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# SOME PHARMACOLOGICAL ACTIONS OF DIETHYLDITHIOCARBAMATE ON RABBIT AND RAT ILEUM

BY

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Disulfiram, an inhibitor of the enzyme dopamine-β-hydroxylase, was reported by Thoenen, Haefely, Gey & Hürlimann (1965) to reduce the response of both the nictitating membrane and the spleen of the cat to sympathetic nerve stimulation, and it was considered that there was a decreased release of noradrenaline, disulfiram having lowered the tissue noradrenaline levels (Musacchio, Kopin & Snyder, 1964; Musacchio, Goldstein, Anagoste, Poch & Kopin, 1966). The activity of disulfiram probably depends on its reduction to diethyldithiocarbamate (Goldstein, Anagoste, Lauber & McKereghan, 1964; Goldstein, Lauber & McKereghan, 1965). The present experiments were carried out to determine the effects of diethyldithiocarbamate (DDC) on the catecholamine content of rat and rabbit ileum, and to study its effects on the response of the isolated ileum of the rabbit to sympathetic nerve stimulation.

## METHODS

### *Whole animal experiments*

Injections of DDC were made subcutaneously into the scruff of the neck of either adult Wistar rats (body weight, 220–250 g) or litter-mate young rabbits (body weight, 800–1,000 g). For comparison, 3-iodo-L-tyrosine (an inhibitor of tyrosine hydroxylase) and reserpine (an agent which depletes catecholamines) were injected similarly but only into rats. Animals were killed at different times after the DDC injection and segments of ileum were removed for extraction and assay of catecholamines and 5-hydroxytryptamine, as described below. Rats treated with 3-iodo-L-tyrosine or reserpine were killed 4 hr after injection and the ileum was removed for subsequent extraction and assay. Untreated rats and rabbits were killed to obtain control values.

### *Incubation experiments*

Segments of rabbit ileum weighing about 1 g were washed in McEwen's (1956) solution and incubated for different times at 37° C in flasks containing a mixture of McEwen's solution (20 ml.),

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ascorbic acid (20 mg/l.) and ethylene diamine tetra-acetate (disodium salt, 10 mg/l.). In this mixture, the levels of noradrenaline and dopamine did not alter during 4 hr incubation. Drugs under investigation were added 5 min before incubation commenced. Segments were removed at different times, rinsed in ice-cold McEwen's solution, dried between filter paper, weighed and stored at −10° C until extracted and assayed for their catecholamine content.

*Stimulation of rabbit ileum*

The standard Finkleman (1930) preparation using a segment of rabbit ileum with mesentery attached was set up in a 50 ml. organ bath of McEwen's solution at 37° C, aerated with 5% carbon dioxide and 95% oxygen. The sympathetic nerves were threaded through a bipolar platinum electrode and stimulated with square-wave shocks (pulse width 1 msec, frequency 30–50 sec, supramaximal voltage usually 10 V) for periods of 30 sec every 4 min. In these conditions, the inhibitory responses to sympathetic nerve stimulation remained unaltered over 4 hr. Different concentrations of DDC alone or in the presence of cocaine (4 $\mu$g/ml.) were added to the bath and left in contact with the preparation for 2 hr after which reduction of the response of the tissue to nerve stimulation was measured. In other experiments, a concentration of DDC (usually 100 $\mu$g/ml.) was left in contact with the preparation to produce about a 50% blockade of the response to sympathetic nerve stimulation and electrical stimulation was then discontinued; the sympathomimetic amine under investigation was then added to the organ bath and left in contact for 15 min. After washing, electrical stimulation was recommenced in the continued presence of the enzyme inhibitor, and the extent of the reversal of the blockade was measured.

*Extraction and estimation of the catecholamines and 5-hydroxytryptamine*

Tissues were prepared for extraction using the method of Callingham & Cass (1963). Catecholamines were extracted into butanol saturated with sodium chloride and 0.01 N-HCl (Shore & Olin, 1958) and their subsequent displacement into an aqueous phase was effected by the addition of a non-polar liquid (*n*-heptane). Tissue homogenates were added to mixtures of sodium chloride (8 g) and salt-saturated butanol (30 ml.) in glass-stoppered bottles which were shaken in a modified Kahn shaker for 60 min; they were then centrifuged at 2,000 rev/min for 5 min and 25 ml. aliquots of the butanol were removed to a second series of glass-stoppered bottles containing mixtures of *n*-heptane (50 ml.) and 0.01 N-HCl (5 ml.). These were shaken for 5 min to displace the catecholamines into the aqueous phase and then centrifuged at 2,000 rev/min for 5 min. Samples (4.5 ml.) of the aqueous phase were removed for the catecholamine estimations. Dopamine was determined by the method of Carlsson & Waldeck (1958) as modified by Carlsson (1959), and noradrenaline was estimated by the method of Shore & Olin (1958) using one-quarter amounts of the reagents they recommended.

