Journal of Pharmacy and Pharmacology

Published by
The Pharmaceutical Society
of Great Britain

Volume 19 No. 6
June 1967

Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN

17 Bloomsbury Square, London, W.C.1. HOLborn 8967

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J. Pharm. Pharmac., 1967, **19**, 345–349 Received January 19, 1967

Uptake and storage of catecholamines in rabbit brain after chronic reserpine treatment

A. DAHLSTRÖM, K. FUXE, B. HAMBERGER AND T. HÖKFELT

The brains of rabbits treated chronically with small doses of reserpine have been examined by the histochemical fluorescence method for dopamine, noradrenaline and 5-hydroxytryptamine. Measurements were made 4 and 24 hr after the last reserpine injection both *in vivo* and *in vitro*. Evidence has been obtained that the small functionally-important pool of amine can be directly visualized in the fluorescence microscope. It was found to be present intraneuronally, probably localized to amine storage granules. The degree of functional recovery was correlated with the recovery, as shown by the increased fluorescence, of a small intraneuronal pool of amine and with the ability of amine storage granules to take up monoamines.

STRONG support has been obtained, in biochemical studies (Häggendal & Lindqvist, 1963, 1964) on brains of chronically reserpine-treated animals, for the concept that there exists a small, labile store of monoamine which is important for the immediate function of the monoaminergic neuron, whereas the largest store is not so important. Thus, the recovery of this small pool—but not of the normal amine levels—may be related to recovery of normal behaviour of the rabbit previously given reserpine. In the sympathetic nervous system it has been shown that the recovery from the pharmacological effects of reserpine coincide with the recovery of the ability of adrenal medullary granules (Lundborg, 1963; Carlsson, Jonasson & Rosengren, 1963) and peripheral adrenergic tissues (Andén, Magnusson & Waldeck, 1964; Andén & Henning, 1966) to take up noradrenaline. These findings are consistent with the view that the refilling of a small labile pool is—partially if not mainly—responsible for the early recovery of monoaminergic neurotransmission.

The purpose of the present investigation was to demonstrate the cellular localization of the small amounts of catecholamines and 5-hydroxytryptamine (5-HT) found in brains of chronically reserpine-treated rabbits (Häggendal & Lindqvist, 1963; 1964) 24 hr after the last injection. The presence of catecholamines and 5-HT was demonstrated using the histochemical fluorescence method (see Hillarp, Fuxe & Dahlström, 1966; Corrodi & Jonsson, 1967). Furthermore, *in vitro* studies on brain slices from these rabbits were made to see if the time-course of recovery of this small, labile pool of amines could be correlated with that of the ability of the central catecholamine terminals to take up and store noradrenaline.

Experimental

MATERIAL AND METHODS

Albino rabbits of 1·5–2·5 kg were used. Ten rabbits were treated with daily injections of reserpine (Serpasil) for 4–8 weeks (0·2 mg/kg, s.c.). Five of these rabbits were killed 4 hr, and the rest 24 hr after the last injection by an intravenous injection of air. Large parts of the telencephalon (including the caudate nucleus and putamen, the septal area and the

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hippocampal formation), of the diencephalon (mainly the hypothalamus and the subthalamus) and of the mesencephalon (mainly the tegmentum) and also the pons and the medulla oblongata were dissected out. The brain pieces were freeze-dried and treated with formaldehyde gas for 1 hr, embedded and sectioned as previously described (Dahlström & Fuxe, 1964; Hamberger, Malmfors & Sachs, 1965).

From the same animals the slices were made from the neocortex, the caudate nucleus, putamen, the hypothalamus, the vas deferens and/or submaxillary gland. The slices from each area of all the rabbits were incubated either with (-)-α-methylnoradrenaline (Corbasil), 0·1 and 1 μg/ml, or (−)-noradrenaline (0·1 and 1 μg/ml) for 20 min (Hamberger & Masuoka, 1965; Hamberger, 1967). After incubation the slices were freeze-dried (Thieme, 1965) and treated with formaldehyde gas as for the rest of the brain specimens.

Results

HISTOCHEMISTRY

4 *hr after the last reserpine injection*

In vivo. Practically no catecholamine and 5-HT nerve terminals were observed in the pieces studied except in two of the animals in the brains of which a number of weak to medium green-fluorescent catecholamine nerve terminals were observed, scattered mainly in the lateral hypothalamus and the retrochiasmatic area. The dopamine, noradrenaline and 5-HT cell bodies in the brain showed only a very weak to weak green or yellow fluorescence respectively.

In vitro. Previous to incubation practically no dopamine, noradrenaline or 5-HT terminals were observed though certain individual differences were found. After incubation with α-methylnoradrenaline (0·1 and 1 μg/ml) catecholamine nerve terminals with a medium to strong intensity could be observed in all these areas. If, instead, noradrenaline was administered to the incubation bath, higher concentrations (1 μg/ml) had to be used and even then only a weak green fluorescence could be observed in the catecholamine nerve terminals.

24 *hr after the last reserpine injection*

In vivo. The number and intensity of the noradrenaline nerve terminals in the hypothalamus had increased considerably in two out of five rabbits, most of the terminals having a very weak to weak green fluorescence intensity. In the brains of the other rabbits no certain signs of increase in fluorescence were observed. In the two rabbits mentioned above, weakly fluorescent catecholamine terminals could be seen also in the lower brain stem (e.g. in the ventral part of the griseum centralis, the dorsal motor nucleii of the vagus nerves and the nuclei of the tractus solitarii) and the dopamine nerve terminals of the caudate nucleus and putamen showed up with a distinct fluorescence. The varicose appearance of the terminals was the same as in normal animals. However, in none of the animals was fluorescence found in the noradrenaline nerve terminals of, for example,

the neocortex and the reticular formation. No 5-HT terminals were detected in any part of the brain studied.

The catecholamine and 5-HT cell bodies usually showed some recovery, especially in those rabbits which showed increased amounts of catecholamine nerve terminals. Large individual differences were observed, however, among the cell bodies within each of the various monoamine cell groups, probably reflecting different rates of granule formation (Dahlström, Fuxe & Hillarp, 1965).

In vitro. The slices from the hypothalamus and the caudate nucleus and putamen, but not those from the neocortex, showed weakly fluorescent catecholamine nerve terminals even before incubation with the amines. At this time interval, uptake in catecholamine terminals was found after incubation with α-methylnoradrenaline (1 and 0·1 μg/ml) just as after 4 hr. Also after noradrenaline (0·1 μg/ml) a clear uptake was found in catecholamine terminals but not in non-terminal axons. A comparison of the 4 and 24 hr tissue sections showed the main difference to be that a clear uptake occurred in catecholamine terminals after incubation with noradrenaline (0·1 μg/ml) in the second but not in the first instance.

BEHAVIOUR

Four hr after the last reserpine injection the rabbits exhibited a fully developed reserpine syndrome (see Carlsson, 1966), whereas after 24 hr the animals usually showed normal gross behaviour. The rabbits with functional recovery were the same animals showing the best recovery of catecholamine fluorescence with increased amounts of fluorescent dopamine and noradrenaline nerve terminals.

Discussion

The present findings support the view that the small pool of dopamine and noradrenaline, which was discovered by Häggendal & Lindqvist (1964) in the brains of chronically reserpine-treated rabbits, is present in various dopamine and noradrenaline nerve terminal systems respectively. The fact that no recovery was observed in the 5-HT nerve terminals 24 hr after injection may be due to technical reasons since the 5-HT nerve terminals are very thin, the fluorescence is highly ultraviolet-sensitive, and the fluorescence yield for 5-HT is considerably less than for catecholamine (Dahlström & Fuxe, 1964; Corrodi & Jonsson, 1967); also the labile 5-HT fraction may well lie in 5-HT nerve terminals. Individual variations were observed in the degree of recovery of the catecholamine nerve terminals 24 hr after reserpine treatment. Thus, in some of the rabbits it was not possible to see any recovery of fluorescence in the catecholamine nerve terminals in spite of good functional recovery. A probable explanation for this may be that the labile amine fraction in the rabbits with a poor recovery of fluorescence was too small to be visualized with the present technique. It is also possible that the receptor sites for the amines after chronic reserpine treatment have developed a supersensitivity for the amines according to the "law of denervation" (see Rosenbluth, 1949), which may contribute to the

functional recovery. Häggendal & Lindqvist (1964) have shown that the normetanephrine level in the brains of rabbits killed 24 hr after the final reserpine injection (at full functional recovery) was also lower than in normal rabbits. This may indicate that in reserpine-treated rabbits less noradrenaline is released by nervous activity than in normal rabbits.

The incubation experiments now reported provide evidence that the recovery of the small amine fraction within the specific catecholamine nerve terminals coincides with the ability of the catecholamine storage granules to take up and store low concentrations of noradrenaline; this noradrenaline was taken up to a greater extent by the catecholamine nerve terminals at 24 hr than at 4 hr after the last injection. This is in contrast to the results obtained with α-methylnoradrenaline (resistant to monoamine oxidase), which was taken up by the catecholamine nerve terminals to the same degree at both 4 and 24 hr after the last reserpine injection. Thus 24, but not 4, hr after reserpine administration low concentrations of noradrenaline can be taken up by the dopamine and noradrenaline nerve terminals and stored in a site protected from monoamine oxidase. Since no definite fluorescence was observed in the non-terminal axons of the catecholamine neurons after incubation with noradrenaline, even 24 hr after reserpine injection, this site may represent the catecholamine storage granules since very few of these are found in the non-terminal parts. On the other hand granules are highly concentrated in the varicosites, which represent the presynaptic structures (Fuxe, 1965; Hillarp & others, 1966). This view is also supported by the fact that it seems to be necessary for the noradrenaline to be present in the granules in order to be released by nerve impulses (Malmfors, 1965). At the present time it is not possible to decide whether the small amine fraction observed in the present experiments is located in the small ATP-free pool, like the one first discovered by Hillarp (1960) in the adrenal medullary granules, or not. Nor is it possible to decide whether this store lies in amine granules which previously have been blocked by reserpine and which regain their ability to take up and store catecholamines, or whether the store lies in newly formed granules, which have been produced in the cell bodies and rapidly transported to the terminals (Dahlström & others, 1965; Dahlström, 1967; Dahlström & Häggendal, 1966). This question remains till other experiments, e.g. axonal interruptions, have been made. It may be, however, that small repeated doses do not damage the granules in the same way as does a single high dose.

Taken together, the present results support the view that the small pool of dopamine, noradrenaline and 5-HT immediately important for function and which was discovered in brain by Häggendal & Lindqvist (1963, 1964), can be directly visualized within the various dopamine and noradrenaline nerve terminal systems of the brain. Also, functional recovery is correlated not only with restoration of a small, probably intragranular, pool of amine, but also in all probability with a partial recovery of uptake of noradrenaline into the amine storage granules. A similar, partial recovery of uptake has also been observed to be correlated with the recovery of function in the nictitating membrane of the cat after reserpine (Andén &

Henning, 1966). The fact that at the time of full functional recovery there is only a partial recovery of uptake of amine in the adrenergic nerve terminals (Andén & Henning, 1966) and in the central catecholamine nerve terminals, seems to favour the possibility that newly formed amine storage granules transported along the axon (Dahlström, 1967) are the structures into which the amines are taken.

Acknowledgements. For generous supplies of drugs we are indebted to the Swedish Ciba, Stockholm, Sweden (Serpasil) and Hoechst Anilin AB, Göteborg, Sweden (Corbasil). The present investigation was supported by grants (B67-12X-714-02, B67-12X-711-02) from the Swedish Medical Research Council.

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J. Pharm. Pharmac., 1967, **19**, 350–354 Received February 6, 1967

Adrenoceptive receptors in the duodenum, aorta and atria of the rabbit

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pA_{10} values for the α-receptor blocking drug phentolamine against adrenaline, noradrenaline and phenylephrine were determined in rabbit aorta and duodenum, and for the β-receptor blocking drug propranolol, against adrenaline, noradrenaline and isoprenaline in rabbit atria and duodenum. The values for phentolamine against different amines were found to be similar, varying by less than half a log unit, between or within the tissues, as were the values for propranolol. These data provide quantitative evidence that α-receptors in the aorta and duodenum are similar, as are the β-receptors in the atria and duodenum, even though both types of receptor serve divergent functions in different tissues.

ACCORDING to current concepts, α-adrenoceptive receptors subserve stimulation in most tissues but inhibition in the intestines, while β-receptors subserve inhibition in most tissues but excitation in the heart. Such divergence in function of either type of adrenoceptive receptors in different tissues calls for quantitative evidence to support the view that a particular type of receptor is the same even though it serves opposite functions in different tissues.

Drug receptors are best identified by specific antagonists. Schild (1949) introduced 'pA' as an index for measuring specificity of antagonism and Arunlakshana & Schild (1959) used this method to show that the acetylcholine receptors in skeletal muscle were different from those at the muscarinic sites while they were the same in a variety of muscarinic sites. In the present study we have determined pA_{10} values for an α- and a β-adrenergic blocking agent (phentolamine and propranolol respectively) against selected sympathomimetic amines in different tissues of the rabbit, to obtain quantitative data about the interaction between active drugs and antagonists at the α- and β-receptors.

Experimental

METHODS

Three isolated tissue preparations obtained from albino rabbits weighing between 1·5 and 3 kg were used: spirally cut aortic strips for the excitatory effect through α-receptors, excised atria for the excitatory effect through β-receptors, and duodenal segments for the inhibitory effect through both α- and β-receptors. The required tissue was removed quickly and placed directly into chilled McEwen solution (McEwen, 1956). The preparations were cleared of extraneous tissue and suspended in baths containing McEwen solution (10 ml at 37° for aortic strips and duodenal segments, and 30 ml at 29° for the atria) bubbled with a mixture of oxygen 95% and carbon dioxide 5%.

Isotonic contractions of duodenal segments and aortic strips against 4 g tension and magnified ninefold were recorded by a frontal writing lever on a smoked drum. Isometric contractions of excised atria were

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recorded on a polygraph by means of a force displacement transducer (Grass FT–03). Before making any tests, aortic strips were suspended for 90 min and duodenal segments and atria were allowed to contract for 60 min. The bathing fluid was changed every 15 min during this waiting period.

pA_{10} DETERMINATION

The dose of each agonist producing approximately 50% of the maximal effect was determined in preliminary experiments on aortic strips, and then used as the unit dose in the determination of pA_{10} values in all tissues. The effects of these doses of agonists varied widely in different tissues, but were always submaximal. The effect of this selected dose was tested repeatedly in each experiment until steady responses were obtained. The effect of a tenfold unit dose (10x) was then tested in the presence of a certain concentration of the antagonist to which the tissues had been exposed for 3 min. After allowing time for recovery of the tissues from the effect of the antagonist, the effect of the 10x dose of the agonist was measured again in the presence of a higher concentration of the antagonist. In this way the concentration of the antagonist was increased stepwise by approximately half a log unit at a time, until that concentration was reached which reduced the effect of the 10x dose of the agonist to less than the effect of the unit dose in the absence of the antagonist.

DRUGS AND SOLUTIONS

Four sympathomimetic amines were used: (−)-noradrenaline bitartrate monohydrate and phenylephrine hydrochloride as predominantly α-agonists, isoprenaline bitartrate dihydrate as a predominantly β-agonist, and (−)-adrenaline bitartrate as a potent agonist on both receptor systems. Propranolol hydrochloride, phentolamine hydrochloride and 2-bromolysergic acid diethylamide were the antagonists used. Solutions were made in 0·9% saline solution and stored in the frozen state.

Phenoxybenzamine was used in a few experiments on the duodenum. The stock solution of phenoxybenzamine hydrochloride was made in acidified propylene glycol and stored at 4°. Dilutions when required were made in 0·9% saline solution.

Results

Preliminary experiments indicated that the pA_{10} values for phentolamine against different agonists were between 7 and 6 in both tissues and the pA_{10} values for propranolol were between 6 and 5. For the final determination of the pA_{10} values, therefore, closely spaced concentrations of the antagonists within the range of 1 to 1·5 log units were used. Fig. 1 shows the protocol of a typical experiment. After washing out the antagonist and 10x dose of the agonist, the unit dose of the agonist was added every 30 min to determine when the tissue had recovered from the effect of the previous dose of the antagonist (only the first and the last of these responses are shown in Fig. 1).

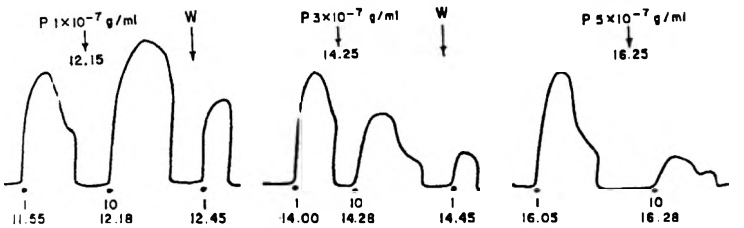

FIG. 1. pA_{10} determination of phentolamine against adrenaline on rabbit aortic strip. All contractions with adrenaline (1) 3×10^{-8} and (10) 3×10^{-7} g/ml. At P ↓ the indicated concentration of phentolamine was added 3 min before testing the effect of 10x concentration of adrenaline. At W ↓ phentolamine was washed out. The time sequence is also shown.

In the experiments on aortic strips, the effect of a 10x dose of the agonist in the presence of different concentrations of the antagonist was calculated as a percentage of the effect of the unit dose in the absence of the antagonist. With duodenal segments and atria, the effect of the unit dose as well as 10x dose of the agonist was expressed as a percentage change in the height of spontaneous contraction. The results from two experiments, one with duodenum and another with aorta, have been plotted in Fig. 2 to illustrate the method used for deriving the concentra-

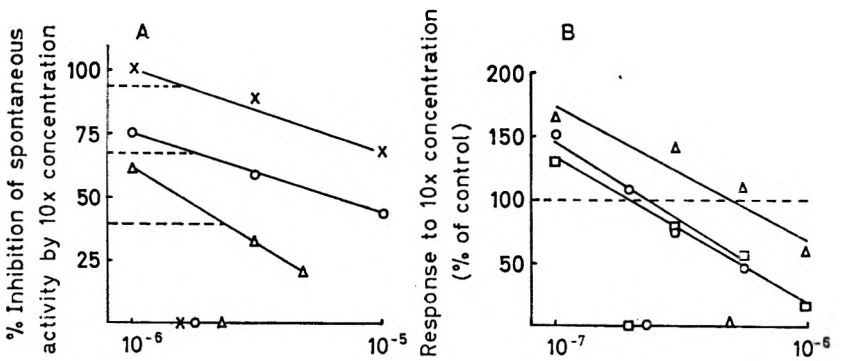

FIG. 2. Graphic derivation of the concentration equivalent to pA_{10} of (A) propranolol (g/ml) on duodenum and (B) phentolamine (g/ml) on aortic strip; ○ adrenaline, × isoprenaline, △ noradrenaline, □ phenylephrine. The broken lines are drawn through the effect of the unit dose in the absence of the antagonist. The symbols on the abscissae indicate the concentrations of either antagonist corresponding to the pA_{10} values against each agonist.

tion from which pA_{10} was calculated. Using this procedure, pA_{10} values for each agonist-antagonist pair were determined in 3–6 experiments in each tissue and the results are presented in Table 1.

pA_{10} values of phentolamine against each agonist were lower in the aorta than in the duodenum. Moreover the values for noradrenaline in either tissue were lower by 0·3–0·6 log units than the values for adrenaline or phenylephrine. For propranolol, the values for all the agonists within each tissue were nearly identical, but the values in the duodenum compared to the atria were consistently lower by about

TABLE 1. pA_{10} VALUES ± S.E. OF PHENTOLAMINE AND PROPRANOLOL AGAINST DIFFERENT SYMPATHOMIMETIC AMINES IN RABBIT AORTA, DUODENUM, AND ATRIA

	Phentolamine	
	Aorta	Duodenum
Adrenaline	6·14 ± 0·06 (6)	6·49 ± 0·02 (3)
Noradrenaline	5·74 ± 0·07 (5)	6·17 ± 0·25 (3)
Phenylephrine	6·12 ± 0·08 (6)	6·50 ± 0·23 (3)
	Propranolol	
	Atria	*Duodenum
Adrenaline	5·71 ± 0·08 (3)	5·14 ± 0·36 (3)
Noradrenaline	5·75 ± 0·41 (3)	5·10 ± 0·38 (5)
Isoprenaline	5·65 ± 0·33 (3)	5·06 ± 0·13 (5)

* Values determined on duodenal segments pre-exposed to 10^{-6} g/ml of phenoxybenzamine for 15 min. Figures in parentheses are number of experiments.

0·5 log units. The values for propranolol in the duodenum were obtained with segments pretreated for 15 min with phenoxybenzamine 10^{-6} g/ml, because in the absence of the latter drug there was wide variation from experiment to experiment in the degree of blockade produced by propranolol. Vanov (1963) has also noted inconsistent blockade by pronethalol against sympathomimetics in the rabbit duodenum. Exposure to this concentration of phenoxybenzamine did not appreciably alter the doses of agonists nor the spontaneous activity of the preparations.

Phenylephrine caused stimulation instead of inhibition in some duodenal segments after pretreatment with phentolamine (α-receptor blockade), the tone of these segments being increased while the amplitude of rhythmic contractions was reduced. The degree of stimulation caused by a constant dose of phenylephrine was related directly to the concentration of phentolamine. Furthermore this stimulatory effect was not modified by propranolol added before or during exposure to phenylephrine. However 2-bromolysergic acid diethylamide (BOL) in a concentration of 10^{-6} g/ml completely abolished this stimulatory effect of phenylephrine.

Discussion

It is generally accepted that receptors of similar configuration are concerned in situations where an antagonist gives the same pA_x against different agonists on a given tissue or where it gives the same pA_x against a given agonist in different tissues. The theoretical basis for this relationship has been discussed by Arunlakshana & Schild (1959), Ariëns (1964) and Furchgott (1964), the crucial point being that pA is strictly a measure of affinity of the antagonist. The question, however, is what degree of difference in the pA values indicates different receptors. Clark & Raventos (1937) noted a difference of more than 4 log units in the pA_{10} values of atropine–acetylcholine between the frog rectus muscle and the frog auricles. Arunlakshana & Schild (1959) reported a difference

of 3 log units between the pA_2 values of atropine against acetylcholine and histamine. Similarly Kohli & Innes (1964) reported a difference of nearly 3 log units between the pA_2 values of BOL against 5-hydroxytryptamine (5-HT) and noradrenaline. In the present study, the pA_{10} values of phentolamine against the various sympathomimetic amines varied by less than 0·5 log unit both within and between the tissues. Similarly the values for propranolol were close, both within and between the tissues. These results therefore suggest (*a*) that the sympathomimetics examined all act on the same receptor in a given tissue, and (*b*) that α-receptors in the aorta and duodenum are similar, as are the β-receptors in the atria and the duodenum.

Because few quantitative studies of this type are available, it is difficult to say what the significance of the small differences in the pA_{10} values between different tissues might be (Table 1). Furchgott (1964) has pointed out that pA values calculated on the basis of drug concentrations in the bath fluid may not be true. Therefore the differences in the pA values between the tissues may not be real but may be explained by the difference in the partition coefficients of the tissues between the aqueous phase and the biophase. Another possible explanation for this difference in pA_{10} values may be that sympathomimetic amines can interact with more than one type of receptor and the different tissues may differ in the proportion of different receptors on which these agonists can act. In this regard it is interesting that phenylephrine caused contraction instead of relaxation after previous exposure to phentolamine and that this stimulatory effect was not modified by propranolol but was abolished by BOL. This observation suggests that phenylephrine might produce this stimulatory effect, directly or indirectly, through 5-HT receptors. The ability of some sympathomimetics to interact with 5-HT receptors in other tissues has been reported previously (Vane, 1960; Innes, 1963; Kohli, 1965).

