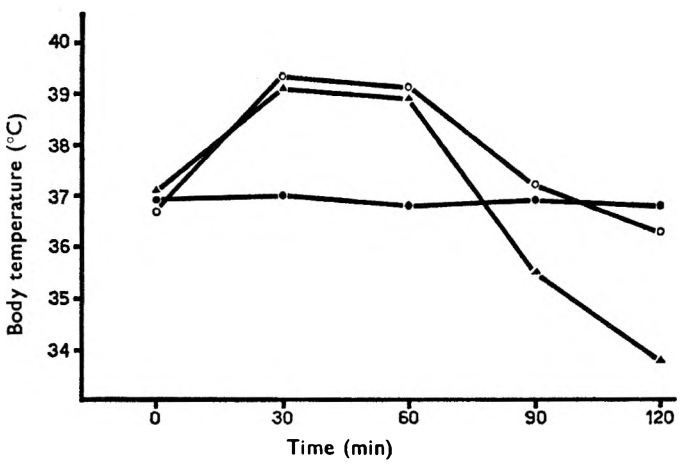

FIG. 7. Increase of body temperature in rats (with a blockade of monoamine oxidase: pheniprazine 10 mg/kg i.p. 16 hr before test) induced by dopa (200 mg/kg i.p.) with or without desipramine 1 hr before at a dose of 15 mg/kg. —●—Desipramine. —○—Desipramine + dopa. ▲ Dopa.

EFFECT IN RESERPINISED ANIMALS

Desipramine raised the body temperature in hypothermic reserpinised mice. The effect of 7·5 mg/kg of desipramine was similar to that elicited by 500 mg/kg of dopa A combination of both drugs resulted in a more prolonged effect (see Fig. 8).

When rats were injected with reserpine it was possible to observe in the first 2 hr an increase in body temperature of about 1·5°, which was more evident and reproducible when the animals were restrained (unpublished results from this laboratory). Desipramine and nortriptyline given before reserpine enhance, and more frequently prolong, this hyperthermia (see Fig. 9).

EFFECT ON OTHER TYPES OF HYPERTHERMIA

To ascertain the significance of this interaction between imipramine-like drugs and catecholamines it was considered of interest to investigate the effect of desipramine on other types of drug-induced hyperthermia. Dexamphetamine showed a clear hyperthermic effect in rats and in mice.

Table 1 summarises data obtained in mice. Desipramine prevented the effect of low doses of dexamphetamine (5 mg/kg) but prolonged the hyperthermia induced by 15 mg/kg. In rats, an inhibition was always observed at the beginning (first hour) followed by a potentiation of the hyperthermic response (see Table 2). These data showed large variations

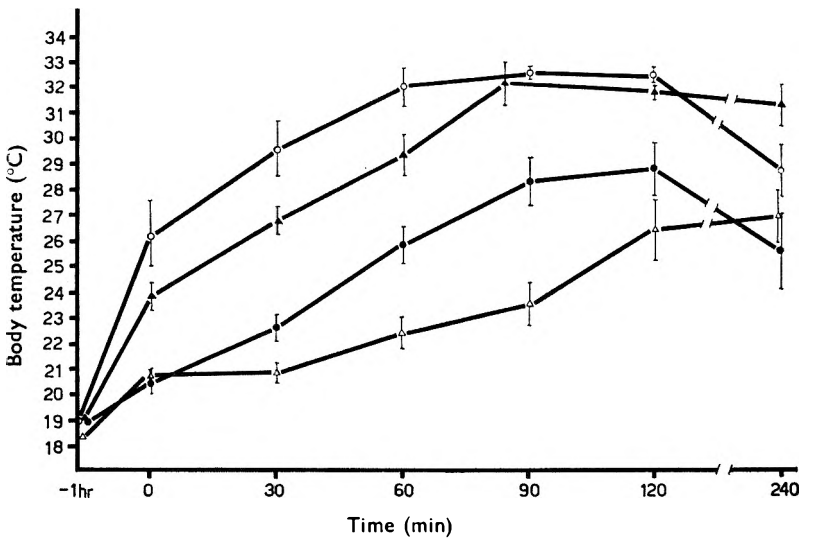

FIG. 8. Increase of body temperature in mice treated 16 hr before the test with reserpine (2·5 mg/kg i.p.) at a room temp. of 20° C. Dopa was given at a dose of 500 mg/kg i.p. and desipramine at a dose of 7·5 mg/kg i.p. —▲—Desipramine + dopa. —○—Desipramine. —▲—Control. —●—Dopa.

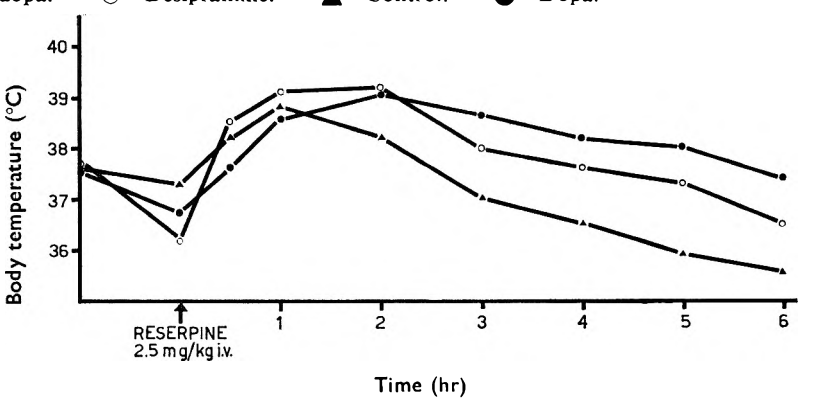

FIG. 9. Effect of desipramine and nortryptiline at a dose of 15 mg/kg i.p. on the hyperthermia induced in rats by an intravenous injection of reserpine (2·5 mg/kg i.v.) at a room temp. of 22° C. —●—Desipramine. —○—Nortryptiline. —▲—Control.

in the different experiments but the trend was similar. The hyperthermia induced by phenethylamine in iproniazid-treated rats was significantly reduced when desipramine was given. Finally the increase of body temperature following a subcutaneous injection of yeast was not affected by desipramine (see Table 3).

Table 4 presents a summary of the effects induced by desipramine on the various types of hyperthermia studied.

TABLE 1. EFFECT OF DESIPRAMINE ON THE HYPERTHERMIA INDUCED BY DEXAMPHETAMINE IN MICE

No. of mice	Treatment	mg/kg i.p.	Body temperature ° C after min				
			−60	0	30	60	90
20	Saline	—					
	Dexamphetamine	5	37·2	36·9	38·5	37·0	36·3
20	Desipramine	7·5					
	Dexamphetamine	5	37·0	35·6	35·5	35·4	35·6
20	Saline	—					
	Dexamphetamine	5	37·2	36·8	39·1	37·3	35·6
10	Nortriptyline	7·5					
	Dexamphetamine	5	37·3	36·8	38·8	36·3	35·7
10	Saline	—					
	Dexamphetamine	15	38·7	37·8	40·0	38·2	38·0
10	Desipramine	7·5					
	Dexamphetamine	15	38·4	37·1	39·5	39·0	39·2
10	Desipramine	7·5	38·4	36·8	36·4	35·9	35·7
5	Saline	—	38·7	37·9	37·8	—	37·4

Dexamphetamine was given at 0 and desipramine 60 min before infusion.
Animals were housed 5 per cage (10 × 20 × 15 cm).

TABLE 2. EFFECT OF DESIPRAMINE ON THE HYPERTHERMIA INDUCED BY DEXMPHETAMINE IN RATS

Treatment	mg/kg	Body temperature ° C after min								
		−60	0	30	60	90	120	240	300	360
Dexamphetamine	15	36·9 ±0·3	36·3 ±0·1	38·4 ±0·2	39·3 ±0·4	39·6 ±0·3	39·0 ±0·2	37·4 ±0·1	37 ±0·2	36·8 ±0·1
Desipramine + dexamphetamine	15	36·9 ±0·3	35·9 ±0·2	37 ±0·4	38 ±0·6	38·9 ±0·5	38·9 ±0·4	39·1 ±0·2	38·7 ±0·1	39·1 ±0·4
Desipramine + dexamphetamine	7·5 15	36·9 ±0·2	36·2 ±0·1	37·6 ±0·4	38·7 ±0·6	38·9 ±0·3	38·5 ±0·2	38·2 ±0·4	37·8 ±0·4	38·3 ±0·6
Dexamphetamine	7·5	36·9 ±0·2	37·5 ±0·1	39·4 ±0·2	39·6 ±0·2	38·7 ±0·2	38·7 ±0·2	37·1 ±0·1	37·2 ±0·1	37·5 ±0·1
Desipramine + dexamphetamine	15 7·5	36·9 ±0·2	36·7 ±0·2	37·3 ±0·3	38·3 ±0·4	38·5 ±0·4	38·4 ±0·4	38·2 ±0·2	38·4 ±0·5	38·4 ±0·5
Desipramine + dexamphetamine	7·5 7·5	37 ±0·1	37·2 ±0·2	38 ±0·4	38·8 ±0·2	39·3 ±0·3	39·1 ±0·3	38·1 ±0·6	38·1 ±0·5	38·2 ±0·6

Desipramine was given 60 min before dexamphetamine.

Discussion and conclusions

The results show that desipramine enhances the hyperthermic effect induced by a number of adrenergic agents. A significant potentiation and prolongation of the body temperature increase was detected when imipramine, or its congeners, was given before an infusion of noradrenaline, adrenaline and isoprenaline. These results are consistent with biochemical findings demonstrating that imipramine reduced the uptake of labelled noradrenaline (Hertting & others, 1961; Axelrod & others, 1962; Titus & Spiegel, 1962). However, with isoprenaline, it is not yet known if there is any uptake and binding.

The hyperthermia occurring shortly after the administration of reserpine seems to be related to a release of catecholamines because we have found it to be inhibited by adrenergic blocking agents (unpublished results). Imipramine and its congeners prolong this hyperthermia and this fact suggests that the previously reported inhibition of the hypothermia induced by reserpine (Garattini & others, 1962) may only be the result of the prolongation of the hyperthermic phase. The present experiments are not in disagreement with the hypothesis that imipramine enhances the effect of reserpine by potentiating endogenous catecholamines which may be released. Similarly the symptomatology described when desipramine is combined with a short-acting reserpine derivative (Ro 4.1284) may be due to a central potentiation of catecholamines in relation to the speed of

TABLE 3. EFFECT OF DESIPRAMINE ON THE HYPERTHERMIA INDUCED BY PHENETHYLAMINE AND YEAST IN RATS

Treatment	mg/kg	Body temperature ° C after, min						
		−60	0	30	60	90	120	150
Phenethylamine	5	37·1±0·3	37·1±0·3	39·3±0·7	39·3±0·9	39·3±0·9	38·7±0·4	37·9±0·3
Desipramine + phenethylamine	15 5	36·9±0·1	36·1±0·3	36·6±0·4	36·7±0·5	37·0±0·6	37·2±0·7	37·2±0·4
Desipramine + phenethylamine	7·5 5	36·8±0·2	36·4±0·1	38·3±0·2	38·6±0·2	38·8±0·2	—	38·5±0·3
		0	5 hr	6 hr	7 hr 30 min	8 hr 30 min		
Yeast	1,500	36·2±0·2	37·9±0·1	37·8±0·2	37·7±0·2	36·9±0·1		
Desipramine + yeast	15 1,500	35·8±0·3	37·5±0·1	36·5±0·2	36·8±0·2	36·7±0·2		

Desipramine was given 60 min before phenethylamine and 5 hr after yeast.
Rats received 200 mg/kg of iproniazid phosphate 16 hr before phenethylamine.

TABLE 4. EFFECT OF DESIPRAMINE ON VARIOUS TYPES OF HYPERTHERMIA

Agent	Types of effect observed
Noradrenaline	enhancement and prolongation
Adrenaline	" " "
Isoprenaline	" " "
Dopa*	" " "
Reserpine	" " "
Dexamphetamine	prolongation
Phenethylamine*	inhibition
Yeast	no effect

* In animals pretreated with a monoamine oxidase inhibitor.

their release (Sulser, Bickel & Brodie, 1964). This interpretation may also be a basis for reconciling the discrepancies observed in various laboratories about the degree of antagonism between desipramine and reserpine.

The potentiation of the dexamphetamine-induced hyperthermia is in agreement with many data already available concerning an increased effect of amphetamine in animals pretreated with imipramine-like drugs (Carlton, 1961; Stein, 1962; Theobald, Buch, Kunz, Morpurgo, Stenger & Wilhelms, 1964; Morpugo & Theobald, 1965). More difficult to interpret is the inhibition of the hyperthermia induced by phenethylamine in rats receiving desipramine. This result, and the fact that desipramine

does not interfere with the changes of body temperature induced by yeast, suggests that desipramine is not a general potentiator of hyperthermic responses (Loew, 1964).

The results described here permit us to broaden the pharmacological knowledge about imipramine and its congeners. The potentiation of catecholamine hyperthermia is specific for these antidepressant agents and it is not shared by a tranquilliser like chlorpromazine. While the hyperthermia induced by noradrenaline is potentiated by monoamine oxidase inhibitors (unpublished data from this laboratory) that induced by isoprenaline is not potentiated by pheniprazine. This suggests another test enabling antidepressant agents belonging to the monoamine oxidase inhibitors or to the imipramine class to be distinguished.

At present the potentiation of the catecholamine hyperthermic responses seems to be a characteristic feature of the imipramine-like drugs which should be added to the already known interactions with reserpine and amphetamine.

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Quantitative separation of riboflavine from vitamin mixtures

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A method for separating riboflavine from vitamin mixtures is described. The acidified aqueous solution is passed through a small column of large particle size talc on which the riboflavine is selectively and quantitatively adsorbed. Successive washings with 0·01 N hydrochloric acid and 5–10% dioxan remove the other components almost completely. The vitamin, eluted with 20% dioxan, is measured spectrophotometrically.

VITAMIN formulations usually contain riboflavine in amounts large enough to permit determination using the distinguishing physical properties of fluorescence and light absorption at certain wavelengths. For simple preparations, where there is no interference from other components, direct measurement is possible; a typical example is the B.P.C. spectrophotometric assay of compound aneurine tablets.

With more complex formulations elaborate physical or chemical measures, or both, are necessary to eliminate the effects of other substances (see for example Koschara, 1935; Hodson & Norris, 1939; Brealey & Elvidge, 1956; Ferrebee, 1940; Conner & Straub, 1941; Klatzkin, Norris & Wokes, 1949).

We describe a successful separation of riboflavine from vitamin mixtures through its selective adsorption on a column of talc. The vitamin is then quantitatively eluted in a sufficiently pure state to permit viewing its full absorption spectrum.

