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Selective depletion of noradrenaline: a proposed mechanism of the adrenergic neurone-blocking action of bretylium

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Summary

1. The effects of bretylium have been investigated on the content and subcellular distribution of noradrenaline in cat spleen and on the overflow of noradrenaline in response to stimulation of the splenic nerve.

2. Bretylium, 15 min after its administration, produces a significant depletion of noradrenaline in only the supernatant fraction of an homogenate; at this time adrenergic neurone blockade is evident. This depletion of noradrenaline is apparent up to 18 h later but has disappeared 7 days after the administration of bretylium when nerve function is also restored.

3. Both the development of the neurone blockade and the depletion of noradrenaline are prevented by previous administration of (+)-amphetamine.

4. In bretylium-pretreated cats the noradrenaline content of the supernatant fraction is replenished and the neurone blockade is abolished after treatment with (+)-amphetamine.

5. The depletion of noradrenaline, which is evident 30 min, 60 min and 18 h after treatment with bretylium, from other subcellular fractions—especially the high-speed particulate fraction—appears to be unassociated with adrenergic neurone blockade.

6. It is concluded that bretylium produces its adrenergic neurone-blocking activity by depleting noradrenaline from a " store " whose amine appears in the supernatant fraction after homogenization. Whilst bretylium is present this " store " cannot refill with noradrenaline.

Introduction

Many adrenergic neurone-blocking agents such as bretylium, bethanidine and debrisoquine have been shown not to cause depletion of catecholamines in acute experiments when gross tissue-levels have been examined (Brodie & Kuntzman, 1960; Cass & Spriggs, 1961; Boura & Green, 1963; Moe, Bates, Palkoski & Banziger, 1964). Even with guanethidine, which causes depletion of catecholamines (Sheppard & Zimmerman, 1959; Cass, Kuntzman & Brodie, 1960), Cass & Spriggs (1961), Gaffney, Chidsey & Braunwald (1963) and Chang, Costa & Brodie (1965) showed that there was a time dissociation between catecholamine depletion and adrenergic neurone blockade.

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Abbs (1965) showed that there was a substantial increase of catecholamines in splenic venous blood immediately following the intravenous injection of 10 mg/kg of either xylocholine, bretylium or guanethidine. Abbs (1966) and Carlsson (1966) suggested that the released catecholamine might be coming from a small part of the " store " which is essential for the proper functioning of adrenergic nerves. If the amine released from this " store " were not replaced a small localized depletion would result ; such a depletion might be responsible for the adrenergic neurone-blocking activity exhibited by bretylium.

The present experiments, employing subcellular fractionation procedures, were designed to test the above hypothesis. Some of the present results were communicated to the British Pharmacological Society (Abbs & Robertson, 1969).

Methods

Preparation of animals

Male, female or neutered adult cats were used. Anaesthesia was induced with ether and maintained with chloralose (80 mg/kg, intravenously, dissolved in 0·9% NaCl). A tracheal cannula was inserted, the animals were eviscerated from mid-duodenum to rectum, blood pressure was recorded from the left femoral artery and injections were made through a polyethylene cannula into the right femoral vein.

In acute experiments in which the subcellular distribution of noradrenaline was examined spleens were removed at appropriate times after the injection of 0·9% NaCl or of the drugs under test.

In experiments in which the noradrenaline content of plasma, from splenic venous blood, was measured the animals were further prepared as described by Blakeley, Brown & Ferry (1963) and heparin (1,000 i.u./kg ; heparin injection B.P. 1,000 i.u./ml) was injected.

In experiments in which the effects of bretylium were to be studied 18 h and 7 days after its administration, the drug was given intravenously during light ether anaesthesia. The cats were then allowed to recover and lead a " normal " existence until they were fully anaesthetized for spleen removal or overflow determination 18 h or 7 days later.

Details of doses and time schedules are given in the appropriate section of results.

Subcellular fractionation of the spleen

Spleens were placed in ice-cooled saline for 5 min, blotted, cut into small pieces and homogenized at 0° to 4° C, with an Ultra-Turrax homogenizer driven at 24,000 rev/min. 10 volumes of 0·25 M sucrose containing 0·001 M $MgCl_2$ and 0·005 M phosphate buffer, pH 7·4, was used per g wet weight of tissue.

