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THE RELEASE OF CATECHOL AMINES BY CHOLINE 2,6-XYLYL ETHER, BRETYLIUM AND GUANETHIDINE

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Choline 2,6-xylyl ether (xylocholine), bretylium, and guanethidine produce initial sympathomimetic effects when injected subcutaneously or intravenously into animals (Exley, 1957; Boura & Green, 1959; Maxwell, Plummer, Schneider, Povalski & Daniel, 1960) and similar actions have been reported for bretylium and for guanethidine upon intravenous injection into man (Taylor & Donald, 1960; Lichtlen, Schaub & Bühlmann, 1960).

Many workers have tried to associate these sympathomimetic effects with the release of catechol amines. Exley (1957), for example, showed that the pressor response caused by the intravenous injection of xylocholine into cats was abolished by piperoxan, and Gillis & Nash (1961) showed that tolazoline blocked the response to intravenously injected bretylium or guanethidine in the rat. From these observations and from other indirect evidence of a similar nature (Yelnosky & Mortimer, 1961; Bhagat & Shideman, 1963) it has been concluded that the initial sympathomimetic effects are associated with catechol amine release.

Little attention has been given to the direct estimation of the individual catechol amines in blood or in effluents from isolated perfused tissues in order clearly to show their release. In particular the possible release of 3,4-dihydroxyphenylethylamine (dopamine) by these compounds has not been studied.

The object of the present experiments was to examine the effects of xylocholine, bretylium, and guanethidine on the output of adrenaline, noradrenaline and dopamine in cat plasma.

METHODS

Preparation of animals

Male, female or neutered adult cats were used. Anaesthesia was induced with ether and maintained with chloralose (80 to 100 mg/kg, dissolved in 0.9% saline) injected through a polyethylene cannula into the right femoral vein. A tracheal cannula was inserted. The animals were then prepared essentially according to the method of Brown & Gillespie (1957) except that nerves were not prepared for stimulation. The temperature was maintained as near to 37° C as possible throughout the experiment. After removal of the gut and the adrenal glands the abdomen was closed. The animal was left for 1 hr to settle down.

At the end of the 1 hr rest period the abdomen was opened and a clamp was placed on the cut stump of the superior mesenteric vein close to its junction with the splenic vein and a cannula was tied into it. Heparin (1,000 I.U./kg; heparin injection B.P. 1,000 I.U./ml.) was injected into the right femoral vein; the animal was then ready for blood-sample collection.

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Collection and treatment of blood samples

With control cats collection began after the cannulation of the superior mesenteric vein. A clamp was placed on the portal vein just above its junction with the splenic vein and the clamp on the superior mesenteric vein was removed. Blood from the splenic vein was allowed to flow for 2 min through the cannula into ice-cooled cellulose nitrate tubes containing 2 ml. of a 1% solution of disodium edetate in 0.9% saline (Vendsalu, 1960).

At the end of the collection period the clamp was replaced on the superior mesenteric vein and that on the portal vein was removed. The cannula was washed out with 0.9% saline. Further blood samples were removed from the cats at 20, 40 and 60 min.

Once the blood had been collected all further processing, up to the time of putting the deproteinized plasma on the ion-exchange columns, was done in a cold room at 0 to 4° C. The whole 2 min sample of collected blood, mixed with the anticoagulant by inverting the tube, was centrifuged without delay. The plasma was removed from the corpuscles, care being taken not to disturb the buffy coat, and its volume was measured. It was then deproteinized with 1.2 ml. 4 N-perchloric acid (Vendsalu, 1960) and the samples were allowed to stand for 30 min. The precipitated plasma protein was centrifuged and the clear supernatant fluid was either adjusted to *p*H4 and passed through the cation-exchange columns immediately or stored at − 20° C until required.

With drug-treated cats the first sample after the resting period was collected in the usual way and served as a control. At 19 min after this control sample had been collected, 10 mg/kg of one of the drugs was injected intravenously (femoral vein) and collection of the blood sample was started at 20 min. A further two samples were collected, one at 40 min and the other at 60 min. All these samples were then treated in the same way as those from the control cats.