The procedure was developed in the course of investigating the use of purified talc for the chromatographic separation of cyanocobalamin as a step in its determination. It is well known that talc has such a tenacious affinity for vitamin B_{12} as to preclude its use as a filter aid or lubricant for tablets (Merck Index, 1960). On the other hand talc is considered among the substances on which riboflavine is not adsorbed (Sebrell & Harris, 1954). Using talc, we found that riboflavine was adsorbed at the top of the column from aqueous solutions of vitamin B complex. Cyanocobalamin was then adsorbed as an immediately contiguous band and the remaining constituents passed through with the effluent. Cyanocobalamin was eluted with 10% dioxan while riboflavine required 20% dioxan, but the method was not generally applicable to the determination of vitamin B_{12}.

Experimental

Materials. Reagent grade dioxan checked to ensure that its absorbance at 267, 375 and 444 mμ does not exceed 0·03 (lower grades are sometimes effectively purified by charcoal treatment). Purified talc B.P. Hydrochloric acid 0·01 N.

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Effect of particle size. The degree of fineness of the talc was important; very fine talc, contrary to expectation, had little retentive power. This was observed initially; the yellow riboflavine band appearing a few mm below the top of the bed, leaving a white layer of the finest talc. In addition, the band of riboflavine adsorbed on ordinary purified talc was broad, easily washed down, and the eluate showed much extraneous spectrophotometric absorption. On coarse talc, left after elutriation, the riboflavine band was narrower, less easily eluted and the eluate gave curves more typical of the vitamin. For precision, reproducibility and to ensure a reasonable flow rate, all fine powder, therefore, had to be removed.

Preparation of talc bed. As much as 100–120 g of talc may be necessary to make a suitable bed because the coarse fraction forms only a small proportion of it. The mesh size is not critical.

After soaking the talc with water in a suitable Erlenmeyer flask, suspend it in a large volume of water and allow to settle. Decant the unsettled portion after 5 min. Repeat this operation several times until the supernatant no longer shows opalescence or suspended fine particles.* Pour a slurry of the talc sufficient to form a bed of about 80 mm into a column 12 × 200 mm with a stop cock connected to a suction pump and with a small cotton wool pledget at the bottom. Apply gentle suction to give a flow rate of about 80–100 drops (4–6 ml)/min and wash with water until the washings are clear.

The column may be used for several estimations provided each time it is adequately washed with water followed by about 30 ml each of 20% ethanol and water; this especially after passing coloured solutions such as B complex and liver extract, coloured tablets or aqueous preparations of fat and water soluble vitamins.

Effect of other substances. The adsorptive capacity of talc was reduced to a variable degree by the other materials present in solution with the riboflavine. This desorbing effect could be overcome by dilution, the extent of which was related to the nature and concentration of these materials. Thus an injection solution containing 10% of vitamin B_1, 20% of nicotinamide, 1% of vitamin B_6, 2% of procaine hydrochloride in addition to 0·2% of riboflavine, had to be diluted 100 times, while the same solution to which 10% of dry liver extract had been added required 400 times dilution to ensure that the riboflavine was held high enough on the column to permit adequate washing. A solution of lipotropic components containing acetylmethionine equivalent to 10% methionine, 10% inositol and 1% choline chloride with small amount of B complex factors including 0·03% riboflavine required only 4 times dilution. An aqueous mixture of fat and water soluble vitamins required 300 times dilution; it contained in 100 ml, 2 g of nicotinamide and vitamins A (as acetate or palmitate ester) 1 mega i.u.; D (calciferol) 0·2 mega i.u.; E

* The talc used in most of this work was British Drug House's "Talc, Purified by Acids"; measurement of the particle size by the Sedimentation Balance "Prolabo" revealed that 75% was larger than 4·8 μ, 50% than 8·1 μ and 10% than 15·8%. A sample of this talc, after processing as described, measured microscopically, 20–50μ.

((±)-α-tocopheryl acetate) 0·2 g; B_1 2·5 g; B_2 0·1 g; B_6 0·3 g; C 2·5 g together with polysorbate 80 5 g and propylene glycol 40 g. Solutions containing significantly higher concentrations of the surface-active agent could not be assayed by this method as the capacity of the talc to adsorb the riboflavine was then completely lost. Sugar and glycerol used to sweeten B complex syrups had a little desorbing effect and dilution was necessary mainly to reduce viscosity.

Preliminary testing. Initially, sufficient sample is dissolved or diluted to contain 1 mg% of riboflavine. A preliminary test is then made to determine the dilution which ensures optimum adsorption while not rendering the final volume too large to take an unnecessarily long time to flow. A dilution higher than 1 mg% is made only if the adsorption area proves too broad or if partial elution does not permit sufficient washing for the complete removal of interfering substances.

The sample is passed through the talc and followed by sufficient 0·01 N hydrochloric acid and 10% dioxan to elute the other constituents. The amount of washing necessary must be determined for each type of product. The riboflavine is then eluted with 20% dioxan and the eluate, usually 10 ml or less, diluted to a concentration of 1 mg% before spectrophotometric examination at the three standard wavelengths of 267, 375 and 444 mμ against a blank solution prepared by appropriate dilution of the same eluant. The use of a special buffer for dilution is desirable but not essential since the pH of the eluates always lie between 2–8 within which range variation in spectral characteristics of riboflavine at these wavelengths is negligible (Brealey & Elvidge, 1956; Daglish, Baxter & Wokes, 1948).

The ratio of absorbance of the solution at 375 and 444 mμ to that at 267 mμ should lie within the limits 0·314 to 0·333 and 0·364 to 0·388 respectively as stated by the British Pharmacopoeia, otherwise the conditions should be readjusted.

General assay procedure. Carry out operations in a subdued light. Prepare a solution of the sample to contain about 1 mg% of riboflavine using 0·01 N hydrochloric acid. Filter through paper known not to adsorb riboflavine. If necessary dilute a sample size equivalent to 0·25 mg and allow to pass through the talc column at the rate of about 4–6 ml/min. Use a little water to wash the column walls free of sample into the bed then pass 30–60 ml of 0·01 N hydrochloric acid and 30–60 ml of 10% dioxan as determined by preliminary experiments. Add 20% dioxan until the yellow band nears the bottom of the column. When the coloured band reaches the stopcock, collect the effluent until all the yellow colour has flushed through the stem. Measure the eluate, add 2 ml of 0·01 N hydrochloric acid and dilute to 25 ml with water. Prepare a blank of the same volume of 20% dioxan diluted similarly to 25 ml.* Measure the absorbance at the maximum at 444 mμ and calculate the result using $E(1\%\ 1\ cm) = 320$.

* If spectroscopically pure dioxan is available, omit measuring the eluate and directly dilute to 25 ml with 20% dioxan after adding 2 ml of 0·01 N hydrochloric acid; modify the blank accordingly.

Confirm the result and identity of riboflavine by measuring the absorbance at 375 and 267 mμ.

Results and discussion

The method was applicable to all the vitamin preparations examined; the results in Table 1 show that the recovery of riboflavine is 97–102%.

TABLE 1. ABSORPTION OF RIBOFLAVINE COMPARED WITH THAT OF THE VITAMIN MIXTURES FROM WHICH IT HAS BEEN SEPARATED

Product	Final concentration mg %	Absorption 267 mμ	Absorption 375 mμ	Absorption 444 mμ	Recovery %
Standard solution					
Direct	1	0·845	0·265	0·315	
Recovered	1	0·845	0·263	0·310	97
Parenteral solutions					
*B complex	1	over 2	0·360	0·322	
Riboflavine recovered	1	0·840	0·263	0·314	98·1
*B complex (same without procaine)	1	over 2	0·266	0·318	
Riboflavine recovered	1	0·830	0·264	0·308	96·3
*B complex with liver	1·1	over 2	0·655	0·475	
Riboflavine recovered	1·1	0·970	0·305	0·350	100
*B complex with lipotropic factors	1	over 2	0·325	0·350	
Riboflavine recovered	1	0·825	0·260	0·309	96·6
Oral liquid preparations					
B complex & vitamin C, Syrup	1	over 2	0·380	0·335	
Riboflavine recovered	1	0·840	0·260	0·310	97
*Aqueous mixture of fat & water soluble vitamins, Drops	1	over 2	1·200	0·395	
Riboflavine recovered	1	0·890	0·280	0·330	103·0
Tablets					
B complex (weak), uncoated	1·25	over 2	0·410	0·450	
Riboflavine recovered	1·25	1·10	0·350	0·410	102·5
B complex (weak), coated, coloured	1·25	over 2	0·376	0·440	
Riboflavine recovered	1·25	1·045	0·328	0·400	100
B complex (strong), coated, coloured	1·22	over 2	0·355	0·415	
Riboflavine recovered	1·22	1·05	0·330	0·390	99·9
Capsule					
B complex & lipotropic factors	1·25	over 2	0·456	0·440	
Riboflavine recovered	1·25	1·13	0·360	0·410	102·5

* Composition in the text.

In most of the methods cited for estimating riboflavine in complex vitamin mixtures fluorimetry is employed after either chemical treatment or a combination of chromatographic separation and chemical treatment. So far as could be traced no published procedure seems capable of giving a final solution sufficiently free from interfering substances to allow direct spectrophotometric measurement of riboflavine. Separation on talc as described by us makes this possible. This could be because talc, being one of the weakest adsorbents, allows the other constituents to pass freely and yet has sufficient affinity for riboflavine to provide a specific means for its separation. The method combines the advantages of a simple and rapid chromatographic separation with the speed and accuracy of spectrophotometry; it is also more specific and flexible than fluorimetric procedures.

The method gives a full spectral view of the riboflavine separated with its characteristic multiple peaks so that it is not necessary to check the results against a standard solution nor to compare them with those of

microbiological assays. Indeed, the method involves its own means of identifying the riboflavine and confirming its purity.

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Effect of choline on the release of acetylcholine and on its tissue stores in the phrenic nerve-diaphragm preparation treated with dyflos

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The release of acetylcholine from the guinea-pig phrenic nerve-diaphragm preparation treated with different concentrations of dyflos during four successive stimulation periods, was detected. At 38°, after 50 and 200 μg/ml of dyflos, the amount of acetylcholine released fell abruptly after the first stimulation period; a less evident decrease in the output was observed from the preparations preincubated with 500 and 1,000 μg/ml of dyflos. A reasonably constant release was detected at 28° after 1,000 μg/ml of dyflos; at this temperature the initial output was about 40% of that at 38°. Choline chloride (3 μg/ml) added to the perfusion fluid prevented the decreasing output only in the preparations incubated with the anticholinesterase at concentrations of 500 and 1,000 μg/ml. Dyflos did not affect the time course of acetylcholine release at 28°, the initial output of acetylcholine and the release at rest under any experimental conditions or the tissue stores of acetylcholine at the end of the fourth period of stimulation. A probable impairment by dyflos of the choline transfer system is discussed.

THE rat and guinea-pig phrenic nerve-diaphragm preparation, treated with neostigmine or physostigmine, maintains a steady release of acetylcholine in response to repeated stimulation periods (Straughan, 1960; Matthews & Quilliam, 1964); no significant increase in the acetylcholine released is detectable when choline is added to the perfusion fluid (Straughan, 1960). In the cat superior cervical ganglion kept in the usual Locke solution containing physostigmine, a decreasing output has been observed (Birks & McIntosh, 1961); the release becomes reasonably constant in choline-supplemented perfusion fluid (Birks & McIntosh, 1961; Matthews, 1963). The acetylcholine released from the guinea-pig phrenic nerve-diaphragm preparation pretreated with diisopropylfluorophosphonate (dyflos; DFP) gradually decreases during successive stimulation periods (Beani, Bianchi & Ledda, 1964). We now report the results of experiments made to examine the effect of choline on acetylcholine release and on its tissue stores in the guinea-pig phrenic nerve-diaphragm preparation treated with various concentrations of dyflos.

Experimental

All the experiments were made on the guinea-pig phrenic nerve-diaphragm preparation according to the method previously described (Beani & others, 1964); the preparations (1/4 of the whole hemidiaphragm) were kept in oxygenated Tyrode solution with glucose 2 g/litre, at 28° and 38°. The nerve was stimulated by supramaximal rectangular impulses 0·1 msec duration, at 50 shocks/sec. In a few control experiments neostigmine 10^{-5} was used as a cholinesterase inhibitor, the preparations being preincubated with the drug for 150 min before the experiment. The acetylcholine released was assayed on the leech dorsal muscle kept in

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Locke solution diluted 1 to 1·4 with distilled water containing physostigmine 10^{-5} and morphine hydrochloride 2×10^{-5} (Murnagham 1958).

In most experiments we used different concentrations of dyflos (50, 200, 500 and 1,000 μg/ml) to inhibit the esterase; the method of estimating the acetylcholine release was that of Beani & others (1964).

The experiments consisted of four successive 10 min stimulation periods, with 10 min intervals of rest; a 3 min conditioning stimulation period (Straughan, 1960) at 50/sec was given before the first collecting period.

When the effect of choline was tested, one preparation was kept in normal perfusion fluid and the contralateral one, from the same animal, in choline supplemented Tyrode solution. The tissue acetylcholine was extracted from the control and from the choline-treated preparations at the end of the fourth stimulation period, using the method of Beani, Bianchi & Ledda (1962). The drugs used were: acetylcholine chloride, Roche; neostigmine, Roche; dyflos, Boots; choline chloride, Merck; physostigmine sulphate, B.D.H.

Results

The release of acetylcholine from neostigmine-treated preparations. As previously described by Straughan (1960), a steady release was observed when neostigmine 10^{-5} was employed as esterase inhibitor. The average release (3 experiments) was 36·8 ng in the first stimulation period, 39 ng in the second, 40·1 ng in the third and 39·7 ng in the fourth period. The samples collected in rest periods were not assayed.