Acknowledgements. We wish to thank Mrs. N. Beament and Mrs. M. Ibbett for their valuable technical assistance. We are grateful to the following for gifts of drugs: Ciba Limited for phentolamine, Sandoz Limited for 2-bromolysergic acid diethylamide, Smith Kline & French Inter-American Corporation for phenoxybenzamine, and Sterling Winthrop Research Institute for phenylephrine and (−)-adrenaline.

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J. Pharm. Pharmac., 1967, **19**, 355–366 Received January 2, 1967

The mechanism of the inhibition of dehydrogenases by salicylate

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Salicylate inhibits rabbit muscle lactate dehydrogenase, horse liver alcohol dehydrogenase, pig heart malate dehydrogenase and pig heart isocitrate dehydrogenase *in vitro*. The inhibitions are reversible, involving competition with NAD, $NADH_2$ or NADP. The results are discussed with reference to some of the *in vivo* actions of the drug.

SALICYLATE causes an increased incorporation of [^{14}C] from labelled succinate into malate and citrate of mitochondrial preparations (Bryant, Smith & Hines, 1963) by inhibiting malate and isocitrate dehydrogenase activities. It was found that salicylate also inhibited several other dehydrogenase enzymes *in vitro* (Hines & Smith, 1964). It was suggested that the mechanism of inhibition of these enzymes involves a reversible competition with either NAD or NADP since the inhibitory effects of the drug were reduced by the further addition of the appropriate coenzyme to the reaction mixtures (Hines & Smith, 1964). The present paper describes kinetic studies, using purified lactate, alcohol, malate and isocitrate dehydrogenases, designed both to establish the validity of this mechanism of inhibition and to determine the inhibitor constants.

Experimental

MATERIALS

Rabbit muscle lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27), horse liver alcohol dehydrogenase (alcohol: NAD oxidoreductase, EC 1.1.1.1), pig heart malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37), pig heart isocitrate dehydrogenase (L_S-isocitrate: NADP oxidoreductase EC 1.1.1.42), NAD, $NADH_2$, NADP, sodium pyruvate and tris were obtained from Boehringer Corporation (London) Ltd. L-Lactic acid (Grade 1), the monosodium salt of L-malic acid and the trisodium salt of DL-isocitric acid (Type 1) were obtained from the Sigma Chemical Co., St. Louis. Other chemicals were of analytical grade and deionized water was used throughout.

ENZYME ASSAYS

Lactate, alcohol, malate and isocitrate dehydrogenase activities were determined by measuring the formation of either oxidized or reduced coenzyme. Mixtures containing substrate, coenzyme, buffer and salicylate, when present, were allowed to come to thermal equilibrium in a 1 cm cell fitted into a constant-temperature cell housing. Full experimental details for each enzyme were as follows.

Lactate dehydrogenase. The reaction mixture contained L-lactate, 47·5 mM; NAD, 0·46 mM; sodium salicylate, 0–20 mM; in a total volume of 3 ml 0·0067M tris-acetate buffer, pH 8·0. The reaction was started by the addition of 10 μl of a solution containing approximately 1 μg of

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lactate dehydrogenase. The commercial lactate dehydrogenase preparation had previously been dialysed against the assay buffer, the dialysing medium being replaced three times over a period of 24 hr.

Alcohol dehydrogenase. The reaction mixture contained ethanol, 2·2 mM; NAD, 0·10 mM; sodium salicylate, 0–5 mM; in a total volume of 3 ml 0·067M glycine-sodium hydroxide buffer, pH 10·0. The reaction was started by the addition of 10 μl of a solution containing approximately 12 μg of alcohol dehydrogenase. The commercial alcohol dehydrogenase had previously been dialysed against 0·1 μ potassium phosphate buffer, pH 8·0, the dialysing medium being replaced three times over a period of 24 hr.

Malate dehydrogenase. The reaction mixture contained L-malate, 0·62 mM; NAD, 0·48 mM; sodium salicylate, 0–10 mM; in a total volume of 3 ml 0·067 M glycine-NaOH buffer, pH 10·0. The reaction was started by the addition of 1 μl of a solution containing approximately 0·5 μg malate dehydrogenase.

Isocitrate dehydrogenase. The reaction mixture contained DL-isocitrate, 0·028 mM (L$_S$-isocitrate, 0·014 mM); NADP, 0·024 mM; $MgCl_2$, 2 mM; and sodium salicylate, 0–20 mM; in a total volume of 9 ml 0·033M tris buffer, pH 7·3. The reaction was started by the addition of 2 μl of a solution containing approximately 20 μg isocitrate dehydrogenase. A portion of the mixture was poured into a 1 cm silica cell for measurement.

The reactions were followed by measuring the changes in extinction at 365 mμ in a Unicam SP800 recording spectrophotometer. Measurements of extinction were made at 365 mμ, not at 340 mμ, to avoid interference caused by the absorption of salicylate at the lower wavelength. The initial rates (v) were determined from the tracings obtained with an external recorder. The concentrations of substrates and coenzymes in solutions were determined enzymatically (Bergmeyer, 1963).

DIALYSIS EXPERIMENTS

In general the enzyme solutions, in either the absence or the presence of salicylate, were placed in cellulose tubing (inflatable diameter 6 mm, Visking Co.) and dialysed at 0° against the appropriate buffer, which was changed several times. The enzyme activities were measured before and after dialysis. Experimental details for each enzyme were as follows.

Lactate dehydrogenase. Enzyme solutions containing 10 μg enzyme protein and 1 mg serum albumin per ml of 0·1M tris-acetate buffer, pH 8·0, were mixed with equal volumes of either the same tris buffer or the buffer containing 60 mM sodium salicylate. Aliquots (1 ml) of the enzyme solutions, both before and after dialysis, were assayed in a 3 ml reaction mixture containing 47·5 mM L-lactate, 0·46 mM NAD and tris buffer at pH 8·0.

Alcohol dehydrogenase. Enzyme solutions containing 12 μg enzyme protein per ml of 0·1 μ phosphate buffer, pH 8·0, were mixed with equal volumes of either phosphate buffer or the same buffer containing 60 mM sodium salicylate. Aliquots (1 ml) of the variously treated enzyme

solutions were assayed in a 3 ml reaction mixture containing 2·2 mM ethanol, 0·1 mM NAD and glycine buffer, pH 10·0.

Malate dehydrogenase. Freshly diluted enzyme solutions containing 6 μg enzyme protein and 10 mg serum albumin per ml of 0·1M phosphate buffer, pH 7·5, were mixed with equal volumes of either the same buffer or the buffer containing 60 mM sodium salicylate. Aliquots (1 ml) of the enzyme solutions were assayed in a 3 ml reaction mixture containing 1·0 mM L-malate, 1·0 mM NAD and glycine buffer at pH 10·0.

Isocitrate dehydrogenase. Freshly prepared enzyme solutions containing 0·1 mg enzyme protein and 10 mg serum albumin per ml of 0·05M tris chloride buffer, pH 7·3, were mixed with equal volumes of either tris buffer or the buffer containing 120 mM sodium salicylate. Aliquots (1 ml) of the various enzyme solutions were assayed in a 3 ml reaction mixture containing 0·1 mM DL-isocitrate, 0·1 mM NADP, $MgCl_2$ and tris buffer, pH 7·3.

Analysis of kinetic data

Dalziel (1957, 1963) has derived a steady state initial rate equation for a compulsory order mechanism, with or without rate-limiting ternary complexes, in which the coenzyme forms a binary complex with the dehydrogenase enzyme but the substrate does not, and which may be written

$$\frac{E}{v} = \Phi_0 + \frac{\Phi_1}{[C]} + \frac{\Phi_2}{[S]} + \frac{\Phi_{12}}{[C][S]} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where E represents the total enzyme concentration, [C] and [S] are the initial concentrations of coenzyme and substrate respectively, and v is the initial rate. In the presence of an inhibitor (I), which competes with the coenzyme and forms an inactive complex (EI) with a dissociation constant of K_I, the initial rate equation becomes

$$\frac{E}{v} = \Phi_0 + \frac{\Phi_1\left(1 + \frac{I}{K_I}\right)}{[C]} + \frac{\Phi_2}{[S]} + \frac{\Phi_{12}\left(1 + \frac{I}{K_I}\right)}{[C]\,[S]} \quad .. \quad .. \quad .. \quad (2)$$

In the present work equations (3) and (4) were derived from equation 2 and primary plots, $\frac{[C]}{v}$ against [C] and $\frac{[S]}{v}$ against [S], were obtained by the Type B method described by Webb (1963) because this procedure allowed a greater significance to be attached to the results of the more accurate experiments using the higher substrate concentrations.

$$\frac{[S]E}{v} = \Phi_2 + \frac{\Phi_{12}\left(1 + \frac{I}{K_I}\right)}{[C]} + \left\{\Phi_0 + \frac{\Phi_1\left(1 + \frac{I}{K_I}\right)}{[C]}\right\}[S] \quad .. \quad (3)$$

$$\frac{[C]E}{v} = \Phi_1\left(1 + \frac{I}{K_I}\right) + \frac{\Phi_{12}\left(1 + \frac{I}{K_I}\right)}{[S]} + \left(\Phi_0 + \frac{\Phi_2}{[S]}\right)[C] \quad .. \quad (4)$$

To calculate the kinetic constants, secondary plots were made in which the slopes and intercepts from the primary plots were plotted against inhibitor concentrations. From equation 3 it can be shown that for a constant concentration of coenzyme [C_c] and for several concentrations of inhibitor, the slopes and intercepts on the $\frac{[C]}{v}$ axis of the primary plot can be represented by the following equations:

$$\text{Slopes of primary plot} = \Phi_0 + \frac{\Phi_1\left(1 + \frac{I}{K_I}\right)}{[C]} \quad .. \quad .. \quad (5)$$

$$\text{Intercepts of primary plot} = \Phi_2 + \frac{\Phi_{12}\left(1 + \frac{I}{K_I}\right)}{[C]} \quad .. \quad .. \quad (6)$$

Similarly for a constant concentration of substrate [S_c] the following equations can be derived:

$$\text{Slopes of primary plot} = \Phi_0 + \frac{\Phi_2}{[S]} \quad .. \quad .. \quad .. \quad (7)$$

$$\text{Intercepts of primary plot} = \Phi_1\left(1 + \frac{I}{K_I}\right) + \frac{\Phi_{12}\left(1 + \frac{I}{K_I}\right)}{[S]} \quad .. \quad (8)$$

It follows from equation 7 that the slopes of the primary plots must be constant, i.e. the plots are parallel lines, unless the inhibitor also competes with the substrate as well as the coenzyme. If the inhibitor competes with the substrate, the primary plots concerned in equation 5 should be parallel. The interpretation of the slopes and intercepts of the secondary plot are given in Table 1; from this Table the following kinetic parameters (see Dalziel, 1963) were calculated: K, Φ_0, Φ_1, Φ_2, Φ_{12}, $K_{mc}\left(=\frac{\Phi_1}{\Phi_0}\right)$, $K_{ms}\left(=\frac{\Phi_2}{\Phi_0}\right)$ and $K_c\left(=\frac{\Phi_{12}}{\Phi_2}\right)$.

TABLE 1. INTERPRETATION OF SECONDARY PLOTS FROM PRIMARY PLOTS. THE FOLLOWING EXPRESSIONS ARE DERIVED FROM EQUATIONS 5–8

		Slopes of secondary plots	Intercepts of secondary plots
[C_c]	Slopes of primary plots/[I] ..	$\frac{\Phi_1}{[C_c]\,K_I}$	$\Phi_0 + \frac{\Phi_1}{[C_c]}$
	Intercepts of primary plots/[I] ..	$\frac{\Phi_{12}}{[C_c]\,K_I}$	$\Phi_2 + \frac{\Phi_{12}}{[C_c]}$
[S_c]	Slopes of primary plots/[I] ..	0	$\Phi_0 + \frac{\Phi_2}{[S_c]}$
	Intercepts of primary plots/[I] ..	$\frac{\Phi_1}{K_I} + \frac{\Phi_{12}}{[S_c]\,K_I}$	$\Phi_1 + \frac{\Phi_{12}}{[S_c]}$

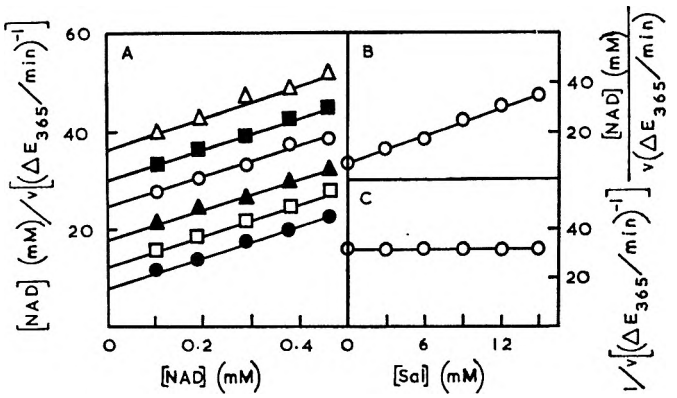

FIG. 1. Lactate dehydrogenase, kinetics with a constant concentration of lactate. The experimental conditions were as given in the text except that the coenzyme [NAD] and salicylate [Sal] concentrations were varied as shown. (A) Primary plot. ●, Control; □, 3mM salicylate; ▲, 6mM salicylate; ○, 9mM salicylate; ■, 12mM salicylate; △, 15mM salicylate. (B) Secondary plot of intercepts of (A). (C) Secondary plot of slopes of (A).

Results

The results in Table 2 show that salicylate inhibits the activities of lactate, alcohol, malate and isocitrate dehydrogenases and that the degree of inhibition increases with salicylate concentration. The results of the dialysis experiments (Table 3) show that complete reactivation of the inhibited enzymes occurred after dialysis. The effects of varying the concentrations of NAD and salicylate in the presence of a constant concentration of lactate on the reaction rate of lactate dehydrogenase are shown in Fig. 1. Both the primary and secondary plots (see analysis of kinetic data) are presented in Fig. 1 and in subsequent figures. The results

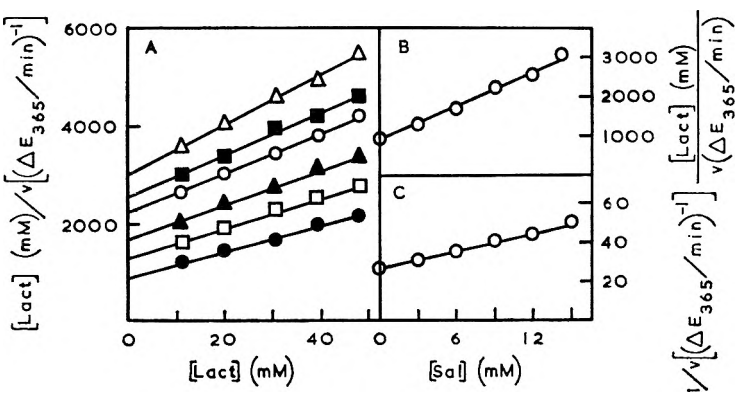

FIG. 2. Lactate dehydrogenase, kinetics with a constant concentration of NAD. The experimental conditions were as given in the text except that the L-lactate [Lact] and salicylate [Sal] concentrations were varied as shown. (A) Primary plot ●, Control; □, 3mM salicylate; ▲, 6mM salicylate; ○, 9mM salicylate; ■, 12mM salicylate; △, 15mM salicylate. (B) Secondary plot of intercepts of (A). (C) Secondary plot of slopes of (A).

of similar experiments in which the concentration of lactate and salicylate were varied and that of NAD remained constant are shown in Fig. 2.

The lactate dehydrogenase reaction was also investigated in the reverse direction; Fig. 3 shows the results of experiments in which the concentrations of $NADH_2$ and salicylate were varied and pyruvate maintained constant and Fig. 4 those in which pyruvate and salicylate were varied in the presence of a constant concentration of $NADH_2$.

The results with alcohol dehydrogenase in which constant concentrations of either NAD or alcohol were maintained are given in Figs 5 and 6, with malate dehydrogenase using constant concentrations of either NAD or malate in Figs 7 and 8, and with isocitrate dehydrogenase and constant concentrations of either NADP or isocitrate in Figs 9 and 10.

The inhibitor constants and other kinetic parameters, calculated from the data contained in Figs 1 to 10, are given in Table 4.

Discussion

The Michaelis constants calculated from the present results (Table 4) are in good agreement with those reported by previous workers for lactate dehydrogenase (Anderson, Florini & Vestling, 1964), alcohol

TABLE 2. EFFECT OF SALICYLATE ON DEHYDROGENASE ENZYMES. Experimental details were as described in the Experimental section. The mean of three separate determinations was used to calculate each percentage inhibition. The initial velocities (ΔE_{365}/min) for the control reaction mixtures were as follows: lactate dehydrogenase, 0·057 ± 0·008; alcohol dehydrogenase, 0·036 ± 0·001; malate dehydrogenase, 0·021 ± 0·001; isocitrate dehydrogenase, 0·022 ± 0·001.

(a) Lactate dehydrogenase		(b) Alcohol dehydrogenase	
Salicylate (mM)	% Inhibition	Salicylate (mM)	% Inhibition
2·5	6	1·0	6
5·0	14	2·0	19
7·5	31	3·0	31
10·0	39	4·0	36
15·0	48	5·0	42
20·0	59		
(c) Malate dehydrogenase		**(d) Isocitrate dehydrogenase**	
Salicylate (mM)	% Inhibition	Salicylate (mM)	% Inhibition
2·5	8	5·0	6
5·0	16	10·0	20
7·5	36	15·0	26
10·0	45	20·0	34

TABLE 3. EFFECT OF DIALYSIS ON THE INHIBITION OF THE DEHYDROGENASES BY SALICYLATE. Experimental details were as described in the Experimental section. The results are expressed as ΔE_{365}/min and represent the mean ± standard deviations. The number of observations is given in parentheses

Dehydrogenase	Before dialysis		After dialysis	
	Control	Salicylate	Control	Salicylate
Alcohol	0·018 ± 0·001 (4)	0·010 ± 0·001 (4)	0·016 ± 0·001 (4)	0·015 ± 0·001 (4)
Lactate	0·063 ± 0·001 (5)	0·038 ± 0·002 (5)	0·032 ± 0·002 (5)	0·033 ± 0·002 (5)
Malate	0·135 ± 0·002 (6)	0·054 ± 0·002 (6)	0·134 ± 0·003 (6)	0·134 ± 0·003 (6)
Isocitrate	0·113 ± 0·003 (6)	0·076 ± 0·003 (6)	0·055 ± 0·001 (6)	0·057 ± 0·001 (6)

TABLE 4. KINETIC PARAMETERS OF LACTATE, ALCOHOL, MALATE AND ISOCITRATE DEHYDROGENASES. The values given here have been calculated from the constants defined under "Analysis of kinetic data." The three independent values of Φ_{12}, obtained during the calculations, have been included to indicate the internal consistency of the experimental data

Dehydrogenase	K_1 mM	Φ_0 $\frac{1}{\Delta E_{365}/min}$	Φ_1 $\frac{mM}{\Delta E_{365}/min}$	Φ_2 $\frac{mM}{\Delta E_{365}/min}$	Φ_{12} $\frac{(mM)^2}{\Delta E_{365}/min}$	K_{mc} mM	K_{ms} mM	K_c mM
Lactate (lactate→ pyruvate) ..	3·79	21·0	2·66	522·5	211, 175, 216	0·127	24·9	0·39
Lactate (pyruvate→ lactate) ..	3·56	30·5	0·21	26·5	0·15, 0·17, 0·10	0·007	0·87	0·0053
Alcohol (ethanol→ acetaldehyde)	1·25	25·0	1·13	35·2	1·62, 2·04, 1·34	0·045	1·41	0·047
Malate (malate→ oxaloacetate) ..	1·33	33·4	2·21	2·88	1·45, 1·99, 1·12	0·066	0·086	0·53
Isocitrate (isocitrate→ oxoglutarate) ..	5·54	38·7	0·22	0·14	0·0013, 0·0016, 0·0026	0·0056	0·0035	0·013

dehydrogenase (Theorell, Nygaard & Bonnischen, 1955), malate dehydrogenase (Wolfe & Nielands, 1956; Cassman & England, 1966) and isocitrate dehydrogenase (Moyle, 1956).

The present work confirms that salicylate inhibits the four dehydrogenase activities *in vitro* (Table 2) and the results of the dialysis experiments (Table 3) show that the inhibitions are reversible. The plots of the kinetic data obtained in the present work demonstrate that salicylate competes with NAD and $NADH_2$ for lactate dehydrogenase (Figs 1C and 3C), with NAD for alcohol and malate dehydrogenases (Figs 5C and 7C) and with NADP for isocitrate dehydrogenase (Fig. 9C). The data exclude a mechanism involving competition with the respective substrates because the relevant plots (Figs 2C, 4C, 6C, 8C and 10C) do not have zero slopes. The pyridine nucleotide coenzymes used in the present experiments were not specially purified although it has been reported (Dalziel, 1963; Dalziel & Dickinson, 1965) that nucleotide impurities in commercial samples of NAD and NADP may act as competitive inhibitors of alcohol dehydrogenase and possibly other dehydrogenases. However, this type of interference only appeared to be of significance below pH 7 (Dalziel, 1963) and all reaction mixtures we used were at pH 7·3 or above.

The inhibitor constants (Table 4) show that salicylate inhibits the dehydrogenases to approximately the same degree as alanine aminotransferase but much more powerfully than aspartate aminotransferase (Gould, Dawkins, Smith & Lawrence, 1966). From Table 4 the ratios of K_c to K_1 give an indication of the relative affinities of the coenzyme and of the salicylate for the enzyme. On this basis, malate dehydrogenase is the most sensitive and isocitrate dehydrogenase is the least sensitive to the inhibitory action of salicylate involving competition with the oxidized forms of the coenzymes. The lactate dehydrogenase $K_c : K_1$

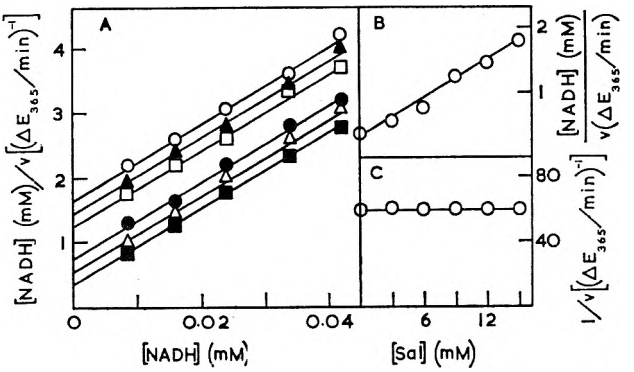

FIG. 3. Lactate dehydrogenase, kinetics with a constant concentration of pyruvate. Mixtures containing 0·93mM pyruvate and varying concentrations of coenzyme [NADH] and salicylate [Sal] as indicated, in a total volume of 3 ml of 0·0067 M tris-acetate buffer, pH 8·0, were allowed to come to thermal equilibrium at 25° in a 1 cm silica cell. The reaction was started by the addition of 10 μl of a solution containing approximately 1 μg of lactate dehydrogenase, which had been dialysed before use as described in Table 2. (A) Primary plot. ■, Control; △, 3mM salicylate; ●, 6mM salicylate; □, 9mM salicylate; ▲, 12mM salicylate; ○, 15mM salicylate. (B) Secondary plot of intercepts of (A). (C) Secondary plot of slopes of (A).

ratios show that salicylate inhibits the conversion of lactate to pyruvate much more strongly than the reverse reaction.