A 20 ml portion of homogenate was centrifuged at 12,000 *g* for 10 min to produce a low-speed particulate fraction (coarse pellet) which was then resuspended in homogenizing medium (5 ml) and recentrifuged. The resulting supernatant fluids were combined and centrifuged at 100,000 *g* for 1 h yielding a high-speed particulate fraction (fine sediment) and a high-speed supernatant layer.

Homogenizing medium was added to the coarse pellet, to the high-speed particulate fraction and to an aliquot (10 ml) of uncentrifuged homogenate so that

the volumes of these three fractions were the same as that of the high-speed supernatant fraction. Each of the four fractions was then deproteinized with 2·3 ml 4 N perchloric acid and the precipitated protein was re-extracted with 10 ml 0·4 N perchloric acid in 0·9% NaCl. 1·4 ml 4% disodium edetate in 0·9% NaCl was added to each of the supernatant fluids after deproteinization and the volume of each was adjusted to 35 ml with 0·4 N perchloric acid in 0·9% NaCl.

13·2 ml portions, in duplicate, of each deproteinized extract were stored overnight at −20° C before separation and assay.

Recoveries of noradrenaline added to extracts of the four fractions were similar and were approximately 70%.

Collection and treatment of blood samples

The superior mesenteric vein was cannulated and clamped. 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 was allowed to flow for 2 min through the cannula into ice-cooled cellulose tubes containing 2 ml of a 1% solution of disodium edetate in 0·9% NaCl. Blood flowing during the first minute of a collection period was designated the resting sample. After one minute the splenic nerve was stimulated at supramaximal voltage with square-wave pulses of 0·5 ms duration at 30 Hz for 30 seconds. The blood flowing during this 30 s and for 30 s thereafter was collected in a second tube and was designated the stimulation sample. 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% NaCl.

Further processing was carried out as described by Abbs (1966) except that deproteinization was effected by the addition of 4 N perchloric acid to give a final concentration of 0·4 N. The deproteinized plasma was stored overnight at −20° C after being mixed with 2 ml of a 1% solution of disodium edetate in 0·9% NaCl and 0·2 ml 4 N perchloric acid.

Separation and assay of noradrenaline and adrenaline

After overnight storage, noradrenaline and adrenaline were separated from other catecholamines—in either spleen or plasma extracts—by means of a strong cation-exchange resin ; they were then assayed as described by Abbs (1966). β-Thiopropionic acid was used only in the assays of samples from deproteinized plasma. Internal standards were always assayed in parallel with the biological samples.

Bretylium and amphetamine were shown not to interfere with the separation, recovery and assay of noradrenaline and adrenaline.

Estimation of deoxyribonucleic acid-phosphorus (DNA-P) in cat spleen

Deoxyribose attached to purine bases in the deoxyribonucleic acid molecule was extracted from the residue of the unfractionated homogenate after the catecholamines had been extracted and was assayed essentially as described by Dearnaley & Geffen (1966).

Bretylium and amphetamine were shown not to interfere with the estimation.

Drugs

Bretylium was used as the tosylate and amphetamine as (+)-amphetamine sulphate. Doses are expressed in terms of the salts.

All drugs were administered intravenously in 0·9% NaCl.

Results

All results quoted are uncorrected for recovery. The adrenaline content of spleen and of plasma was less than 10% of the noradrenaline content.

All tests for significance of differences between means were carried out using Student's *t* test.

The results quoted for P1, P2, S and T are calculated with reference to the whole spleen.

Optimal period of homogenization

The criterion used to assess optimal homogenization was a maximal yield of noradrenaline in the high-speed particulate fraction (P2 fraction) concomitant with a minimum yield of noradrenaline in the coarse pellet (P1 fraction).

Homogenization was carried out for periods of 10 s with an interval of 1 min between each period to prevent overheating. Total periods of homogenization of 30 s, 60 s and 90 s were used.

Homogenizing for 30 s left much of the tissue undisrupted and homogenizing for 90 s resulted in a large increase of noradrenaline in the supernatant fluid (S fraction) at the expense of the P2 fraction (Table 1).