The release of acetylcholine from dyflos-treated preparations. The results of these experiments are reported in Table 1. At 38°, after dyflos 50 μg/ml, the amount of acetylcholine released fell abruptly after the first

TABLE 1. ACETYLCHOLINE RELEASED (NG ± S.D.) DURING FOUR SUCCESSIVE PERIODS OF STIMULATION AT 50/SEC FOR 10 MIN, FROM GUINEA-PIG PHRENIC NERVE-DIAPHRAGM PREPARATIONS PERFUSED EITHER WITH TYRODE SOLUTION OR WITH CHOLINE (3 μG/ML) SUPPLEMENTED TYRODE SOLUTION, AND PREINCUBATED WITH DIFFERENT CONCENTRATIONS OF DYFLOS

Temp. °C	Dyflos µg/ml	No expts	Groups	Stimulation periods			
				1st	2nd	3rd	4th
38	1,000	10	controls	31·8 ± 8·8	29·3 ± 8·2	24·1 ± 8·8	19·7 ± 5·7
		6	choline	30·5 ± 4·8	31·4 ± 4·9	28·3 ± 4·4	27·2 ± 5·9*
	500	7	controls	32·5 ± 5·1	27·1 ± 7·4	21·0 ± 6·3	17·0 ± 9·4
		7	choline	34·4 ± 7·3	34·2 ± 6·6	29·7 ± 7·3	27·1 ± 5·9†
	200	6	controls	29·5 ± 9·6	20·4 ± 8·2	12·0 ± 3·4	11·8 ± 3·5
		—	—	—	—	—	—
	50	5	controls	28·1 ± 7·5	14·6 ± 2·6	11·6 ± 3·9	10·3 ± 2·8
		6	choline	25·0 ± 7·4	18·0 ± 7·4	14·2 ± 6·8	14·2 ± 5·1
28	1,000	5	controls	13·3 ± 5·5	13·9 ± 5·2	13·7 ± 4·7	10·0 ± 3·0
		5	choline	13·9 ± 5·8	15·0 ± 7·4	14·4 ± 7·2	12·4 ± 6·3

* = Statistically different ($0{\cdot}05 > P > 0{\cdot}02$) from the control group.
† = Statistically different ($P = 0{\cdot}05$) from the control group.

stimulation period so that in the fourth period it was only 36·6% of the amount released in the first period; a similar time course of transmitter output was observed after 200 μg/ml, the acetylcholine released in the last period being 40% that of the first period.

The percentage decrease was less evident in preparations preincubated with dyflos, 500 and 1,000 μg/ml: it was 52·3 and 61·9% respectively. At 28°, after dyflos, 1,000 μg/ml, the acetylcholine release was almost constant. The amount released during the first stimulation period was about 40% of that released at 38° in the same period.

Effect of choline on dyflos-treated preparations. Choline chloride 3 μg/ml added to the perfusion fluid improved the transmitter output (see Table 1).

At 38°, the acetylcholine released in the fourth period was 57% of that released in the first period in the preparations incubated with dyflos, 50 μg/ml; it was 78·7 and 89·1% respectively in those incubated with 500 and 1,000 μg/ml. The difference between the average release of the controls and of the treated preparations was statistically significant only in the fourth period, with the 500 and 1,000 μg/ml amounts employed The effect of choline on preparations pretreated with 200 μg/ml of dyflos was not tested because the output detected was similar to that after 50 μg/ml.

Choline had no significant effect on the time course of acetylcholine release in preparations incubated at 28°, where a steady release was present even in normal Tyrode solution; nor did choline affect the amount of transmitter released in the first stimulation period both at 28° and at 38° or the release at rest under every experimental condition (values not given).

Acetylcholine tissue stores. The results of these experiments are reported in Table 2. Both at 28° and at 38° there was no difference between the acetylcholine tissue stores in the control and in the choline-treated preparations, at the end of the fourth stimulation period.

TABLE 2. TOTAL TISSUE ACETYLCHOLINE (NG/G OF FRESH TISSUE) AT THE END OF THE FOURTH PERIOD OF STIMULATION AT 50/SEC, IN HEMIDIAPHRAGMS PERFUSED EITHER WITH TYRODE SOLUTION OR WITH CHOLINE SUPPLEMENTED TYRODE SOLUTION, AND PREINCUBATED WITH DYFLOS 1,000 μG/ML FOR 150 MIN (Every value is the mean of 10 experimental points.)

Temp. °C	Groups	Guinea-pig weight (g ± s.d.)	Hemidiaphragm weight (mg ± s.d.)	Acetylcholine (ng ± s.d.)
28	controls	253 ± 17	193 ± 30	455 ± 106
	choline	253 ± 17	198 ± 21	470 ± 51
38	controls	256 ± 26	200 ± 24	181 ± 61
	choline	256 ± 26	203 ± 20	189 ± 67

Discussion

As previously reported by Straughan (1960), a constant transmitter release was observed in neostigmine-treated phrenic nerve-diaphragm preparations during successive stimulation periods; this suggests that all the elements necessary for an efficient acetylcholine synthesis are available in the preparation itself.

Under identical experimental conditions, the acetylcholine released in the first stimulation period in dyflos-treated preparations was almost equal to that in neostigmine-treated preparations; nevertheless it decreased in the subsequent collecting periods. Dyflos concentrations of 500 μg/ml or more are necessary to ensure a complete and irreversible esterase inhibition (Beani & others, 1964); the release in preparations preincubated with 50 or 200 μg/ml was low, irregular and unaffected by choline. It is remarkable that dyflos, in concentrations as high as 1,000 μg/ml, did not affect the nerve conduction; neither irregular muscle responses to the nerve stimulation nor an acetylcholine release lower than that detected after lower concentrations was observed.

The decreasing output in dyflos-treated preparations was detectable at 38°, but was much less evident at 28°; at this incubation temperature the initial acetylcholine output was about 40% of the amount released at 38°. This is in agreement with the observation of Straughan (1960) who used neostigmine as inhibitor. This finding suggests that dyflos, which is not a pure esterase inhibitor (Holmstedt, 1959), can cause some metabolic damage which may also affect acetylcholine synthesis when the experimental conditions (high temperature and stimulation rate) seriously deplete the reserves of the nerve terminals. It seems likely that this action of dyflos is an impairment of the enzymatic process of the choline transfer to the site of acetylcholine synthesis as it is counteracted by the presence of choline in the medium.

Choline affects the output of acetylcholine but not its tissue stores. This observation agrees with the idea that the extracted tissue acetylcholine after esterase inhibitors is, for the most part, the fraction not readily available for the release provoked by nerve stimulation (Birks & McIntosh, 1961). It is evident that this fraction increases in hypothermia because tissue acetylcholine at 28° is 2·5 times higher than at 38° (Table 2).

Dyflos appears to cause some damage in the metabolic pathways of acetylcholine synthesis; nevertheless we believe that its use in a choline supplemented medium is more suitable than that of reversible esterase inhibitors in experiments made to examine whether a drug is able to modify the transmitter release from motor nerve terminals. Neostigmine or physostigmine, which must be kept in the bath throughout the experiment, may react with acetylcholine receptors (Nachmansohn, 1959) and alter the effect of the drug under investigation.

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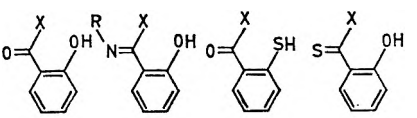
Some pyrroline derivatives as potential anti-inflammatory agents

A. H. BECKETT, CALVIN M. LEE AND J. K. SUGDEN

Eight *N*-substituted-4-carbethoxy-2-oxo-3-hydroxypyrrolines and six *N*-β-phenethyl-2-oxo-3-arylaminopyrrolines were synthesised and tested for possible anti-inflammatory action. Infrared and nuclear magnetic resonance spectral evidence is presented to show that in the solid state and in solution ($CDCl_3$) these compounds exist solely as the tautomer in which the double bond is within the heterocyclic ring. The compounds showed no important pharmacological activity.

THERE are four main types of anti-inflammatory agent in clinical use viz. salicylates, phenylbutazone derivatives, corticosteroids, and quinoline type compounds. None of these are totally satisfactory because of their side-effects. Whitehouse & Bostrom (1962) proposed two prerequisites for anti-inflammatory activity in salicylates; the molecule must chelate with metal ions and it must be lipid soluble. On this basis it was suggested that the structures (Ia-Id, where R = alkyl, X = H, OH, OR) may have anti-inflammatory activity.



Ia Ib Ic Id

The purpose of the present work is to replace the six-membered aromatic structure with a planar five-membered heterocyclic ring suitable for simple modification to improve lipid solubility and chelating ability.

Experimental

PREPARATION OF THE COMPOUNDS

General method of preparation of 1-*substituted*-4-*carbethoxy*-2-*oxo*-3-*hydroxypyrrolines* (*II*). Compounds of type III were prepared from appropriately substituted amines and ethyl acrylate in equimolar amounts (0·1–0·2 mol) by heating under reflux in absolute ethanol (100 ml) for 30 min, and the solution allowed to stand for 24 hr before adding diethyl oxalate. This solution was added portionwise to a solution prepared by dissolving sodium (0·1–0·2 mol) in absolute ethanol (100 ml); the mixture was heated under reflux on a steam-bath for 1 hr and the solvent evaporated off. The solid residue was dissolved in the minimum amount of water and the solution acidified with concentrated hydrochloric acid to yield a precipitate which was recrystallised from ethanol.

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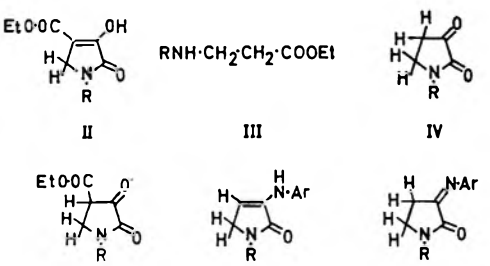
Part of the thesis submitted by J. K. Sugden for the degree of Doctor of Philosophy, University of London, 1964.

The following new compounds were prepared: II, R = *p*-chlorophenethyl, yield 52%, m.p. 172–172·5°. Found: C, 58·6; H, 5·3; N, 4·4%; $C_{15}H_{16}NO_4$ requires C, 58·15; H, 5·2; N, 4·5%; ν 3150, 1670 cm^{-1}. II, R = *p*-methoxyphenethyl, yield 61%, m.p. 152–152·5°. Found: C, 62·8; H, 6·3; N, 4·4%; $C_{16}H_{19}NO_5$ requires C, 62·9; H, 6·2; N, 4·6%; ν 3150, 1660 cm^{-1}. II, R = β-phenylisopropyl, yield 57%, m.p.

TABLE 1. ULTRAVIOLET SPECTRAL DATA OF COMPOUNDS OF TYPE II IN ETHANOL

No.	R =	λ_{max} in mμ	ε
1	Methyl	249	13,500
2	*n*-Butyl	247	11,400
3	Cyclohexyl	247	10,500
4	Benzyl	249	15,100
5	Phenethyl	248	11,300
6	β-Phenylisopropyl	248	14,200
7	*p*-Methoxyphenethyl	226, 249	16,300; 12,600
8	*p*-Chlorophenethyl	221, 247	15,200; 12,300

114–115°. Found: C, 66·4; H, 6·7; N, 4·7%; $C_{16}H_{19}NO_4$ requires C, 66·4; H, 6·6; N, 4·8%; ν 3120, 1660 cm^{-1}. The other compounds recorded in Table 1 were prepared as described by Southwick, Previc, Casanova & Carlson (1956) and Southwick & Crouch (1953); the m.p. values of these materials were in agreement with reported values.



II III IV

V VI VII

General method of preparation of 1-*β-phenethyl-2-oxo-3-arylamino-pyrrolines* (VI, R = phenethyl). 1-β-Phenethyl-2,3-dioxopyrrolidine (IV, R = phenethyl) (0·01 mol) (Southwick & others, 1956) and the appropriate aromatic amine (0·01 mol) were dissolved in absolute ethanol (40 ml) and heated under reflux for 1·0 hr. The solid which separated upon cooling was recrystallised from ethanol. The following new compounds were prepared: VI, Ar = phenyl, yield 1·0 g (36%), m.p. 164–165°. Found: C, 77·4; H, 6·65; N, 10·35%; $C_{18}H_{18}N_2O$ requires C, 77·7; H, 6·5; N, 10·1%. VI, Ar = *p*-nitrophenyl, yield 1·0 g (30%), m.p. 215–216°. Found: C, 67·2; H, 5·2; N, 12·6%; $C_{18}N_{17}N_3O_3$ requires C, 66·9; H, 5·3; N, 13·0%. VI, Ar = *p*-tolyl, yield 1·3 g (34%), m.p. 182–183°. Found: 78·5; H, 7·2; N, 9·8%; $C_{19}H_{20}N_2O$ requires C, 78·1; H, 6·9; N, 9·6%. VI, Ar = *p*-methoxyphenyl, yield 1·6 g (50%) m.p. 177·5–179°. Found: C, 74·1; H, 6·35; N, 9·35%; $C_{19}H_{20}N_2O_2$ requires C, 74·0; H, 6·5; N, 9·1%. VI, Ar = *p*-chlorophenyl, yield 1·3 g (41%) m.p. 206–207°. Found: C, 69·4; H, 5·6; N, 9·35%; $C_{18}H_{17}N_2OCl$ requires C, 69·1; H, 5·5; N, 9·0%. VI, Ar = β-naphthyl, yield 1·5 g (45%),

m.p. 199·5–200·5°. Found: C, 81·0; H, 6·1; N, 8·8%; $C_{22}H_{20}N_2O$ requires C, 80·5; H, 6·1; N, 8·5%.

PHYSICAL MEASUREMENTS

The infrared spectra of the compounds as Nujol mulls were obtained on a Unicam SP200, ultraviolet spectra of the compounds dissolved in absolute ethanol were obtained on a Unicam SP 800, and NMR spectra of the compounds in deuterochloroform using tetramethylsilane as reference were obtained using a Varian A60 instrument.

TABLE 2. NMR SPECTRAL DATA OF COMPOUNDS OF TYPE II IN $CDCl_3$ AT 60 MC. (τ values from tetramethylsilane)

R =	Me	Bu	Benzyl	Phenethyl
Protons:				
C–Me (ester) ..	8·64 (3H, T)	8·68 (3H, T)	8·72 (3H, T)	8·67 (3H, T)
N–Me	6·88 (3H, S)	—	—	—
Ph–CH_2– ..	—	—	—	7·10 (2H, M)
N–CH_2–	—	6·50 (2H, M)	5·32 (2H, S)	6·30 (2H, M)
–CH_2– (ring) ..	6·00 (2H, S)	6·00 (2H, S)	6·13 (2H, S)	6·15 (2H, S)
CH_2–C (ester) ..	5·70 (2H, Q)	5·68 (2H, Q)	5·70 (2H, Q)	5·70 (2H, Q)
Aromatic ..	—	—	2·67 (5H, S)	2·75 (5H, S)
OH*	0·50 (1H, S)	−1·10 (1H, S)	0·75 (1H, S)	0·84 (1H, S)

* Disappears on deuteration.
(S = singlet, D = doublet, T = triplet, Q = quartet, M = multiplet.)

PHARMACOLOGICAL TESTING

Compounds of structure II (Nos. 1, 4, 5, 6, 7, 8 Table 1) were tested for anti-inflammatory activity by measuring the delay in onset of skin erythema following exposure of the depilated backs of guinea-pigs to ultraviolet light (Hardy, Wolff & Goodell, 1952). Only compound 8 showed any activity (at 60 mg/kg); other compounds were inactive at 200 mg/kg. Compound 8, tested by the method of Randall & Selitto (1957), gave an ED50 of 83 mg/kg as compared with 2 mg/kg for phenylbutazone. Compound 1 showed some antipyretic and analgesic activity after 4 hr at a dose of 200 mg/kg.