The present findings also suggest that salicylate is a general inhibitor of all pyridine nucleotide-linked dehydrogenase enzymes. The drug has been shown to interfere with the NADP-dependent glucose 6-phosphate and 6-phosphogluconate activities in preparations of human erythrocytes (Sturman & Smith, 1966) and with the $NADH_2$-cytochrome c reductase

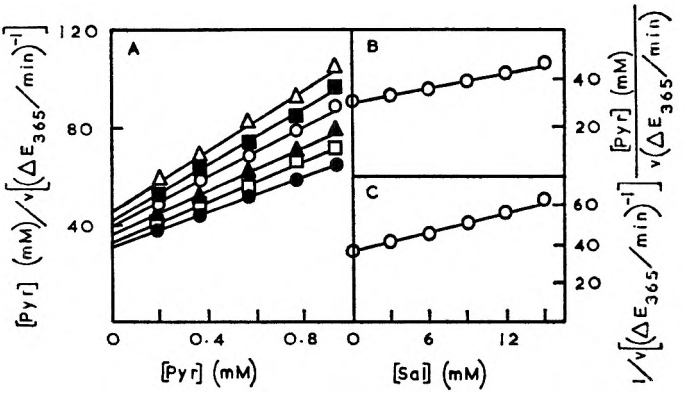

FIG. 4. Lactate dehydrogenase, kinetics with a constant concentration of $NADH_2$. Mixtures containing 0·042mM $NADH_2$ and varying concentrations of pyruvate [Pyr] and salicylate [Sal] as indicated, in a total volume of 3 ml of 0·0067 M tris-acetate buffer, pH 8·0, were allowed to come to thermal equilibrium at 25° in a 1 cm silica cell. The reaction was started by the addition of 10 μl of a solution containing approximately 1 μg of lactate dehydrogenase, which had been dialysed before use as described in Table 2. (A) Primary plot. ●, Control; □, 3mM salicylate; ▲, 6mM salicylate; ○, 9mM salicylate; ■, 12mM salicylate; △, 15mM salicylate. (B) Secondary plot of intercepts of (A). (C) Secondary plot of slopes of (A).

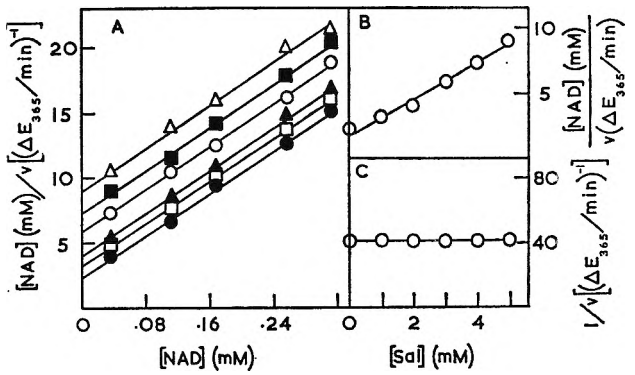

FIG. 5. Alcohol dehydrogenase, kinetics with a constant concentration of ethanol. The experimental conditions were as given in the text except that coenzyme [NAD] and salicylate [Sal] concentrations were varied as shown. (A) Primary plot. ●, Control; □, 1mM salicylate; ▲, 2mM salicylate; ○, 3mM salicylate; ■, 4mM salicylate; △, 5mM salicylate. (B) Secondary plot of intercepts of (A). (C) Secondary plot of slopes of (A).

system in guinea-pig liver mitochondria (Hines, Bryant & Smith, 1963).

The *in vitro* results observed in the present work may have important implications in some of the *in vivo* actions of salicylate. Slater & Sawyer (1966) have reported that the injection of salicylate in the rat causes a rapid decrease in the content of NADP and $NADPH_2$ in the liver. They considered it unlikely that this effect would be due to salicylate displacing the nucleotides from binding sites into free solution, thereby making them more susceptible to enzymic breakdown. However, an increased accumulation of the nucleotides in free solution may enhance their leakage into the circulation especially as the uncoupling action of salicylate on oxidative phosphorylation reactions impairs normal

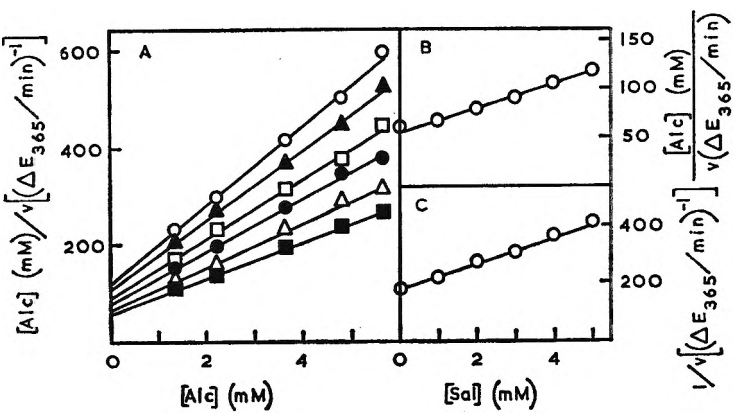

FIG. 6. Alcohol dehydrogenase, kinetics with a constant concentration of NAD. The experimental conditions were as given in the text except that the ethanol [Alc] and salicylate [Sal] concentrations were varied as shown. (A) Primary plot. ■, Control; △, 1mM salicylate; ●, 2mM salicylate; □, 3mM salicylate; ▲, 4mM salicylate; ○, 5mM salicylate. (B) Secondary plot of intercepts of (A). (C) Secondary plot of slopes of (A).

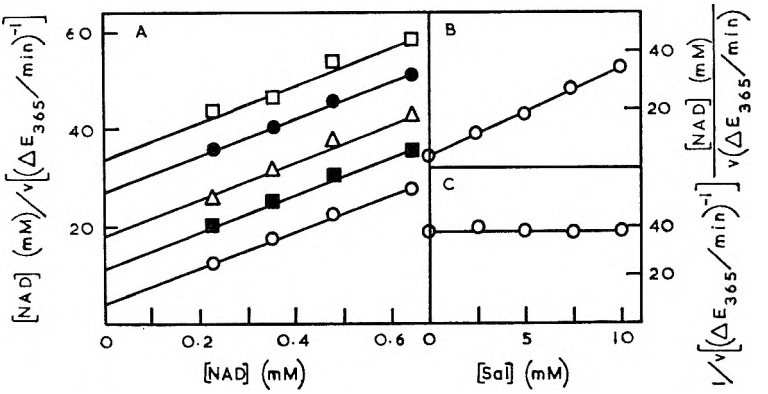

FIG. 7. Malate dehydrogenase, kinetics with a constant concentration of malate. The experimental conditions were as given in the text except that the coenzyme [NAD] and salicylate [Sal] concentrations were varied as shown. (A) Primary plot. ○, Control; ■, 2·5mM salicylate; △, 5mM salicylate; ●, 7·5mM salicylate; ■, 10mM salicylate. (B) Secondary plot of intercepts of (A). (C) Secondary plot of slopes of (A).

membrane permeability (Mitidieri & Affonso, 1959). Such a diminution in intracellular nucleotide concentrations would be expected to reinforce an inhibitory action of the drug on NADP-dependent dehydrogenases.

It has also been shown that, in man and experimental animals, the administration of increasing amounts of salicylate causes a progressive rise in oxygen consumption. A similar effect occurs with isolated tissue preparations, except that the stimulation of oxygen uptake is succeeded by a depression of this function at higher salicylate concentrations. A typical result has been reported with isolated sacs of rat small intestine

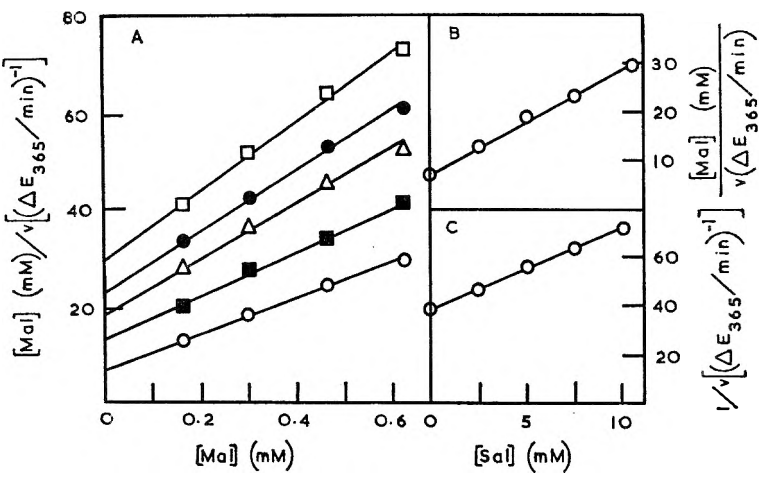

FIG. 8. Malate dehydrogenase, kinetics with a constant concentration of NAD. The experimental conditions were as given in the text except that the L-malate [Mal] and salicylate [Sal] concentrations were varied as shown. (A) Primary plot. ○, Control; ■, 2·5mM salicylate; △, 5mM salicylate; ●, 7·5mM salicylate □, 10mM salicylate. (B) Secondary plot of intercepts of (A). (C) Secondary plot of slopes of (A).

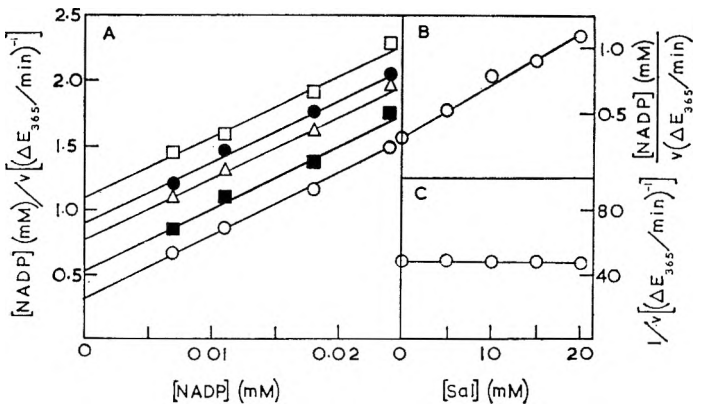

FIG. 9. Isocitrate dehydrogenase, kinetics with a constant concentration of isocitrate. The experimental conditions were as given in the text except that the coenzyme [NADP] and salicylate [Sal] concentrations were varied as shown. (A) Primary plot. ○, Control; ■, 5mM salicylate; △, 10mM salicylate; ●, 15mM salicylate; □, 20mM salicylate. (B) Secondary plot of intercepts of (A). (C) Secondary plot of slopes of (A).

(Smith, 1958). 0 to 1 mM salicylate produced no effect, 1 to 5 mM caused an initial increase in the oxygen uptake and salicylate concentrations above 5 mM produced a marked depression in the oxygen consumption. Similar responses with mouse liver slices (Sproull, 1954) and rat cerebral cortex preparations (Fishgold, Field & Hall, 1951) have been observed. One possible explanation of these effects is that the initial stimulation of oxygen consumption results from the well-known uncoupling action of salicylate on oxidative phosphorylation processes, but that this effect is progressively antagonized and eventually overcome by the inhibitory action of increasing doses of the salicylate on the pyridine nucleotide-linked dehydrogenases.

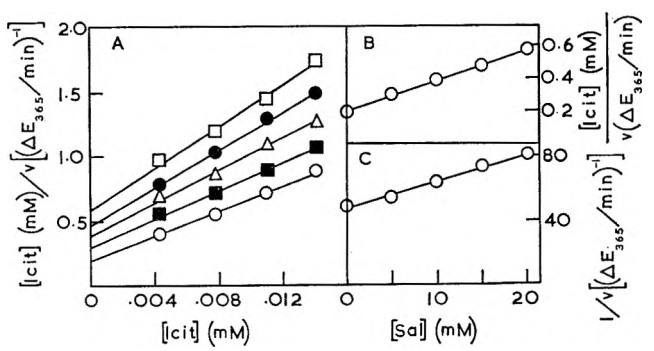

FIG. 10. Isocitrate dehydrogenase, kinetics with a constant concentration of NADP. The experimental conditions were as given in the text except that the isocitrate [Icit] and salicylate [Sal] concentrations were varied as shown. (A) Primary plot. ○, Control; ■, 5mM salicylate; △, 10mM salicylate; ●, 15mM salicylate; □, 20mM salicylate. (B) Secondary plot of intercepts of (A). (C) Secondary plot of slopes of (A).

A further interaction of the inhibitory effect of salicylate on dehydrogenase enzymes and body metabolism may be involved in the acid-base disturbances which occur in salicylate intoxication in man. Toxic amounts of the drug cause three basic actions which affect the acid-base equilibrium directly; an increased alveolar ventilation, an increased metabolic rate and an accumulation of organic anions in the plasma (Smith & Smith, 1966). The net result in an individual patient depends on the relative intensities of these actions and the human infant appears to be particularly vulnerable to the last action, which leads to a state of metabolic acidosis (Done, 1963). It has been shown that the plasma of salicylate-intoxicated infants and young children contains abnormally high concentrations of organic anions (Winters, 1959) although no detailed fractionation of the individual components has been accomplished. An inhibition of pyridine nucleotide-linked dehydrogenases by salicylate would be expected to cause decreased rates of metabolism of hydroxy acids in the tissues, which could lead to an accumulation of their anions in the plasma.

Acknowledgements. We wish to thank the Arthritis and Rheumatism Council and King's College Hospital and Medical School Research Committee for generous financial support and the Nuffield Foundation for a grant towards the purchase of the spectrophotometer.

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J. Pharm. Pharmac., 1967, **19**, 367–373 Received January 6, 1967

Blockade of bradykinin-induced nociception in the rat as a test for analgesic drugs with particular reference to morphine antagonists

G. F. BLANE
with the technical assistance of I. R. MACFARLANE and G. REED

A rat test, recently outlined by Deffenu & others for the evaluation of analgesic drugs and using intra-arterially administered bradykinin as the nociceptive stimulus, has been applied to varied categories of analgesic drugs including, for the first time, morphine antagonists. For purposes of comparison, data obtained with the same drugs using established techniques are also given. The use of the bradykinin-antagonism test as a laboratory model with which to investigate drugs of potential therapeutic value in man is discussed and compared particularly with the mouse phenylquinone-induced writhing test. Attention is drawn to the possibility of correlating the effect of drugs on bradykinin-induced pseudo-affective responses in animals with human pain.

NALORPHINE, pentazocine and related morphine antagonist drugs, which are inactive in established laboratory tests for antinociceptive activity like the rat tail-pressure test, are now known to be active in the mouse phenylquinone-induced writhing test (Taber, Greenhouse & Irwin, 1964; Blumberg, Wolf & Dayton, 1965; Pearl & Harris, 1966), the rat paw-oedema test (Winter & Flataker, 1965) and a modification of the Randall-Siletto rat tail test (Ward, Foxwell & Funderbunk, 1965).

The assessment of analgesic drugs by their ability to block the responses of animals to intra-arterial injections of chemical algesic agents was first discussed by Lim (1960), and was investigated in some depth by Guzman, Braun & Lim (1962) using lightly anaesthetized cats and dogs. Bradykinin, in a minimum effective dose of 1–2 μg, was by far the most potent algesic agent in provoking a "pseudo-affective" response characterized by a fleeting vocal response, hyperpnoea and hypertension. In 1964, Guzman, Braun, Lim & others evaluated some well-known drugs in the conscious dog, using as the criterion for analgesia the block of the vocal response evoked by injection of bradykinin into the splenic artery. Deffenu, Pegrassi & Lumachi (1966) recently adapted the Guzman technique to rats. Small doses of bradykinin injected retrogradely into the right carotid artery of conscious animals by way of a previously implanted catheter resulted in a syndrome consisting of dextrorotation of the head, flexion of the right fore-limb and, occasionally, squeaking. ED50 values were reported for methadone, phenylbutazone, codeine, aspirin and phenacetin. Aminopyrine was also active in this modified test but antagonist-analgesics such as nalorphine were not mentioned.

We report here the results we have obtained using the technique of Deffenu & others and compare them with data obtained using two conventional laboratory techniques for the evaluation of analgesic drugs, and also with the potency of these drugs in man.

From the Pharmacology Laboratory, Reckitt and Sons Ltd., Hull.

Methods

The technique used to cannulate the rats differed only in minor detail from that outlined by Deffenu & others (1966). Male S.P.F.-derived Sprague–Dawley rats of between 250 and 350 g were lightly anaesthetized with ether, and a polythene cannula (internal diameter 0·40 mm, Portland Plastic PP. 25) tied into the right carotid artery with the tip pointing towards the heart. A trochar was used to deliver the free end through the skin of the dorsal surface in the scapular region. Having established that the cannulation was successful, 0·2 ml of saline was injected into the tube and the open end closed with a tightly fitting polythene cap similar to the type used by Weeks & Jones (1960). The skin wound in the neck was closed by application of Michelle clips and the anaesthetic discontinued. Deffenu & others (1966) used their animals 1 hr after recovery from anaesthesia but we allowed at least 4 hr to elapse before testing and mostly tests were made on the day after cannulation.

The minimum dose of bradykinin required to consistently produce dextrorotation of the head and flexion of the right fore-limb was established for each rat. This dose was commonly 0·05 or 0·10 μg and rats not responding to 0·50 μg were discarded. Rats did not always squeak after these threshold doses of bradykinin and disappearance of the head rotation and fore-limb flexion were taken as the criteria for scoring an analgesic effect in animals after drug administration. Bradykinin was given as a solution of the pure synthetic peptide (Sandoz) in 0·2 ml of 0·9% saline and was washed in immediately with a further 0·2 ml of saline. The response developed within about 5 sec and persisted for about a further 10 sec. Compounds under test were administered by oral, subcutaneous or intraperitoneal route, after which the established threshold dose of bradykinin was injected at regular intervals until the response returned. In control animals there was no evidence that tachyphylaxis developed after repeated injections of bradykinin. For any given drug and dose level, the percentage of rats failing to respond to the algesic stimulus could be plotted against time to give a clear presentation of the rate of onset, duration, and decay of activity (Fig. 1).

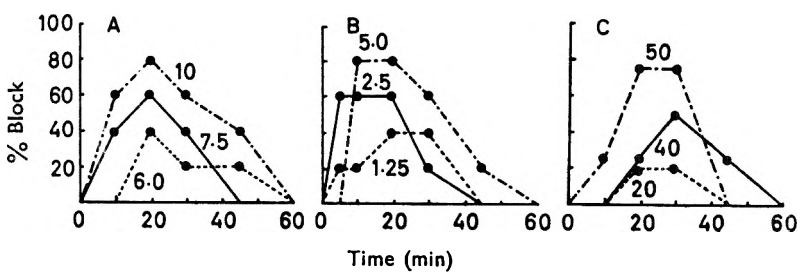

FIG. 1. Representative response curves obtained after the administration of analgesic drugs to rats receiving liminal intra-arterial doses of bradykinin at regular intervals. A. Fentanyl (μg/kg). B. M285 (mg/kg). C. Phenylbutazone (mg/kg). Each curve based on the mean response of 5 rats to a dose of analgesic administered subcutaneously at zero time. Ordinate shows percentile block of the bradykinin-induced nociceptive syndrome.

With all drugs the ED50 values at the time of peak effect and their 95% confidence limits were estimated using the method of Litchfield & Wilcoxon (1949).

Details of the rat tail-pressure test and the mouse anti-writhing test, against which the bradykinin antagonizing effects of drugs have been compared, have been published previously (Boura & Fitzgerald, 1966). The tail-pressure test was essentially similar to that described by Green & Young (1951), where animals were regarded as showing analgesia if they failed to squeal on application of a pressure greater than twice the mean pressure required to cause a vocal response in the control. Relative analgesic efficacy in mice was determined as the dose of drug required to reduce by 50% the number of abdominal stretches caused by the intraperitoneal injection of 2 ml/kg of phenyl-*p*-benzoquinone (Hendershot & Forsaith, 1959).

Narcotic antagonist activity was assessed as the dose of drug which would, in the rat tail-pressure test, reduce to 50% the total analgesia caused by 10 mg/kg morphine sulphate administered subcutaneously (Green, Ruffell & Walton, 1954).

All doses are expressed as the weight of the salt used, where applicable.

Results and discussion

To explore fully the potentialities and limitations of the bradykinin-antagonism technique, representatives from a variety of classes of analgesic drugs were examined in this and the other tests. The drugs and the results obtained are listed and classified in Table 1. The route of administration where possible was the same as that normally used in man. Aspirin, phenylbutazone and codeine, had been examined by Deffenu & others (1966) and our results are in fair agreement with theirs.

Results obtained in some typical bradykinin-antagonism tests are depicted graphically in Fig. 1, and the dose-response lines obtained with four drugs at the time of peak-effect are shown in Fig. 2. Although three different classes of analgesic are represented (strong narcotic—morphine, narcotic antagonist—nalorphine, and antipyretic/anti-inflammatory agents —aspirin and phenylbutazone) the lines do not differ significantly from parallelism at the 95% level of confidence.

The anti-inflammatory analgesics were inactive in the rat tail-pressure test (ED50 $\gg$ 100 mg/kg) but active in the Hendershot & Forsaith test with the relation between the ED50 values for aspirin and phenylbutazone, at least, being of the same order as that of the dose levels which are most commonly used in man. Aspirin and phenylbutazone were active in the anti-bradykinin test only when given intraperitoneally, and the relation between their ED50 values was almost identical to that seen in the Hendershot & Forsaith test. However, the doses required to block the bradykinin-induced syndrome in rats were some five times greater than those needed to antagonize phenylquinone-induced writhing in mice. Mefenamic acid was totally inactive at sub-toxic doses by any route in the bradykinin-antagonism test.