TABLE 1. *Effect of homogenization time on the content and subcellular distribution of noradrenaline in cat spleen*

	Spleen fraction			
	P1	P2	S	T
Time	Noradrenaline content %			Noradrenaline content ng/μmol DNA-P
30 s (*n*=4)	67·43±1·98	18·34±4·12	14·23±2·40	85·66±13·47
60 s (*n*=6)	53·81±3·29	26·29±3·64	19·90±2·64	90·77±12·10
90 s (*n*=4)	48·89±2·56	14·22±2·89	36·89±4·55	93·32±17·52

Results quoted are means ± standard error.
n, Number of observations.
The noradrenaline contents (%) of the P1, P2 and S fractions are expressed as a percentage of the sum of the noradrenaline contents of the three fractions.

TABLE 2. *Content and subcellular distribution of noradrenaline in spleens from control cats*

	Spleen fraction			
	P1	P2	S	T
Treatment	Noradrenaline content (ng/μmol DNA-P)			
None (*n*=12)	35·57±3·25	17·54±2·12	19·87±2·33	86·99±6·84
*0·9% NaCl (1 ml/kg) (*n*=3)	35·26±2·65	16·79±1·78	19·27±3·56	83·70±6·06

Results quoted are means ± standard error. *n*, Number of observations.
*0·9% NaCl was injected intravenously into lightly anaesthetized animals 18 h before removal of the spleen under full anaesthesia.

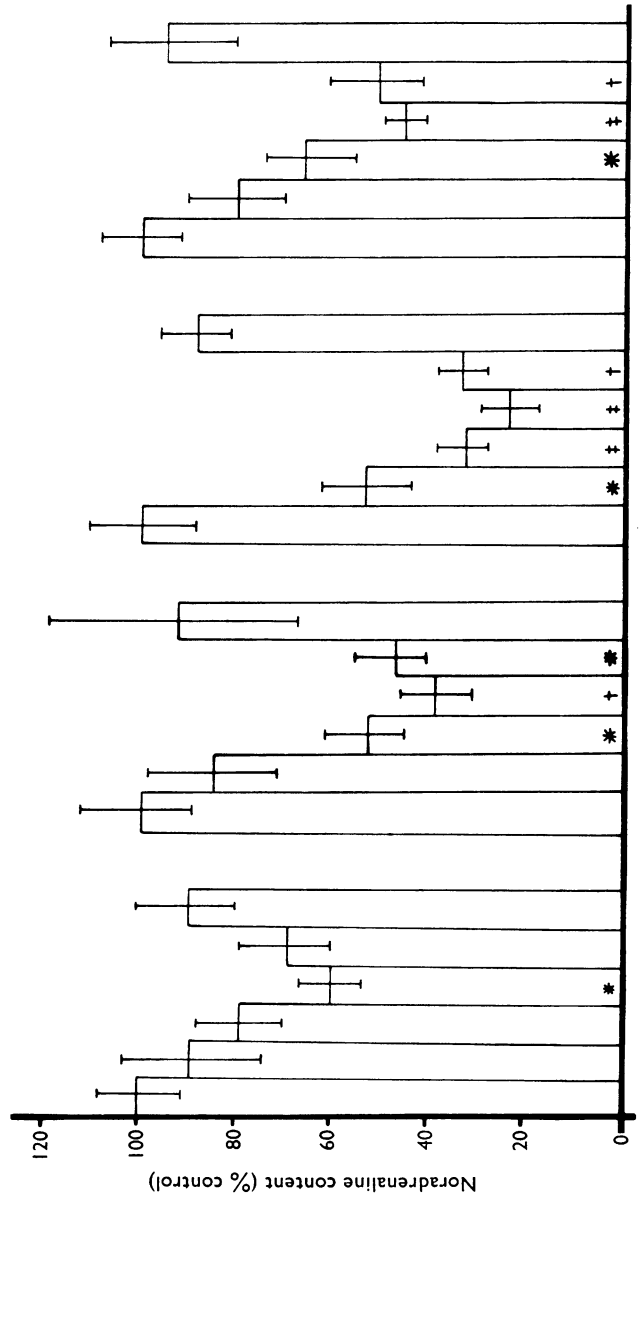

FIG. 1. Effect of bretylium on the content and subcellular distribution of noradrenaline in cat spleen. Results are means ± S.E. C, Controls; B, bretylium (10 mg/kg) treatment; subscripts indicate time in min or hours (h) or days (d) after injection. *$P<0{\cdot}05$, †$P<0{\cdot}01$, ‡$P<0{\cdot}001$.