5-Hydroxytryptamine was extracted by a method similar to that used for the catecholamines except that borate buffer (pH 10, 6 ml.) was included in the saturated butanol (Udenfriend, 1962), and the estimation was made using the method of Udenfriend, Bogdanski & Weissbach (1955). The fluorescence of the samples were read in an Aminco-Bowman spectrophotofluorometer at the following uncorrected activation and emission wavelengths respectively: dopamine (335 and 378 m$\mu$); noradrenaline (400 and 500 m$\mu$); 5-hydroxytryptamine (300 and 345 m$\mu$). Solutions of the pure amines were also extracted and their activation and emission spectra were always compared with those of the tissue extracts. The product of the galvanometer reading and the sensitivity setting represented the relative fluorescence intensity (R.F.I.). The difference between the R.F.I. of the test sample and that of the blank sample gave the absolute fluorescence value. The concentrations of dopamine, noradrenaline and 5-hydroxytryptamine in the tissue extracts were determined by comparing their fluorescence intensities with those of tissue samples to which known amounts of each amine had been added before extraction. The percentages extracted were determined by comparing the fluorescence intensities of these latter samples with those of known amounts of each amine which had not been subjected to the extraction procedure. All experiments were carried out in duplicate and the amine contents are expressed as $\mu$g/g of fresh tissue.

## RESULTS

### *Amine content of rat and rabbit ileum*

Table 1 shows that the noradrenaline concentrations of the ileum of both rat and rabbit are similar and more than three times those of dopamine. The levels of 5-hydroxytryptamine are also similar in the two species and are even higher than those of noradrenaline.

### *Injections of diethyldithiocarbamate*

A single subcutaneous injection of DDC (400 mg/kg) into both rats and rabbits significantly lowers the noradrenaline content and raises the dopamine content of the ileum (Fig. 1). In rabbits, the lowest noradrenaline value occurs 1 hr after injection

TABLE 1

NORADRENALINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE CONTENTS (μg/g FRESH TISSUE) OF RABBIT AND RAT ILEUM

Values shown are the means ± S.E.M. of eight experiments.

| Amine | Rabbit | Rat |
|---|---|---|
| Noradrenaline | 0·41±0·03 | 0·37±0·04 |
| Dopamine | 0·12±0·02 | 0·09±0·02 |
| 5-Hydroxytryptamine | 0·57±0·05 | 0·62±0·04 |

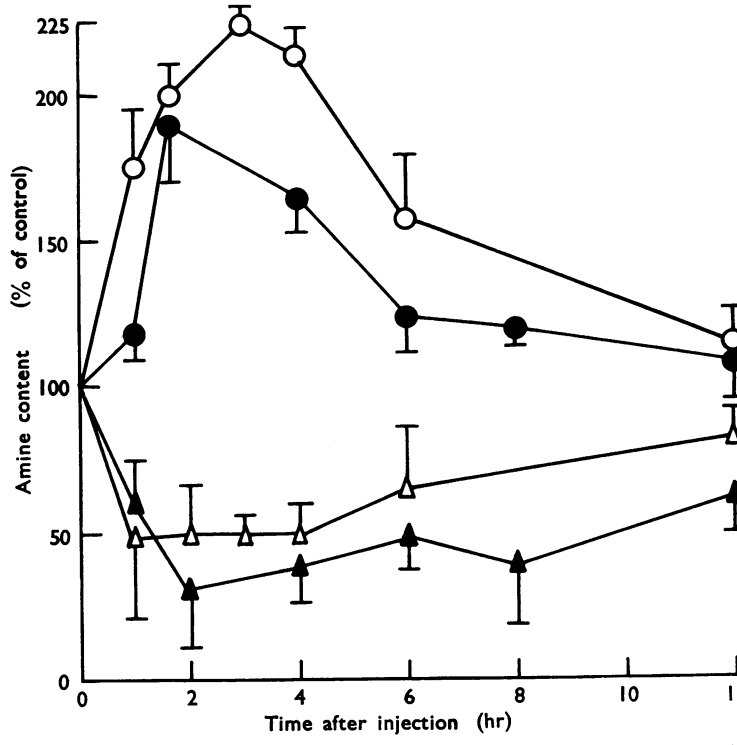


Fig. 1. Effect of a single dose of DDC (400 mg/kg) subcutaneously on the dopamine (circles) and noradrenaline (triangles) contents of the ileum of rats (filled symbols) and rabbits (open symbols). Results are expressed as percentages of the control values of untreated animals and each point is the mean of at least five experiments. Standard errors of the mean are shown by the vertical lines.