TABLE 1. ACTIVITY OF A VARIETY OF ANALGESIC DRUGS IN LABORATORY ANIMAL TESTS AND IN MAN

Class of analgesic	Representative drugs	Animal tests (ED50 mg/kg)				Approximate human analgesic dose
		Rat tail pressure	Mouse H & F	Anti-bradykinin rat	Morphine antagonism	
Anti-inflammatory weak analgesic with peripheral action	Aspirin	⩾100 oral ⩾100 s.c./i.p.	100·0 oral 22·0 s.c. 25·0 i.p.	⩾500 oral 125 i.p.	— —	300–600 mg oral
	Phenylbutazone	⩾100 i.p.	5·6 s.c. 12·0 i.p.	32·0 i.p.	—	100–200 mg oral
	Mefenamic acid	⩾100 i.p.	43·0 i.p.	> 300 i.p.	—	500 mg oral
Weak analgesic central action	Codeine phosphate	17·0 s.c.	5·6 s.c.	38·5 s.c.	—	60 mg oral/s.c.
Strong analgesic central action	Morphine hydrochloride Etorphine hydrochloride (M99) Fentanyl	2·5 s.c. 0·0017 s.c. 0·02 s.c.	0·64 s.c. 0·0004 s.c. 0·034 s.c.	1·1 s.c. 0·00086 s.c. 0·0080 s.c.	— — —	10–15 mg. s.c./i.m. 0·05 mg s.c. 0·5 mg s.c.
Weak narcotic antagonist	Pentazocine M5046 hydrochloride	⩾100 s.c. ⩾100 s.c.	3·0 s.c. 11·5 s.c.	1·85 s.c. 2·50 s.c.	60·0 s.c. 8·0 s.c.	20–30 mg i.m. ?
Strong narcotic antagonist	Nalorphine hydrobromide Levallorphan tartrate M285 hydrochloride M5050 hydrochloride	⩾100 s.c. ⩾100 s.c. ⩾100 s.c. ⩾100 s.c.	2·1 s.c. 2·4 s.c. 0·028 s.c. > 100 s.c.	5·0 s.c. 125·0 s.c. 2·3 s.c. > 100 s.c.	0·48 s.c. 0·30 s.c. 0·015 s.c. 0·004 s.c.	15 mg s.c./i.m. ? 0·5–1·0 mg i.m. ?
Tranquillizer/ analgesic	Methotrimeprazine Chlorpromazine	⩾100 i.m. ⩾100 i.m.	0·39 i.m. 0·79 i.m.	> 50 i.m. > 50 i.m.	— —	15–30 mg i.m. 0

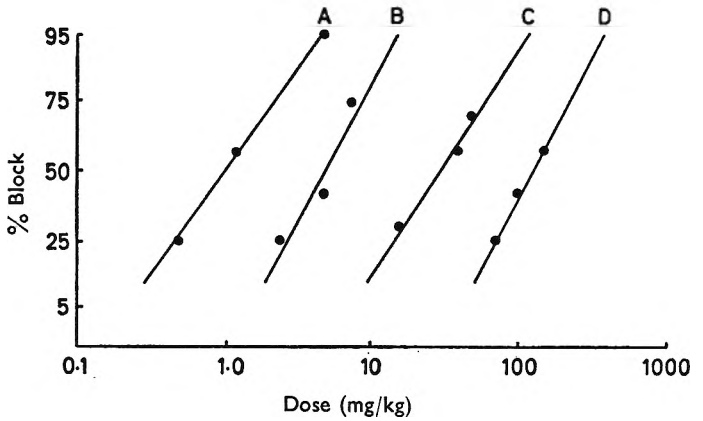

FIG. 2. Dose-response lines obtained with four analgesic drugs in the bradykinin-antagonism test. A. Morphine. B. Nalorphine. C. Phenylbutazone. D. Aspirin. Each point represents the mean response of 5 rats at the time of peak-effect for the analgesic agent. The probit of the proportion of animals in which the bradykinin-induced syndrome is blocked by the analgesic (ordinate) is plotted against logarithm of dose (abscissa). The lines do not deviate significantly from parallelism at the 95% level of confidence.

Weak and strong narcotic analgesics were active in all three tests, the mouse test being the most, and the rat tail-pressure the least sensitive of the three. In all tests, etorphine, fentanyl and morphine were correctly ranked in this descending order of potency. Fentanyl and etorphine appear to be less potent in man, relative to morphine, than might be predicted from the animal test results.

The narcotic antagonists used were consistently inactive in the rat tail-pressure test and, with the exception of M5050 [*N*-(cyclopropylmethyl)-tetrahydro-7(1-hydroxy-1-methlethyl)-6,14-*endo*ethanonororipavine (Bentley, 1967)], active in the Hendershot & Forsaith test. These findings present no novelty but it was of interest to find the antagonists, other than levallorphan and M5050, to be active in the bradykinin-antagonism test. There appears to be no correlation of the potency of these drugs as antagonists of morphine in the rat, and their activity in tests for analgesia; nor can morphine-antagonist activity be related with human analgesic potency where it is known. Thus, for example, the extremely powerful antagonist M5050 appears to be entirely lacking in analgesic activity in the animals, while the very weak antagonist pentazocine is moderately active in both Hendershot & Forsaith and the bradykinin-antagonism tests. Taber & others (1964), Blumberg & others (1965) and Pearl & Harris (1966) reached the same conclusion but found good parallelism between the phenylquinone writhing test and the analgesic potencies reported in man. In general terms we too find the Hendershot & Forsaith test to have good predictive value. It ranks pentazocine, nalorphine and M285 [*N*-(cyclopropylmethyl)tetrahydro-7-(1-hydroxy-1-methylethyl)-6,14-*endo*ethenonororipavine; cyprenorphine hydrochloride

(Bentley, Boura & others, 1965)] in an ascending order of potency, and the values in man follow the same pattern. However, in our experience, levallorphan is almost as potent in the Hendershot & Forsaith test as is nalorphine. This raises an interesting point since Blumberg & others (1965) considered their relatively high ED50 of 25 mg/kg for levallorphan in the Hendershot & Forsaith test to be consistent with the lack of efficacy of this drug as an analgesic in man, quoting Foldes (1964) as their clinical authority. However, in a more recent evaluation in man, Keats & Telford (1966) found 8 mg of levallorphan to be almost equipotent with 10 mg of morphine and hence, by analogy, equipotent also with nalorphine (Lasagna, 1964) as predicted by our results from the Hendershot & Forsaith test.

Since levallorphan is only weakly active as an antagonist of bradykinin in the rat (ED50 125 mg/kg) the predictive value of this test in the antagonist-analgesic group of drugs cannot be considered assured. Much depends on the outcome of scheduled trials in man with M5046 [*N*-cyclopropylmethyl)tetrahydro-7-(1-hydroxy-1-methylethyl)-6,14-*endo*ethanonorthebaine (Bentley, 1967)], which by prediction from the animal test should be active but less potent than pentazocine, and M5050 which appears to be a highly specific antagonist without analgesic activity, as well as the results of Keats and Telford's further studies with levallorphan which are in progress.

We were unable to distinguish between chlorpromazine, which lacks analgesic activity in man, and methotrimeprazine which is reported to have about half the potency of morphine (Montilla, Frederik & Cass, 1963; Pearson & De Kornfeld, 1963; Lim, Miller, Guzman & others, 1966). Both drugs were active in the abolition of phenylquinone-induced writhing of mice but without effect in either rat tail-pressure or the bradykinin-antagonism tests.

In predicting the action in man from that in animals, no special advantage can be claimed for the bradykinin-antagonism test in the rat when compared with the mouse phenylquinone-induced writhing test. The area of particularly uncertain parallelism covers antagonist-analgesics and phenothiazines where the clinical reports are often contradictory. The preparation of the rats is relatively laborious and requires a degree of skill not called for in the mouse test. However, unlike the nociceptive stimuli usually associated with the production of experimental pain, the bradykinin stimulus is liminal and apparently not injurious. A secondary advantage accruing from this is the opportunity to follow the time-course of an analgesic effect in every animal under test.

The possible relation between bradykinin-induced pseudo-affective responses in animals, and pain in man has been emphasized by Guzman & others (1964) and by Lim, Guzman, Rogers & others (1964). Supporting evidence comes from the work of Burch & De Pasquale (1962), and more recently from that of Coffman (1965). These authors have described the sensations of pain produced by injection of small doses of the nonapeptide into the brachial artery of human volunteers and the block effected by normal doses of analgesic drugs. Also relevant are the investigations of

Keele (1960), on the basis of which he has suggested that locally-released bradykinin may act as a transmitter at visceral pain receptor sites.

Acknowledgements. We wish to express our appreciation to Sandoz Ltd. of Basel for the gift of a sample of purified bradykinin, and to thank Mr. A. L. A. Boura for many constructive discussions.

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J. Pharm. Pharmac., 1967, **19**, 374–382 Received December 9, 1966

Histamine release from the mast cells of guinea-pig lung

J. F. EILBECK AND W. G. SMITH

The distribution of mast cells and the effects of antigen on the mast cell population of sensitized guinea-pig lung have been examined. Doses of antigen, phospholipase A, trypsin, and compound 48/80 which released similar amounts of histamine also caused mast cell damage to similar extents following *in vitro* or *in vivo* administration. Pretreatment with ethanolamine, hydrocortisone or theophylline reduced the release of histamine and of SRS-A, and mast cell damage during subsequent anaphylaxis. Whilst there is evidence that the mast cell might be a selective target for the anaphylactic reaction in guinea-pig lung tissues, there is also evidence to suggest that the anaphylactic reaction induces generalized cell damage in these tissues.

IN 1955 Mota & Vugman reported that guinea-pig lung tissues had a high mast cell count and that sensitized animals given anaphylactic shock by the intracardiac injection of antigen exhibited a marked depletion of this mast cell population. Boreus & Chakravarty (1960) examined the *in vitro* effects of antigen on the mast cell content of guinea-pig lung tissues, and observed that the disappearance of mast cells could be correlated with the release of histamine and the slow reacting substance of anaphylaxis (SRS-A) from the tissue. These observations were extended by Boreus (1960, 1961) who made *in vivo* studies of the reactions of mast cells in the nasal mucosa of sensitized guinea-pigs to intra-arterial, intravenous, and topical administration of antigen.

Histamine can be released from sensitized guinea-pig lung tissue by trypsin, phospholipase A, and compound 48/80 as well as by antigen (Marquis & Smith, 1963). It is not known, however, whether the released histamine is in all instances derived from tissue mast cells. The histamine-releasing capacity of these substances has now been examined simultaneously with their effect on the mast cell population of guinea-pig lung tissues. The effect of pretreatment with anti-anaphylactic doses of theophylline (Firth & Smith, 1962), ethanolamine (Smith, 1961; Goadby & Smith, 1965), and hydrocortisone (Goadby & Smith, 1964; 1965) on the subsequent reaction of guinea-pig lung mast cells to anaphylaxis *in vitro* is also reported. These experiments were made to see if they supported the view advanced by Boreus & Chakravarty (1960) that the mast cell is the main, and perhaps the only, source of histamine and SRS-A released from guinea-pig lung tissue by antigen or histamine releasing agents.

Experimental

MATERIALS

Egg white was separated from the yolks of fresh eggs and freeze-dried immediately. The trypsin used was crystalline (B.D.H.). The Russell

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viper venom, used as a preparation of phospholipase A, and compound 48/80 were kindly donated by Messrs Burroughs Wellcome & Co.

INITIAL TREATMENT OF ANIMALS

Guinea-pigs of either sex, weighing between 200 and 250 g, were fed on Diet 18 pellets (Oxo Ltd.) and received 50 mg of ascorbic acid daily in their drinking water. Each animal was sensitized by the intraperitoneal injection of 100 mg of freeze-dried egg white dissolved in 2 ml of normal saline. After a sensitization period of 21 days, the animals were subjected to anaphylactic shock *in vivo* by the method of Herxheimer (1952) in which each animal is exposed to an aerosol of a 1% w/w solution of egg white in a Wright aerosolizer (Wright, 1958) at 15 lb/in.^{2} Animals were removed from the chamber in which they were exposed to antigen immediately before the characteristic convulsions of anaphylaxis. The time in seconds taken by each animal to reach this stage was termed the *collapse time* and was regarded as a measure of the sensitivity of each animal to the anaphylactic reaction. Seven to 10 days after exposure to the aerosol of antigen the animals were weighed and distributed into groups of six, such that the mean values for collapse time and body weight were approximately equal for each experimental group.

HISTOLOGY

Lung tissue was fixed according to Mota & Vugman (1956) by the injection of fixative through the trachea and main bronchi. The fixative solution contained 50% ethanol and 4% lead subacetate and was acidified with 0·5% glacial acetic acid. The tissue was stored in fixative for 24 hr before sections, 50μ thick, were cut routinely from samples (5 samples per lobe from 2 lobes per animal). These were cut on a freezing microtome and then stained with 0·1% ethanolic toluidine blue acidified to pH 4·0 with glacial acetic acid. Paraffin sections, 10μ thick, were also prepared for detailed histological examinations.

The frozen sections (2 sections/tissue sample; 20 sections/animal) were used to count the tissue mast cell population, according to Mota & Vugman (1956), Padawar (1963) and Boreus (1960). Each section was examined microscopically so that each field covered an area of 0·0154 mm^2 of lung tissue. Ten such fields were chosen at random for each section and the number of mast cells recorded. The total number in 100 fields was taken as the *mast cell count* (M.C.C.) for each animal. From these values a *mean mast cell count* (M.M.C.C.) for each group of animals was calculated. Differences in the values obtained for test and control groups were measured by the *mast cell disappearance value* (M.C.D.V.) introduced by Boreus (1960). This is given by:

$$\text{M.C.D.V.} = \frac{\text{M.M.C.C. (control)} - \text{M.M.C.C. (test)}}{\text{M.M.C.C. (control)}} \times 100$$

MEASUREMENT OF HISTAMINE RELEASE *in vitro*

Histamine released from perfused isolated lungs by the action of antigen, phospholipase A, cystalline trypsin, and compound 48/80 was collected by the method of Brocklehurst (1960). Except in the case of antigen, attempts to measure the histamine content of the vascular perfusates so obtained met with difficulty because contamination with histamine releaser interfered with the biological assay of histamine. To overcome this, before assay, perfusates containing histamine releasers other than antigen were extracted according to Barsoum & Gaddum (1935) as simplified by Code (1937). This treatment did not permit accurate assay of histamine in perfusates contaminated with compound 48/80, but the depression of histamine was consistent over the dose range of compound 48/80 used and was not great. A correction factor of 1·19 was calculated from data from control solutions of histamine containing added compound 48/80 and this factor was subsequently applied to all compound 48/80 perfusates. Four-point assays of histamine using a latin square design were made on guinea-pig terminal ileum in the presence of atropine 10^{-7}M. All values for histamine were calculated as histamine base.

ESTIMATION OF HISTAMINE RELEASE *in vivo*

Histamine release following the intracardiac injection of antigen, phospholipase A, trypsin, or compound 48/80 into groups of 6 animals was estimated by collecting 2 ml blood samples from each animal 5 min after injection. The samples from each group of animals were pooled into 20 ml of 10% aqueous trichloroacetic acid which was subjected to Code's extraction procedure. The extract was assayed for histamine content and the mean histamine value per ml of blood calculated. From the known body weights of the animals and the figures for blood volume per kg quoted by Dittmer (1961), the blood volume of each animal was obtained and the mean blood volume for each group of animals calculated. Using the mean blood volume and the estimated mean histamine content per ml, the total blood histamine of each group was calculated.

MEASUREMENT OF LUNG HISTAMINE CONTENT

The histamine content of guinea-pig lung tissues was determined by extraction from weighed samples of tissue after grinding with sand and 10% aqueous trichloroacetic acid solution in the proportions of 10 ml/g of wet tissue. This extract was treated by the procedure of Code (1937). The resulting aqueous solution of histamine was assayed for histamine base on guinea-pig terminal ileum in the presence of atropine 10^{-7}M. From the weights of sample and original whole lung, estimates were made of the total lung histamine content per animal.

MEASUREMENT OF THE SLOW-REACTING SUBSTANCE OF ANAPHYLAXIS

SRS-A was assayed on guinea-pig terminal ileum in the presence of atropine 10^{-7}M and mepyramine 10^{-7}M by comparison with a laboratory standard. In each case, a four-point assay of latin square design was made.

TREATMENT OF ANIMALS WITH ANTI-ANAPHYLACTIC AGENTS

Ethanolamine. Each animal received the equivalent of 200 mg/kg ethanolamine base as the hydrochloride each day for three days by intramuscular injection, the last injection being made 1 hr before death (Smith, 1961).

Hydrocortisone. Each animal was given a single intramuscular injection of 100 mg/kg of hydrocortisone (as the sodium hemisuccinate salt dissolved in water) 18 hr before death (Goadby & Smith, 1964).

Theophylline. A single dose of 80 mg/kg of theophylline (dissolved in water with the aid of ethylenediamine) was given intraperitoneally to each animal 15 min before death (Firth & Smith, 1962).

Results

THE DISTRIBUTION OF MAST CELLS IN GUINEA-PIG LUNG TISSUES

Tissue mast cells were observed to be widely distributed throughout the lung tissues. The cells were spherical or ovoid, between 10 and 25 μ in diameter or length, densely packed with granules which often obscured the nucleus and stained metachromatically. They were especially numerous in the pleura, in pleural invaginations into the parenchyma of the lung, and in the connective tissue surrounding the bronchi and bronchioles. They were much less numerous in the alveolar walls and the mucosa of the large bronchi.

THE RELEASE OF HISTAMINE *in vitro*

Histamine release from perfused sensitized guinea-pig lung tissues

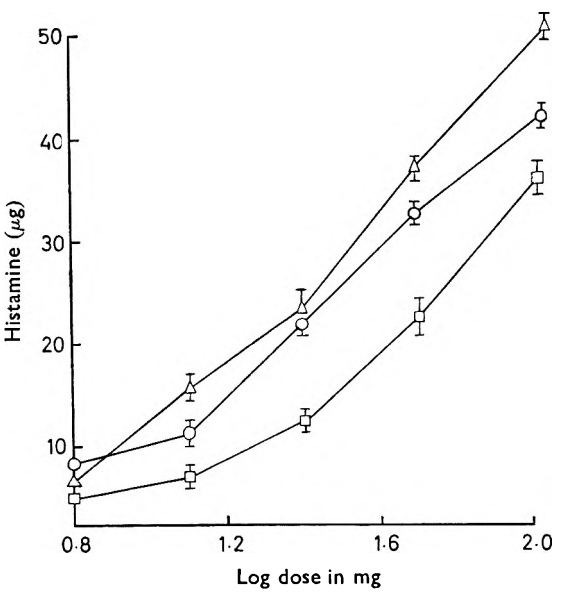

FIG. 1. Histamine released from sensitized guinea-pig lung *in vitro* by increasing doses of antigen ○, phospholipase A □, and trypsin △. Ranges indicated are standard errors.

induced by increasing doses of all four histamine releasers was measured. The results were plotted as log dose-effect curves. (Fig. 1). With antigen, phospholipase A or trypsin, increasing doses of each substance released increasing amounts of histamine. The curve obtained with compound 48/80 (Fig. 2) differed in that, as the dose was increased the

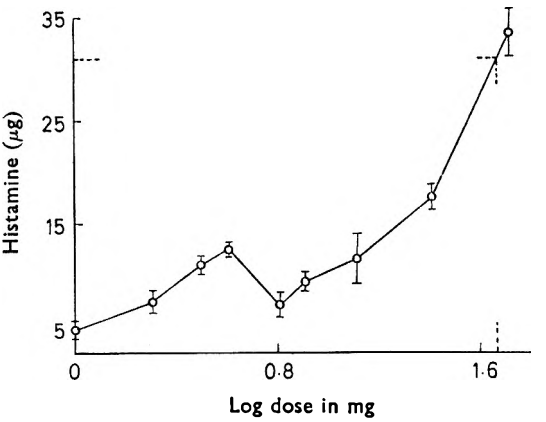

FIG. 2. Histamine released from sensitized guinea-pig lung *in vitro* by increasing doses of compound 48/80. Ranges indicated are standard errors.

release of histamine increased, then decreased, and finally increased again. This *dosage peak* was also examined in relation to mast cell disappearance.

MAST CELL DISAPPEARANCE *in vitro* AND *in vivo*

The mean mast cell counts for groups of animals exposed to increasing doses of antigen decreased. This is shown in Fig. 3 where the effect on the tissue mast cell population is expressed as the mast cell disappearance

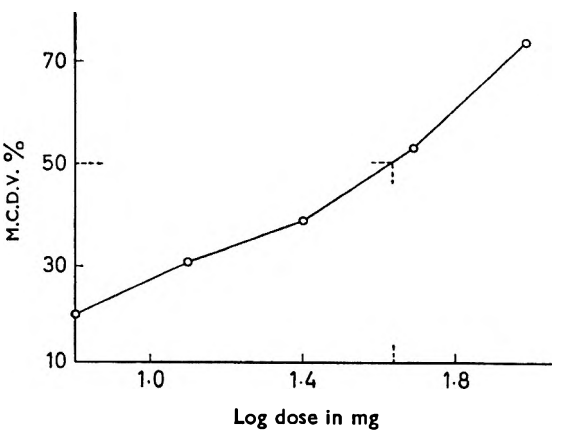

FIG. 3. Effect of increasing doses of antigen on the *mast cell disappearance value* (M.C.D.V.) observed in sensitized guinea-pig lung.

value. This value increases progressively with increasing doses of antigen. Apart from the disappearance of mast cells after anaphylaxis, some of the remaining mast cells were damaged but not totally degranulated, and there were also signs of peribronchial oedema and emphysema in the tissue.

It was found from Fig. 3 that 44 mg of antigen caused the disappearance of 50% of the mast cell population after anaphylaxis. From Fig. 1 it was estimated that this dose of antigen liberated 31 μg of histamine. From this figure it can be calculated that 31 μg of histamine was released by 35 mg of phospholipase A, or 77 mg of trypsin, or 45 mg of compound 48/80. These equiactive doses were then tested *in vitro* and *in vivo* to compare both their histamine-releasing properties and their effects on the mast cell population of guinea-pig lung tissue (Table 1).

TABLE 1. HISTAMINE RELEASE AND MAST CELL COUNTS OBSERVED AFTER THE ADMINISTRATION OF EQUIACTIVE DOSES OF HISTAMINE RELEASERS *in vitro* TO LUNGS REMOVED FROM GROUPS OF 6 SENSITIZED GUINEA-PIGS OR *in vivo* TO GROUPS OF 6 SENSITIZED GUINEA-PIGS

	Control	Antigen 44 mg	Phospholipase A 35 mg	Trypsin 77 mg	Compound 48/80 45 mg
In vitro					
Mean histamine release μg ..	0·35	30·7 (5·6)	33·4 (6·0)	28·8 (3·9)	26·1 (4·3)
Mean mast cell count	206·9 (34·2)	99·5 (19·6)	94·2 (16·3)	117·7 (16·9)	110·8 (1·6)
Mast cell disappearance value %	—	51·9	54·5	43·1	46·4
In vivo					
Estimates of mean circulating blood histamine μg ..	1·9	25·2	28·1	20·8	25·9
Mean mast cell count	206·9 (34·2)	110·2 (31·4)	99·5 (21·9)	111·0 (26·8)	120·8 (20·0)
Mast cell disappearance value %	—	46·7	51·9	46·3	41·6

Figures in parentheses are standard deviations.

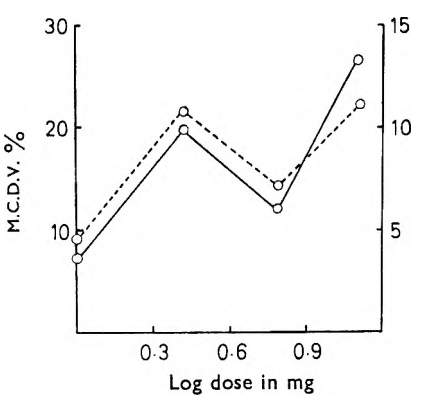

FIG. 4. Mast cell disappearance (O——O) and histamine release (O——O) in guinea-pig lung following increasing dosage with compound 48/80.

In each instance the histamine releasers effected a similar and substantial release of histamine; at the same time there was a large reduction in the numbers of mast cells in the tissue. Using Student's *t* test, it was found that histamine release and mast cell disappearance after equiactive doses of phospholipase A, trypsin, and compound 48/80 were not statistically significantly different from those induced by antigen.

Mast cell counts were also made on tissue removed from animals treated with small doses of compound 48/80 over the range in which an *initial low dosage peak* of activity had been observed. Fig. 4 shows that histamine release and mast cell disappearance followed similar courses over this dose range.