Separation of catechol amines using a strong cation-exchange resin.

Dowex 50W–X4 resin (200 to 400 mesh, weight 400 mg) was used in the hydrogen form and supported on lead-free glass-wool in a small glass column (13 mm^2 × 35 mm) set up in the apparatus as described by Bertler, Carlsson & Rosengren (1958). The resin was washed with 50 ml. 2 N-hydrochloric acid and then with glass-distilled water until the *p*H of the effluent rose to about 5.

A simulated plasma solution containing disodium edetate and perchloric acid in 0.9% saline, in the same amounts as in the treated plasma, was used to determine where the various amines came off the column. The simulated plasma solution with added catechol amines or the deproteinized plasma solution, made up to 13.2 ml. with 0.9% saline, was adjusted to *p*H4 and passed through the column. The column was then washed with 10 ml. of glass-distilled water, 5 ml. of phosphate buffer, 5 ml. of 1 N-hydrochloric acid and 6 ml. of 2 N-hydrochloric acid. Under these conditions 3,4-dihydroxyphenylalanine (dopa) was not retained, adrenaline and noradrenaline were eluted with 5 ml. of 1 N-hydrochloric acid and 3,4-dihydroxyphenylethylamine (dopamine) with 6 ml. of 2 N-hydrochloric acid respectively.

Similar recoveries were obtained with amounts of adrenaline and noradrenaline from 5 to 25 ng and with dopamine from 200 to 500 ng added to plasma. With 10 ng of adrenaline, 10 ng of noradrenaline and 200 ng of dopamine added the recoveries (means and standard errors) were, in five experiments: adrenaline 80.8 ± 7.8%: noradrenaline 76.3 ± 10.8%; and dopamine 75.8 ± 4.2%.

Xylocholine, bretylium, and guanethidine were shown not to interfere with the separation or recovery of the catechol amines.

Assay of catechol amines

Adrenaline and noradrenaline were assayed fluorimetrically, using an Aminco-Bowman spectrofluorimeter, in an aliquot of the 1 N-hydrochloric acid eluate after adjustment to *p*H 6.5. The method used was essentially that of Vendsalu (1960), which incorporates the differential assay proposed by Bertler *et al.* (1958). Activation wavelengths used were 400 and 445 mμ and the fluorescence wavelength was 520 mμ (all wavelengths are uncorrected instrumental values). Several modifications were made in these experiments. Firstly, zinc sulphate was omitted, as it was unnecessary and increased light scatter in the samples. Secondly, false positive values, due to interfering fluorescent material being extracted from the ion-exchange resin, were avoided by omitting the ferricyanide treatment from the sample blank. In the absence of adrenaline and

TABLE 1

ADRENALINE AND NORADRENALINE CONTENT (NG/2 MIN) OF PLASMA SAMPLES FROM CONTROL CATS AND FROM DRUG-TREATED CATS

Drugs were injected at 19 min

Treatment	Time of sampling (min)	Cat 1		Cat 2		Cat 3		Cat 4		Means		
		Adren-aline	Nor-adrenaline	Adren-aline	Nor-adrenaline	Adren-aline	Nor-adrenaline	Adren-aline	Nor-adrenaline	Adren-aline	Nor-adrenaline	Total amine
None	0	3·11	1·35	10·63	2·06	7·02	2·30	1·84	4·31	5·65	2·51	8·16
	20	1·83	2·01	8·49	1·84	13·67	2·58	2·28	3·36	6·57	2·45	9·02
	40	2·61	2·08	14·39	1·45	7·14	2·34	3·08	5·26	6·81	2·78	9·59
	60	3·00	1·31	7·88	1·71	5·20	1·96	3·48	4·41	4·89	2·35	7·24
Xylocholine	0	4·87	4·41	1·83	2·01	1·17	5·15			2·62	3·86	6·48
	20	13·28	11·17	5·30	4·32	5·17	34·54			7·92	16·86	24·60
	40	8·21	2·85	2·25	2·94	3·36	7·83			4·61	4·54	9·15
	60	8·89	1·93	2·67	2·13	1·84	3·58			4·47	2·55	7·02
Bretylium	0	1·28	8·60	2·30	6·83	2·70	5·95			2·09	7·13	9·22
	20	9·06	32·82	14·71	19·55	10·54	102·12			11·44	51·50	62·94
	40	0·09	3·32	1·85	3·92	1·84	3·90			1·26	3·71	4·97
	60	0·09	3·29	1·34	4·81	2·67	2·17			1·37	3·42	4·79
Guanethidine	0	8·65	2·90	1·16	6·20	9·05	2·81			6·29	3·97	10·26
	20	22·27	3·95	0·95	15·07	23·49	6·77			15·57	8·60	24·17
	40	4·02	1·32	1·60	7·32	12·05	2·41			5·89	3·68	9·57
	60	3·58	2·30	1·89	4·33	8·97	1·76			4·81	2·80	7·61