whereas in rats it is reached at 2 hr. In both species, these levels remain significantly lower than the control values for at least 6 hr. The maximum increase in tissue dopamine occurs in both species a little later (at 2–3 hr after injection), and the return to control values also requires at least 6 hr. The injection of DDC does not alter the 5-hydroxytryptamine levels in the ileum of either species.

A single subcutaneous injection of 3-iodo-L-tyrosine (200 mg/kg) into rats significantly reduces both the dopamine and noradrenaline levels in the ileum at 4 hr after injection but does not alter the 5-hydroxytryptamine level (Table 2). Reserpine (5 mg/kg), on the other hand, reduces the levels of all three amines.

TABLE 2

EFFECT OF DDC (400 mg/kg), 3-IODO-L-TYROSINE (200 mg/kg) AND RESERPINE (5 mg/kg) ON THE NORADRENALINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE CONTENTS OF RAT ILEUM 4 HR AFTER THE SUBCUTANEOUS INJECTION OF EACH DRUG

The values, expressed as percentages of the control levels, are the means of five experiments. * Significant at the $P=0{\cdot}05$ level.

| Agent | Noradrenaline | Dopamine | 5-Hydroxytryptamine |
|---|---|---|---|
| DDC | 42* | 162* | 118 |
| 3-Iodo-L-tyrosine | 52* | 77* | 94 |
| Reserpine | 12* | 10* | 14* |

*Incubation with diethyldithiocarbamate*

The noradrenaline content of rabbit ileum is significantly decreased when segments are incubated for 4 hr with DDC (100 μg/ml.) and the dopamine levels are more than doubled (Table 3). Incubation with dopamine (0.5 μg/ml.) alone increases both levels, and when dopamine is included in the incubation medium after the ileum has been in contact with DDC for 2 hr the dopamine level is further increased although the noradrenaline remains at about half the control value (Table 3).

TABLE 3

EFFECT OF DDC (100 μg/ml.), DOPAMINE (0·5 μg/ml.), AND A MIXTURE OF DDC AND DOPAMINE ON THE NORADRENALINE AND DOPAMINE CONTENTS OF ISOLATED RABBIT ILEUM

The values are expressed as percentages of the control levels. Incubation was allowed to proceed for 4 hr and the results are of five different experiments. * Significant at the $P=0{\cdot}05$ level.

| Treatment | Noradrenaline | Dopamine |
|---|---|---|
| None | 100 | 100 |
| DDC | 56* | 218* |
| Dopamine | 134* | 128* |
| DDC+dopamine | 50* | 400* |

*Effect of diethyldithiocarbamate on nerve stimulation*

DDC reduces the response of the isolated rabbit ileum to sympathetic nerve stimulation when concentrations of the enzyme inhibitor in excess of 100 μg/ml. are used and maximum inhibition is produced by 500 μg/ml. (Fig. 2). The noradrenaline content of the ileum is also decreased under these conditions and there is good agreement between the bath concentrations of DDC required to reduce the response of the tissue to nerve stimulation and those required to deplete the ileum of noradrenaline (Fig. 2). When

preparations are treated with cocaine (4 μg/ml.), a drug which inhibits the re-uptake of noradrenaline by sympathetic nerves after its release, the degree of inhibition produced by DDC is significantly potentiated when concentrations of the enzyme inhibitor exceed 150 μg/ml. (Fig. 2).

Adrenaline and noradrenaline, as well as dopamine, reverse the blockade produced by DDC (100 μg/ml.) of the response of the ileum to sympathetic nerve stimulation whereas dexamphetamine and β-phenylethylamine do not have this action (Table 4). The reversal of the blockade is prevented by previously treating the tissue with cocaine (4 μg/ml.).