Optimal homogenization was achieved after 60 s. Homogenization was therefore carried out for 60 s throughout the work.

There were no differences in the total amounts of noradrenaline in the spleens (T fraction) whether periods of 30 s, 60 s or 90 s were used (Table 1).

Content and subcellular distribution of noradrenaline

Control experiments

Spleens were removed from twelve cats after operative procedures and served as controls. A further series of controls was prepared by injecting 0·9% NaCl (1 ml/kg intravenously) into lightly-anaesthetized cats and removing their spleens under full anaesthesia 18 h later. These results are shown in Table 2.

There were no detectable differences in the noradrenaline content of the spleen and of its subcellular fractions between these two types of control experiments. The sum of the noradrenaline contents of the P1, P2 and S fractions was approximately 80% of the experimentally determined total content of noradrenaline (T fraction) both in control experiments and in animals which had received drug treatment. The noradrenaline content, expressed as ng noradrenaline/μmol DNA-P, of spleen fitted a normal distribution.

Bretylium treatment

Fifteen minutes after the administration of bretylium (10 mg/kg) there was a significant depletion of noradrenaline in the supernatant fraction and 15 min later there was a further depletion of noradrenaline from this fraction, but there was little further change at 60 min and at 18 h after injection. There were significant depletions of noradrenaline in the P2 and T fractions first evident 30 min after the injection of bretylium but which were more apparent 30 min later; the noradrenaline was still depleted in these fractions 18 h after drug treatment. Noradrenaline was depleted from the P1 fraction only at 60 min. Seven days after the injection of bretylium the noradrenaline content of the spleen and of its subcellular fractions was within the range found in the control experiments.

The results of these experiments are shown in Fig. 1.

TABLE 3. *Content and subcellular distribution of noradrenaline in spleens from cats injected with 2·5 mg/kg amphetamine*

	Spleen fraction			
	P1	P2	S	T
Treatment	Noradrenaline content (ng/μmol DNA-P)			
None (n=12)	35·57±3·25	17·54±2·12	19·87±2·33	86·99±6·84
Amphetamine 10 (n=5)	29·26±5·47	16·50±3·49	15·74±3·74	69·89±13·38
Amphetamine 40 (n=4)	31·03±6·05	20·66±3·83	17·07±3·77	85·50±18·96
Amphetamine 70 (n=4)	29·27±5·75	19·74±4·41	19·25±4·97	77·49±17·81

Results quoted are means ± standard error.
n, Number of observations.
Figures after amphetamine are times (min) at which the spleens were removed after the injection of amphetamine.

Amphetamine treatment

There were no significant differences in the content and subcellular distribution of splenic noradrenaline between control experiments and experiments in which the spleen was removed 10 min, 40 min or 70 min after the injection of 2·5 mg/kg amphetamine (Table 3).

Bretylium treatment in amphetamine-pretreated animals

Amphetamine, 2·5 mg/kg, was injected and allowed to act for 40 min. Bretylium, 10 mg/kg, was then injected and spleens were removed 30 min later. Pretreatment with amphetamine prevented the bretylium-induced depletion of noradrenaline (Fig. 2).

Amphetamine treatment in bretylium-pretreated animals

Bretylium, 10 mg/kg, was injected and allowed to act for 30 min. Amphetamine, 2·5 mg/kg, was then injected and spleens were removed 10 min, 25 min, or 40 min later (Fig. 3).

Ten minutes after the injection of amphetamine there were significant depletions of noradrenaline in the P2 and T fractions. Some of the noradrenaline—which had been depleted by bretylium—in the S fraction appeared to be replaced at this time although this difference was not significant when the mean results were tested against those obtained 30 min after bretylium and there was still a significant depletion of noradrenaline when tested against controls.