noradrenaline, blanks so treated gave fluorescence readings identical with those of the samples; this was not the case if the "normal" type of oxidized blank was used. Thirdly, false negative values, due to increase in fluorescence of the sample blank and to decay of the fluorescence of adrenaline and noradrenaline with time, were avoided by the addition of β-thiopropionic acid to the ascorbic acid solution.

Dopamine was assayed as described by Udenfriend (1962).

Standardized times for the reading of the fluorescence were used throughout. External and internal standards, sample blanks, reagent blanks and fluorescence spectra were run routinely. Xylocholine, bretylium, and guanethidine were shown not to interfere with the assays. Adrenaline and noradrenaline contents were calculated, using the formula given by Vendsalu (1960). Full details of the analytical technique have been recorded elsewhere (Abbs, 1964).

Drugs were used in the form of salts, xylocholine as the bromide, bretylium as the tosylate and guanethidine as the sulphate. Doses used are expressed in terms of these salts.

RESULTS

All the results quoted are corrected for background fluorescence but are uncorrected for recovery.

Plasma content of adrenaline and noradrenaline

Control cats. Four samples were taken from each of four cats. The adrenaline, noradrenaline and total amine contents in samples obtained from these cats were reasonably constant (Table 1), especially the mean total amine values. The last sample is similar to the first in amine content, thus showing that the blood volumes withdrawn were not large enough to cause haemorrhagic hypotension with a consequent increase in noradrenaline output (Millar, Keener & Benfey, 1959). The ratio of noradrenaline to adrenaline varied considerably from cat to cat but was more constant from sample to sample in any one cat. The mean adrenaline content was greater than the mean noradrenaline content in these control cats, and in only one cat was the ratio of noradrenaline to adrenaline greater than unity.

The arterial blood pressure fell slowly throughout the hour when the samples were being withdrawn from the cats. At the beginning of the collection period the blood pressure was 90 to 100 mm Hg; during the collection period it fell to 60 to 70 mm Hg.

Xylocholine-treated cats. The catechol amine content of the plasma samples from these cats is shown in Table 1. Four samples were collected from each of three cats. The samples collected at 20 min, between 1 and 3 min after injection of xylocholine, contained much more adrenaline and noradrenaline than did the samples collected at zero time which acted as controls. In each cat the amounts both of adrenaline and of noradrenaline were greatly increased. Cat 3 showed this particularly well; there was a fivefold increase in the adrenaline content and a sevenfold increase in the noradrenaline content. The means for adrenaline and noradrenaline in the samples collected at 20 min showed a similar increase in both amines. The mean catechol amine content of the samples collected at 40 min and at 60 min was similar to that of the samples collected at 0 min.

Thus xylocholine increases the plasma content both of adrenaline and of noradrenaline, but only for a short time. No significant increase could be detected at 20 min or at 40 min after injection of the drug.

Bretylium-treated cats. Table 1 shows that the output both of adrenaline and of noradrenaline was greatly increased in the sample collected after injection of the drug but was reduced in samples collected at 20 min and at 40 min after injection. As with xylocholine, however, the magnitude of the increase was very variable. In these cats all but one of the samples contained more noradrenaline than adrenaline.