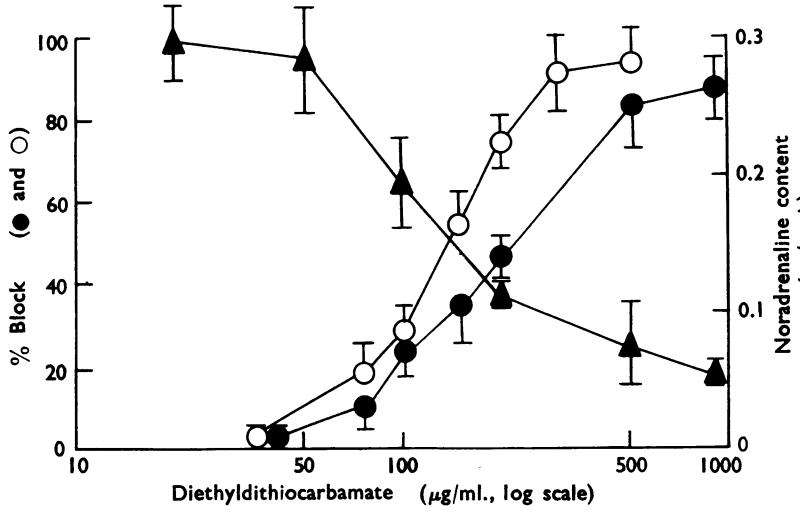


Fig. 2. Effect of different concentrations of DDC in the bath (μg/ml.) on the response of the isolated rabbit ileum to sympathetic nerve stimulation alone (●——●) and in the presence of cocaine (4 μg./ml., ○——○); each value is expressed as a percentage reduction of the control response. The effect of DDC on the noradrenaline content of rabbit ileum (μg/g fresh tissue) is also shown (▲——▲). Each point is the mean ± S.E.M. of at least five experiments.

TABLE 4

ABILITY OF DIFFERENT DRUGS TO REVERSE THE DIETHYLDITHIOCARBAMATE-INDUCED BLOCKADE OF THE RESPONSE OF THE ISOLATED RABBIT ILEUM TO SYMPATHETIC NERVE STIMULATION ALONE AND IN THE PRESENCE OF COCAINE (4 μg/ml.)

+, Reversal of blockade ; o, no effect.

| Drug | Concentration (μg/ml.) | Alone | With cocaine |
|---|---|---|---|
| Dopamine | 0·5–2·0 | + | o |
| Noradrenaline | 0·5–0·5 | + | o |
| Adrenaline | 0·5–0·5 | + | o |
| β-Phenylethylamine | 5–50 | o | o |
| Dexamphetamine | 1–20 | o | o |

## DISCUSSION

DDC has been shown in the present work to produce a dose-dependent blockade of the response of the isolated rabbit ileum to sympathetic nerve stimulation. This blockade is probably the result of noradrenaline loss at the sympathetic nerve endings and may be caused by inhibition of dopamine-β-hydroxylase, the enzyme responsible for its

formation from dopamine. The evidence for this is as follows. First, incubation of segments of rabbit ileum with the enzyme inhibitor reduces the tissue noradrenaline content and this closely parallels the degree of blockade. It is also significant that the effect of the inhibitor in depleting the noradrenaline levels *in vitro* is similar to that found *in vivo*. Second, the effect of the drug is potentiated by cocaine, a drug known to prevent the uptake of noradrenaline by sympathetic nerves (Iversen, 1965 ; Malmfors, 1965). Cocaine probably acts by preventing the reuptake of noradrenaline after its release from the postganglionic sympathetic nerve endings and this enhances the blockade. The fact that only a small potentiation of the blockade occurs at low concentrations of the inhibitor may be a reflection of the increased noradrenaline concentration in the vicinity of the receptor tissues tending to oppose the onset of the blockade. Third, dexamphetamine, a drug known to reverse the effects of adrenergic neurone blocking drugs such as guanethidine (Day & Rand, 1963) is ineffective in reversing the blockade produced by DDC. Similarly, the indirectly acting sympathomimetic amine, $\beta$-phenylethylamine, which is also an effective inhibitor of adrenergic neurone blocking agents (Day, 1962), is ineffective in reversing the DDC-induced blockade of transmission. Furthermore, we have found in other experiments that DDC possesses no ganglion blocking or adrenergic neurone blocking activity in the cat's nictitating membrane preparation, and no $\alpha$- or $\beta$-receptor blocking activity on the isolated rabbit ileum preparation.