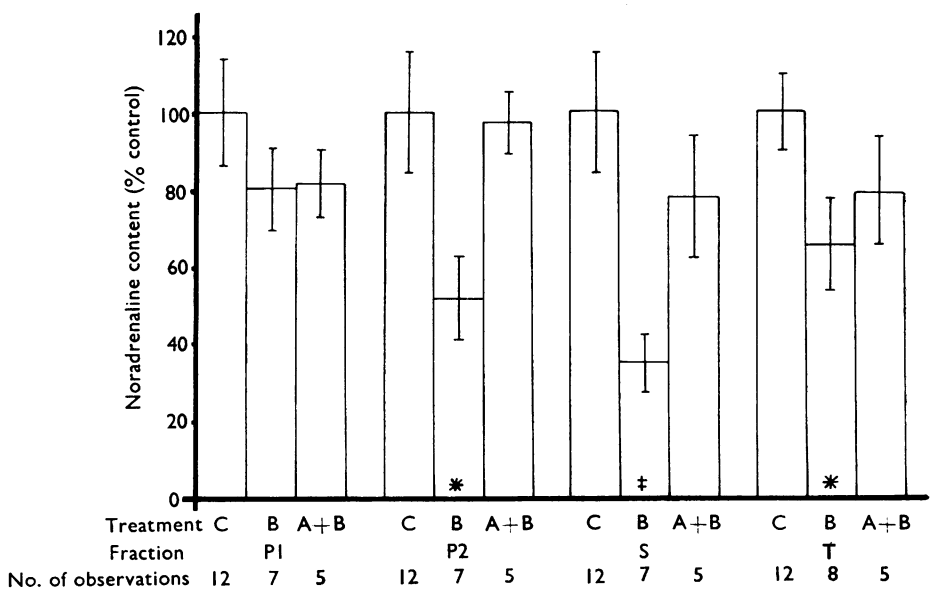

FIG. 2. Prevention of the noradrenaline-depleting action of bretylium in cat spleen by pretreatment with amphetamine. Results are means ± S.E. C, Controls; B, results obtained 30 min after the injection of 10 mg/kg bretylium; A + B, results obtained 30 min after the injection of bretylium (10 mg/kg) into cats injected 40 min previously with 2·5 mg/kg amphetamine. *$P<0.05$, ‡$P<0.001$.

After 25 min, however, there was a significant repletion of noradrenaline in the S fraction ; the noradrenaline content was now within the control range. The noradrenaline content of the unfractionated spleen was also within the control range, but in contrast there was still a significant depletion of noradrenaline from the P2 fraction.