Compounds of structure VI (Nos. 9, 10, 11 and 14 Table 3) were also tested by the ultraviolet light induced erythema test. Only compound 11 showed any activity at a dose level of 200 mg/kg. Compounds 10, 11 and 14 showed slight sedative effects at doses above 1 g/kg.

Results and discussion

PREPARATION OF THE COMPOUNDS

Attempts to hydrolyze the ester group in compounds of type II failed. Refluxing in 6N hydrochloric acid resulted in decarboxylation and rearrangement to the corresponding 2,3-dioxopyrrolidine (IV), except in (II, R = Me) where no product could be isolated. The reaction of 2,3-dioxopyrrolidine (IV, R = phenethyl) with aromatic amines was used to prepare 1-substituted-2-oxo-3-arylaminopyrrolines (VI, R = phenethyl) [cf. the reaction of 1-benzyl-2,3-dioxopyrrolidine with aniline to form VI, R = benzyl, Ar = phenyl (Southwick & others, 1956)]. Compounds of structure II and VI gave coloured complexes with ferric or cupric ions.

SPECTRAL DATA AND STRUCTURAL CONSIDERATIONS

On the basis of pK_a and infrared spectral data, it was suggested by Southwick & others (1956) that compounds of type II existed mainly in the enolic form rather than the ketonic form V. Present evidence based on infra-red and NMR data (Table 2) shows that compounds of type II exist in the solid state and in solution ($CDCl_3$) solely in the enolic form; the evidence is as follows.

TABLE 3. ULTRAVIOLET AND INFRARED SPECTRA OF COMPOUNDS OF TYPE VI

No.	Ar =	Ultraviolet (ethanol) mμ/extinction		Infrared (Nujol) cm^{-1}
9	Phenyl	243 15,000	291 9,600	3250, 1660, 1630
10	*p*-Nitrophenyl	250sh 7,900	374 12,100	3300, 1660, 1640
11	*p*-Tolyl	243 17,000	293 13,000	3300, 1670, 1640
12	*p*-Methoxy	241 16,500	296 10,400	3300, 1670, 1640
13	*p*-Chlorophenyl	247 14,000	292 14,600	3300, 1670, 1640
14	β-Naphthyl	265sh 17,000 309 13,600	275sh 12,300 350 2,600	3300, 1680, 1640, 1620

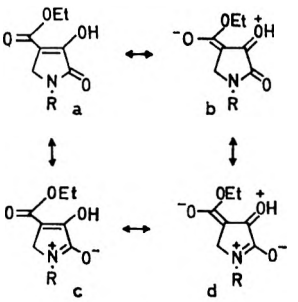
sh = shoulder.

The hydrogen-bonded hydroxyl group of II is indicated in the solid state by the medium strength band around 3100 cm^{-1} in the infrared spectrum (Nujol mull) of these compounds; the carbonyl group is shown by the broad band at 1650 $\pm$ 20 cm^{-1}. The hydroxyl proton in structure II is also shown by the broad band (1 proton) in the NMR spectra of the four 1-substituted 4-carbethoxy-2-oxo-3-hydroxypyrrolines examined (Table 2); this signal disappears on deuteration, under neutral conditions. Supporting evidence is the two-proton *singlet* (*ca* 6·0 τ) which is assigned to the C_5 protons; this signal is again consistent only with structure II. [The predicted NMR spectrum of V would show no active hydrogen and have the signal of the aliphatic proton on C_4 split into a multiplet by the two adjacent C_5 protons; the signal of these latter protons would also be split by the adjacent C_4 proton.] The NMR spectra and their integrals show no detectable amount of tautomer V in $CDCl_3$ solutions of compounds of type II.

Other aspects of the NMR spectra include an unsplit two-proton singlet in the spectrum of the *N*-benzyl analogues which is assigned to the methylene group of the benzyl side-chain. In the phenethyl analogues, multiplets appear at 7·10 and 6·30 τ each representing 2 protons. The 7·10 τ peak is assigned to the methylene group next to the benzene ring.

The tautomer present in ethanol solution cannot be deduced unequivocally from the ultraviolet spectra of solutions of compounds (II) due to the lack of suitable model compounds. However, since from the infra-red and NMR evidence the enolic structure II is present in the solid state and in deuterochloroform solution, the high ultraviolet absorption at about 248 mμ (ϵ ca. 13,000, Table 1) may be assigned with reasonable

certainty to the enolic form and to resonance between the hybrids (VIIIa←→VIIId; R = benzyl).



VIII

The preference of 1-substituted-4-carbethoxy-2-oxo-3-hydroxypyrrolines for the enolic form II may be due to the additional resonance stabilisation (VIIIa←→VIIIb←→VIIId) which is not possible with the ketonic form V. [The NMR spectrum of the compound without the ester group (IV, R = phenethyl) shows only a complicated set of peaks for eight protons indicating that the ketonic form of this compound is present in deuterochloroform solution.]

On the basis of infra-red evidence (Southwick & others, 1956), structure VI and not VII (R = benzyl, Ar = phenyl) was assigned to 1-benzyl 2-oxo-3-phenylaminopyrroline. Our infra-red and NMR evidence (Tables 3 and 4) indicates that the six new phenethyl analogues we report exist in the solid state and in solution ($CDCl_3$) as structure VI. The evidence is as follows.

TABLE 4. NMR SPECTRA OF N-SUBSTITUTED 2-OXO-3-PHENYLAMINOPYRROLINES (VI, Ar = PHENYL) IN $CDCl_3$ AT 60 MC (τ values from tetramethylsilane)

	R = Benzyl	R = Phenethyl
Protons:		
$-CH_2$ (adjacent to Ph)	—	7·08 (2H, multiplet)
$-CH_2$ (ring)	6·18 (2H, doublet)	6·20 (4H, multiplet)
$-CH_2$ (adjacent to N) ..	5·30 (2H, singlet)	
Olefinic	4·08 (1H, triplet)	4·17 (1H, triplet)
N-H*	3·34 (1H, broad singlet)	3·40 (1H, broad singlet)
Aromatic	2·84 (10H, multiplet)	2·90 (10H, multiplet)

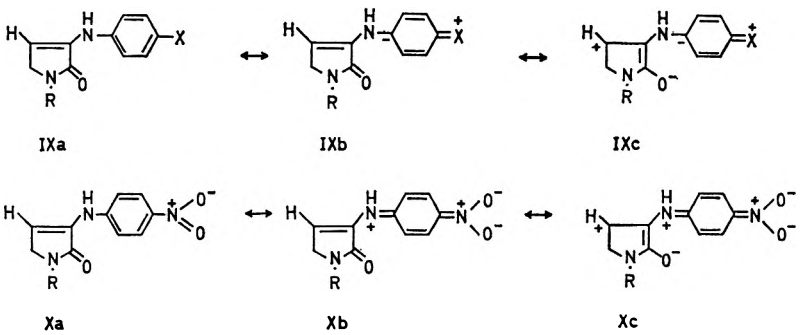
* Disappears on deuteration.

The presence of the imino-group in structure VI is shown in the solid state by the N-H bond 3300 cm^{-1} (Table 2) in the infra-red spectrum (Nujol mull); the lactam carbonyl and the carbon-carbon double bond bands are at 1670 and 1640 cm^{-1} respectively. For NMR measurements, 1-benzyl-2-oxo-3-phenylaminopyrroline was used as a prototype of the series since the phenethyl group resonance partly obliterates the ring proton signals in the corresponding 1-phenethyl analogues (see Table 4). The N-H proton is indicated by a low broad one-proton signal at 2·34 τ which disappears upon deuteration. A one-proton triplet at 4·08 τ (J = 2·5 cps) can only be the signal of the olefinic proton at C_4 split by the two ring protons at C_5; these latter appear as a doublet at 6·18 τ

(identical J). [The predicted NMR spectrum for structure VII would have no active proton, no olefinic proton, and a complicated pattern for four ring protons.] The position, multiplicity, and integral of the NMR signals are consistent only with Structure VI; there is no detectable amount of structure VII.

The ultraviolet spectra of these compounds in ethanol cannot distinguish unequivocally between the two tautomers because both VI and VII are equally conjugated. The ultraviolet data in Table 3 show that substitution in the *para* position of the benzene ring of compounds of type VI leads in some instances, e.g. compounds 12 and 13, Table 3, to only small changes in wavelength, while the substitution of a *p*-NO_2 group leads to a bathochromic shift of 80 mμ. Since the infra-red and NMR data indicate structure VI in the solid state and $CDCl_3$ solution, it is concluded that the *p*-OMe and *p*-Cl may exert a mesomeric effect on the benzene ring which is not transmitted to the pyrroline double bond (i.e. IXa$\leftrightarrow$IXc; R = phenethyl, X = Cl or OMe); thus only small wavelength shifts are produced by these substitutions.

On the other hand, the *p*-NO_2 group (No. 10 Table 2) can interact with the double bond in the pyrroline ring forming a system encompassing four conjugated double bonds (Xa$\leftrightarrow$Xb$\leftrightarrow$Xc; R = phenethyl) and this causes the large bathochromic shift.



IXa IXb IXc

Xa Xb Xc

PHARMACOLOGICAL RESULTS

The lack of significant pharmacological activity in compounds of type II and VI precludes any structure-activity correlations.

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The effect of humidity and temperature on the equilibrium moisture content of powders

E. SHOTTON AND N. HARB

The equilibrium moisture content of maize, wheat and potato starches, alginic acid, tragacanth, acacia, lactose, dextrose, sucrose and hexamine has been determined at different relative humidities and temperatures. Samples were exposed to an atmosphere of controlled temperature and humidity until equilibrium was attained. The moisture content was determined by drying. The moisture content of each powder at equilibrium was dependent on the relative humidity.

ALTHOUGH water vapour is usually considered to be one of the minor constituents of the atmosphere, it can be of great importance in the manufacture and storage of materials.

Moisture gained by a solid material can be held in different ways and stages (Geary, 1956). It can be simply acquired by the physical process of sorption and is known as "free water", or it can be held by a chemical bond and is termed "bound water" (Briggs, 1932).

The sorption of water vapour by powders has been examined by many workers. Edgar & Swan (1922) found that sorption of moisture varies with the specific nature of the material, the exposed surface area of the solid, the temperature and the velocity of the movement of the moist air.

The uptake of moisture by starch was determined by Browne (1922), who found that anhydrous starch reached its saturation point after about 12 days and contained 24·37% moisture. Hellmann & Melvin (1948) found that moisture sorption by 10 corn starches was not affected by origin, condition, preparatory procedure, drying procedure and time of storage.

The sorption of water by maize and potato starches has been studied by Sair & Fetzer (1944). These authors agree with Katz (1917) that starches of different origin vary markedly in sorptive capacity and that water was held by physical sorption between relative humidities of 20 and 90%. This opinion differs from that of Freeman (1942), who stated that water in starch was in the bound form. Ulmann (1957) declared that water sorbed by starch was held by adsorption, by binding, and by two unspecified kinds of association.

Craik & Miller (1958) showed that sucrose deliquesced when exposed above a relative humidity of approximately 80% at 30°. Browne (1922) found the equilibrium moisture contents of dried samples of sucrose, dextrose, and lactose exposed at a relative humidity of 60% to be 0·03, 0·07 and 1·23% respectively, equilibrium being attained after 9 days.

Experimental

EQUILIBRIUM MOISTURE CONTENTS

Apparatus. A thermostatically controlled humidity cabinet was constructed so that air could be circulated over suitably chosen saturated

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solutions to attain a given humidity and then over the samples of powders. The humidity was checked with a wet and dry bulb hygrometer (Harb, 1963).

Materials. The materials examined were maize, wheat and potato starches, alginic acid, tragacanth, acacia, lactose, anhydrous dextrose, sucrose and hexamine.

Method. Samples were spread in a uniform layer about 3 to 5 mm thick in Petri dishes 10 cm diameter and placed in the humidity cabinet. At 1, 2, 3 and 4 hr after exposure to the humidified air each sample was mixed thoroughly. After 24 hr the powders were well mixed and the

TABLE 1. EQUILIBRIUM MOISTURE CONTENTS OF THE POWDERS AT DIFFERENT HUMIDITIES AND TEMPERATURES

(Expressed as a percentage of the dry weight)

Temp. °C	Relative humidity	Maize starch	Wheat starch	Potato starch	Tragac.	Acacia	Alginic acid	Lactose	Dextrose	Sucrose
25	34	10·8	10·9	13·5	8·5	8·2	8·7	—	—	—
	55	13·2	13·0	16·4	13·7	12·2	10·9	—	—	—
	66	15·2	15·3	19·2	19·5	15·4	12·3	—	—	—
	87	19·8	19·4	26·4	32·4	28·9	18·9	—	9·6	—
	100	27·4	28·0	35·6	gel	gel	30·3	0·1	soln.	soln.
30	33	10·9	10·4	13·7	9·6	8·9	9·0	—	—	—
	44	12·7	12·7	16·1	12·8	11·3	11·3	—	—	—
	65	15·4	15·4	19·4	19·9	15·7	12·9	—	—	—
	80	19·1	18·9	24·1	27·7	25·9	16·2	—	10·0	—
	86	19·6	20·7	26·9	31·8	—	19·2	—	10·0	—
	92·5	24·8	25·9	33·0	42·5	gel	25·1	—	soln.	soln.
	100	26·8	29·6	35·6	gel	soln.	30·5	0·1	soln.	soln.
40	32	8·07	9·6	13·1	10·8	9·8	9·2	—	—	—
	50	12·9	12·6	16·0	14·9	12·9	11·6	—	—	—
	63	15·3	14·8	18·9	19·9	16·2	13·6	—	—	—
	75·5	17·1	16·7	21·5	25·2	20·4	15·9	—	3·7	0·05
	89·5	20·1	19·9	26·2	32·7	gel	22·3	—	soln.	0·2
	100	30·5	29·2	36·3	gel	soln.	31·4	0·2	soln.	soln.
50	32	9·3	9·2	12·5	9·8	9·3	8·1	—	—	—
	47	11·5	11·5	15·2	12·5	11·1	9·8	—	—	—
	67	15·3	15·2	19·1	19·7	15·5	13·8	—	—	—
	76	17·6	17·3	22·9	24·1	24·3	15·6	—	10·2	—
	87	21·1	20·9	26·3	34·2	gel	20·1	0·3	soln.	soln.
	100	30·3	30·9	37·7	gel	soln.	31·6	0·6	soln.	soln.

moisture content determined. This was repeated at 24 hr intervals until equilibrium was attained as shown by a constant moisture content. Temperatures of 25°, 30°, 40° and 50° were used with relative humidities between 30 and 100% at each temperature.

The moisture content of the starches was determined by drying in a hot air oven at 105° for 3 hr. Samples of tragacanth, acacia, alginic acid, and the sugar samples were dried in a vacuum oven at 10 mm Hg and 70° to constant weight (approximately 2 hr).