Guanethidine-treated cats. Table 1 shows that guanethidine, like xylocholine and bretylium, increased the amount both of adrenaline and of noradrenaline after injection, but again this increase was not maintained. The mean increases in adrenaline and noradrenaline were of a similar magnitude after guanethidine.

Plasma content of dopamine

Control cats. Analyses for dopamine were carried out in four samples from each of twenty cats. Dopamine could not be detected in any sample. The smallest amount of dopamine which could have been satisfactorily detected was 80 ng per 2 min plasma sample.

Drug-treated cats. No dopamine was found in any sample from any of the drug-treated cats.

Identification of adrenaline and noradrenaline in plasma

Because of the small amounts of adrenaline and noradrenaline in control plasma samples, where no fluorescence peak is discernible, identification by the methods used in this work is very difficult. After treatment of the cats with drugs, however, sufficient material was present in the plasma to give a fluorescence peak. In the two experiments illustrated (Figs. 1 and 2), one with bretylium and one with xylocholine, the material liberated had a fluorescence spectrum similar to that produced by noradrenolutine and it gave a similar fluorescence intensity when activated either at 400 or 445 mμ, as did noradrenolutine. It also came off the ion-exchange column in the same 1 N-hydrochloric acid eluate as noradrenaline. This evidence, although not proof, strongly suggests that in these two experiments the material liberated by the drugs is largely noradrenaline. As the spectra produced by adrenolutine and noradrenolutine are qualitively similar when activated either at 400 or at 445 mμ, final identification as one or the other of the two amines, or as a mixture of both, depends on analysis of the ratio of fluorescence intensities produced when the lutines are activated at these two wavelengths. With an activating wavelength of 400 mμ the ratio of fluorescence of adrenolutine to noradrenolutine is approximately 1 and with 445 mμ is approximately 5. Such an analysis showed that in these two experiments the material liberated was largely noradrenaline.

When there is insufficient material in plasma to obtain a fluorescence peak, as in control samples, we may still rely on the evidence that the material under investigation came off the ion-exchange resin with 1 N-hydrochloric acid as adrenaline and noradrenaline would. Furthermore, the adrenaline and noradrenaline contents of control samples agreed closely with those of other workers who used chromatographic separation and then biological assay. The results for adrenaline and noradrenaline in control samples are similar to those obtained by Lockett & Eakins (1960), who used cats anaesthetized with chloralose and with the adrenals excluded from the circulation.

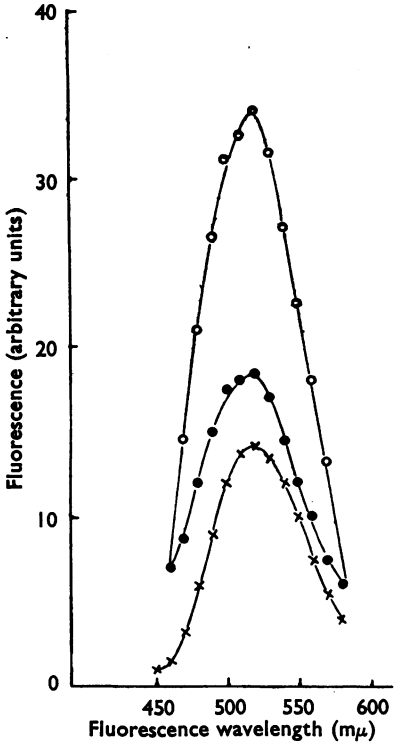

Fig. 1. Fluorimetric identification of the material present in the sample collected at 20 min from bretylium-treated cat No. 3. ×—×, 25 ng of noradrenaline; ●—●, sample from bretylium-treated cat; ○—○, sample + 25 ng of noradrenaline. The activating wavelength was 400 mμ.