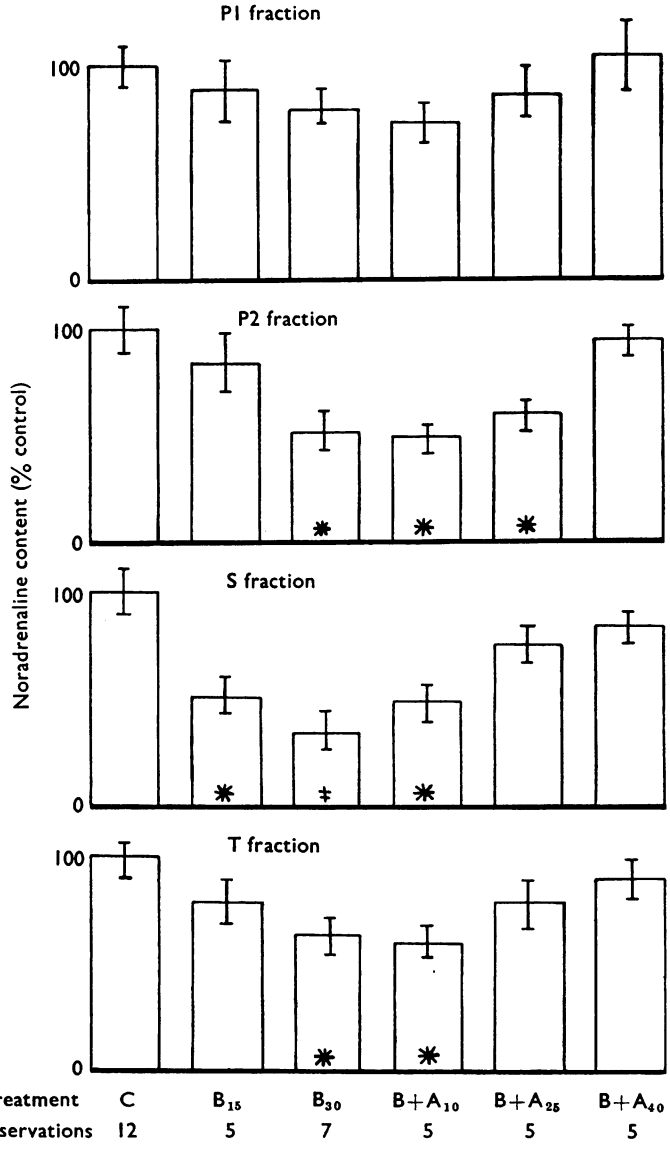

FIG. 3. Reversal of the noradrenaline-depleting action of bretylium in cat spleen by amphetamine. Results are means ± S.E. C, Controls ; B, treatment with bretylium (10 mg/kg) ; subscripts following B indicate time (min) after injection of bretylium ; B+A, results obtained by injecting 2·5 mg/kg amphetamine into cats which had received an injection of bretylium (10 mg/kg) 30 min previously ; subscripts following B+A indicate time (min) after the injection of amphetamine. *$P<0.05$, ‡$P<0.001$.

Overflow of noradrenaline after stimulation of the splenic nerve

The overflow of noradrenaline was calculated as follows: the noradrenaline concentration (ng/ml) was determined in the resting sample. The volume of the stimulation sample was measured and a figure for its background noradrenaline content was computed by reference to the concentration of noradrenaline in the resting sample. This computed background level of noradrenaline in the stimulation sample was subtracted from the noradrenaline content of the stimulation sample. The difference between these figures was termed overflow, expressed as ng, of noradrenaline.

Nerve stimulation caused a visible contraction of the spleen in all experiments except those in which bretylium had been administered alone. The collections of stimulation samples were characterized by an initial increase in blood flow compared with the flow in resting samples. This was also true for samples collected after the administration of bretylium, indicating perhaps that the splenic contraction in response to nerve stimulation may not be completely abolished by bretylium. The volume of blood in a stimulation sample was generally greater than that of the corresponding resting sample. The plasma volume was generally smaller; stimulation resulted in the expulsion of a large number of red cells from the spleen. The mean volumes of plasma from successive stimulation samples in control experiments were 2·1 ml, 2·0 ml, 1·7 ml, 1·7 ml, and 1·6 ml; similar plasma volumes were obtained after drug treatment.

Because of variations in overflow in different cats it was necessary to measure the first overflow in each experiment and to express subsequent overflows as a percentage of the initial one.

Control experiments

Two series of control experiments were performed (Fig. 4). In each series an initial stimulation was made and was followed 15 min later by an injection of 0·9% NaCl (1 ml/kg). In the first series (Fig. 4a) the stimulations 2–4 inclusive were given at intervals of 30 min after the injection of 0·9% NaCl. In the second series (Fig. 4b) the second and third stimulations were given at intervals of 15 min after the injection of 0·9% NaCl; stimulation 4 was given 60 min after the injection of 0·9% NaCl.

The overflows (%) of noradrenaline resulting from stimulations 2, 3 and 4 were closely similar in these two series of control experiments. The differences in time intervals between stimulations 1, 2 and 3 in the two series seem therefore to be of little importance.

There was a progressive decline in the overflow of noradrenaline from stimulations 1–4. Stimulation 2, for example, gave an overflow of approximately 70% of that of the first stimulation, whereas stimulation 4 gave an overflow of approximately 30%. This was probably as a result of the gradual fall in blood pressure which occurred in these experiments.

Further experiments, in which 0·9% NaCl (1 ml/kg) was injected into lightly-anaesthetized animals 18 h before nerve stimulation, were also performed (Fig. 4c). The relation between the overflows from the first four stimulations was similar to that of control experiments in which there had been no light anaesthesia 18 h previously.

The noradrenaline concentrations (ng/ml) in resting samples whose collections were started 14 min, 29 min and 59 min after the administration of 1 ml/kg 0·9% NaCl were as follows:

Results (means and standard errors) were at 14 min, $0{\cdot}1 \pm 0{\cdot}1$; at 29 min, $0{\cdot}5 \pm 0{\cdot}2$; at 59 min, $1{\cdot}4 \pm 0{\cdot}5$. Numbers of observations were 6, 11 and 8 respectively.