VAPOUR-PRESSURE OF SATURATED SOLUTIONS

Apparatus. The apparatus used was essentially that of Smith & Menzies (1910) except that a larger bulb was used. Values were obtained at 25°, 30°, 40° and 50° for lactose, dextrose, sucrose and hexamine.

Results

Equilibrium moisture contents of the powders at different humidities and temperatures are given in Table 1. The results are expressed as percentage of the dry weight.

On exposing the samples to relative humidities from 32 to 67% at the different temperatures, all the powders kept their normal characteristics, showing little or no agglomeration and caking.

At a relative humidity of 75% the starches and acacia began to cake whereas the other materials remained powdery and free-flowing.

At a relative humidity of 80% potato starch caked slightly but maize and wheat starches showed no change. Acacia and tragacanth swelled and caked. The sugars remained powdery.

TABLE 2. HYGROSCOPICITY DATA OF SUGARS AND HEXAMINE

		Lactose		Dextrose		Sucrose		Hexamine	
°C	Vapour pressure of distilled water (Lange)	Vapour pressure of saturated solution mm Hg 0°C	Relative humidity of air in equilib. with saturated solution	Vapour pressure of saturated solution mm Hg 0°C	Relative humidity of air in equilib. with saturated solution	Vapour pressure of saturated solution mm Hg 0°C	Relative humidity of air in equilib. with saturated solution	Vapour pressure of saturated solution mm Hg 0°C	Relative humidity of air in equilib. with saturated solution
25	23·756	22·27	93·1	19·29	81·1	18·37	77·41	17·12	72·1
30	31·824	29·50	92·7	25·75	80·6	24·57	77·1	22·47	70·6
40	55·324	50·25	90·8	43·98	79·5	42·77	77·3	39·37	71·2
50	92·51	83·29	90·4	71·12	77·2	70·33	76·0	69·56	75·2

Between relative humidities of 86 to 89·5% the starches caked. Acacia changed from its powdery form to a thick sticky paste and tragacanth caked. Hexamine, sucrose and dextrose started to deliquesce and form solutions while lactose showed no change.

The caked starches showed mould growth on the surface of the samples within a few days at a relative humidity of 92·5%; lactose became moist and alginic acid darkened in colour.

As the absolute humidity increases with rise of temperature at a constant relative humidity, this work was carried out at approximately the same relative humidity at each temperature.

There is a linear relationship between the logarithm of the moisture content and the relative humidities for the starches and alginic acid. Tragacanth and acacia behave similarly at the lower relative humidity values but at higher values they pass into solution. The moisture sorption for most of the materials is not materially affected by temperature. Table 2 gives the hygroscopicity data for the sugars and hexamine. The relative humidity of air in equilibrium with the saturated solution of the substance was calculated since it is at this relative humidity that the substance should commence to deliquesce.

Discussion

Equilibrium was reached when the vapour pressure of water at each relative humidity and temperature equalled the equilibrium vapour pressure of the substance under the stated conditions. Air circulation accelerated the rate of moisture sorption as in the work of Yee & Davis (1944). Lactose did not form a solution even at a relative humidity of 100%; this may be because of its rate of solution or because this degree of relative humidity was not actually attained.

With rise of relative humidity the sorptive capacity of potato starch and maize and wheat starches showed a clear divergence. Potato starch was more hygroscopic than either maize or wheat starches, which had similar sorption properties. The values of equilibrium moisture content for maize starch are similar to those obtained by Hellman & Melvin (1948).

The results for the sorption of water vapour by starches are similar to those of Sair & Fetzer (1944) above relative humidities of 33%. Desorption isotherms given by these authors suggest that water is retained by starch between relative humidities of 20 and 90% in the adsorbed form only. Further evidence suggesting that starch retains water by adsorptive forces alone is found in heat of adsorption data (Winkler & Geddes, 1931).

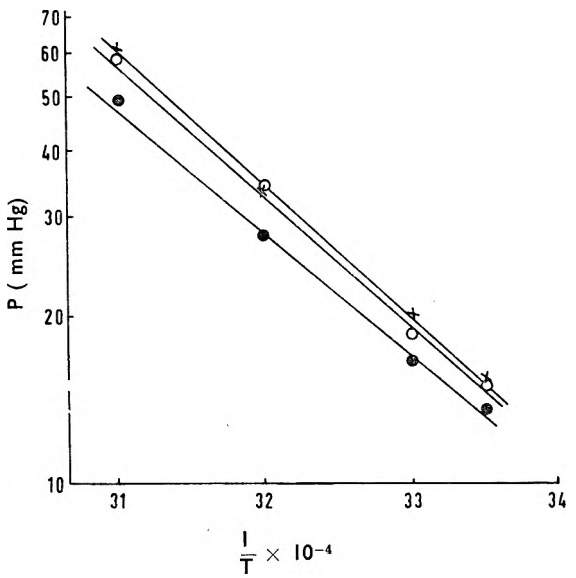

FIG. 1. Relationship between log P and 1/T for the moist powders. T is absolute temperature. P is the partial pressure of water vapour in equilibrium with the powders containing the moisture content given in Table 3. ○ Maize and wheat starch. × Potato starch. ● Tragacanth, acacia and alginic acid.

The heat of adsorption is a measure of the strength of the bond between the water molecules and the sorbent molecules. The heat sorption was calculated from plots of log P against 1/T (Fig. 1) using the Clapeyron equation, where T is the absolute temperature and P is the partial pressure of water vapour in equilibrium with the powders containing the moisture content given in Table 3. Using the data from Fig. 1, the heat of sorption of water vapour on the solid was compared with the average latent heat of vaporisation of water from 25 to 50° (Hodgman, 1962–63) (see Table 3).

The values of heat of sorption obtained are little different from the heat of vaporisation of water and this indicates a weak bond is formed which is suggestive of physical adsorption. According to the results, water is retained by the powders by simple adsorption from 32 to 100% relative

humidity. The emulsifying agents showed more avidity for moisture than the starches. This is probably related to their hydrophilic nature and to their water solubility. At relative humidities below that at which they deliquescence, acacia and tragacanth behave similarly to the starches in having values for heat of sorption characteristic of physical adsorption.

TABLE 3. HEAT OF SORPTION OF MOIST POWDER CALCULATED FROM FIG. 1

Substance	Slope from Fig. 1	Heat of vaporisation kcal/mol. H_2O	Heat of sorption kcal/mol. H_2O
Maize starch (15% moisture)	2927	10·96	0·58
Wheat starch (15% moisture)	2927	10·96	0·58
Potato starch (20% moisture)	2417	11·06	0·68
Tragacanth (15% moisture)	2420	11·07	0·695
Acacia (15% moisture)	2420	11·07	0·695
Alginic acid (15% moisture)	2420	11·07	0·695

Heat of vaporisation of water = 10·38 kcal/mol. (Hodgman).

Lactose, anhydrous dextrose, sucrose and hexamine at a relative humidity below 65% contained negligible moisture, but absorbed small amounts of moisture between 65 and 85%. Above 85% all except lactose deliquesced. Anhydrous dextrose formed the monohydrate at relative humidities of 87% at 25°, 80% at 30° and 77% at 50°. The sugars and hexamine deliquesced in atmospheres having relative humidities at which the partial pressure of water vapour was equal to the vapour pressure of their saturated solutions.

For a given relative humidity, temperature seemed to have little effect on the equilibrium moisture content for the starches and the emulsifying agents used. For dextrose, the formation of the monohydrate (approximately 10% moisture) occurred at increasingly lower humidities the higher the temperature. The deliquescence of dextrose and sucrose was affected by temperature. At 86–87% relative humidity and 25° and 30° both sugars were still powdery, but at a similar relative humidity at 40° and 50° they formed solutions.

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Pharmacological properties of 3-dimethylamino-2-methyl-1-phenyl-1-*o*-tolylpropanol (SK&F 70463-A)

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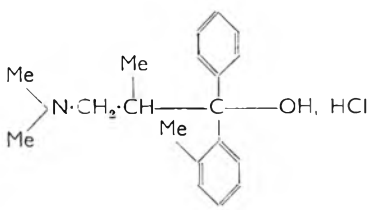
3-Dimethylamino-2-methyl-1-phenyl-1-*o*-tolylpropanol hydrochloride (SK&F 70463-A) produces both stimulant and depressant effects in laboratory animals. In particular, it possesses marked anticonvulsant activity. Diuretic effects have also been observed.

IN examining a series of diarylpropanolamines of general formula $R^1R^2{\cdot}N{\cdot}CH_2{\cdot}CHR^3{\cdot}CAr^1Ar^2OH$ which possess both diuretic and anticonvulsant activity, our interest at first centred on the diuretic properties of the compounds and various structural modifications were made to enhance this activity. However, it was found that the diuretic effects were always accompanied by effects on the central nervous system and these came to be regarded as the most interesting feature.

3-Dimethylamino-2-methyl-1-phenyl-1-*o*-tolylpropanol hydrochloride



(SK&F 70463-A)

(SK&F 70463-A) proved to be the most active compound in the series and was therefore selected for a more detailed investigation of its pharmacological properties. Its activity in various tests was compared with that of suitable reference compounds, including diphenylhydantoin, imipramine, chlorpromazine and amphetamine.

Methods

General. Male Schofield albino mice, approximately 20 g, male Wistar and Sprague Dawley albino rats, 100–160 g, cats, 2–4 kg, mongrel and beagle dogs, approximately 10 kg, and rhesus monkeys, approximately 2 kg, were used. The compounds, dissolved, or suspended in 5% acacia were administered by stomach tube at varying dose levels to groups of 20 animals, unless otherwise stated. LD50 or ED50 values and their confidence limits ($P = 0{\cdot}95$) were calculated using the method of Litchfield & Wilcoxon (1949). Dose volumes were adjusted to 2·5 ml/100 g for mice and rats, and 2 ml/kg for dogs and monkeys. Cats were given the compounds in hard gelatine capsules.

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Acute toxicity. The compounds were administered to mice and rats and the LD50 values calculated from the total mortalities occurring in the seven days after dosing.

Behavioural studies. Behavioural studies were undertaken using cats, mongrel dogs and monkeys. In these species, changes in behaviour and condition of the animals were recorded both on the day of the experiment and, if necessary, on subsequent days. One animal was used at each dose level.

Prevention of reserpine-induced ptosis. The method was based on that used by Costa, Garattini & Valzelli (1960) to demonstrate antagonism of reserpine-induced ptosis by imipramine in rats. Reserpine (1·5 mg/kg) was injected intravenously 2 hr after administration of the compounds to Wistar rats. The ED50 value was calculated from the numbers of rats without ptosis after a period of 45 min, ptosis being defined as not less than $\frac{2}{3}$ closure of the palpebral fissure persisting for more than 15 sec.

Potentiation of picrotoxin convulsions. The procedure was based on an observation by Tedeschi (personal communication) that central nervous system stimulants (e.g., dexamphetamine) and antidepressants (e.g., imipramine, tranylcypromine) potentiate a sub-threshold dose of picrotoxin to cause facial or forelimb clonus or both. Sprague-Dawley rats were injected intravenously with an aqueous solution of picrotoxin (1·35 mg/kg) 2 hr after receiving the compounds. They were then observed for 30 min and the ED50 calculated from the numbers of animals with clonus.

Stimulation of confinement motor activity. Two hr after dosing with the compounds, Wistar rats were placed in Confinement Motor Activity Units (Tedeschi, Fowler, Cromley, Pauls, Eby & Fellows, 1964) which detect minor changes in locomotor activity such as are produced by caffeine and tranylcypromine. Following an acclimatization period of 5 min, two successive 10 min activity counts were recorded. Mean activity counts for each period were calculated and the logarithms of these values plotted against log dose. The Stimulant Dose 200 (SD 200) for each period was determined graphically and is defined as that dose of a compound which increases by 200% the mean 10 min activity count of control animals tested concomitantly.

Anorexic activity. Wistar rats were trained to consume the normal daily requirement of food within 6 hr. During the training period, lasting 14 days, the animals were housed in individual cages. Food (Diet 41B cubes) was supplied in excess at 10 am each day and the surplus removed at 4 pm. Trained animals were then dosed with the test compounds or the vehicle alone and 1 hr later were presented with a weighed amount of food (approximately 5 g) for a period of 1 hr. Any food remaining was then removed, weighed and the food consumption calculated. The mean food consumption for each treated group was expressed as a percentage of that for the control group and plotted against log dose. The dose required to reduce the food consumption to 50% of the control value was then determined.

Protection against amphetamine toxicity in aggregated mice. The method was based on the studies of Lasagna & McCann (1957). Ten mice were used for each dose group and 1 hr after administration of the compounds the mice were given (±)-amphetamine sulphate (20 mg/kg) by subcutaneous injection. The animals were then placed two groups to a cage in a constant temperature cabinet maintained at approximately 27°. The number surviving in each dose group was recorded 24 hr later and the ED50 calculated.

TABLE 1. COMPARISON OF SK&F 70463-A AND VARIOUS REFERENCE DRUGS IN TESTS FOR CENTRAL NERVOUS SYSTEM ACTIVITY AFTER ORAL ADMINISTRATION

Test	Parameter	SK&F 70463-A mg/kg	Dexamphetamine sulphate mg/kg	Imipramine hydrochloride mg/kg	Chlorpromazine hydrochloride mg/kg	Diphenylhydantoin mg/kg
Acute toxicity (mice)	LD50	245 (211–284)	110 (85–143)	324 (289–363)		
Acute toxicity (rats)	LD50	185 (145–237)	210 (159–277)	490 (398–603)		
Prevention of reserpine-induced ptosis	ED50	27 (17–43)		3·6 (2·5–5·3)		
Potentiation of picrotoxin convulsions	ED50	16 (10–24)	10 (6·6–15)	34 (23–51)		
Anorexic activity	Dose giving 50% reduction in food consumption	13	2·1			
Confinement motor activity: 1st count: 2nd count:	SD200 SD200	19 13	1·8 1·9			
Protection against amphetamine toxicity (aggregated mice)	ED50	18 (14–23)		54 (35–83)	2·4 (2·0–2·8)	
Block of conditioned avoidance response	ED50	11 (8·8–14)			6·0 (4·7–7·7)	
Block of unconditioned avoidance response	ED50	16 (13–20)			23 (14–36)	
Prevention of apomorphine-induced chewing	ED50	7·5 (4·2–13)			4·7 (3·4–6·6)	
Potentiation of hexobarbitone sleeping time	Dose giving 600% increase	31·6			5·8	
Prevention of maximal electroshock seizures	ED50	2·4 (1·7–3·3)		30 (22–41)		2·4 (1·9–3·1)
Prevention of maximal leptazol seizures	ED50	3·6 (2·7–4·9)		95 (65–124)		6·6 (4·7–9·2)

Confidence limits (P = 0·95) are shown in parentheses

Blockade of a conditioned avoidance response. The method was essentially that described by Cook & Weidley (1957) using Wistar rats. At 1, 2 and 3 hr after dosing with the compounds, previously conditioned animals were subjected to the sound of the buzzer. Failure to climb the pole within 30 sec was indicative of blockade of the conditioned response. They were then subjected to the shock and buzzer together for 30 sec and failure to climb the pole under these conditions indicated that the unconditioned response was blocked. The ED50 values for blockage of both the conditioned and the unconditioned responses were calculated.