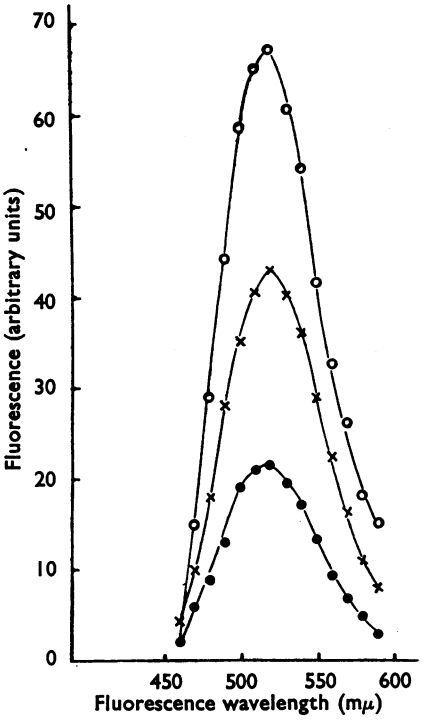

Fig. 2. Fluorimetric identification of the material present in the sample collected at 20 min from xylocholine-treated cat No. 3. ×—×, 25 ng of noradrenaline; ●—●, sample from xylocholine-treated cat; ○—○ sample + 25 ng of noradrenaline. The activating wavelength was 400 mμ.

Internal standards (10 to 25 ng of adrenaline or noradrenaline) always gave 80 to 120% of the expected value and this showed that other substances were not interfering with the assay.

DISCUSSION

Adrenalectomized cats were used to avoid liberation of adrenaline from the adrenal glands due to haemorrhagic hypotension (Millar & Benfey, 1958). Not more than 10 to 12 ml. of blood was removed from the cat in any one sample to avoid the release of noradrenaline from the adrenergic nerves (Millar *et al*, 1959), which might also have partially masked the effects of the drugs. With blood volumes greater than 10 to 12 ml. light scattering became a serious problem in the fluorimetric assays. It is impossible to say how much of the increase in adrenaline and noradrenaline output is coming from the spleen and how much is coming from extra-splenic sources.

Xylocholine, bretylium, and guanethidine cause an initial increase in both the adrenaline and noradrenaline content of plasma but this increase is not maintained at 20 and at 40

min after injection. The increase could be due either to decreased tissue uptake or to a direct release of adrenaline and noradrenaline. It is unlikely to be due to preservation of adrenaline and noradrenaline by inhibition of catechol *O*-methyltransferase, because, *in vitro* at least, none of the drugs inhibits the enzyme (Abbs, 1962). Whatever the ultimate mechanism of the increase there is enough adrenaline and noradrenaline released to account for the initial sympathomimetic effects of these drugs.

Tissue binding is probably the most important inactivation process for catechol amines at physiological dose levels (Iverson & Whitby, 1962) so that any agent which interferes with this process would have a pronounced effect on the content of adrenaline and noradrenaline in plasma. Decreased tissue uptake might therefore account for the increased amounts of adrenaline and noradrenaline found after the injection of guanethidine and bretylium, for Hertting, Axelrod & Patrick (1962) showed that in the rat bretylium and guanethidine decreased tissue uptake of [^{3}H]-noradrenaline. Bhagat & Shideman (1963) disagree with this for bretylium but agree with the finding for guanethidine. Xylocholine does not decrease tissue uptake, at least in the rat (Bhagat, 1963).

If direct release does occur it would be of interest to know the part of the "store" from which liberation is occurring because this may give an insight to the mechanism of action of these drugs. Only a small portion of the store seems to be essential for the proper functioning of adrenergic nerves, for nerve stimulation is still effective until reserpine has depleted the store to below 10% of its original content (Gaffney, Chidsey & Braunwald, 1963).