Bretylium treatment

The overflow of noradrenaline was reduced almost to zero at 15 min, 30 min and 60 min after the injection of 10 mg/kg bretylium (Figs. 5a and b). This was not because of an enhanced fall in blood pressure, for after the initial pressor response had subsided the blood pressure remained at or above the levels in control experiments.

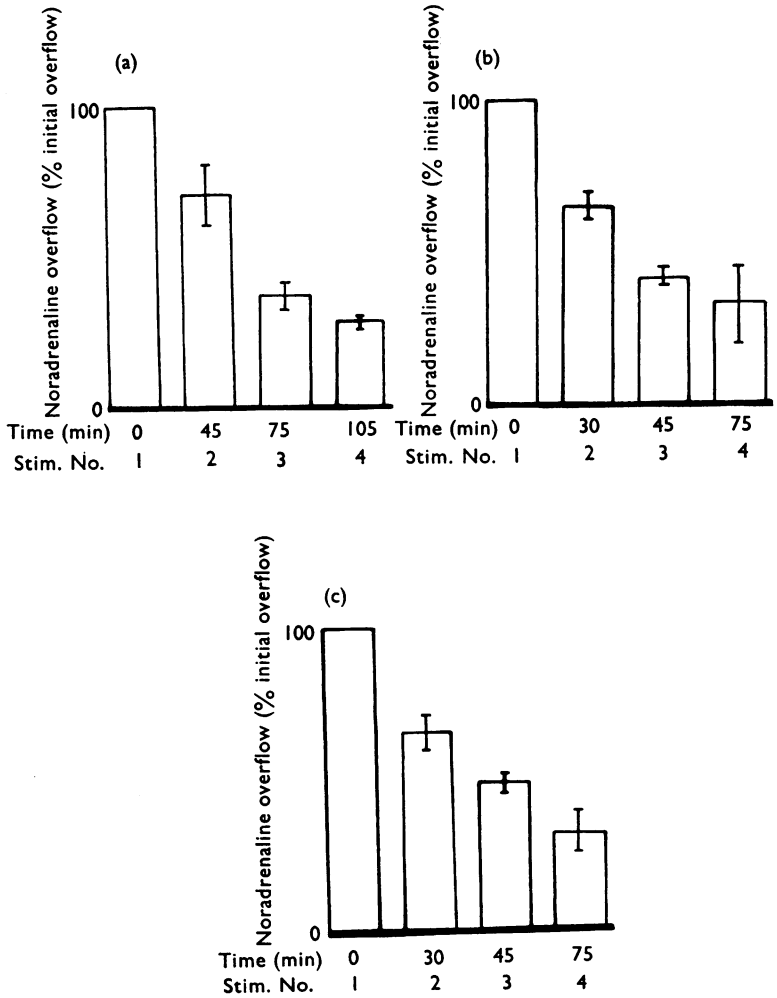

FIG. 4. Overflow of noradrenaline after splenic nerve stimulation. Results are means ± S.E. In (a) and (b), 0·9% NaCl (1 ml/kg) was injected 15 min after the first stimulation. In (c), 1 ml/kg 0·9% NaCl was injected 18 h before the first stimulation. Numbers of observations: (a)=5, (b)=6 ; (c)=3.

The overflow of noradrenaline was again almost zero 18 h after the injection of bretylium (10 mg/kg) and a similar level was found 30 min later (Fig. 5c). Blood pressure was again within the range of that found in control animals.

Seven days after a single dose of bretylium (10 mg/kg), the overflow of noradrenaline was similar to that of control animals (Fig. 5d).

The noradrenaline concentrations (ng/ml) were elevated in resting samples whose collections were commenced at 14 min, 29 min and 59 min after the administration of 10 mg/kg bretylium.