Potentiation of hexobarbitone sleeping time. Two hr after dosing with the test compounds, mice were injected intravenously with a solution of hexobarbitone sodium (40 mg/kg) in normal saline. Each animal was immediately placed on its back or side and left until it succeeded in righting itself, the sleeping time being noted. The mean sleeping time for each treatment group was expressed as a percentage increase over that of controls given the vehicle alone. These values were plotted against log dose and the dose causing a 600% increase in sleeping time was determined.

Prevention of apomorphine-induced chewing. The procedure followed was essentially that described by Janssen, Niemegeers & Jageneau (1960). Two hr after dosing with the compounds, Wistar rats were injected intravenously with apomorphine (0·25 mg/kg) and were then observed for a further 30 min. The ED50 was calculated from the numbers in which licking or chewing or both did not occur.

Prevention of apomorphine-induced vomiting. Female beagles were injected once weekly for three consecutive weeks with apomorphine hydrochloride (40 μg/kg, intravenously). This treatment produced emesis in all dogs at each trial within 2–3 min. The animals were dosed with the test compound during the fourth week, two animals being used at each dose level. The animals were fed 2 hr after dosing and apomorphine was injected 1 hr later. The production or prevention of emesis was noted.

Maximal electroshock seizure test. The prevention of maximal electroshock seizures in mice was determined by a modification of the method of Swinyard, Brown & Goodman (1952), the current strength being reduced to 25 mA. ED50 values were estimated from the numbers of mice in which tonic extension of the hind limbs was prevented at 1, 2 or 3 hr after dosing.

Maximal leptazol seizure test. The procedure was essentially as described by Goodman, Grewal, Brown & Swinyard (1953), the test compounds being administered to mice 2 hr before the intravenous injection of leptazol (60 mg/kg). ED50 values were estimated from the numbers of animals in which tonic extension of the hind limbs was prevented.

Diuretic activity. Wistar rats, 110–220 g, were uniformly distributed by weight into groups of eight. The compounds were administered as solutions, or suspensions in 5% (w/v) acacia in normal saline, control animals receiving the vehicle alone. Each group was then placed in a metabolism cage standing over a stainless steel funnel and the urine was collected for 5 hr after which urine volume and pH were recorded, and aliquots of each sample were taken for electrolyte analysis. The concentrations of sodium and potassium ions were estimated by flame photometry and of chloride ions by the iodimetric method of Sendroy (1937). The residual anion excretion was calculated by subtraction of the chloride ion excretion from the total cation excretion.

Inhibition of carbonic anhydrase. The procedure *in vitro* was that described by Maren (1960), canine erythrocytes being used as the enzyme source. *In vivo* assessment was made with mice, 20–30 g, divided into

groups of ten. One hr after dosing with the test compounds, the mice were killed by dislocating the neck, and the brains and kidneys were removed, rinsed in chilled normal saline, dried between blotting paper, and weighed. The tissues were homogenised in nine volumes of 0·25M sucrose, and the homogenates cleared by precipitation of proteins with 50% saturated ammonium sulphate. The carbonic anhydrase activity of the cleared homogenates was then determined as above, the volume of homogenate containing one unit of enzyme activity (i.e., the volume required to induce a colour change in the phenol red indicator in half the time taken for a boiled aliquot of the homogenate to produce a similar colour change) being obtained. The number of units per gram of tissue was calculated.

Results

Acute toxicity. The acute toxicities of SK&F 70463-A, imipramine hydrochloride and dexamphetamine sulphate in the mouse and rat are shown in Table 1.

Behavioural studies. After the administration of SK&F 70463-A to cats, dogs and monkeys, the first effects were stimulation of the central nervous system, typified by tremors and convulsions. These were followed by prostration at higher doses, and several of the animals remained in a severely depressed state for several days. Complete recovery was observed after a dose of 25 mg/kg in cats, 10 mg/kg in dogs, and 40 mg/kg in monkeys.

Prevention of reserpine-induced ptosis. SK&F 70463-A has approximately one-seventh of the activity of imipramine hydrochloride (see Table 1).

Potentiation of picrotoxin convulsions. The activity of SK&F 70463-A in this test, as shown in Table 1, is approximately twice that of imipramine hydrochloride and two-thirds that of dexamphetamine sulphate.

Stimulation of confinement motor activity. SK&F 70463-A produces stimulation of confinement motor activity but is less active than dexamphetamine sulphate (see Table 1). It appeared to have a greater effect on activity during the second period of observation than during the first. Tedeschi & others (1964) have reported a similar finding with amphetamine but this was not confirmed by us.

Anorexic activity. SK&F 70463-A has approximately one-sixth of the activity of dexamphetamine sulphate (Table 1). It appears that the ratios of the doses effective in the anorexic and confinement motor activity tests are approximately the same for the two compounds, suggesting possibly a similar mechanism of action. This is supported by parallelism of the dose-response curves.

Protection against amphetamine toxicity in aggregated mice. The activity of SK&F 70463-A in this test has been compared with that of the major tranquilliser, chlorpromazine hydrochloride, and the antidepressant, imipramine hydrochloride. The results in Table 1 indicate that it possesses an intermediate degree of activity, being one-eighth as active as chlorpromazine but three times as active as imipramine.

Blockade of a conditioned avoidance response. Although SK&F 70463-A is capable of blocking a conditioned avoidance response and is approximately one-half as active as chlorpromazine hydrochloride (see Table 1), this appears to be a relatively non-specific effect. Not only was the conditioned response blocked but so also was the unconditioned response at a dose level only marginally greater. This is in contrast to the results with chlorpromazine, with which there was an approximately fourfold difference in doses in this case. It was evident that the animals given SK&F 70463-A were markedly excited and inability to climb the pole would seem to have been a measure of a state of agitation rather than of tranquillisation.

TABLE 2. EFFECT OF SK&F 70463-A, CHLOROTHIAZIDE AND ACETAZOLAMIDE ON WATER AND ELECTROLYTE EXCRETION FOLLOWING ORAL ADMINISTRATION TO SALINE-LOADED RATS

Compound	Dose mg/kg	No. of rats	Mean body weight (g) and range	Urinary pH	Water excretion (ml/kg/5 hr)	Electrolyte excretion (m-equiv./kg/5 hr)				
						Na	K	Cl	HCO_3	Na/K
—	—	24	162·8 (115–211)	6·05 5·75 5·70	8·42	1·77	0·81	2·25	0·33	2·20
Chlorothiazide	5	24	163·4 (122–218)	6·00 5·85 5·70	15·30	3·05	1·03	3·81	0·27	2·95
	15	24	165·0 (125–217)	6·10 5·65 5·80	20·70	4·10	1·25	5·41	0·07	3·29
Acetazolamide	1	24	162·8 (115–209)	7·00 6·90 6·70	12·78	2·46	1·05	2·56	0·55	2·35
	3	24	164·1 (119–214)	7·65 7·50 7·45	16·37	3·43	1·28	3·40	1·31	2·69
SK&F 70463-A	10	24	164·5 (114–210)	6·50 6·00 6·25	12·16	2·46	1·08	2·95	0·89	2·28
	20	24	163·4 (119–215)	6·90 6·40 7·20	19·63	2·89	0·98	3·09	0·78	2·93

Potentiation of hexobarbitone sleeping time. SK&F 70463-A is approximately one-fifth as active as chlorpromazine hydrochloride (see Table 1).

Prevention of apomorphine-induced chewing. The activity of SK&F 70463-A is approximately two-thirds that of chlorpromazine hydrochloride (see Table 1).

Prevention of apomorphine-induced vomiting. No activity against apomorphine-induced emesis in dogs could be demonstrated with SK&F 70463-A at doses up to 10 mg/kg. Higher doses were not examined because of the likelihood of clonic convulsions and prostration. This result is of particular interest since Janssen & others (1960) reported that all compounds which they had found to be active in preventing apomorphine-induced chewing in the rat also prevented the emetic effect of apomorphine in the dog.

Maximal electroshock seizure test. SK&F 70463-A was found to be equal in activity to diphenylhydantoin and twelve times as active as imipramine hydrochloride (see Table 1).

Maximal leptazol seizure test. SK&F 70463-A is slightly less effective against leptazol than against electroshock seizures (see Table 1), but it is almost twice as active as diphenylhydantoin and more than twenty-five times as active as imipramine hydrochloride.

Diuretic activity. The results of three experiments comparing SK&F 70463-A with chlorothiazide and acetazolamide have been combined and are summarised in Table 2. The slope of the log dose-urinary volume response curve for SK&F 70463-A differs from those of the reference compounds, so that an estimation of the relative potencies is not possible. Parallel log dose-response curves are, however, obtained with sodium output and it appears that SK&F 70463-A has one-thirteenth of the natriuretic activity of acetazolamide and one-fifth that of chlorothiazide. Qualitatively, whereas chlorothiazide increased the urinary excretion of water, sodium, potassium and chloride, SK&F 70463-A and acetazolamide also increased excretion of "residual anion" (presumably bicarbonate). SK&F 70463-A had the least effect on potassium excretion and the loss was less at the higher dose than at the lower.

TABLE 3. EFFECT OF SK&F 70463-A AND ACETAZOLAMIDE ON CARBONIC ANHYDRASE ACTIVITY *IN VITRO*

	Concentration (μM) of added compound	Time (sec) for colour change
Enzyme preparation alone	—	10
Enzyme preparation .. .. .. plus acetazolamide	6·5 650	39 40
Enzyme preparation .. .. .. plus SK&F 70463-A	4·0 400	11 16
Boiled enzyme preparation alone ..	—	40

Inhibition of carbonic anhydrase: in vitro. Table 3 records the time of passage of a constant flow of carbon dioxide gas until the indicator changed colour. Whilst acetazolamide completely abolished enzyme activity at a concentration of 6·5 μM, the time being increased to that observed when the enzyme preparation was inactivated by boiling, SK&F 70463-A was without effect at concentrations up to 400 μM.

TABLE 4. EFFECT OF SK&F 70463-A AND ACETAZOLAMIDE ON THE CARBONIC ANHYDRASE ACTIVITY OF MOUSE KIDNEY AND BRAIN

Drug	Dose (mg/kg) orally	No. of units of enzyme activity per g tissue	
		Kidney	Brain
—	—	420	480
Acetazolamide ..	30	40	80
SK&F 70463-A ..	30	350	360

In vivo. The carbonic anhydrase activity of tissues obtained from mice treated with acetazolamide or SKF 70463-A is presented in Table 4. Acetazolamide (30 mg/kg) caused a marked reduction in activity whereas a similar dose of SK&F 70463-A had relatively little inhibitory effect on the carbonic anhydrase of either kidney or brain.

Discussion

It is apparent that SK&F 70463-A produces a mixture of stimulant and depressant effects on the central nervous system. The results of the anti-reserpine, picrotoxin potentiation, confinement motor activity and anorexic tests, together with the gross observations made in the behavioural studies, indicate that the compound has CNS stimulant effects which appear to resemble those of amphetamine. However, the duration of hexobarbitone anaesthesia in mice was prolonged. A number of other tests for CNS depressant effects (anti-amphetamine, conditioned avoidance response, anti-apomorphine, maximal electroshock seizure, maximal leptazol seizure) also gave positive results, and it becomes evident, therefore, that the compound has a spectrum of pharmacological activity which, resembles to some extent that of imipramine hydrochloride.

In the diuretic studies, the increased pH of the urine, together with the increase in "residual anion" excretion suggested that SK&F 70463-A might act by inhibition of carbonic anhydrase. The carbonic anhydrase inhibitor acetazolamide has been used successfully in the treatment of epilepsy (Goodman & Gilman, 1954) and there is evidence (Millichap & Woodbury, 1954) that its anticonvulsant activity is a direct result of the inhibition of brain carbonic anhydrase. Since SK&F 70463-A also possesses marked anticonvulsant activity, the possibility existed that both its diuretic and anticonvulsant properties could likewise be due to carbonic anhydrase inhibition. Carbonic anhydrase studies *in vitro* and *in vivo* fail to support this hypothesis.

The main clinical effects reported were ataxia and drowsiness and when stimulant activity was observed it closely resembled that due to amphetamine but the incidence of side-effects was so high that investigations of the potential anticonvulsant actions of SK&F 70463-A were not made.

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Letters to the Editor

Traumatic and endotoxin shock in rats

SIR,—Previous investigations have shown that an increase in the generalised capillary permeability plays an important role in the pathogenesis of traumatic shock. Histamine production may be enhanced by activating the enzyme, L-histidine decarboxylase (Karady, Gecse & Horpacsy, 1962) and a fibrin film may develop on the interior surface of some of the capillaries (Jancsó, 1961), with the accompanying loss of fibrinogen. The administration of a substance Phlogodym, which decreases both fibrinogenesis and capillary permeability, was therefore tested in traumatic shock in rats. As endotoxins are considered to play an important role in the pathogenesis of traumatic shock (Fine, Frank, Schweinburg, Jacob & Gordon, 1952), a study has also been made of the effect of Phlogodym on endotoxin shock induced in rats. Phlogodym is a complex of pyrocatechin disulphonic acid and neodymium (Jancsó, 1961).

Traumatic shock. Rats weighing 180–250 g and of both sexes were used. Traumatic shock was elicited using the Noble-Collip technique (rotating drum method, 15 min at 40 rotations/min). Blood from the tail vein was collected and its fibrinogen concentration was determined using Schneider's method (1952). The extent of fibrinolysis in the blood was measured using the method of Burdon, McGovern, Barkin & Meyers (1961) and fibrinogen B was estimated by Cummine & Lyons' method (1948). Phlogodym (50 mg/kg) was injected 30 min before the traumatic shock, whereas heparin (200 units/kg) was given 15 min before and 15 min after the shock. ϵ-Aminocaproic acid was used in an intravenous dose of 1,000 mg/kg. The activity of endothelial system was reduced for 12 hr in a few experiments using saccharated iron oxide (2 g/kg intravenously).

There was a moderate decrease (50%) in the fibrinogen levels after traumatic shock. When Phlogodym was used before the shock, the fibrinogen level decreased almost to zero and the mortality rate increased sharply. The disappearance of the fibrinogen from the blood was confirmed by electrophoretic and immunological studies. The administration of ϵ-aminocaproic acid (which inhibits fibrinolysis) did not prevent the disappearance of the fibrinogen, but heparin (which enhances fibrinolysis) inhibited the reaction. This antagonism of Phlogodym by heparin was the result of the heparin removing fibrinogen B formed by Phlogodym, thereby inhibiting intravascular fibrin formation. When the endothelial system was blocked, Phlogodym was much more effective in potentiating the mortality rate after trauma.