Disulfiram was previously shown to decrease the noradrenaline levels (Musacchio *et al.*, 1964 ; Musacchio *et al.*, 1966) and increase the dopamine content of rat brain (Goldstein & Nakajima, 1966). DDC, the active metabolite of disulfiram (Goldstein *et al.*, 1964 ; Goldstein, Lauber & McKereghan, 1965) has also been reported to lower noradrenaline levels in the ileum (Collins, 1965) and brain and adrenal glands of rats (Carlsson, Lindqvist, Fuxe & Hökfelt, 1966). DDC does not seem to be exerting a reserpine-like action in the present experiments because it depletes only noradrenaline whereas reserpine depletes dopamine, noradrenaline and 5-hydroxytryptamine from tissues ; furthermore, the 5-hydroxytryptamine content after the injection of DDC remains unchanged and, of even greater significance, the dopamine level increases. Inhibition by 3-iodo-L-tyrosine of the enzyme tyrosine hydroxylase, the enzyme responsible for the ring-hydroxylation of tyrosine to form DOPA in the biosynthesis of noradrenaline, lowers both the dopamine and noradrenaline tissue contents (see also Udenfriend, Zaltzman-Nirenberg & Nagatsu, 1965 ; Goldstein, Anagoste & Nakajima, 1965 ; Goldstein & Weiss, 1965). DDC (Goldstein *et al.*, 1964) and disulfiram (Goldstein *et al.*, 1964 ; Musacchio *et al.*, 1964 ; Musacchio *et al.*, 1966), however, do not modify the uptake or the binding of noradrenaline, and disulfiram has been shown to be ineffective in releasing noradrenaline from rat hearts (Musacchio *et al.*, 1966). Thus it seems that DDC is depleting noradrenaline levels, and hence causing a blockade of the response of the isolated rabbit ileum to sympathetic nerve stimulation, by specifically inhibiting the enzyme dopamine-$\beta$-hydroxylase.

As DDC is a chelating agent and may inhibit dopamine-$\beta$-hydroxylase by forming a stable chelate with copper, a metal essential for hydroxylase activity, there is also the possibility that it is acting by chelating calcium-ions which are known to be of importance in the release of noradrenaline from postganglionic sympathetic nerve endings (Boullin, 1966). Comparison of the effect of various concentrations of DDC on the response of

the isolated rabbit ileum to sympathetic nerve stimulation with that of equimolar concentrations of EDTA (disodium salt), however, showed that in the conditions of the experiment, EDTA did not cause a blockade of the response of the tissue. It may thus be assumed that any calcium-chelating activity which DDC may possess does not contribute to its effects on the rabbit ileum at the concentrations tested.

The mechanism of action of dopamine, noradrenaline and adrenaline in reversing the blockade of the response of the isolated rabbit ileum to nerve stimulation by DDC is of interest. Noradrenaline and adrenaline are probably taken up by the sympathetic nerves and released on nerve stimulation, thereby inhibiting the pendular activity of the ileum. This conclusion is substantiated by the fact that cocaine prevents this reversal, presumably by inhibiting the uptake of the amine by the sympathetic nerves. Dopamine also reverses the blockade produced by the enzyme inhibitor, and as with noradrenaline and adrenaline, this reversal is prevented by cocaine. Sympathetic nerves may accumulate dopamine (Hamberger, Malmfors, Norberg & Sachs, 1964; Hillarp & Malmfors, 1964; Malmfors, 1965; Norberg, 1965) and the question arises whether dopamine is released from the sympathetic nerve endings of the rabbit ileum by electrical stimulation or whether it first has to be metabolized to noradrenaline. If DDC is effectively inhibiting the conversion of dopamine to noradrenaline, the reversal of blockade produced by dopamine in the presence of DDC may be the result of the dopamine being taken up by the sympathetic nerves and released unchanged on nerve stimulation. Incubation of segments of ileum with dopamine alone results in a significant increase of the tissue content of both dopamine and noradrenaline, and when DDC and dopamine are both present in the incubation medium, a greater accumulation of dopamine occurs although this is not accompanied by a corresponding increase in the tissue noradrenaline. It is concluded from these results that the concentration of DDC used is effectively inhibiting the conversion of dopamine to noradrenaline. It is unlikely that the dopamine is displacing the inhibitor from its site of action; an attempt made to detect any displacement of DDC from rabbit ileum *in vitro* using a spot test for DDC (Feigl, 1966) showed that no release occurred, even when concentrations of dopamine up to 100 $\mu$g/ml. were used.

## SUMMARY

1. Subcutaneous injections of sodium diethyldithiocarbamate, an inhibitor of the enzyme dopamine-$\beta$-hydroxylase, reduce the noradrenaline content and increase the dopamine content of the ileum of both rats and rabbits.

2. When segments of rabbit ileum are incubated with diethyldithiocarbamate, the noradrenaline levels are decreased and the dopamine values are raised.

3. Diethyldithiocarbamate reduces the response of the isolated rabbit ileum to sympathetic nerve stimulation. Dopamine, noradrenaline and adrenaline reverse this blockade, possibly by being taken up by the nerves and then being released on stimulation.

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