MAST CELL DISAPPEARANCE IN ANIMALS PRETREATED WITH ANTI-ANAPHYLACTIC AGENTS

Treatment of sensitized animals with ethanolamine or theophylline did not alter the lung histamine content or the mast cell population. Animals treated with hydrocortisone, however, differed from untreated animals in that lung histamine levels were significantly lower (at P = 0·95) and the mast cell count had the lowest mean value of any recorded in these experiments although it was not statistically different from the control value (Table 2). The effects of these three compounds on a

TABLE 2. THE EFFECT OF ANTI-ANAPHYLACTIC AGENTS ON THE HISTAMINE CONTENT AND MAST CELL COUNT OF LUNGS FROM GROUPS OF 6 SENSITIZED GUINEA-PIGS

Treatment	Total histamine content μg	Mean mast cell count
Nil	82·3 (10·3)	209·5 (38·2)
Ethanolamine	88·5 (20·4)	217·5 (39·8)
Hydrocortisone	69·8* (3·2)	184·0 (26·7)
Theophylline	73·3 (9·5)	201·0 (24·6)

Figures in parentheses are standard deviations.

* Difference from control is significant at P = 0·95.

subsequent anaphylactic reaction *in vitro* were similar (Table 3). Histamine and SRS-A release were reduced. At the same time the shock-induced fall in mast cell count was significantly reduced by all three anti-anaphylactic agents.

TABLE 3. THE EFFECT OF ANTI-ANAPHYLACTIC AGENTS ON THE RELEASE OF HISTAMINE, THE RELEASE OF SRS-A, AND THE MEAN MAST CELL COUNTS OF LUNGS TAKEN FROM GROUPS OF 6 GUINEA-PIGS AND SUBJECTED TO ANAPHYLAXIS *in vitro*

Treatment	Histamine release μg	SRS-A Release units/ml	Mean mast cell count
Nil	33·4 (4·7)	24·5 (9·3)	95·5 (17·9)
Ethanolamine	21·5* (4·8)	14·8* (4·3)	125·8* (18·6)
Hydrocortisone	16·2* (9·4)	14·3* (9·4)	131·2* (26·2)
Theophylline	20·2* (5·2)	14·7* (8·1)	121·2* (23·7)

Figures in parentheses are standard deviations.

* Difference from control is significant at P = 0·95.

Discussion

Doses of phospholipase A, trypsin and compound 48/80 causing the release of similar amounts of histamine from guinea-pig lung tissue either *in vitro* or *in vivo*, did not differ significantly in their ability to cause mast cell disappearance. In groups of guinea-pigs pretreated with anti-anaphylactic agents, anaphylactic shock *in vitro* resulted in the release of reduced amounts of histamine and SRS-A and a reduction in the fall in mast cell count. Collectively, these results support the hypothesis advanced by Boreus & Chakravarty (1960) that the mast cell is the main source of histamine and SRS-A released from guinea-pig lung by anaphylaxis or histamine-releasing agents.

From this evidence it might be argued that the mast cell in guinea-pig lung tissue is a selective target for the antigen-antibody reaction of anaphylaxis or histamine-releasing agents. However, equi-active doses of these agents do not produce identical effects on the lipid content of the tissue (Marquis & Smith, 1963). Phospholipase A and compound 48/80, like antigen, provoke a loss of phospholipid, but trypsin does not. Whereas antigen induces a fall in lung cholesterol, the other three agents do not have this effect. It therefore appears that the loss of lipid which occurs during histamine release is not confined to mast cells, but must be regarded as an index of more widespread cell damage.

Mast cells are fragile. They can be readily degranulated in mesentery if tension is applied to this tissue when spreading it on microscope slides. In view of this, the occurrence of mast cell degranulation in a tissue as the result of biochemical injury is a reasonable supposition. Other evidence is being accumulated in favour of a concept whereby anaphylaxis produces non-specific cell damage, rather than specific damage to mast cells. Guinea-pig lung subjected to anaphylaxis rapidly resynthesizes its lost phospholipid (Goadby & Smith, 1966). This recovery occurs in a few hours, and is unlikely to be a function of a mast cell population which requires at least 3 weeks for restoration to normal levels.

The demonstration of Riley & West (1953) that histamine is found in tissue mast cells has been repeatedly confirmed, and the present findings indicate that mast cell histamine is the source of histamine released in anaphylaxis. The same might be true of SRS-A. Whilst mast cells might also be the source of SRS-A, evidence for its occurrence in them is lacking; and although it is easy to assume that like histamine, SRS-A is released only from mast cell sources during anaphylaxis, the data so far obtained do not preclude the possibility that SRS-A is a product of more widespread cell damage. The histamine releasers examined also release a slow reacting substance (Marquis & Smith, 1963) but not the same substance for each releaser; a situation more compatible with widespread cell damage than with specific damage to one particular type of cell.

Thus it seems that an anaphylactic reaction in guinea-pig lung tissue induces two effects; a generalized cell damage involving loss of lipid from the tissue, and a disappearance of mast cells. If the latter effect

is dependent on the former, ethanolamine, hydrocortisone, and theophylline could be considered to exert their anti-anaphylactic effect by reducing the severity of the generalized cell damage and this would be manifest from one aspect as a reduction in mast cell disappearance.

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The excretion of [^{14}C] butylated hydroxytoluene in the rat

L. G. LADOMERY, A. J. RYAN AND the late S. E. WRIGHT

The excretion of small doses of [^{14}C]labelled butylated hydroxytoluene has been examined in the rat. Parenteral doses are excreted slowly in urine and faeces over 4 days. Biliary excretion is rapid. The biliary radioactivity is absorbed readily from the gut and a rapid enterohepatic cycle has been found to operate over at least 96 hr.

BUTYLATED hydroxytoluene (BHT, 3,5-di-t-butyl-4-hydroxytoluene) is an antioxidant used widely in foods. It is therefore important that the toxicology, distribution, metabolism and excretion of this compound should be well understood. Dacre (1961) and Akagi & Aoki (1962) studied its metabolism in rabbits. The quantitative urinary excretion of isotopically labelled BHT has been examined by several workers. Golder, Ryan & Wright (1962) recovered only 33% of the radioactivity in urine from rats given small doses of tritiated BHT. This result was confirmed by Ladomery, Ryan & Wright (1963) using [^{14}C]BHT. Daniel & Gage (1965), using higher doses of [^{14}C]BHT found similar urinary excretion and also noted high faecal excretion. All these authors found a slow rate of excretion, which was not complete even after a week.

The present paper is concerned with the biliary excretion by rats of small doses of [^{14}C]BHT.

Experimental

[^{14}C]3,5-Di-t-butyl-4-hydroxytoluene (2,6-di-t-butyl-*p*-cresol; [^{14}C]BHT) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. It was used as 0·02% solution in 50% aqueous ethanol.

METABOLIC EXPERIMENTS

White male rats of 300–350 g were given [^{14}C]BHT (100 μg, except where otherwise stated) in aqueous ethanol (0·5 ml) either by intraperitoneal or intravenous injection. Urine and faeces were collected in a metabolic cage fitted with an all-glass separating device. Bile was collected from cannulated rats maintained under urethane anaesthesia. Ringer solution (1 ml) was given every 3 hr to replace lost water and the animals were kept warm. The "linked animal experiment" was carried out by passing the bile cannula from one rat into the duodenum of a second rat. This cannula was cut and joined with a short section of a hypodermic needle in order to check bile flow. The first rat was raised 5 cm above the second to assist the flow of bile into the duodenum. Bile was collected from the second rat.

RADIOACTIVE COUNTING

Urine and bile were counted directly by the addition of 0·1–0·2 ml to 10 ml of scintillation solvent consisting of 2,5-diphenyloxazole (5·0 g), 1,4-bis-2-(5-phenyloxazolyl)benzene (0·05 g), absolute ethanol (300 ml)

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and toluene (800 ml). Faeces were dried, weighed and a powdered aliquot extracted continuously with methanol. Aliquots of the methanolic solution were counted as above. Tissues were homogenized in ethanol, centrifuged and the supernatant assayed. The contents from the small intestine were diluted with methanol, centrifuged and the supernatant counted. The contents of the large intestine were treated in the same way as the faeces. The samples were counted in a Packard Scintillation Counter, model 3314. Counting efficiencies were determined with an external source.

Results

The low urinary excretion of [^{14}C]BHT observed earlier (Ladomery & others, 1963) led us to examine the faeces of dosed animals for radioactivity. Table 1 shows that over 4 days 37% of a parenteral dose of

TABLE 1. DAILY URINARY AND FAECAL EXCRETION OF RADIOACTIVITY AFTER A SINGLE INTRAPERITONEAL DOSE OF [^{14}C]BHT

Day	% Excreted in urine*	% Excreted in faeces*
1	5·7 ± 0·5	16·7 ± 1·0
2	10·5 ± 0·8	7·5 ± 0·3
3	10·3 ± 0·6	7·3 ± 0·1
4	5·5 ± 0·4	5·4 ± 0·4
Total	32·0 ± 0·6	36·9 ± 1·2

* ± Standard deviation; 6 rats used.

[^{14}C]BHT was excreted by this route. The urinary excretion agreed with our previous results. Since only about 70% of the dose was accounted for, the general distribution of radioactivity from [^{14}C]BHT in rats was examined. The results are in Table 2. Four groups of six rats were

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN RATS AFTER A SINGLE INTRAPERITONEAL DOSE OF [^{14}C]BHT. Values are the percentage of the administered dose; urine and faeces are cumulative

Day	Urine	Faeces	Contents of small intestine	Contents of large intestine	Liver	Spleen	Kidney	Intestinal wall	Total accounted for
1	4·5	7·6	16·9	6·1	2·1	0·03	0·09	7·5	44·8
2	15·6	20·9	9·8	4·7	1·8	0·02	0·08	4·9	57·8
3	26·3	26·1	9·6	3·9	1·0	0·02	0·02	2·9	69·8
4	31·2	37·9	7·4	2·1	0·9	0·14	0·10	1·6	81·3

dosed. Urine and faeces were pooled at 24-hourly intervals. At the same time a group of rats was killed and some of the tissues examined for radioactivity. The urinary and faecal excretion agree well with those already found. Little activity appeared in the liver, kidney or spleen. However, significant amounts appeared in the intestinal contents and the intestinal wall. This totalled about a third of the radioactivity remaining in the body.

From these results it seemed likely that there was some biliary excretion of radioactivity from [^{14}C]BHT and possibly an enterohepatic cycle. The

biliary excretion of [^{14}C]BHT was examined and found to be surprisingly high. After 6 hr, radioactivity equivalent to 95% of an intravenous dose and 52% of an intraperitoneal dose appeared in the bile (Table 3).

TABLE 3. BILIARY EXCRETION OF RADIOACTIVITY BY RATS AFTER SINGLE DOSES OF [^{14}C]BHT

Time (hr)	% Excreted from 100 μg given intravenously*	% Excreted from 100 μg given intraperitoneally*	% Excreted from 10 mg given intravenously*
1	46·6 ± 7·5	16·0 ± 4·5	28·5 ± 7·8
2	25·0 ± 8·0	15·5 ± 4·6	28·9 ± 6·5
3	12·6 ± 2·6	8·9 ± 1·1	8·2 ± 2·0
4	4·7 ± 1·8	5·2 ± 1·3	3·3 ± 0·5
5	2·9 ± 0·6	3·4 ± 0·7	1·5 ± 0·4
6	1·9 ± 0·4	2·8 ± 0·2	1·0 ± 0·2
Total	93·7 ± 11·6	51·8 ± 6·6	71·4 ± 10·4

* ± Standard deviation; 6 animals used in each experiment.

These high recoveries supported the idea of an enterohepatic cycle of BHT metabolites. This cycling was confirmed using pairs of rats with the bile duct cannulated, in which the cannula from one rat (A) was inserted into the duodenum of the second (B). Bile was collected from rat B after rat A was given an intravenous dose of [^{14}C]BHT. Nearly 30% of the dose given to rat A was excreted by rat B after 10 hr (Table 4).

The efficiency of the biliary excretion of BHT was shown when 10 mg was given intravenously. In this instance over 70% of the dose was recovered in the bile after 6 hr (Table 3).

TABLE 4. BILIARY EXCRETION OF RADIOACTIVITY BY LINKED RATS AFTER A SINGLE DOSE OF [^{14}C]BHT

Time (hr)	% dose excreted*	% dose excreted per hr
2	0·2 ± 0·07	0·10
4	5·1 ± 1·4	2·53
6	7·6 ± 0·6	3·80
8	8·0 ± 1·8	4·01
10	9·0 ± 1·3	4·50
Total	29·9 ± 2·7	

* ± Standard deviation; 5 pairs of rats used.

With this demonstration of the enterohepatic cycling of BHT metabolites it became important to determine for how long the cycle functioned. Sixteen rats were given parenteral doses of [^{14}C]BHT and in four of them the bile duct was cannulated every 24 hr. Radioactivity appeared in significant amounts in the bile even 96 hr after dosing, when nearly 10% of the dose was excreted (Table 5).

The efficiency of the biliary excretion was demonstrated in an additional experiment. Bile obtained from a rat given [^{14}C]BHT was injected intravenously (1 ml) into rats. Bile collected from these animals contained 93·0 ± 2·0% of the injected radioactivity after 6 hr. None was found in the bladder contents. This emphasizes the remarkable affinity of the liver for BHT derivatives. The bile contains mainly conjugates (Ladomery, Ryan & Wright, 1967) and it might be expected

TABLE 5. THE RATE OF BILIARY EXCRETION OF RADIOACTIVITY AT 24-HOURLY INTERVALS BY RATS AFTER A SINGLE INTRAPERITONEAL DOSE OF [^{14}C]BHT

Time after dosing (hr)	% dose excreted in 6 hr*	% dose excreted/hr*	Predicted % dose excreted/hr	Rate of excretion as % dose remaining/hr
24	26·0 ± 4·1	4·3 ± 0·7	3·9	4·8
48	20·5 ± 3·0	3·4 ± 0·5	2·9	5·3
72	13·8 ± 2·0	2·3 ± 0·3	2·2	4·8
96	9·6 ± 1·6	1·6 ± 0·3	1·4	5·1
				mean 5·0 ± 0·2

* ± Standard deviation; 4 groups of 4 rats used.

that intravenous injection would lead to more urinary excretion than was found. However, Williams, Millburn & Smith (1965) have found high biliary excretion of certain glucuronides. It is clear that there is no simple relation between polarity and excretion route.

Discussion

The present work has confirmed the low urinary excretion of tritiated BHT found by Golder & others (1962). The slow urinary excretion of [^{14}C]BHT led us to examine the faecal excretion of activity. After a single parenteral dose urinary excretion is about 32–35% and faecal excretion is 35–37%, accounting for 70% of the radioactivity after 4 days (Table 2).

The result suggested that there might be some accumulation of BHT in tissues. However, under our dosage conditions little radioactivity was found in the organs examined. The liver with 2% contained most. Examination of the intestinal contents, however, revealed that these, together with the gut wall, contained about 30% of the activity remaining in the body after allowing for the urinary and faecal excretion (Table 3). Tye, Engel & Rapien (1965) found only a small amount of BHT in the tissues at much higher dose levels (44 mg/kg) than used in the present study. Daniel & Gage (1965) accounted for about 80% of oral doses of BHT. Faecal excretion in their experiments was much higher than that found in this work. However, this may be due to poor absorption of the BHT since it was given in olive oil, in which it is readily soluble. The laxative properties of olive oil could also be a factor in these results. The slow excretion and the constancy of the radioactive pool in the gut suggested that biliary excretion of BHT might be important. This was shown to be the case when 95% of an intravenous dose of [^{14}C]BHT appeared in the bile of cannulated rats (Table 3). An intraperitoneal dose of [^{14}C]BHT was excreted to the extent of 52% in the bile. This slower rate of excretion of a parenteral dose may be due to the rate of absorption from the peritoneal cavity. However, in both instances the rate of biliary excretion of radioactivity is greater than the daily urinary or faecal excretion. Coupled with the remarkable constancy of the proportion of radioactivity in the gut it is clear that enterohepatic cycling takes place.

Proof of this was obtained from the "linked animal experiment" (Table 4). The 30% of radioactivity recovered in the bile of rat B indicates that the circulation between bile and intestines is rapid. The

steady state attained after about 8 hr (Table 4) could be due to an active intestinal absorption process which becomes saturated. This is supported by the fact that intravenous injections of [^{14}C]BHT at 100 times our usual dose level are excreted to the extent of 71% of the dose in bile (Table 3) showing that the biliary system is freely permeable to the BHT metabolites. From Table 4 the steady state excretion of BHT metabolites in the bile approaches 5% of the residual radioactivity per hr. Using this figure, the biliary excretion of radioactivity was calculated for various time intervals after a parenteral dose. Table 5 shows the results obtained with rats given [^{14}C]BHT and cannulated at the bile duct 24–96 hr after dosing compared with the predicted excretion. A plot of the logarithm of the per cent dose/hr excreted (Wagner, 1961) against mean collection time for the experimental and predicted figures in Table 5 gives two straight and parallel lines. This provides further support that the enterohepatic circulation of BHT metabolites follows first order kinetics at the rate of approximately 5% of the remaining radioactivity per hr.

Acknowledgements. This work forms part of a research programme supported by the National and Medical Research Council of Australia and Grant EF-258 of the U.S. National Institutes of Health.

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J. Pharm. Pharmac., 1967, **19**, 388–394 Received January 2, 1967

The biliary metabolites of butylated hydroxytoluene in the rat

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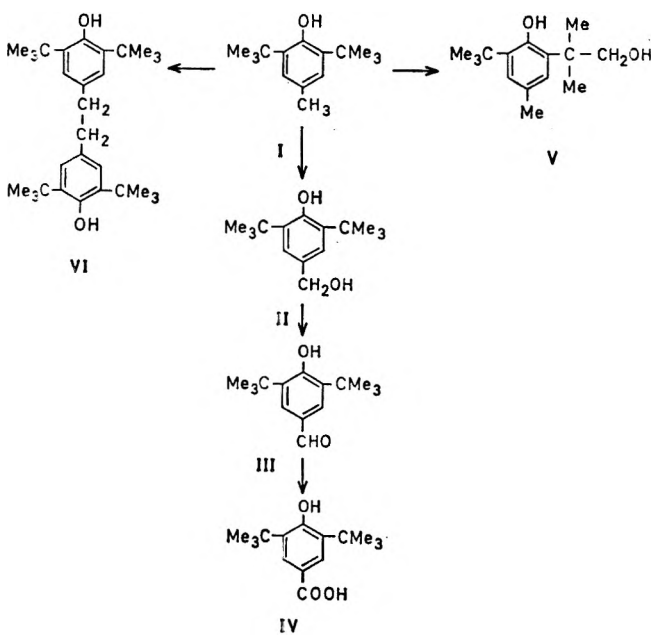
The biliary metabolites from intravenous and intraperitoneal doses of small amounts of [^{14}C]butylated hydroxytoluene have been separated and estimated. The metabolites recognized were the alcohol (II), aldehyde (III) and acid (IV) together with small amounts of the diphenyl ethane (VI). Three other metabolites appear to be present in relatively large amounts. The pattern of metabolites in bile and urine has been compared. It is suggested that the relatively low excretion of the acid (IV) in the urine compared to bile is due to a selective reabsorption of this compound after biliary excretion.

IN a previous paper, Ladomery, Ryan & Wright (1967) described the excretion of small doses (100 μg) of butylated hydroxytoluene (BHT; I) in the rat. They found that BHT was excreted slowly in urine and faeces because of a rapid enterohepatic circulation. This indicated that BHT was being rapidly metabolized since BHT itself is a non-polar molecule which would not be expected to be excreted by the liver unchanged. This paper describes the detection and estimation of the biliary metabolites from rats given small doses of [^{14}C]BHT. Previous metabolic work on BHT (Dacre, 1961; Akagi & Aoki, 1962) is summarized in Fig. 1.



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Experimental

MATERIALS

[^{14}C]3,5-*Di-t-butyl-4-hydroxytoluene* (2,6-di-t-butyl-*p*-cresol; [^{14}C]BHT) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. It was used as 0·02% solution in 50% aqueous ethanol.

3,5-*Di-t-butyl-4-hydroxybenzoic acid* (IV) was prepared by passing isobutylene into a well-stirred mixture of methyl *p*-hydroxybenzoate (6·16 g) and concentrated sulphuric acid (12·0 g) at 100°. When the theoretical quantity of gas (4·5 g) had been taken up (about 36 hr), the mixture was extracted with chloroform-light petroleum (b.p. 60–80°) (1 : 1, 150 ml) and the solution washed with 10% aqueous sodium hydroxide (4 × 50 ml). The organic phase was evaporated to give methyl 3,5-di-t-butyl-4-hydroxybenzoate (2·0 g), m.p. 167–168° from methanol. Dacre (1961) reports m.p. 165°.

The ester was hydrolysed by refluxing with an excess of 0·5N methanolic sodium hydroxide under N_2 for 4 hr. Dilution with water and acidification precipitated the acid, m.p. 219–220° from light petroleum (b.p. 60–80°). Dacre (1961) reports 210°; Yoke, Dunbar & others (1956) report m.p. 218°.

3,5-*Di-t-butyl-4-hydroxybenzaldehyde* (III) was prepared by the method of Campbell & Coppinger (1953), m.p. 189° (lit., m.p. 189°). The semicarbazone had m.p. 227° from ethanol. Found: C, 65·8; H, 8·3; N, 14·2%. $C_{16}H_{25}N_3O_2$ requires C, 66·0; H, 8·6; N, 14·4%.

3,5-*Di-t-butyl-4-hydroxybenzyl alcohol* (II) was prepared by reduction of the aldehyde with sodium borohydride, m.p. 145–146° from methanol or light petroleum (b.p. 60–80°) (Bolle & Tomaszewski, 1963, report 140°). Treatment of the alcohol with pyridine and acetic anhydride gave 3,5-di-t-butyl-4-hydroxybenzyl acetate, m.p. 99–100° from light petroleum (b.p. 60–80°). Akagi & Aoki (1962) report m.p. 98–99°.

1,2-*Bis*(3,5-*di-t-butyl-4-hydroxyphenyl*)*ethane* (VI) was prepared by hydrogenating 3,5,3′,5′-tetra-t-butylstilbene-4,4′-quinone (Cook, Nash & Flanagan, 1955) in ethanol at 35° and 200 lb/in^2 with Raney nickel. Evaporation of the ethanol gave the diphenylethane, m.p. 173–174° from aqueous acetone. (Cook, 1953, reports m.p. 174–175°).

CHROMATOGRAPHY

Bile samples were spotted on Whatman No. 1 paper and chromatographed in n-butanol–water (1 : 1) (solvent A). Spots were visualized with Gibbs reagent. Radioactive areas were detected by scanning the paper chromatogram with a Vanguard model 880 Autoscanner. Substances eluted from the paper were hydrolysed with acid and the ether soluble fraction chromatographed on Whatman No. 1 paper using cyclohexane–methanol (2 : 1) (solvent B). Spots were detected as before. These extracts were also chromatographed on thin-layers of silica gel with ether–light petroleum (1 : 1) (solvent C). Radioactive areas on thin-layer plates were detected by exposure to X-ray film.

METABOLIC EXPERIMENTS

Urine and bile were collected as described by Ladomery & others (1967). Hydrolyses were made by refluxing bile with 2N hydrochloric acid under nitrogen for 3 hr. Metabolites were extracted continuously using peroxide-free ether.