Endotoxin shock. Groups of 10 albino rats (body weight about 200 g) were injected intravenously with 200 μg typhoid endotoxin after the animals had received either Phlogodym (50 mg/kg) or neodymium nitrate (100 mg/kg) intravenously 30 min previously. The results showed that both substances aggravated the endotoxin shock. For example, the mortality rate recorded over 24 hr rose from 20 to 80% with Phlogodym and to 90% with neodymium. On the other hand, in rats receiving the intravenous dose of endotoxin every second day in increasing amounts from 50 to 200 μg so as to achieve endotoxin resistance, the mortality rate only increased from 0% to 11% with Phlogodym.

The fibrinogen levels measured by Schneider's method (1952) were significantly decreased with Phlogodym and with neodymium but only about 4 hr after

the endotoxin. For example, the decrease with endotoxin alone was 50% but with endotoxin and each of the two agents the values were reduced almost to zero (minimum reduction was 90%). In rats made resistant to endotoxin, there was only a 50% decrease in the fibrinogen level using Phlogodym.

Thus, Phlogodym renders rats more sensitive to traumatic shock. The blood fibrinogen level falls dramatically, probably due to the promotion of intra-capillary thrombi formation. As Phlogodym is an anticoagulant and an agent decreasing capillary permeability other factors are also involved.

As endotoxin enhances coagulation and reduces the fibrinogen (McKay, Shapiro & Shanberge, 1958), Phlogodym may exert a similar effect in both traumatic and endotoxin shock, thereby aggravating both the reactions.

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Some effects of propranolol on the central nervous system

SIR,—Propranolol, 1-isopropylamino-3-(1-naphthyloxy)-2-propanol, has been described by Black, Crowther, Shanks, Smith & Dornhurst (1964) as a potent β-adrenergic blocking agent. We wish to report the results of some experiments which show that propranolol has sedative and anti-convulsant properties (Table 1).

TABLE 1. DOSES AND ROUTES OF ADMINISTRATION FOR EACH OF THE EFFECTS MEASURED. THE VALUES QUOTED ARE THE MEANS AND 95% CONFIDENCE LIMITS FROM GROUPS OF 10 ANIMALS

Test	Species	Route	Dose mg/kg
Toxicity	mouse	i.v.	LD50 = 38·0 (26·0–55·5)*
	,,	s.c.	LD50 = 244 (224–289)*
Potentiation of narcosis	,,	,,	ED_{min} = 10**
,, ,,	rat	,,	ED_{min} = 25†
Ataxia	mouse	,,	ED50 = 17·4 (12·9–23·3)*
Muscular hypotonus	,,	,,	ED_{min} = 20‡
Partial blocking of electroshock	,,	,,	ED50 = 6·0 (4·55–7·93)*
,, ,, ,,	rat	,,	ED50 = 4·9 (3·20–8·96)*
Complete inhibition of electroshock	mouse	,,	ED50 = 14·4 (11·8–17·6)*
Antagonism of nicotine toxicity	,,	,,	ED50 = 10·0 (6·72–15·1)*
Antagonism of strychnine toxicity	,,	,,	ED50 = 38·5 (22·8–65·0)*
Antagonism of amphetamine grouped toxicity	,,	,,	ED_{min} = 5

*Results calculated from experimental data by the method of Litchfield & Wilcoxon (1949); **$P < 0.02$; †$P < 0.001$; ‡$P < 0.05$.

Narcosis was induced in mice by propranolol in a dose of 100 mg/kg, or more, administered subcutaneously. The duration of sleeping time in rats or

mice after intravenous hexobarbitone was measured with or without pretreatment with propranolol. The sleeping times of 180 ± 58 sec in mice induced by 40 mg/kg hexobarbitone sodium and 393 ± 219 sec in rats due to 30 mg/kg of the same hypnotic were significantly prolonged (Student's "*t*" test) by propranolol given subcutaneously 30 min before the barbiturate.

Ataxia was measured by the inability of mice to remain on a rotating rod (9 revolutions/min) for a 2 min test period. The maximum effect was reached 30 min after the subcutaneous injection of propranolol and this effect began to decrease after 60 min. These findings may be related to the muscular hypotonia measured by Fleury's technique (1957). 30 min after the subcutaneous injection of 20 mg/kg propranolol, the maximum weight that mice were able to support was reduced from the control level of 52 g to 38 g (a 27% reduction; $P < 0.05$). This effect also diminished with time.

Anticonvulsant properties were investigated by observing the effects of propranolol on electroshock induced through bitemporal electrodes in mice and rats by impulses of 0·2 sec 2–4 mA and 1·0 sec 10–12 mA respectively. The inhibition of tonic extensor seizures of the hind legs was regarded as partial blocking of seizures and the abolition of clonic seizures of all four extremities was the criterion for complete inhibition of electroshock.

Lethal doses (LD100) of nicotine bitartrate (8 mg/kg) and strychnine nitrate (0·6 mg/kg) were injected intravenously into mice. Propranolol was given subcutaneously 30 min before the intravenous toxicity test and survival was considered to be a sign of a protective action. Smaller doses of propranolol were necessary for antagonism of the lethal action of nicotine than for the antagonism of strychnine. Convulsions due to leptazol were not influenced even by 75 mg/kg of subcutaneously injected propranolol.

Antagonism to amphetamine toxicity was studied by the method of Burn & Hobbs (1957) on mice kept in groups of ten, or caged individually. Grouped and individually-caged mice were given (±)-amphetamine phosphate subcutaneously in doses of 100 mg/kg and 200 mg/kg respectively. The mortality rate was assessed 24 hr later. 5 mg/kg propranolol subcutaneously reduced the mortality in grouped mice from the 80% control level to 40%; a 10 mg/kg dose effected a further reduction to 10%. In individually-caged mice the same doses of propranolol effected similar percentage reductions in the mortality due to amphetamine.

These experiments indicate that propranolol has central nervous system depressant and anticonvulsive properties. This is interesting because dichloroisoprenaline, which shows some similarity with propranolol in the chemical structure of its side chain, causes excitement and has no anticonvulsant action. This change in the biological effect may be attributed to the presence of a naphthyl group in the molecule of propranolol. Our experiments give no answer to the question of whether there is any connection between the β-adrenergic blockade and the observed central nervous effects. Antagonism to amphetamine toxicity might be the test in which the adrenergic blocking action of the compound is asserted. Nevertheless, considering the overall effects, we believe that the drug has two independent actions, but further experiments are required to clarify this problem.

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Examination of cinnamon by direct thin-layer chromatography

SIR,—Cassia bark (*Cinnamomum cassia*) is the commonest substitute for cinnamon (*C. zeylanicum*), and whilst it should be possible to distinguish one from the other by macroscopical and microscopical means, analysts have expressed a need for aids to their identification. Dutta (1961) suggested mucilage ash values. It seems logical to attempt to make a distinction by examining the active principles, the essential oils. For this purpose thin-layer chromatography may be used as it has previously been applied to umbelliferous fruits (Betts, 1964). The oils of both barks contain cinnamaldehyde as the principal constituent, but cinnamon oil, unlike cassia oil, contains eugenol. Both of these constituents are not resolved on the usual silicic acid thin-layer plates, and eugenol is not easily detected in the presence of cinnamaldehyde by the spot detection methods previously described.

The modification to the previous technique (Betts, 1964) are as follows: magnesium silicate (TLC, Woelm), after mixing with absolute ethanol (12 g to 50 ml) was spread on plates and left to dry in air. Bark, 500 mg, was extracted with 2·5 ml acetone and spotted on the plates. After the solvent run eugenol was detected as a slate-blue spot, Rf approximately 0·45, by spraying with Folin & Ciocalteu's reagent (Hopkin & Williams Ltd.), as recommended for phenols by Waldi (1965). This was followed by spraying with dinitrophenylhydrazine solution to reveal cinnamaldehyde, Rf approximately 0·55. Folin & Ciocalteu's reagent provides a more sensitive test for eugenol than previous techniques and reveals the presence of eugenol in whole or powdered cinnamon.

An examination was made of various commercial (but not official) specimens of "cinnamon". Three samples of whole bark were obviously not cinnamon B.P. as they consisted of thick pieces, with much cork remaining, in single, double and a few compound quills. Microscopically they corresponded to a specimen of *C. burmanni* bark, kindly supplied by the Museum of the Pharmaceutical Society. This is an adulterant mentioned in the B.P.C. The diameter of the fibres was less than 30 μ, corresponding to true cinnamon, but calcium oxalate was present as small prisms instead of the normal acicular crystals. Chromatographically the samples were devoid of eugenol but contained cinnamaldehyde as did the Museum specimen. Museum samples of true cinnamon readily yielded a eugenol spot.

Seven commercial samples of powdered "cinnamon" were also examined. All the samples when chromatographed showed a cinnamaldehyde spot, but three were devoid of eugenol. These all contained fibres of diameter greater than 40 μ, and were probably samples of *C. cassia*. The large fibres were present in samples containing eugenol. Eugenol was also observed in a specimen which contained small calcium oxalate prisms. The cost of the samples was no guide to their quality. Only one sample appeared to be cinnamon B.P.

Of some Museum specimens of other *Cinnamomum* spp. barks examined chromatographically, *C. loureirii* (another adulterant mentioned in the B.P.C.) contained cinnamaldehyde without eugenol. *C. pedatinervium* and *C. sintok* contained eugenol without cinnamaldehyde, the former source being the

richest. These two eugenol-containing barks both contained acicular calcium oxalate crystals, but their fibres were greater than 40 μ in diameter. Hoppe (1958) gives eugenol as the main constituent of the oil of *C. culilawan*, but the specimen I examined yielded neither eugenol nor cinnamaldehyde.

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Effects of the amphetamine group on intraneuronal brain amines *in vivo* and *in vitro*

SIR,—Previous studies have shown that (+)-amphetamine in large doses causes a decrease in the brain content of noradrenaline (Smith, 1965). The present investigation was made to study the action of (+)-amphetamine at the cellular level with the help of the histochemical fluorescence method of Hillarp & others. The existence of central dopamine, noradrenaline and 5-hydroxytryptamine (5-HT) neurones has recently been demonstrated by this technique. These neurones have been shown to contain specific mechanisms for uptake and storage of the amines. They have an uptake mechanism, probably localized at the level of the cell membrane and sensitive to, for example, desipramine or cocaine (see review by Hillarp, Fuxe & Dahlström, 1965). Furthermore, they possess a reserpine-sensitive storage mechanism localised in specific granules.

Single injections (i.p.) of (+)-amphetamine (5–60 mg/kg), (±)-amphetamine (15–60 mg/kg), methamphetamine (30 mg/kg) and benzylamphetamine (30 mg/kg) have been given to male, albino rats (Sprague-Dawley, 200–300 g). The animals were killed at 1, 2 or 3 hr after the injection. Pieces from all parts of the brain were dissected, freeze-dried and treated with formaldehyde gas (Dahlström & Fuxe, 1964). Fluorescence microscopic examination showed that (+)-amphetamine, 15–60 mg/kg, caused a fairly marked to marked decrease in number and intensity of the very fine catecholamine (mainly noradrenaline) terminals in, for example, the neocortex, the gyrus cinguli and the formatio reticularis of the lower brain stem. The fine to fairly thick catecholamine terminals of the hypothalamus, on the other hand, remained unaffected even with the higher doses. The dopamine and 5-HT terminals exhibited a normal appearance after all doses except with 60 mg/kg. After this dose, the dopamine terminals showed a distinct decrease in their amine contents. Somewhat less marked changes occurred after (±)-amphetamine and methamphetamine, while benzylamphetamine did not cause any definite decrease in the intraneuronal amine levels of the noradrenaline terminals.

These results are supported by *in vitro* studies on the central monoamine neurone with brain slices (for technical details, see Hamberger & Masuoka, 1965). Noradrenaline terminals in brain slices from neocortex after preincubation with noradrenaline, 10 μg/ml, and rinsing were markedly decreased in

number and intensity with a concentration of 1 μg (+)-amphetamine/ml in the incubation medium, while the noradrenaline terminals in hypothalamic brain slices were not significantly affected even with a concentration of 100 μg/ml. The dopamine terminals in the nucleus putamen were found to be intermediate in sensitivity, showing a distinct decrease of endogenous amine with a concentration of 10μg/ml.

Biochemical determination of noradrenaline and dopamine (Bertler, Carlsson & Rosengren, 1958; Carlsson & Waldeck, 1958; Carlsson & Lindqvist, 1962) in whole brain after treatment with large doses of amphetamine (15–30 mg/kg, 1 and 2 hr before killing) demonstrated a distinct decrease in the brain content of noradrenaline (down to 50% of the normal value) but no certain changes of the dopamine levels.

TABLE 1. EFFECT OF (+)-AMPHETAMINE ON L-DOPA INDUCED MONOAMINE ACCUMULATION IN RAT BRAIN AND HEART

Drugs mg/kg			Heart		Brain		
Nialamide i.p.	(+)-Amphetamine i.p.	Dopa s.c.	Noradrenaline	Dopamine	Noradrenaline	Dopamine	3-Methoxytyramine
150	15	50	0·05	6·21	0·06	2·31	3·02
150	0	50	0·13	7·44	0·21	4·70	2·22
100	5	25	0·03	1·46	0·04	0·65	1·49
100	0	25	0·11	1·74	0·14	1·10	0·94
100	5	25	0·04	1·48	0·05	0·87	1·38
100	0	25	0·10	2·14	0·15	1·08	0·79

(+)-Amphetamine was given 45 min, dopa 30 min before killing. The rats were pretreated with reserpine (10 mg/kg i.p.) and nialamide (in doses indicated) 22 and 4 hr before death, respectively. Control rats were treated in the same way as the experimental animals except that no (+)-amphetamine was given. The values are single values of 3 pooled organs expressed in μg/g tissue.