The sympathomimetic effects produced by xylocholine, bretylium and guanethidine are quickly followed by the onset of adrenergic-neurone blockade but at this time there is no tissue depletion of noradrenaline (Cass & Spriggs, 1961; Bhagat, 1963). As there is no measurable depletion of noradrenaline from tissues, the amount of amine which might be directly liberated by xylocholine, bretylium, and guanethidine is probably quite small in relation to the whole store. Such a small amount might be the 10% of the store which seems essential for the functioning of adrenergic nerves. A 10% depletion in the total tissue store might well be undetected by the usual techniques employed for the assay of catechol amines. If the release is coming from the store essential for nerve activity it is only necessary to assume that these agents—xylocholine, bretylium and guanethidine—first stimulate the nerve ending, release noradrenaline or adrenaline and then prevent further release. Green (1962), however, concluded—from the observation that the sympathomimetic effects of bretylium were still present, and indeed actually increased, after the adrenergic nerves had been blocked—that the sympathomimetic effects of bretylium were due to catechol amine release from sites peripheral to the adrenergic-nerve blockade. Xylocholine, bretylium and guanethidine might, however, trigger off the release of a small amount of catechol amine from the "nerve store" each time they were applied and yet still prevent noradrenaline from being released on nerve stimulation. Indeed, Costa, Kuntzman, Gessa & Brodie (1962) proposed that tissue depletion caused by guanethidine, after a short delay period, was a consequence of continuous activation of the physiological release mechanism. Brodie (1963) suggested that the appearance of the neurone blockade, at a time when there was no measurable tissue depletion, may be caused by the inability of the nerve endings to release noradrenaline more quickly than it is already being released by guanethidine.

A single dose of bretylium or xylocholine might also cause a similar initial stimulation but, in contrast to the action of guanethidine, the effect is not maintained, for neither of the drugs causes tissue depletion of catechol amines in acute experiments (Bhagat, 1963); repeated doses might cause stimulation in a similar manner. The sympathomimetic effects would be expected to increase because of the tissue sensitization to adrenaline and noradrenaline.

Adrenaline and noradrenaline were found both in control cats and in drug-treated cats. The origin of the noradrenaline is not difficult to explain; it probably comes from adrenergic nerves. Exactly where the adrenaline is coming from—especially as the cats had been adrenalectomized—is, however, not so obvious. It may be from extramedullary chromaffin tissue or from the tissue stores of catechol amines which are closely connected with adrenergic nerves. One would expect the larger part of the amine coming from the nerve stores to be noradrenaline. [^{3}H]-Adrenaline, however, can be taken up by the isolated gracilis muscle of the dog and can be released both spontaneously and on stimulation of the adrenergic nerves about 30 min later (Rosell, Axelrod & Kopin, 1964). At 2 hr after the adrenaline infusion about 60% of the radioactivity found in the muscle was due to adrenaline. Although tritiated exogenous material need not necessarily behave as endogenous amine would, there is a good deal of evidence that it does (Potter & Axelrod, 1963; Wolfe & Potter, 1963).

Some of the circulating adrenaline liberated from the adrenal medulla might therefore be taken up by the stores and be released later. Thus the amount of circulating adrenaline or noradrenaline in adrenalectomized animals at any one time would depend on the ratio of the amines in the tissue stores. Conversely, the amount of adrenaline in the tissue stores would depend on the amount of circulating adrenaline a short time before. If this is true the stores would contain a reasonable amount of adrenaline after the handling of the adrenal glands during adrenalectomy. This would explain the origin of the adrenaline found in the samples from control cats and in the samples before and after treatment with drugs.

No dopamine was found in the plasma either from control or from drug-treated cats. The minimum amount which could be detected by the method used in these experiments was 80 ng in a plasma sample. If, however, dopamine were released from adrenergic nerves, where 50% of the total catechol amine is dopamine (Schümann, 1956), and if this were the mechanism whereby these drugs produced adrenergic-neurone blockade, one might expect amounts as large as this to be liberated. *In vivo* these drugs might liberate dopamine and thus prevent its conversion to noradrenaline but the results obtained in acute experiments do not support this hypothesis. A slow prolonged release of small amounts of dopamine which could not be detected by the method used might, however, account for the catechol amine depletion found after chronic administration of bretylium (Green, 1962) or xylocholine (Coupland & Exley, 1957).

SUMMARY

1. The effects of xylocholine, bretylium and guanethidine on the catechol amine content of plasma samples from the splenic vein of adrenalectomized, eviscerated cats were studied, using fluorimetric analysis.

2. Xylocholine, bretylium, and guanethidine initially increase the output both of adrenaline and of noradrenaline but this increase is not maintained.

3. The increased output of adrenaline and noradrenaline can account for the initial sympathomimetic effects of the drugs.

4. None of the drugs releases dopamine into plasma.

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