REVERSE ISOTOPE DILUTIONS

These were made by adding the required compound to bile. Determinations on unhydrolysed bile were made by adding ether (10 ml) and shaking well. The mixture was left standing at 3° for 48 hr with frequent shaking. The compound was re-isolated from the ether and purified to constant count. Hydrolysis experiments were made by refluxing the bile and reference compound with an equal volume of 4N hydrochloric acid. The hydrolysate was extracted with ether, the compound isolated and purified to constant count.

RADIOACTIVITY

This was assayed by liquid scintillation counting. Biological fluids and chromatogram eluates were accurately pipetted into scintillation fluid. Pure compounds were accurately weighed into vials and dissolved in the scintillator. Radioactive areas from thin-layer plates were removed and suspended in the scintillator. They were kept in the dark for 24 hr before counting.

Results

Paper chromatography of rat bile containing the metabolites from an intravenous injection of [^{14}C]BHT is summarized in Tables 1 and 2. Six

TABLE 1. PAPER CHROMATOGRAPHY OF UNHYDROLYSED RAT BILE AFTER INTRAVENOUS INJECTION OF [^{14}C]BHT

	Unhydrolysed bile; solvent system A		
Spot	Rf value	% Radioactivity excreted	Compound Rf value reported*
A	0·01	3·9	
B	0·08	4·3	
C	0·54	47·5	BHT-hippuric acid 0·5–0·55
D	0·60		BHT-ester glucuronide 0·8
E	0·83	28·0	conjugated phenols 0·95–1·0
F	0·96	16·1	
Total		99·8	95% of dose excreted

* Dacre (1961).

radioactive areas were detected. Spot C, which overlapped with D, corresponded to the glycine conjugate* of 3,5-di-t-butyl-4-hydroxybenzoic

* Wright, Akintonwa & others have been unable to detect this compound as a metabolite of (II) and have questioned Dacre's (1961) findings. We do not wish to imply a positive identification of any biliary conjugate in this present paper.

TABLE 2. CHROMATOGRAPHY OF ELUTED AND ACID HYDROLYSED BILIARY METABOLITES IN SYSTEM B

Spot hydrolysed	Rf value of hydrolysate	Reference compounds (Fig. 1)	Rf value
A	—*	IV	0·49
B	0·06	III	0·67
C + D	0·13, 0·52, 0·81	I	0·85
		VI	0·88
E	0·47		
F	0·43, 0·70, 0·89		

* Not detected after hydrolysis.

acid (IV) (47·5% of total activity) and spot E to its ester glucuronide (28%) (Dacre, 1961). Spot F was probably unconjugated phenols which amounted to about 16% of the total activity. This was confirmed by reverse isotope dilution (Table 3) which showed that the acid (IV),

TABLE 3. ESTIMATION OF [^{14}C]BHT METABOLITES IN RAT BILE AFTER INTRAVENOUS INJECTION OF [^{14}C]BHT

Compound	% Dose in unhydrolysed bile	% Dose in hydrolysed bile
I	0·5	0·5
VI	0·4	0·4
II	1·0	1·6
III	5·5	5·5
IV	4·4	39·5
Total	11·8	47·5

* 95% of administered radioactivity was excreted.

aldehyde (III) and alcohol (II), BHT dimer (VI) and BHT (I) together accounted for 12% of the biliary radioactivity before hydrolysis. Spots A, B and D were unidentified.

Elution of the radioactive areas from the paper chromatograms, acid hydrolysis and chromatography of the hydrolysates gave the results summarized in Table 2. Spot A was lost, spot B appeared to be unaltered. Spots C and D gave the acid (IV) and two unidentified spots. Spot E gave only the acid, while spot F gave the expected acid (IV), aldehyde (III) and dimer (VI). The alcohol (II) was probably in too low a concentration to be detected. Reverse isotope dilution studies (Table 3) of the bile hydrolysate showed that the acid (IV) was a major constituent, being nearly 40% of the total. The aldehyde (III), alcohol (II), BHT (I) and the dimer (VI) were minor constituents. Exactly half (47·5%) of the total activity was accounted for as these substances.

Because of a lack of reference conjugates it was decided to work with the bile hydrolysates. At this time it was found that better resolution of the biliary metabolites was achieved by thin-layer rather than paper chromatography. The chromatography of hydrolysed bile extracts after an intravenous injection of [^{14}C]BHT is shown in Table 4. Six radioactive areas were detected with X-ray film. Spot B did not appear in this bile, but was detected in "late" (72 hr) bile (see below) The relation between the unidentified compounds detected on thin-layer plates and on the paper

TABLE 4. THIN-LAYER CHROMATOGRAPHY OF ACID HYDROLYSED BILE AFTER AN INTRAVENOUS INJECTION OF [^{14}C]BHT (SOLVENT C)

Radioactive spot	Rf value	% Dose excreted	Reference compound (see Fig. 1)	Rf value
A	0·0	35·0		
B*	0·1	—		
C	0·25	37·1	IV	0·23
D	0·52	1·5	II	0·52
E	0·65	11·0		
F	0·71	4·5	III	0·71
G	0·88	1·1	VI	0·92
			I	1·0
Radioactivity residual		4·0		
Total†		94·2		

* Metabolite B was found only in bile collected 72 hr after dosing.
† 95% of dose excreted in bile.

chromatograms is unknown. Spot C was the acid (IV), D the alcohol (II), F the aldehyde (III) and G was either BHT (I) or the dimer (VI). Spots A and E were unidentified.

This thin-layer technique was then applied to separate and estimate the metabolites appearing in bile collected for 6 hr immediately after an intraperitoneal injection ("early" bile) and also in the bile collected 72 hr after injection ("late" bile). The results are summarized in Table 5.

TABLE 5. ELUTION AND ESTIMATION OF [^{14}C]BHT METABOLITES ON THIN-LAYER CHROMATOGRAMS OF HYDROLYSED BILE AFTER INTRAPERITONEAL ADMINISTRATION

Compound	% Radioactivity excreted	% Dose excreted
0–6 hr bile (51·9% of dose excreted)		
A	33·8	17·5
B	—	—
IV	36·2	18·8
II	2·8	1·4
E	1·8	0·9
III	3·2	1·7
VI	4·3	2·2
I		
Residual activity*	13·4	7·0
Total	95·5	49·5
72–78 bile (11·6% of dose excreted)		
A	15·0	1·7
B	15·6	1·8
IV	52·6	6·1
VI	4·0	0·5
Residual activity*	11·1	1·3
Total	98·3	11·4

* Radioactivity remaining on plate after removal of radioactive areas.

After being revealed by exposure to X-ray film, the radioactive areas were scraped off the plates and counted.

In "early" bile the acid (IV) was the major component identified. The aldehyde (III), alcohol (II), BHT (I) and dimer (VI) accounted for only about 10% of the excreted radioactivity. Spot E was also a minor

component. Spot A, however, was present to the same extent as the acid (IV).

In "late" bile a simpler metabolic pattern appeared. Spot A was accompanied by an equal amount of a new spot, B. The acid (IV), was found and also a spot which, in view of the time elapsed since dosing, must have been the dimer (VI).

A complicating factor in this work was that not all the radioactivity spotted on to the plates was accounted for by the areas detected on X-ray film. A count of the activity of the residual silica gel showed this to contain the remaining radioactivity. The reason for this is not known. However, other workers in these laboratories (R. E. Thomas, private communication) have observed similar effects with tritiated cardiac glycosides and genins. The errors introduced by this retention of activity by the gel may be relatively small since there was good agreement between the reverse isotope dilution studies and elution from the plate. It is possible that part of the streaking of radioactivity is due to traces of decomposition products.

Vigorous acid hydrolysis caused loss of metabolite B from "late" bile (Table 6), but did not appreciably alter the amounts of the remaining metabolites. Table 6 also contains a comparison of pooled 4-day urine metabolites and "late" bile metabolites. It is interesting to note that the acid (IV) is not a major urinary constituent as it is in bile.

TABLE 6. ESTIMATION OF [^{14}C]BHT METABOLITES IN POOLED URINE AND LATE BILE HYDROLYSED WITH 6N HYDROCHLORIC ACID

Compound (see Fig. 1)	1–4 Day urine*		72–78 hr bile†	
	% Excreted radioactivity	% Dosed radioactivity	% Excreted radioactivity	% Dosed radioactivity
A	20·2	6·3	15·9	1·7
B	—	—	—	—
IV	16·2	5·1	52·5	6·1
II	24·6	7·7	—	—
E	—	—	—	—
III	10·4	3·3	—	—
I, VI	—	—	6·0	0·5
Residual activity	24·0	7·2	20·1	2·3
Total	95·4	29·6	94·4	10·6

* 31·2% of dose excreted in urine.
† 11·6% of dose excreted in 72–78 hr bile.

Discussion

The differing proportions of metabolites in "early" and "late" bile as well as in pooled 4-day urine suggest a selective process of biliary and urinary excretion. However, as yet, we have little information on the nature of the conjugates excreted in bile, and none on the nature of the compounds re-absorbed from the gut. In addition, nothing is known of the faecal metabolites. Yet it is interesting to speculate about the high proportion of the acid (IV) which appears in the bile. "Early" bile contains 34% and "late" bile 53% of the total excreted activity as (IV),

while pooled 4-day urine contains only 16%. The acid (IV) is mainly excreted as the ester glucuronide by rats (Wright & others, 1965). It would be hydrolysed by plasma esterases and therefore preferentially excreted by the liver. Ether glucuronides would probably be more stable to esterase action and would therefore be excreted more efficiently by the kidney. This assumption agrees with the minor amounts of the alcohol (II) found in bile and the high proportion (25%) in urine. More aldehyde (III) is found in "early" bile than pooled urine. It is possible that there is an equilibrium between the alcohol and aldehyde effected by the reversible alcohol dehydrogenase systems of liver during enterohepatic circulation.

It is interesting that Wright & others (1965) found a slow excretion of label from doses of 3,5-di-t-butyl-4-hydroxybenzyl alcohol[^{14}C] (II). Rats and dogs both excreted this compound in urine and faeces over several days. The major metabolite was the acid (IV) together with minor amounts of unidentified metabolites. In the light of the present work and its distribution studies, it is clear that the metabolites from (II) also undergo extensive enterohepatic circulation. Wright & others did not detect any unchanged (II) in the excreta.

Because of the agreement between the urinary excretion of tritiated BHT (Golder, Ryan & Wright, 1962) and [^{14}C]BHT (Ladomery, Ryan & Wright, 1963) the latter workers suggested that there might be little attack at the methyl group of BHT. It was known that 50% of the label in tritiated BHT was in the methyl group. It is now clear from the present work that, although the proportion of the acid (IV) is low in the urine, this is due to a process of selective excretion rather than selective oxidation.

Among the metabolites detected by Dacre (1961) was the alcohol, β-(3-t-butyl-2-hydroxy-5-methylphenyl)-$\beta\beta$-dimethylethanol, formed by oxidation of the t-butyl group. This compound does not seem to have been detected in the present work. It could not be isolated from rabbit urine using Dacre's (1961) method.

Acknowledgements. This work forms part of a research programme supported by the National and Medical Research Council of Australia and Grant EF-258 of the U.S. National Institutes of Health.

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Mode of action of methyldopa

SIR,—Varma (1967) has recently shown that methyldopa produces its usual antihypertensive effect in immunosympathectomized rats made hypertensive by metacorticoid treatment. The author believed this observation to be inconsistent with the hypothesis of Day & Rand (1963) that methyldopa produced its antihypertensive effect by substituting a less active "false transmitter substance" for noradrenaline in the sympathetic nervous system.

We would suggest that the results of Varma (1967) could be interpreted as confirmatory rather than contradictory to our hypothesis for the following reasons. It is known that immunosympathectomy does not completely remove the sympathetic nervous system (Levi-Montalcini & Angeletti, 1962; Iversen, Glowinski & Axelrod, 1966). This explains Varma's findings of reduced (but not abolished) myocardial catecholamine content and urinary catecholamine excretion. The mean resting blood pressure in the immunosympathectomized rats was no different from that of control animals, and thus vascular tone is presumably still under sympathetic control. Immunosympathectomized hypertensive rats showed a larger mean fall in blood pressure after methyldopa (66 mm Hg) than did control hypertensive animals (40 mm Hg), although the number of observations in Varma's experiments is small. This suggests that the vascular sympathetic innervation in immunosympathectomized rats, being more sparse than in control rats, is more susceptible to the partial sympathetic nerve block produced by methyldopa than is the sympathetic innervation in control animals.

We would also like to comment on the published work of others cited by Varma (1967) as inconsistent with the false transmitter hypothesis. It has been reported that methyldopa does not reduce the effects of sympathetic stimulation (Stone, Ross & others, 1962; Varma & Benfey, 1963). However, Day & Rand (1964) showed that methyldopa did impair responses to sympathetic stimulation in their experiments but the impairment was confined to low frequencies of stimulation. This observation has since been confirmed by Farmer (1965). It is believed that physiological impulse rates in the sympathetic nervous system are low. Varma (1967) also quoted the work of Davies (1966) as being inconsistent with our hypothesis since this worker noticed no reduction in noradrenaline output on sympathetic stimulation from the cat spleen after methyldopa. Davies (1966), however, measured "noradrenaline" output by assaying his plasma samples on the pithed rat blood pressure which we find does not differentiate noradrenaline from its α-methyl analogue. Moreover, Muscholl & Maitre (1963) showed that after methyldopa treatment sympathetic nerve stimulation in rabbit isolated perfused heart preparations released a mixture of noradrenaline and α-methylnoradrenaline. These latter workers assayed the perfusate in their experiments by a specific fluorimetric method.

We would therefore suggest that the false transmitter hypothesis to explain the antihypertensive effect of methyldopa is still tenable.

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Inhibition of noradrenaline uptake by angiotensin

SIR,—It has been postulated that angiotensin contracts vascular smooth muscle, in part, by releasing noradrenaline from sympathetic nerve endings (Distler, Liebau & Wolff, 1965), and Feldberg & Lewis (1964) demonstrated that angiotensin liberated noradrenaline from adrenal medulla. This effect has been used to explain the potentiation of response to sympathetic nerve stimulation after the administration of this peptide (Benelli, Della Bella & Gandini, 1964), although Hertting & Suko (1966) and Thoenen, Hürlimann & Haefely (1965) could not measure increased release of noradrenaline after angiotensin administration.

Recently we demonstrated that angiotensin prevents the uptake of noradrenaline in rat brain (Palaic & Khairallah, 1967) by acting at the level of the "membrane pump" defined by Carlsson (1966). By acting in a similar manner to cocaine, angiotensin was also postulated to block re-uptake, causing supersensitivity to noradrenaline. We have now made experiments with spleen slices and rat aortae, and compared the results with those on brain stem slices.

Female Sprague-Dawley rats (ca 200 g) were decapitated. Spleen, thoracic aorta and brain stem were rapidly removed, chilled, and 0·4 mm thick slices were prepared from spleen and brain stem. Blood vessels were carefully cleaned of extraneous fat tissue and cut spirally. Sections were incubated at 37° in 5 ml Krebs solution (6·9 g NaCl, 2·1 g $NaHCO_3$, 0·35 g KCl, 0·28 g $CaCl_2$, 0·11 g $MgCl_2$, 0·14 g Na_2HPO_4, and 2·0 glucose per litre) and aerated with oxygen 95%, carbon dioxide 5%. A duplicate section was used as control.

Tissues were first equilibrated for 10 min followed by another 30 min incubation in the presence of 0·5 μg [^{14}C]noradrenaline (specific activity 254 μc/mg). Angiotensin was added to the incubation medium at the beginning, 10 min before noradrenaline. The final concentration of angiotensin was deliberately high (100 μg in 5 ml), since spleen and brain contained high levels of angiotensin destroying enzymes. At the end of incubation, the tissue was rapidly washed twice with 0·9% saline, blotted dry and weighed. After drying overnight in an oven, the tissue was burned (Kalberer & Rutschmann, 1961) and the [$^{14}CO_2$] trapped and counted by liquid scintillation.

The amount of radioactivity taken up by the three different tissues varied, being lowest in blood vessels and highest in brain (Table 1). Since nerve endings are the usual storage sites for noradrenaline, we would like to ascribe the different levels of radioactivity to different amounts of sympathetic nerve endings in these tissues. Pease (1962) reported that aorta is relatively poor in sympathetic innervation. Angiotensin inhibited uptake of noradrenaline in the

TABLE 1. EFFECT OF ANGIOTENSIN ON NORADRENALINE UPTAKE. Tissue slices and blood vessel strips were incubated with [^{14}C]noradrenaline (0·1 μg/ml) and angiotensin (20 μg/ml). Results are expressed in counts/mg tissue; averages ± standard error of mean, and P values express degrees of significance between control and angiotensin treated tissues.

Aorta		Spleen		Brain	
Control	Angiotensin	Control	Angiotensin	Control	Angiotensin
173	113	256	83	661	257
171	121	256	114	641	273
125	86	376	86	540	502
123	53	310	129	1120	480
124	105	244	222	846	450
126	106	329	192	540	430
141	116	281	198	551	178
141		314			327
141 ± 7	100 ± 8	296 ± 8	146 ± 21	700 ± 81	362 ± 42
$P < 0.0025$		$P < 0.0005$		$P < 0.0025$	

three tissues, with a 30% inhibition in aorta and a 50% inhibition in spleen and brain slices.

Thus this inhibition of uptake of noradrenaline by angiotensin seems to be a generalized phenomenon, also occurring in isolated blood vessels. This can explain the findings of Benelli & others (1964), and also results reported by McCubbin, deMoura & others (1965). In the former case nerve stimulation releases noradrenaline. Under normal circumstances, cessation of the sympathetic effect is mainly due to re-uptake of the catecholamines. Angiotensin prevents this, thus potentiating the effect of stimulation. In the latter case, tyramine releases stored noradrenaline, which normally would be taken up again. Angiotensin again prevents this, thus potentiating the tyramine effect on blood pressure.

Acknowledgements. This work was partially supported by a grant from the Life Insurance Medical Research Fund, and in part by a U.S. Public Health grant HE-6835, National Institutes of Health.

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March 28, 1967

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The stereospecificity of noradrenaline uptake by cat atria

SIR,—Kopin & Bridgers (1963) noted that there was a difference in the rate of disappearance of (±)-[^{3}H]noradrenaline from rat hearts *in vivo* when compared with (−)-[^{14}C]noradrenaline, and this was shown subsequently to be due to different binding forces or rates of release for the (+)- and (−)-isomers of noradrenaline (Beaven & Maickel, 1963). Iversen (1963, 1965) showed that the uptake kinetics for (+)- or (−)-noradrenaline into whole rat hearts were also different at low concentrations of noradrenaline (less than 0·5 μg/ml), but were not dissimilar at higher concentrations. Since we previously reported a lack of stereospecificity for (+)- or (−)-noradrenaline uptake into particulate fractions of cat atria using high total noradrenaline concentrations (Mueller & Shideman, 1964), it was felt desirable to see if in this species stereospecificity could be observed at low total noradrenaline concentrations. Because specificity could exist at either the cell membrane or the storage vesicle membrane, or at both, it was desirable to fractionate the atrial homogenates obtained after exposure of the intact spontaneously beating organ to (±)-7[^{3}H]noradrenaline* [^{3}H]-NA. Since adequate control of exposure to and washout of [^{3}H]-NA from tissues seemed essential, isolated superfused cat atria were employed. In the superfused preparations the atria were bathed only by solution flowing over the outside of the tissue.

Cats of either sex (1–2 kg) were anaesthetized with sodium pentobarbitone (35 mg/kg i.p.). The heart was removed rapidly and placed in an oxygenated modified Tyrode solution having the following composition in g/litre: NaCl, 7·00; KCl, 0·354; $CaCl_2.2H_2O$, 0·350; KH_2PO_4, 0·081; $MgSO_4.7H_2O$, 0·147; $NaHCO_3$, 2·1; glucose, 0·9; ascorbic acid, 0·02. The atria were dissected free from ventricular muscle and placed in a superfusion chamber similar to that described by Gaddum (1953) and Cambridge & Holgate (1955). The muscle was covered with 100 ml of the modified Tyrode solution, and a resting load of 1·0 g was applied. After superfusion was begun, the bath was drained and the resting tension was increased by the weight of the atria under study. The rate of superfusion was held constant with two Dual Syringe Feeders (Modern Metalcraft) which delivered 3·5 to 3·8 ml/min. Atria were first superfused for 10 or 50 min with modified Tyrode solution, then exposed to modified Tyrode solution containing the [^{3}H]-NA, and finally washed for 3 min with solution containing no [^{3}H]-NA. The atria were rapidly removed from the apparatus, blotted dry, and homogenized in 0·075 M phosphate buffer (pH 7·5). An initial sediment was prepared by centrifuging the homogenates at 5000 × *g* for 5 min (IS); the supernatant fluid was then centrifuged at 105,000 × *g* for 60 min yielding a pellet (P) and final supernatant (S) fraction. Since the [^{3}H]-NA isolation procedure (Whitby, Axelrod & Weil-Malherbe, 1961) does not separate (+)- from (−)-[^{3}H]-NA, the estimated radioactivity reflects the amounts of both labelled isomers taken up by the tissue fractions. The (−)-isomer of noradrenaline was obtained from Calbiochem Corporation as the (−)-noradrenaline (+)-bitartrate monohydrate $[\alpha]_D^{25}$ −9·6, and the (+)-isomer of noradrenaline was obtained from Sterling Winthrop Laboratories as the (+)-noradrenaline (+)-bitartrate $[\alpha]_D^{25}$ +36·8.

At 1 μg/ml of (+)- or (−)-noradrenaline, 2·3 ng/ml of (±)-[^{3}H]-NA (1 μc/86 ng) was added to the superfusing medium, resulting in an 881-fold dilution of the isomer under study. At concentrations of 20 ng/ml of (+) or (−)-noradrenaline, 2·9 ng/ml of (±)-[^{3}H]-NA (1 μc/29 ng) was added to the superfusing fluid resulting in a 14-fold dilution of the (+)- or (−)-isomer.

* 2-Amino-1-(3,4-dihydroxyphenyl)-[1-^{3}H]ethanol.

When atria were superfused for 10 min with modified Tyrode solution containing (±)-[^{3}H]-NA after a 50 min control superfusion period (Table 1, Exp. series 1), dilution of either (−)- or (+)-[^{3}H]-NA contained in the superfused fluid by addition of large amounts (1 μg/ml) of unlabelled (+)- or (−)-noradrenaline caused an equal depression of the [^{3}H]-NA taken up into the P fraction. Since it was thought possible that a 50 min period of control superfusion might destroy a stereospecific mechanism, the experiment was repeated using only a 10 min period of superfusion before superfusing with Tyrode solution containing [^{3}H]-NA (Experimental series 2). Under these conditions the amount of [^{3}H]-NA taken up was significantly lower than with 50 min control period of superfusion. However, the addition of 1 μg/ml of unlabelled (+)- or (−)-noradrenaline again produced an equal depression of the amount of (+)-[^{3}H]-NA taken up into the P fraction and the total [^{3}H]-NA taken up by the heart. The experiment was repeated again (Experimental series 3) using the 10 min control period and a much smaller final concentration of diluting unlabelled (+)- or

TABLE 1. THE EFFECT OF (+)- AND (−)-NORADRENALINE ON UPTAKE AND SUBCELLULAR DISTRIBUTION OF (±)-[^{3}H]NORADRENALINE IN SUPERFUSED CAT ATRIA. Cat atria were superfused with modified Tyrode solution during the control period. All atria were superfused with the solution containing [^{3}H]-NA for 10 min. Atrial homogenates were fractionated by an initial sedimentation at 5000 × *g* for 5 min (IS); the supernatant thus obtained was sedimented at 105,000 × *g* for 60 min, yielding a pellet (P), and final supernatant solution (S).