To study the effect of the (+)amphetamine on the so called "membrane pump" another kind of experiment was made. Rats of the same stock and the same techniques were used. (+)-Amphetamine, 5–20 mg/kg i.p., was administered to animals pretreated with reserpine (10 mg/kg i.p., 24 hr before killing) and nialamide (100 mg/kg, i.p., 4–6 hr before killing), 15 min before a dose of 3,4-dihydroxyphenylalanine (L-dopa) (50 mg/kg s.c., 30 min before killing). Brains of animals treated only with reserpine - nialamide - L-dopa showed in the fluorescence microscope large amounts of strongly green fluorescent dopamine and noradrenaline terminals in most parts, due in all probability to an uptake and decarboxylation of L-dopa (*cf.* Fuxe, 1965). The amines formed are, however, not bound to the granules, since these have been blocked by reserpine. If (+)-amphetamine is administered in doses down to 5 mg/kg, and possibly lower, before the L-dopa injection, the fluorescence microscopic picture is found to be quite different. The noradrenaline and dopamine terminals become only weakly fluorescent after the L-dopa injection or remain non-fluorescent. Thus, considerably less amounts of catecholamines are detected in the terminals. Biochemical determinations showed (Table 1) that the noradrenaline and dopamine levels in the brain were considerably reduced and that the levels of 3-methoxytyramine (Carlsson & Lindqvist, 1963; Carlson & Waldeck, 1964) were increased after reserpine-nialamide-(+)-amphetamine-L-dopa treatment compared to the levels obtained after reserpine-nialamide-L-dopa treatment. The noradrenaline levels in the heart were also reduced, while no consistent effect on the dopamine levels was observed.

These results support the view that amphetamine at least partly acts by blocking the membrane pump of both the noradrenaline and dopamine neurones. This conclusion is also supported by the results from *in vitro* studies on brain

slices from neocortex and brain stem of reserpinized animals, which showed a marked to complete inhibition of uptake of α-methyl noradrenaline (1 μg/ml) in the catecholamine terminals if amphetamine was present in the bath. The concentration of amphetamine ranged from 100 μg/ml down to 1 μg/ml.

Studies on peripheral adrenergic terminals in slices of vas deferens from reserpinized animals show that the uptake of α-methylnoradrenaline (1 μg/ml) is markedly inhibited also after incubation with (+)-amphetamine (1 μg/ml). These results agree with previous studies on the effect of (+)-amphetamine on the peripheral adrenergic nerve terminals (Carlsson & Waldeck, 1965; Malmfors, 1965), demonstrating a block of the membrane pump after treatment with (+)amphetamine. Thus, no fundamental differences seem to exist between the peripheral and central catecholamine neurones in their reaction to amphetamine.

The present investigation indicates that at least one of the sites of action of amphetamine is on the membrane pump. The experiments also suggest an effect of large doses of the drug directly on the granules. This assumption is based on the fact in contrast to (+)-amphetamine the potent membrane blocker desipramine has never been observed to decrease the noradrenaline levels of the brain, not even with very high doses. The present experiments also demonstrate differences in sensitivity towards amphetamine between the various noradrenaline terminal systems of the brain, which cannot be accounted for by differences in impulse flow, since these differences were present also *in vitro*. There remains to be explained, however, why the pharmacological effects of amphetamine occur after reserpine treatment (Rech, 1964; Smith, 1965). This need not be due to a direct stimulatory effect on the receptor site since the synthesis of amines continues in spite of reserpine treatment and, thus, (+)-amphetamine by means of its action on the membrane pump or some other unknown actions may release sufficient quantities of amines to act on the postsynaptic membrane.

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An improvement on Vane's stomach strip preparation for the assay of 5-hydroxytryptamine

SIR,—Vane (1957) described a practical and sensitive preparation for the assay of 5-hydroxytryptamine (5-HT) using a strip of fundus from the rat stomach. Since then, this preparation has been widely used by others. Two difficulties were encountered in practical application: the slowness of the muscle to relax after responding to 5-HT and the fluctuation in the resting length of the muscle. These were overcome by stretching the muscle for 15 sec after each contraction. A working cycle required at least 4 min with this procedure.

Because of a need to assay over 30 samples of intestinal perfusate for 5-HT at one time, we have modified Vane's preparation to obviate the need for stretching the muscle after each contraction and also to shorten the time cycle.

The modifications we have made are:

(1) The manner of cutting the stomach strip was slightly different, five instead of six incisions being made, three from the fundus end of the opened-out plate of tissue alternating with two incisions made from the pyloric end of the plate.

(2) An auxotonic frontal writing pendulum lever was used. The small counterweight can be varied to give the required tension to the strip. The baseline position of the lever was set at about 10° below the horizontal and had a load of 2·5 g at this position.

(3) On setting up the preparation, it was left to stretch in the organ bath for 2 hr with the physiological solution flowing through at a rate of about 30 drops/min.

(4) A magnesium-free Krebs solution was used: (g/litre) NaCl, 6·92; KCl, 0·353; $CaCl_2$, 0·282; KH_2PO_4, 0·161; $NaHCO_3$, 2·1; glucose, 2·0. The solution was oxygenated with a 3% carbon dioxide and 97% oxygen mixture.

(5) A temperature of 39·5° for the organ bath was used. At this temperature the muscle responded and relaxed more rapidly than at 37°.

Under these conditions the sensitivity of response of the muscle to 5-HT was adequate, a 1 ng dose (in 6·5 ml bath volume) normally causing a recorded

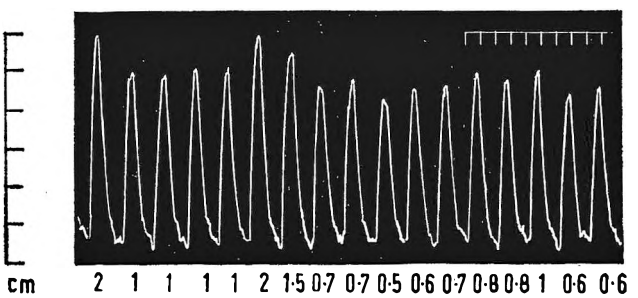

FIG. 1. Record of response of the stomach strip to various doses of 5-HT in ng added to the organ bath (volume 6·5 ml). Time in min. Scale in cm.

contraction of 3 cm. The muscle responded within 10 to 15 sec of adding the 5-HT to the bath and reached maximum contraction in 45 sec; relaxation to the baseline occurred 1 min after 5-HT was washed out; another dose of 5-HT could be added after 2 min. The preparation attained maximum sensitivity after 10 to 12 additions of 5-HT and its response remained consistent for at least 3 hr. Methysergide (Sandoz) in a concentration of 10^{-7} g/ml completely abolished the response to 10^{-9} g/ml of 5-HT.

Doses and concentrations are in terms of base.

A tracing showing graded response by the rat stomach strip preparation with the above modifications to various doses of 5-HT is shown in Fig. 1. We have not been able to determine precisely the cause for this improvement. However, we would like to share this experience with others who may wish to make use of this preparation for the rapid assay of 5-HT.

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Autoxidation of linoleic acid

SIR,—Mehta (1962) has earlier reported on a possible mechanism for the "autoxidation of linoleic rich oils in emulsion". In that report the emulsions studied contained poppy seed oil, safflower oil, and methyl linoleate ester, and were prepared using acacia and tragacanth as emulsifying agents. The mechanism indicated that in the early stages of autoxidation oxygen appeared to add to the double bond to form cyclic peroxides, which were then converted to conjugated dienoic hydroperoxides. The amount of conjugated trienes was insignificant in the oils and ester from which the emulsions were prepared and in all the systems after preparation. The trienes did not develop to any significant extent even after 42 days of autoxidation. The samples were stored at $25° \pm 2$ in ground glass stoppered bottles. Conjugated dienes and trienes were estimated before and after isomerisation by the method of Hilditch (1951).

We have now examined pure linoleic acid*. The surfactant, Brij 35†, 5 g, was used to obtain a solubilised and an emulsion system, containing linoleic acid 1·07 g and 1·33 g respectively, with distilled water to 25 ml. Samples were stored as before.

In both systems, the amount of conjugated dienes reached a maximum value after about 10 days and then the dienes were further oxidised. E (1%, 1 cm) at 268 mμ for the unisomerised sample (corresponding to the conjugated trienes) was 6·16 for the linoleic acid. The occurrence of trienes corresponded to the disappearance of the dienes. After 20 days of autoxidation, E (1%, 1 cm) at 268 mμ was 17·15 and 14·92 for the emulsion and the solubilised system respectively. The formation of significant amounts of trienes was thus indicated. The 30 day values indicated that the trienes were further autoxidised.

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† Brij 35, Atlas Chemical Industries, Inc., Wilmington, Delaware, U.S.A.

Inhibition of dopamine-β-oxidase by diethyldithiocarbamate

SIR,—Hydroxylation of dopamine to noradrenaline by dopamine-β-oxidase has been postulated by Goldstein & Contrera (1961) as the rate-limiting step in the biosynthesis of noradrenaline. If this hypothesis is correct, then the tissue levels of noradrenaline should decrease when this enzyme is inhibited. However, Nikodijevic, Creveling & Udenfriend (1963) using benzyloxyamine and benzylhydrazine analogues were unable to obtain significant decreases in the noradrenaline contents of guinea-pig tissues. More recently, Goldstein, Anagoste, Lauber & McKereghan (1964) found that diethyldithiocarbamate, a metabolite of disulphiram, was a potent inhibitor of dopamine-β-oxidase. In the present work we have shown that diethyldithiocarbamate markedly reduced the noradrenaline levels and simultaneously increased those of dopamine in the small intestine of both the rat and rabbit.

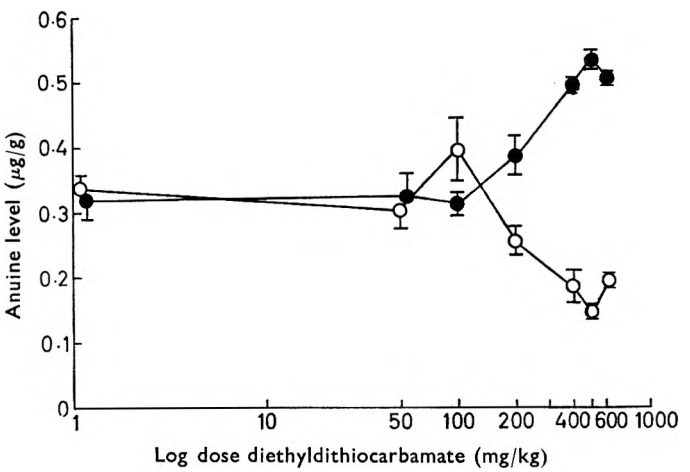

FIG. 1. The effect of different doses of the sodium salt of diethyldithiocarbamate (mg/kg), subcutaneously) on the noradrenaline (○——○) and dopamine (●——●) contents (μg/g ± s.e.) of rat ileum. Each point is the mean of 5 determinations.

The *in vivo* experiments were made by injecting groups of five rats subcutaneously with varying doses of the sodium salt of diethyldithiocarbamate and killing them at hourly intervals over 6 hr. Pieces of ileum were removed, washed, dried, weighed and stored at −4° until assayed for catecholamines. In other experiments, segments of cleaned ileum from untreated rats and rabbits were incubated at 33·5° in McEwen's Ringer solution containing the sodium salt of diethyldithiocarbamate (2×10^{-5}) and aerated with 95% oxygen and 5% carbon dioxide. Segments were removed at different times and treated as described above. The catecholamines (noradrenaline, adrenaline and dopamine) were extracted from tissues by the method used by Shore & Olin (1958). The

content of noradrenaline and adrenaline was estimated as noradrenaline, whereas the dopamine in the final acid extract was estimated by the method of Carlsson & Waldeck (1958, 1959).

The noradrenaline and dopamine content of ileum (μg/g $\pm$ s.e.) of uninjected rats was 0·381 $\pm$ 0·01 (24 animals) and 0·388 $\pm$ 0·011 (30 animals) and of rabbits was 0·357 $\pm$ 0·022 (9 animals), 0·237 $\pm$ 0·029 (7 animals) respectively. The minimal dose of diethyldithiocarbamate required to produce a significant fall in noradrenaline levels in rat ileum was 200 mg/kg, the maximal decrease being at 500 mg/kg when the level was only 43·7% of the control value (see Fig. 1). The corresponding maximal increase in dopamine level also occurred at 500 mg/kg, the value then being 161·3% of the control value.

Incubation for 6 hr resulted in a maximal decrease in the noradrenaline level to 36·7% of the control values in the rat ileum and 45·6% in the rabbit ileum. The corresponding maximal increases in the dopamine levels were 175·6 and 219·1% respectively.

Thus, diethyldithiocarbamate produces significant decreases in noradrenaline levels. It is unlikely that this is due to the release of noradrenaline; firstly, because Goldstein & others (1964) and Musacchio, Kopin & Snyder (1964) showed that the binding and uptake of tritiated noradrenaline was unaffected by disulphiram; secondly, the loss of noradrenaline in the present experiments was accompanied by an increase in dopamine levels; and, thirdly, the mean rate of loss of noradrenaline was estimated as 0·0221 $\pm$ 0·0009 μg/g/hr which was much less than that found with noradrenaline-releasing compounds (Kärki & Paasonen, 1959). The fact that diethyldithiocarbamate produced a significant reduction in endogenous noradrenaline supports the hypothesis that the conversion of dopamine to noradrenaline may be rate-limiting under physiological conditions.

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BOOK REVIEW

INTRODUCTION TO CHEMICAL PHARMACOLOGY. Second Edition. By R. B. Barlow. Pp. viii + 452 (including Index). Methuen & Co. Ltd., London, 1964. 84s.

It is a pleasure to record that the high standard set by Dr. Barlow in the first edition of his book has been well maintained, perhaps even surpassed, in this second edition. The subject matter has been reduced in breadth, but the increased depth of treatment has lengthened the book by some 50%. It would probably be true to say that this is no longer an introduction to, but has become rather a treatise on, chemical pharmacology. The author's intention is that his book should serve primarily the interests of chemists, but it is certain to prove just as useful to the pharmacologist. The subject matter is concerned mainly within the confines of "autonomic pharmacology", with excursions into the actions of local anaesthetics and of drugs affecting histamine receptors. Throughout, Dr. Barlow has attempted to integrate the biological and chemical aspects of his subject with considerable success. There is a useful introduction to enzyme kinetics and receptor theory, both of which have been advanced in recent years to the extent of being fully comprehensible only to the specialist within the field. Although this chapter does not set out to be more than an introduction, it is a timely one. The principles of quantitative experimental methods are also surveyed briefly.

The main body of the book is devoted to relations of structure to activity in autonomic drugs. Introducing each chapter is a brief appraisal of the historical background and present concepts of physiological activity at each site—the neuromuscular junction, autonomic ganglia, postganglionic cholinergic receptors, drugs affecting cholinesterases and adrenergic receptors. In the main there is little to fault in the contents of these chapters. Some expansion of the chemical pharmacology of presynaptic events would have been desirable both for cholinergic and adrenergic nerves, because of the increasing interest in these sites. There are two other minor criticisms. The first concerns the references, which are listed alphabetically in relation to each chapter, but collected between text and appendices at the end of the book. This makes reference finding more of a trial. Either the lists should close each chapter or (less satisfactorily) there should be a single alphabetical list at the end of the book. Secondly, from the dates of the references cited the text would appear to have been some two years in appearing in print, which may reflect publishing difficulties. However, the thread of Dr. Barlow's argument loses little by either of these drawbacks. There are some books, of which each new edition is awaited with anticipation. This is one of them.

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