Exp. ser.	No. of exp.	Control period before [^{3}H]-NA (min)	Unlabelled isomer of NA added to superfusing solution	Conc. isomer μg/ml	Total [^{3}H]-NA mμc/g in atria at end of superfusion mean ± s.e.	% of total [^{3}H]-NA (Mean ± s.e.) Subcellular fraction IS	P	S
1	4	50	None		68·4 ± 8·6	15·8 ± 0·5	12·2 ± 0·6	72·3 ± 0·8
	4	50	(−)	1	63·3 ± 10·5	20·3 ± 2·1	6·0 ± 1·1[a]	73·8 ± 1·8
	4	50	(+)	1	63·3 ± 11·2	20·0 ± 3·0	7·0 ± 0·8[a]	73·0 ± 2·5
2	5	10	None		26·9 ± 0·7	17·2 ± 0·8	18·1 ± 1·4	64·7 ± 0·6
	7	10	(−)	1	15·8 ± 2·5[a]	22·8 ± 5·5	13·4 ± 1·3[a]	63·9 ± 5·3
	5	10	(+)	1	14·5 ± 1·0[a]	17·9 ± 2·0	11·8 ± 4·6[a]	70·3 ± 6·5
3	4	10	None		21·4 ± 1·2	11·4 ± 0·3	21·6 ± 1·1	67·0 ± 1·1
	6	10	(−)	0·02	11·3 ± 0·8[a]	14·3 ± 2·0	14·8 ± 0·9[a]	71·0 ± 2·3
	4	10	(+)	0·02	21·2 ± 2·3	11·4 ± 1·0	24·1 ± 1·2	64·7 ± 1·3

[a] Indicates value significantly different ($P < 0.05$) from comparable value of hearts superfused with (±)-[^{3}H]-NA containing neither added (+)- or (−)-noradrenaline.

(−)-isomer. In this series the (−)-isomer caused a highly significant depression of both the percentage uptake into the P fraction, and in the total [^{3}H]-NA taken up per gram of tissue. Neither change was seen when unlabelled (+)-isomer was used to dilute the labelled (+)-isomer of (±)-[^{3}H]-NA. This would indicate sterospecific uptake both at the cell membrane and the storage vesicle membrane.

These results indicate that the myocardial adrenergic nerve terminals of the cat, like those of the rat (Iversen 1963, 1965) and isolated bovine splenic nerve granules (Euler & Lishajko, 1964; Stjärne & Euler, 1965) exhibit stereospecificity only at low concentrations of noradrenaline, but not at high concentrations. This could be the result of 2 separate uptake mechanisms or one mechanism whose characteristics are such that at high concentrations of noradrenaline stereospecificity is not detectable.

Acknowledgements. This research was supported by USPHS grant No. HE 07939. The authors gratefully acknowledge the able technical assistance of Mr. Robert McElmury.

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The effect of (±)-*p*-chloroamphetamine on the susceptibility to seizures and on the monoamine level in brain and heart of mice and rats

SIR,—In recent experiments we established that α-methyldopa inhibits the convulsion-facilitating effect and the brain noradrenaline-depleting effect of reserpine. The 5-hydroxytryptamine (5-HT)-depleting effect of reserpine was not influenced by α-methyldopa (Pfeifer & Galambos, 1965). We assumed that the changes in brain noradrenaline level played an important role in the susceptibility to seizures but that changes in the 5-HT level did not. Pletscher, Bartholini & others (1964) and Fuller, Hines & Mills (1965) reported the fact that *p*-chloro-*N*-methylamphetamine and *p*-chloroamphetamine lowered the 5-HT level in rat brain without lowering the concentration of noradrenaline. The compounds did not decrease the 5-HT level in the brain in mice. On the basis of these observations *p*-chloroamphetamine seemed to be a useful tool for investigating further the role of 5-HT and catecholamines in the susceptibility to seizures.

Wistar rats and Swiss mice were used in these experiments. The convulsive threshold was determined by the slow intravenous infusion of leptazol (Orloff, Williams & Pfeiffer, 1949). Noradrenaline, dopamine and 5-HT levels in brain and heart were measured by spectrophotofluorimetry (Bogdanski, Pletscher & others, 1956; Drujan, Sourkes & others, 1959).

(±)-*p*-Chloroamphetamine (10 mg/kg) much increased the convulsive threshold in mice and also in rats after 30 min, and the effect was seen even after 8 hr. There was no change in the brain 5-HT levels in mice. In rats the brain 5-HT level decreased to about 40%, and even after 18 hr when the convulsive threshold had returned to the control value, the 5-HT level was still low (Table 1). The brain noradrenaline and dopamine levels in rats and mice were unchanged.

The anticonvulsive effect of (±)-*p*-chloroamphetamine also developed in the presence of reserpine. The mice received 2·5 mg/kg reserpine intraperitoneally and 90 min later chloroamphetamine 10 mg/kg. The leptazol convulsion threshold was estimated after 30 min. In these circumstances the convulsion-facilitating effect of reserpine was not seen. When the mice were treated with chloroamphetamine 2 hr before reserpine and the convulsive threshold was

determined 2 hr after the reserpine, the anticonvulsive effect of the chloroamphetamine could be observed to an even greater extent. The phenomenon was similar to the reversal of the reserpine effect in the presence of a monoamine oxidase inhibitor.

In recent experiments we demonstrated that α-methyl-*m*-tyrosine did not influence the convulsive threshold, but in the presence of a monoamine oxidase inhibitor it greatly increased the convulsive threshold (Pfeifer & Galambos, 1967). When the mice were pretreated with the chloroamphetamine, α-methyl-*m*-tyrosine further increased the anticonvulsive action of chloroamphetamine, just as did reserpine. The chloroamphetamine did not influence the monoamine-depleting effect of reserpine, but diminished the noradrenaline- and dopamine-depleting effect of α-methyl-*m*-tyrosine in brain, but not in heart (Table 2).

TABLE 1. THE EFFECT OF (±)-*p*-CHLOROAMPHETAMINE (10 MG/KG) ON THE CONVULSIVE THRESHOLD AND ON BRAIN 5-HT LEVEL IN MICE AND RATS. Values are means with standard deviation; the number of experiments is shown in parentheses.

Time after treatment hr	Mouse		Rat	
	Convulsive threshold leptazol ml/10 g	Brain 5-HT μg/g	Convulsive threshold leptazol ml/10 g	Brain 5-HT μg/g
0	0·177 ± 0·044 (30)	0·520 ± 0·02 (3)	0·488 ± 0·186 (5)	0·525 ± 0·1 (3)
0·5	0·270 ± 0·04* (10)			
4	0·237 ± 0·073* (19)	0·465 ± 0·053* (4)	1·061 ± 0·154* (5)	0·304 ± 0·05* (4)
8	0·268 ± 0·064* (8)	0·429 ± 0·166 (3)	0·946 ± 0·126* (5)	0·212 ± 0·03* (4)
18	0·152 ± 0·054 (10)	0·468 ± 0·038 (4)	0·643 ± 0·210 (5)	0·238 ± 0·05 (3)

* Significantly different ($P < 0·01$) from control animals.

TABLE 2. THE EFFECT OF (±)-*p*-CHLOROAMPHETAMINE (10 MG/KG, I.P.) ON THE BRAIN NORADRENALINE, DOPAMINE, 5-HT LEVEL, ON THE HEART NORADRENALINE LEVEL AND ON CONVULSIVE THRESHOLD IN CONTROL MICE, IN MICE TREATED WITH RESERPINE (2·5 MG/KG I.P.) AND WITH α-METHYL-*m*-TYROSINE (50 MG/KG I.P.), RESPECTIVELY. Chloroamphetamine was given 2 hr before reserpine or α-methyl-*m*-tyrosine and the determinations were made 2 hr after. Values are means with standard deviation; the number of experiments is shown in parentheses.

Treatment	Animals not given chloroamphetamine					Animals given chloroamphetamine				
	Brain			Heart	Convulsive threshold leptazol ml/10 g	Brain			Heart	Convulsive threshold leptazol ml/10 g
	Noradrenaline μg/g	Dopamine μg/g	5-HT μg/g	Noradrenaline		Noradrenaline μg/g	Dopamine μg/g	5-HT μg/g	Noradrenaline	
—	0·445 ± 0·08 (13)	0·997 ± 0·20 (13)	0·565 ± 0·06 (4)	0·776 ± 0·256 (7)	0·177 ± 0·044 (50)	0·514 ± 0·10 (13)	1·044 ± 0·19 (12)	0·551 ± 0·10 (4)	0·646 ± 0·147 (5)	0·237* ± 0·073 (20)
Reserpine	0·087 ± 0·03 (5)	0·134 ± 0·04 (5)	0·199 ± 0·02 (4)		0·118 ± 0·029 (20)	0·149 ± 0·07 (5)	0·178 ± 0·09 (5)	0·152 ± 0·02 (4)		0·304* ± 0·081 (20)
α-Methyl-*m*-tyrosine	0·179 ± 0·05 (8)	0·332 ± 0·07 (8)		0·244 ± 0·09 (5)	0·161 ± 0·043 (20)	0·376* ± 0·14 (7)	0·598* ± 0·04 (8)		0·318 ± 0·103 (6)	0·331* ± 0·057 (15)

* Significantly different ($P < 0·01$) from animals not given chloroamphetamine.

TABLE 3. THE EFFECT OF (±)-*p*-CHLOROAMPHETAMINE (20 MG/KG I.P.) ON THE BRAIN NORADRENALINE AND DOPAMINE LEVEL AND ON THE HEART NORADRENALINE LEVEL IN MICE. Values are means with standard deviation; the number of experiments is shown in parentheses.

Time after treatment (hr)	Brain		Heart
	Noradrenaline µg/g	Dopamine µg/g	Noradrenaline
0	0·523 ± 0·09 (2)	0·916 ± 0·172 (12)	0·633 ± 0·183 (12)
4	0·548 ± 0·132 (7)	1·190 ± 0·392 (7)	0·450 ± 0·173 (6)
26	0·408 ± 0·136 (5)	0·580 ± 0·211* (5)	0·195 ± 0·114* (5)
48	0·440 ± 0·099 (5)	0·756 ± 0·118 (5)	0·204 ± 0·07* (5)

*Significantly different ($P < 0·01$) from control animals.

Chloroamphetamine in a larger dose (20 mg/kg) decreased slightly the brain noradrenaline and dopamine level, and more so the heart noradrenaline level. The heart noradrenaline level decreased to 30% of the control, an effect which lasted more than 48 hr (Table 3).

These results support our view that changes in brain 5-HT levels do not affect the susceptibility to seizures; chloroamphetamine increases the convulsive threshold with normal brain 5-HT levels in mice and with low brain 5-HT levels in rats. As the anticonvulsive effect of chloroamphetamine took place in the presence of reserpine without affecting the low noradrenaline and dopamine level we may presume that the compound acted directly on the noradrenaline receptors. This hypothesis is supported by the fact that chloroamphetamine diminished the noradrenaline- and dopamine-depleting effect of α-methyl-*m*-tyrosine. The cause of the catecholamine-depleting effect of α-methyl-*m*-tyrosine is thought to be that metaraminol, which is formed from α-methyl-*m*-tyrosine, replaces the depleted noradrenaline stoichiometrically (Carlsson & Lindquist, 1962; Shore, Busfield & Alpers, 1964). It can be presumed that (±)-*p*-chloroamphetamine inhibits the uptake of metaraminol.

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Central hypotensive effect of α-methyldopa

SIR,—The hypotensive action of L-α-methyl-3,4-dihydroxyphenylalanine (α-methyldopa) is rather difficult to demonstrate in laboratory animals. Only large doses of the drug, administered to conscious dogs (Goldberg, Da Costa & Ozaki, 1961; Kroneberg, 1963), to renal hypertensive rats (Muscholl, 1966) or to conscious normotensive rats (Henning, unpublished observations) significantly decrease blood pressure. As far as we know, an acute hypotensive effect has never been demonstrated in the anaesthetized cat. Although in man and animals the administration of α-methyldopa gives rise to decreased peripheral resistance, the mechanism of this effect is still poorly understood. Infusion of α-methyldopa into the brachial artery in man does not cause any increase of the blood flow of the corresponding forearm or hand (Henning & Johnsson, unpublished experiments). One of us (M. H.) finds that α-methyldopamine, which does not pass through the blood brain barrier, depletes noradrenaline from the peripheral sympathetic nerves but has no effect on the blood pressure of conscious rats. Therefore, a central effect of α-methyldopa might be the cause of the decrease of the peripheral resistance. The present communication affords evidence that in the cat a centrally mediated hypotensive effect may be demonstrated for α-methyldopa.

Recently, van Zwieten, Bernheimer & Hornykiewicz (1966) have demonstrated that the injection of low doses of reserpine into the vertebral artery of the cat much decreased the concentration of noradrenaline and dopamine in the brain without affecting the amine content of the heart. This effect was accompanied by a decrease in blood pressure which was probably of central origin. The same experimental method was used for our investigations on the central effect of α-methyldopa.

Cats of either sex (2·0–4·5 kg) were anaesthetized with chloralose (80 mg/kg intraperitoneally). The thorax was opened by severing the first three ribs from the left side of the sternum. The left subclavian artery and its side branches were carefully exposed. All side branches with the exception of the vertebral artery were ligated. A polyethylene catheter was introduced into the subclavian artery and pushed forward in the direction of the heart, until its tip reached the proximal end of the vertebral artery. Thus, solutions infused slowly into this catheter will chiefly flow into the vertebral artery and finally reach those regions in the brain where the pressure regulation centres are located. The blood flow in the subclavian artery most likely prevents the transition of the drugs administered via this route to the peripheral circulation. Artificial respiration was applied throughout the experiments via a tracheal cannula. The drug was dissolved in saline and infused either into the vertebral artery or into the right femoral vein over a period of approximately 1 hr. The blood pressure was taken from the left femoral artery and recorded by a Grass Polygraph via a Statham pressure transducer type P23Dc. About 3 hr after the end of the infusion the animals were killed. The noradrenaline content of the right ventricle of the heart and that of the brain (cerebellum removed) was measured (Bertler, Carlsson & Rosengren, 1958). The dopamine and 5-hydroxytryptamine (5-HT) contents of the brain were measured by the methods described by Carlsson & Lindqvist (1962) and Andén & Magnusson (1967), respectively. Control animals were sham operated. Saline was infused either into the vertebral artery or into the left femoral vein under the same circumstances as those used for the infusion of the drug.

In one series of experiments 20 mg/kg α-methyldopa, dissolved in 5–7 ml saline, was infused into the vertebral artery. As shown in Table 1 the

noradrenaline content of the brain was significantly lowered, whereas that of the heart (right ventricle) remained unaffected. The dopamine and 5-HT levels of the brain underwent no significant changes. Approximately 1-2 hr after the end of the infusion a slow but pronounced hypotensive effect was observed. The blood pressure was reduced on the average by 55 mm Hg (s.e.m. = 13, n = 6). The same low dose (20 mg/kg) of α-methyldopa *intravenously* infused, did not affect the blood pressure. However, the brain noradrenaline was depleted to approximately the same extent as observed after administration o the drug into the vertebral artery. The noradrenaline level of the heart remained normal. Intravenous infusion of a higher dose, 200 mg/kg, gave rise to a reduction of arterial blood pressure in 3 animals out of 4 (mean reduction 28 mm s.e.m. = 15).

The results demonstrate that the infusion of small doses of α-methyldopa into the vertebral artery causes a clearcut fall in blood pressure. Since intravenous infusion of the same low dose does not affect blood pressure, it is uncertain whether the accompanying reduction in brain noradrenaline is related to the hypotensive effect of α-methyldopa which was observed after infusion of the drug

TABLE 1. EFFECTS OF INFUSION OF α-METHYLDOPA ON BLOOD PRESSURE, HEART NORADRENALINE AND BRAIN NORADRENALINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE

Treatment	Hypotensive effect 3 hr after treatment mm Hg	Heart noradrenaline µg/g	Brain		
			Noradrenaline µg/g	Dopamine µg/g	5-HT µg/g
Infusion of saline into vertebral artery (control). (Means ± s.e.m., 4 experiments)	none	1·05 ± 0·196	0·21 ±0·018	0·29 ± 0·034	0·24 ± 0·019
Infusion of α-methyldopa (20 mg/kg) into vertebral artery. (Means ± s.e.m., 6 experiments)	55 ± 13	1·17 ± 0·101	0·14* ± 0·014	0·24 ± 0·023	0·22 ± 0·013
Infusion of α-methyldopa (20 mg/kg) into femoral vein. (Means ± s.e.m., 4 experiments)	none	1·30 ± 0·218	0·13** ± 0·006	0·22 ± 0·010	0·20 ± 0·017
Infusion of α-methyldopa (200mg/kg) into femoral vein. (Means ± s.e.m., 4 experiments)	28 ± 15	0·86 ± 0·051	0·13* ± 0·008	0·15** ±0·013	0·12** ± 0·018

* Differs significantly from the control value (analysis of variance), $P < 0.005$.
** As above $P < 0.001$.

into the vertebral artery. It is remarkable that upon intravenous infusion of such a low dose (20 mg/kg) the noradrenaline in the brain is lowered, whereas that in the heart remains unaffected. Although the depletion in the brain is of the same order of magnitude as that observed after infusion of the drug into the vertebral artery, it may be possible that administration via the two different routes gave rise to depletion in different parts of the brain. Until now only the whole brain (after removal of the cerebellum) has been analysed.

For reserpine-induced depletion the heart seems more sensitive than the brain (Carlsson, 1965). The fact that a relatively high intravenous dose (200 mg/kg) of α-methyldopa shows but a slight hypotensive effect is in agreement with previous observations made in anaesthetized animals (for review, see Muscholl, 1966).

Acknowledgements. We are greatly indebted to Miss Inger Pettersson for skilful technical assistance. This work was supported by grants from Wenner-Grenska Samfundet, Stockholm, Läkemedelsindustriföreningen, Stockholm, the

Swedish National Association against Chest and Heart Diseases, and the Faculty of Medicine, University of Göteborg, Göteborg.

For a generous supply of α-methyldopa we thank Merck, Sharp and Dohme.

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Reversal of α-methyltyrosine-induced behavioural depression with dihydroxyphenylalanine and amphetamine

SIR,—The time course and the degree of behavioural depression following administration of α-methyltyrosine correlates with the reduced brain levels of noradrenaline and dopamine (Hanson, 1965; Moore, 1966). Nevertheless, many factors must be considered before this behavioural deficit can be causally related to a lack of brain catecholamines. For example, toxicity (Weissman & Koe, 1965) and direct depressant actions of α-methyltyrosine could contribute to the behavioural effects. However, with proper precautions it can be shown that these factors do not play a major role in the behavioural effects of this drug. Multiple injections of small doses of α-methyltyrosine produced behavioural depression and catecholamine depletion without concomitant toxicity (Rech, Borys & Moore, 1966). The importance of a direct depressant action of α-methyltyrosine was minimized by the finding that pretreatment with monoamine oxidase inhibitors reduced both the catecholamine-depleting and behavioural depressant effects of this drug without altering the concentration of α-methyltyrosine in the brain (Moore & Rech, 1967). Further efforts to implicate brain catecholamines in the central actions of α-methyltyrosine are described in this communication. It will be shown that both dihydroxyphenylalanine (L-dopa), which serves as an immediate precursor for dopamine and noradrenaline, and (+)-amphetamine, which mimics the actions of catecholamines, at least at peripheral sites, reverse α-methyltyrosine-induced behavioural depression.

Female rats (CD_1, Charles River Animal Farm), 175–200 g, were trained to perform in a shuttle box. Each trial was initiated by activating a small light on the side of the cage occupied by the animal. After 5 sec of light the grid floor on the same side of the cage was electrified for 5 additional sec. If the rat moved to the unlighted side during the initial 5 sec, the response was termed

an "avoidance"; if the animal shuttled during the latter 5 sec of the trial the response was termed an "escape." The trials were repeated every 30 sec; 20 such trials constituted a test session. After 10 training sessions 25 rats, which averaged better than 16 avoidance responses per session, were selected for the experiment. After the test session at zero time each rat received 3 intraperitoneal injections of an aqueous suspension of α-methyltyrosine (50 mg/kg) at 4 hr intervals (at 0, 4 and 8 hr) indicated by the black arrows in Fig. 1. This dosage schedule was previously shown to cause a progressive reduction of brain stores of noradrenaline and dopamine and to impair conditioned avoidance behavioural responses; the maximum effect was seen at 12 hr (4 hr after the last injection) and recovery was complete by 24 hr (Rech & others, 1966). Fifteen animals were tested in the shuttle box at 12 hr and then injected with saline (5 rats), 100 mg/kg L-dopa (8 rats) or with 0·5 mg/kg of (+)-amphetamine sulphate (7 rats). All drugs were injected intraperitoneally. One-half hr later these rats were retested in the shuttle box. As seen in Fig. 1, 3 injections of α-methyltyrosine much reduced the number of avoidances per session and caused a loss of 1–4 escape responses. There was no change in this behaviour pattern after saline (Fig. 1a) but both L-dopa and (+)-amphetamine (Fig. 1b, c) restored escape responses and caused a significantly greater number of avoidance responses. That is, in these animals the number of avoidances per session were significantly higher ($P < 0{\cdot}05$) at $12\frac{1}{2}$ hr than at 12 hr (Student's t test). In another 5 rats, α-methyltyrosine was administered in the same manner but the animals were tested in the shuttle box at $11\frac{1}{2}$ hr after the first dose (Fig. 1d). The extent of depression was about the same as in the previous tests. Immediately after this test each animal was injected with 100 mg/kg L-dopa and tested 1 hr later. At this time ($12\frac{1}{2}$ hr after the first α-methyltyrosine injection) the depression from α-methyltyrosine was completely antagonized.

α-Methyltyrosine blocks catecholamine biosynthesis at the tyrosine hydroxylase step; the subsequent enzymatic steps are unaffected (Udenfriend, Zaltman-

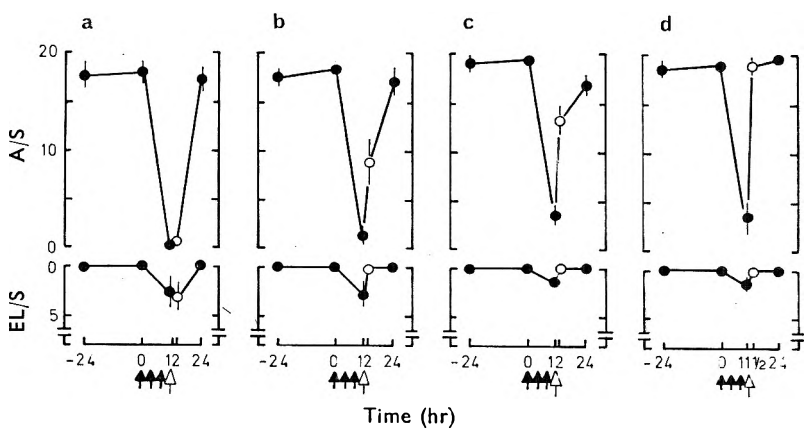

FIG. 1. Effects of L-dopa and (+)-amphetamine on depressed conditioned avoidance behaviour in rats treated with α-methyltyrosine. α-Methyltyrosine (50 mg/kg) was injected at 0, 4 and 8 hr (black arrows). In a, b and c, rats were injected with saline, L-dopa (100 mg/kg) or (+)-amphetamine (0·5 mg/kg) respectively at 12 hr (open arrows) and tested 30 min later. In d, rats were injected with L-dopa at 11·5 hr, and retested 1 hr later. The points represent mean values for each group and the vertical lines the standard errors. Where no line is shown the standard error is less than the radius of the point. A/S avoidance per session; EL/S escapes lost per session.

Nirenberg & others, 1966). Thus L-dopa should enter the biosynthetic pathway below the block and through the processes of decarboxylation and hydroxylation form dopamine and noradrenaline. Using the same dose as reported here, Corrodi, Fuxe & Hökfelt (1966) showed that L-dopa restored the noradrenaline and dopamine content of brains that had been depleted by α-methyltyrosine. The reversal of the behavioural depression by L-dopa may well be secondary to the restoration of these catecholamines in the brain.

Although α-methyltyrosine has been reported to possess potent anti-amphetamine actions (Weissman, Koe & Tenen, 1966) it is obvious that under the conditions of the present experiments amphetamine adequately reversed the depression it induced. Poschel & Ninteman (1966) reported that methylamphetamine reversed the suppression of self-stimulation it caused. The mechanism of the amphetamine reversal is poorly understood. Perhaps (+)-amphetamine mimics the effects of noradrenaline or dopamine at central receptor sites or is able to release residual stores of catecholamines from nerve terminals in the brain. In any case, it is of interest that certain behavioural responses that are depressed by α-methyltyrosine are restored by (+)-amphetamine, while in other behavioural situations a stimulant effect of (+)-amphetamine is antagonized by pretreating with α-methyltyrosine.

These results, along with the reports cited above, strengthen the proposal that α-methyltyrosine-induced behavioural depression occurs as a consequence of the lack of noradrenaline or dopamine, or both, in the brain.

Acknowledgements. This work was supported by Grants AM 11083 and NB 06013 from USPHS.

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Inhibitory responses to transmural stimulation in isolated intestinal preparations

SIR,—Transmural electrical stimulation of guinea-pig isolated ileum elicits contractile responses due to activation of parasympathetic nerve elements within the muscle wall (Paton, 1955, 1957). During the course of experiments to determine the nature of the cholinergic fibres associated with the periarterial nerves in the rabbit intestine (Gillespie & Mackenna, 1961; Day & Rand, 1961; Bentley, 1962) we used transmural stimulation in segments of rabbit isolated intestine. We were surprised to note that in most of the preparations transmural stimulation caused a complex response consisting of immediate inhibition of spontaneous activity followed by a marked contractile response. In about half of the preparations the contractile response was followed by a second inhibitory phase. In Fig. 1 the effects of sympathetic (periarterial) and transmural stimulation are compared in a segment of rabbit isolated ileum suspended in Tyrode solution at 37°. Sympathetic stimulation produced a complete inhibition of the pendular movements which outlasted the stimulation period. Complete recovery of the spontaneous movements occurred after several minutes. In contrast, when the same stimulus was applied transmurally, an inhibitory response occurred which changed during the stimulus to a contractile response outlasting the stimulus period by several minutes.

We have attempted to analyse this complex response to transmural stimulation by means of blocking drugs. The inhibitory phase of the response was prolonged, or in those preparations where it was absent initially, it was revealed after the addition of atropine or hyoscine (10^{-7} to 10^{-4} g/ml) to the bath (Fig. 2B). These drugs did not affect the excitatory phase and produced either no effect on the responses to sympathetic stimulation, or caused only a slight impairment. The initial inhibitory effect of transmural stimulation was unaffected, or in some preparations partly blocked by guanethidine in concentrations (10^{-6} to 10^{-5} g/ml) which completely abolished the responses to sympathetic stimulation (Fig. 2C). The response to transmural stimulation was markedly altered when the bath temperature was lowered, the inhibitory phase being prolonged and the excitatory phase reduced or abolished (Fig. 2D). Both phases of the response to transmural stimulation were unaffected, or occasionally slightly reduced by the anti-adrenaline agents phentolamine and propranolol, added to the bath individually or simultaneously in concentrations (10^{-7} to 5×10^{-7} g/ml) which abolished the responses to added catecholamines and to sympathetic nerve stimulation.

From the results obtained with anti-adrenaline agents and with guanethidine we conclude that the inhibitory responses to transmural stimulation are unlikely to be due entirely to activation of sympathetic adrenergic nerve elements within the muscle wall. However, the following preliminary observations suggest to us that the inhibitory responses are nervously mediated.

The local anaesthetic agent cocaine abolished both phases of the response to transmural stimulation in concentrations (2×10^{-5} to 6×10^{-5} g/ml) similar to those which abolished the responses to sympathetic nerve stimulation.

All phases of the response to transmural stimulation were present when pulse widths as low as 0·1 msec, which are unlikely to affect smooth muscle directly, were used. Moreover, it was shown that the optimal frequency for the inhibitory component was lower (10 to 20 pulses/sec) than the optimal frequency for sympathetic relaxations (30 to 50 pulses/sec).

The complex responses to transmural stimulation were strikingly similar to the effects of the automatic ganglion stimulants nicotine and tetramethyl ammonium in isolated preparations of rabbit ileum.

The characteristics of the inhibitory responses to transmural stimulation

described here are essentially similar to those recently described in the cat stomach (Martinson, 1965a,b), in the guinea-pig isolated taenia coli (Burnstock, Campbell & Rand, 1966), and in the guinea pig isolated stomach (Campbell, 1966) and suggest the presence of non-adrenergic inhibitory neurons in the gastrointestinal tract.

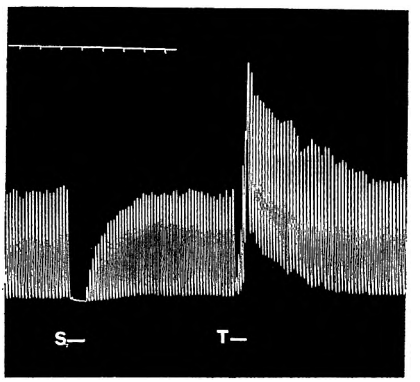

FIG. 1. Longitudinal contractions of rabbit isolated ileum suspended in aerated Tyrode solution at 37°. Sympathetic nerve stimulation (at S) and transmural stimulation (at T) each applied for 20 sec periods with 2 msec 20 V rectangular pulses at a frequency of 50 pulses/sec. Time: 30 sec intervals.

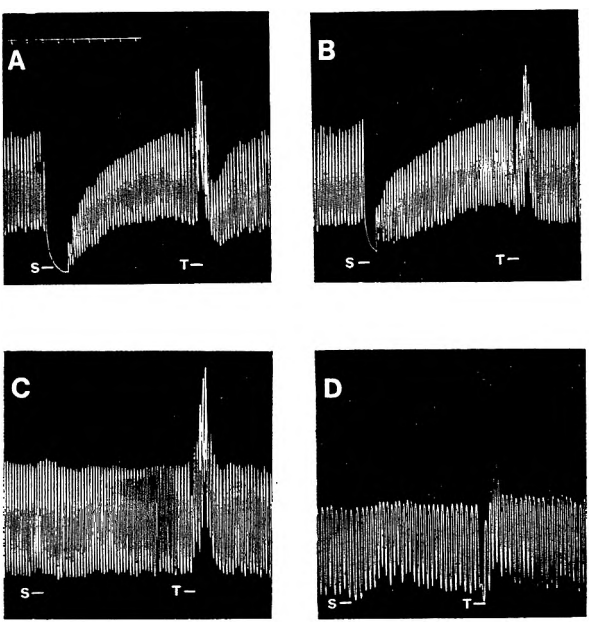

FIG. 2. Rabbit isolated ileum preparations; sympathetic stimulation (at S) and transmural stimulation (at T) applied as in Fig. 1. Control responses in A, 20 min after adding hyoscine (10^{-6} g/ml) in B, 30 min after adding guanethidine (10^{-6} g/ml) in C. In D, in the presence of guanethidine and hyoscine, responses repeated after reducing bath temperature from 37 to 31°.

We have repeated our experiments using transmural stimulation in intestinal preparations taken from duodenum, ileum and colon of the rabbit and cat. In all these preparations, transmural stimulation produced initial inhibitory responses. In some preparations of cat intestine transmural stimulation produced only inhibition which was not abolished by guanethidine. In those preparations of cat intestine showing a mixed response of inhibition and excitation, the excitatory phase was abolished by low concentrations of hyoscine or atropine. The atropine sensitivity of the motor component in cats, and the lack of sensitivity in rabbits, is consistent with the hypothesis that this part of the response is due to activation of parasympathetic nerve elements within the myenteric plexus, since the parasympathetic nerves to rabbit intestine are relatively insensitive to atropine (Ambache, 1951; Ambache & Edwards, 1951) whilst those of the cat are readily susceptible (Gillespie & Mackenna, 1960; Ambache, 1951).

The work of Martinson (1965a,b) and Campbell (1966) using stomach preparations, suggests that the connections of these inhibitory neurons with the central nervous system may be via the vagus nerves. However, since we have obtained inhibitory responses to transmural stimulation in colon preparations it may be that the sacral parasympathetic outflow also contains preganglionic fibres forming connections with non-adrenergic inhibitory fibres within the muscle wall.

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The micellar properties of some non-ionic surface-active agents in polar solvents

SIR,—Reported here are some preliminary results for the apparent critical micellar concentrations (CMC) of three non-ionic surface-active agents in water where applicable, in mixtures of water and formamide and in formamide. The surface-active agents were dodecyl tetraoxyethylene glycol ($C_{12}E_4$), dodecyl hexaoxyethylene glycol ($C_{12}E_6$) and dodecyl octaoxyethylene glycol ($C_{12}E_8$). Details of their syntheses and purification will be published elsewhere.

Critical micellar concentrations obtained from measurements made at 21° ± 0·5° by the drop-volume surface tension method and applying the required correction factors, are given in Table 1. No value could be obtained for the CMC of $C_{12}E_4$ in water as this compound is insoluble at temperatures higher than about 15°.

TABLE 1. THE CRITICAL MICELLE CONCENTRATIONS (MOLES/LITRE) OF $C_{12}E_4$, $C_{12}E_6$, $C_{12}E_8$ IN VARIOUS SOLVENTS

Compound	Solvent Systems expressed as % w/w formamide			
	0%	55%	90%	100%
$C_{12}E_4$	—	$1{\cdot}06 \times 10^{-3}$	$9{\cdot}33 \times 10^{-3}$	$2{\cdot}53 \times 10^{-2}$
$C_{12}E_6$	$0{\cdot}90 \times 10^{-4}$	$1{\cdot}53 \times 10^{-3}$	$9{\cdot}70 \times 10^{-3}$	$3{\cdot}06 \times 10^{-2}$
$C_{12}E_8$	$1{\cdot}41 \times 10^{-4}$	$2{\cdot}04 \times 10^{-3}$	$10{\cdot}60 \times 10^{-3}$	$3{\cdot}50 \times 10^{-2}$

The apparent CMC's increase with increasing concentrations of formamide. Schick (1964) found a similar increase in the CMC's of nonionic surfactants in aqueous solution in the presence of large quantities of urea. He attributed this increase to the ability of urea to disrupt the structure of water thus making more water molecules available for the hydration of the polyglycol side-chains of the surface-active agents. This, he said, brought about an increased surfactant solubility and an increase in CMC. As formamide is chemically similar to urea, and as both these materials raise the dielectric constant of water, a similar effect may be occuring in the mixed water–formamide solvents. However, the much greater solubility of the monodispersed surfactants in formamide alone, as shown by the very high CMC's relative to water, may also be important in determining CMC's in the mixed solvents. More recently it has been reported (Corkill, Goodman & others, 1967) that the presence of urea in aqueous solutions of surfactants brings about a decrease in the apparent heats of micellization, Δ Hm. Similar results have been found with the systems described here, and as the concentration of formamide increases, Δ Hm, as measured by the change of CMC with temperature, changes from positive to negative in sign. Full details of these results will be published elsewhere.

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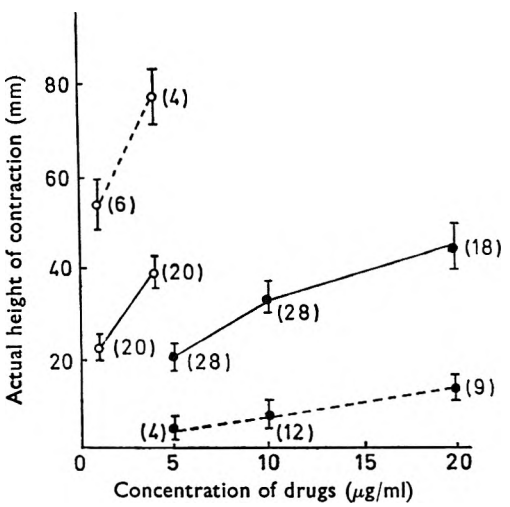
Modification by physostigmine of response to ganglion stimulant drugs

SIR,—Recently it has been shown that pharmacological actions of physostigmine cannot be explained wholly by its cholinesterase inhibition (Holmstedt, 1965). During a study of the pharmacological effects of 4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride (McN-A-343) and *N*-benzyl-3-pyrrolidyl acetate methobromide (AHR-602), which are known sympathetic ganglion stimulants (Jones, 1963), it was found that physostigmine inhibited the responses to McN-A-343 in the frog rectus abdominis isolated muscle, and that physostigmine reversed an inhibitory response of the isolated atria of the tortoise to both drugs (Kim & Shin, 1965).

As already shown by Roszkowski (1961), the frog rectus skeletal muscle was contracted by McN-A-343 (5–20 μg/ml), an action not antagonized by atropine. In the presence of physostigmine sulphate (100 μg/ml) in the bath, the contraction to McN-A-343 was much inhibited, whereas the acetylcholine contraction was potentiated (Fig. 1).

The isolated atria of the tortoise responded with a gradual depression of spontaneous movements to 100–200 μg/ml of McN-A-343 and to AHR-602. After exposing the atria to physostigmine (1 μg/ml) for more than 15 min, the response to McN-A-343 and AHR-602 was changed. An augmentative response was produced in 8 out of 20 experiments to McN-A-343 and 11 out of 15 experiments to AHR-602. The inhibitory effect of acetylcholine bromide (0·1 μg/ml) was more marked in the physostigmine-treated atria. Atropine sulphate (0·01 μg/ml) did not affect the augmentative response in the presence of physostigmine. Atropine or physostigmine changed the depressive response to both substances to an augmentative one, but scarcely affected the acetylcholine response.



FIG, 1. Effects of physostigmine on contracture of frog rectus skeletal muscle by McN-A-343 (●) and acetylcholine (○). Actual height of contraction on drum (mm) was plotted against the dose (μg/ml) of each drug. Each point denotes the mean from the indicated number (in brackets) of preparations with the standard error. The difference in the response to the same dose of each drug was statistically significant. Continuous lines represent control response; broken lines represent response in the presence of physostigmine.

The augmentative response of physostigmine-treated atria to AHR-602 was abolished by pretreatment of the preparations with pronethalol (2 μg/ml). Of the atria from 12 tortoises treated with reserpine (0·1 mg of reserpine phosphate/100 g body weight i.p. 48 to 72 hr previously), two responded to AHR-602 with augmentation in the presence of physostigmine. In the controls, which were given saline instead of reserpine, 6 out of 8 responded to AHR-602 with a positive inotropic effect. The difference was statistically significant ($P < 0.05$).

It is obvious that the modification by physostigmine of the responses to McN-A-343 and AHR-602 shown in the present experiment was not related to its anticholinesterase property. Rather, the action of physostigmine on the tortoise atria mimics that of atropine, and the action on the frog rectus skeletal muscle mimics that of (+)-tubocurarine. Smith (1966) reported inhibition by physostigmine of the pressor response to McN-A-343 in the cat arterial blood pressure by an action on the sympathetic ganglia. The present experiment indicates that the antagonism is not confined only to the sympathetic ganglia.

It is noteworthy that the response to McN-A-343 and AHR-602 in the isolated atria of the tortoise was reversed by physostigmine, and that the reversed effects are related to the sympathetic activity. These facts imply that the pharmacological properties of McN-A-343 and AHR-602, as well as those of physostigmine, are diverse.

Acknowledgements. We are grateful to the following for gifts of drugs: Dr. A. P. Roszkowski (McNeil) for McN-A-343, Dr. B. V. Franko (A. H. Robins) for AHR-602, and Dr. J. W. Black (I.C.I.) for pronethalol.

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The interaction of dexamphetamine with inhibitors of noradrenaline biosynthesis in rat brain *in vivo*

SIR,—It is known that sodium diethyl dithiocarbamate (DDC) is an inhibitor of dopamine-β-hydroxylase (Goldstein, Anagoste & others, 1964) and that DDC decreases tissue noradrenaline content, presumably as a consequence of this action (Collins, 1965; Carlsson, Lindqvist & others, 1966). Similiarly 3-iodo-L-tyrosine has been shown to be a potent inhibitor of the enzyme tyrosine hydroxylase (Goldstein & Weiss, 1965; Ikeda, Levitt & Udenfriend, 1965) and to decrease tissue levels of both noradrenaline and dopamine (Goldstein, Anagoste & Nakajima, 1965). In addition, Anden, Fuxe & Hökfelt (1966) have demonstrated the importance of impulse flow for the depletion of brain amines by synthesis-inhibitors. Thus an increase in impulse flow in any neuron will cause a more rapid depletion of amines from that neuron if biosynthesis is inhibited. Corrodi, Fuxe & Hökfelt (1966) have recently made use of this principle to demonstrate the inhibitory effect of barbiturates on dopamine turnover in the brain. I have applied a similar technique to study the effect of the central stimulant drug, dexamphetamine, on turnover of brain catecholamines.

Although dexamphetamine at high dose levels is known to cause a decrease in brain noradrenaline and dopamine, Smith (1965) has pointed out that the excitant action of the drug can be produced with doses which cause no change in brain noradrenaline levels and which raise brain dopamine levels. It was thought that these low doses of dexamphetamine might be producing some change in impulse flow in noradrenaline and dopamine-containing neurons which was not reflected in changes in total brain catecholamines. Accordingly, the interaction of a low dose of dexamphetamine with the noradrenaline synthesis inhibitors DDC and 3-iodo-L-tyrosine has been examined. The results are in Table 1.

Adult, male, white rats of 180–220 g weight were used. All injections were made subcutaneously in volumes of 1 ml. Estimations of noradrenaline and dopamine were by the method of Brownlee & Spriggs (1965).

TABLE 1. EFFECTS OF DEXAMPHETAMINE ON THE CHANGES IN RAT BRAIN CATECHOLAMINE CONTENT PRODUCED BY INHIBITORS OF NORADRENALINE BIOSYNTHESIS. Percentages, and standard errors are compared with uninjected controls. (Each value represents the mean of eight determinations.) The absolute values for control brains were noradrenaline 0·38 ± 0·02 μg/g, and dopamine 0·62 ± 0·05 μg/g.

Treatment	Duration (hr)	% Noradrenaline	% Dopamine
3-Iodo-L-tyrosine 200 mg/kg s.c. ..	1	60 ± 2·5	50·5 ± 3·5
	3	62·5 ± 3·5	55·0 ± 2·0
3-Iodo-L-tyrosine 200 mg/kg + dexamphetamine 2 mg/kg s.c. ..	1	82 ± 3·0	79 ± 4·7
	3	64 ± 3·2	75 ± 4·2
DDC 500 mg/kg s.c.	1	60 ± 2·6	99 ± 5·7
	3	50 ± 1·0	146 ± 7·0
DDC 500 mg/kg + dexamphetamine 2 mg/kg s.c. ..	1	87 ± 2·7	147 ± 7·5
	3	60·5 ± 2·0	144 ± 8·2
Dexamphetamine 2 mg/kg s.c.	1	97 ± 2·5	126 ± 6·[illegible]
	3	89 ± 4·5	118 ± 5·2

3-Iodo-L-tyrosine caused a fall both in the dopamine and in the noradrenaline content of rat brain; DDC caused a fall in noradrenaline and a rise in brain dopamine levels. A small dose of dexamphetamine (2 mg), which when administered alone caused no significant change in brain noradrenaline levels, reduced the rate of depletion of brain noradrenaline by both synthesis inhibitors when injected with them. The depletion of brain dopamine by 3-iodo-L-tyrosine

was also inhibited but in view of the increased brain dopamine levels produced in rat brain by this dose of dexamphetamine, this result is of doubtful significance. The rise in brain dopamine after DDC was not affected consistently by the dose of dexamphetamine.

There are several possible explanations for these results. Dexamphetamine may have interfered with the passage of the synthesis-inhibitors to the site of catecholamine biosynthesis or may have modified their action in some other way. The comparatively low dose of dexamphetamine which I used reduces the likelihood of competition with the synthesis-inhibitors for any site of action or uptake. Alternatively, the results may arise from a reduction in transmitter output from central noradrenaline neurons. If this is so, then it is unlikely that the sympathomimetic properties of dexamphetamine can be ascribed either to an increase in adrenergic transmitter release or to a decrease in transmitter re-uptake (Glowinski & Axelrod, 1965), since both these actions would have the effect of accelerating the depletion of noradrenaline after the inhibition of synthesis. It seems more likely that dexamphetamine has a direct effect on central noradrenaline receptors.

The apparent reduction in noradrenaline turnover may originate in either an inhibitory feedback from the central receptors or in a direct effect of dexamphetamine on the neuronal membrane—which inhibits the passage of noradrenaline across it.

Acknowledgements. This work has been supported in part by a grant from the Medical Research Council.

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Book Review

GADAMERS LEHRBUCH DER CHEMISCHEN TOXIKOLOGIE UND ANLEITUNG ZUR AUSMITTELUNG DER GIFTE. VOL. II. ALLGEMEINE METHODEN. Edited by E. Graf and Fr. R. Preuss. Pp. xii + 720 (including Index). Vandenhoeck & Ruprecht, Göttingen, 1966. DM.198.

The second volume of the new edition of this textbook, published in German, is concerned with general methods for the recognition and determination of poisonous drugs. In sixteen papers, experts from Germany, Austria and Czechoslovakia describe not only techniques already in general use in toxicology, but also those discovered more recently.

Individual contributions deal with the applications of paper, thin-layer, column and gas chromatography and phase separation methods. In addition spot-test and ring-oven techniques, micro-thermal methods, absorption spectroscopy in the infrared, visible and ultraviolet ranges, spectrophotometric examination of atomic emission and absorption, optical crystallography and polarography are treated in considerable detail. Furthermore, there are sections on enzymatic methods, hetero-element detection, nuclear radiation detection and radio-activation analysis.

The systematic lay out adopted in each chapter ensures that there is a clear and concise exposition of the particular subject. After discussions of the relevant theory, experimental methods and examples of their practical applications are described in detail, so that it is not necessary to refer to the original literature in order to use the techniques. An additional and most helpful feature is the description of the relevant apparatus, together with information on where individual items may be obtained. Identification tables and procedures for systematic separation complete each section so that the book is not only a textbook but is also a useful book of reference.

The inclusion of physico-chemical methods, which have only been applied to toxicology in recent years and for which applications may still be found by further research, make this volume the most modern publication available on the subject and certainly the most interesting.

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Journal of Pharmacy and Pharmacology

JUNE 1967 VOL. 19 No. 6

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Letters to the Editor

PRINTED BY W. HEFFER & SONS LTD., CAMBRIDGE, ENGLAND.