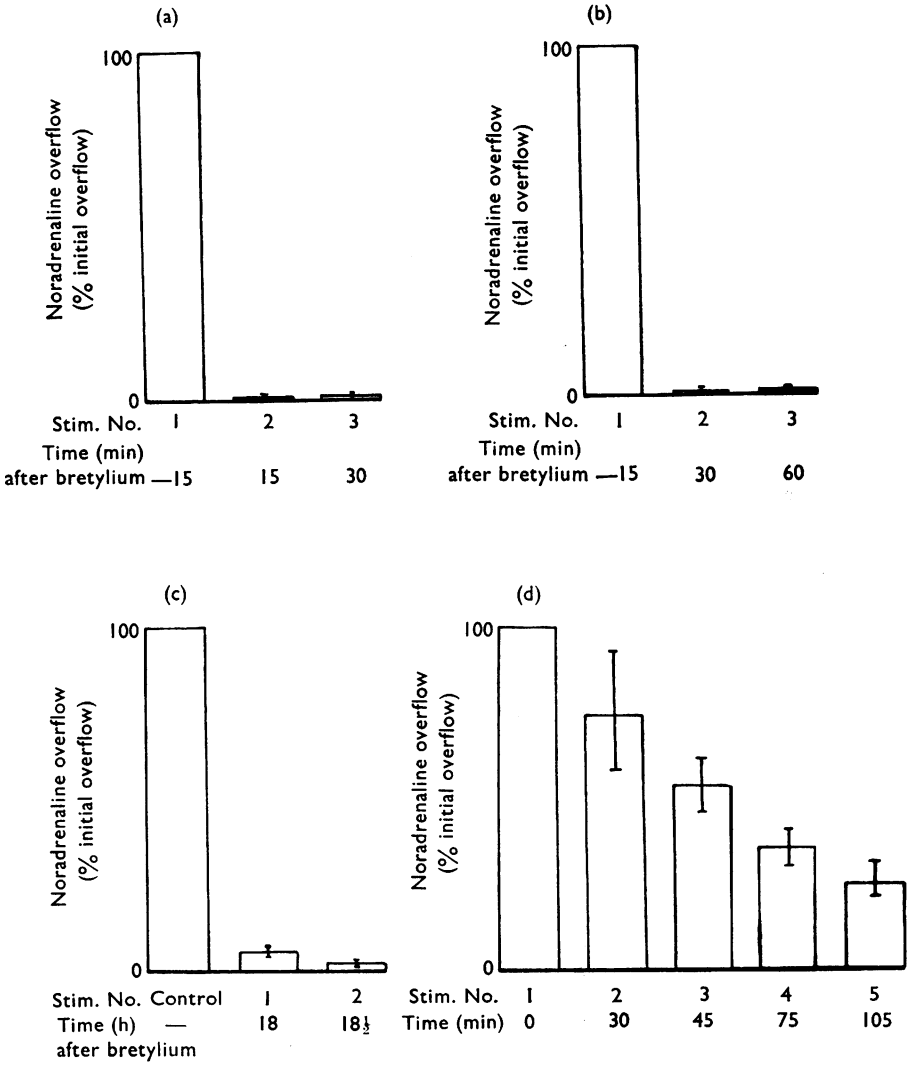

FIG. 5. Effect of bretylium on the overflow of noradrenaline in cat spleen. Results are means ± S.E. (a) and (b) show the action of 10 mg/kg bretylium in acute experiments. In (c), bretylium (10 mg/kg) was injected under light anaesthesia 18 h before the first stimulation; the mean results are expressed as % of the mean results of thirty-six control experiments in which the initial stimulation was made before drug treatment. (d), Effect of 10 mg/kg bretylium administered 7 days before the first stimulation. Numbers of observations: (a)=5, (b) and (d)=4, for stimulations 1 and 2 in (c)=3.

Results (means and standard errors) were, at 14 min, $2{\cdot}9 \pm 0{\cdot}4$; at 29 min, $2{\cdot}9 \pm 0{\cdot}6$; at 59 min, $5{\cdot}2 \pm 1{\cdot}7$. Numbers of observations were 5, 9 and 3 respectively.

Statistically significant differences ($P<0{\cdot}01$) from the resting levels in animals injected with 0·9% NaCl were demonstrable only for the 14 min and for the 29 min samples.

Amphetamine treatment

Although the mean overflows of noradrenaline 10 min, 40 min and 70 min after the injection of amphetamine (2·5 mg/kg) were not statistically different from those found in the control experiments the mean overflow from the second stimulations

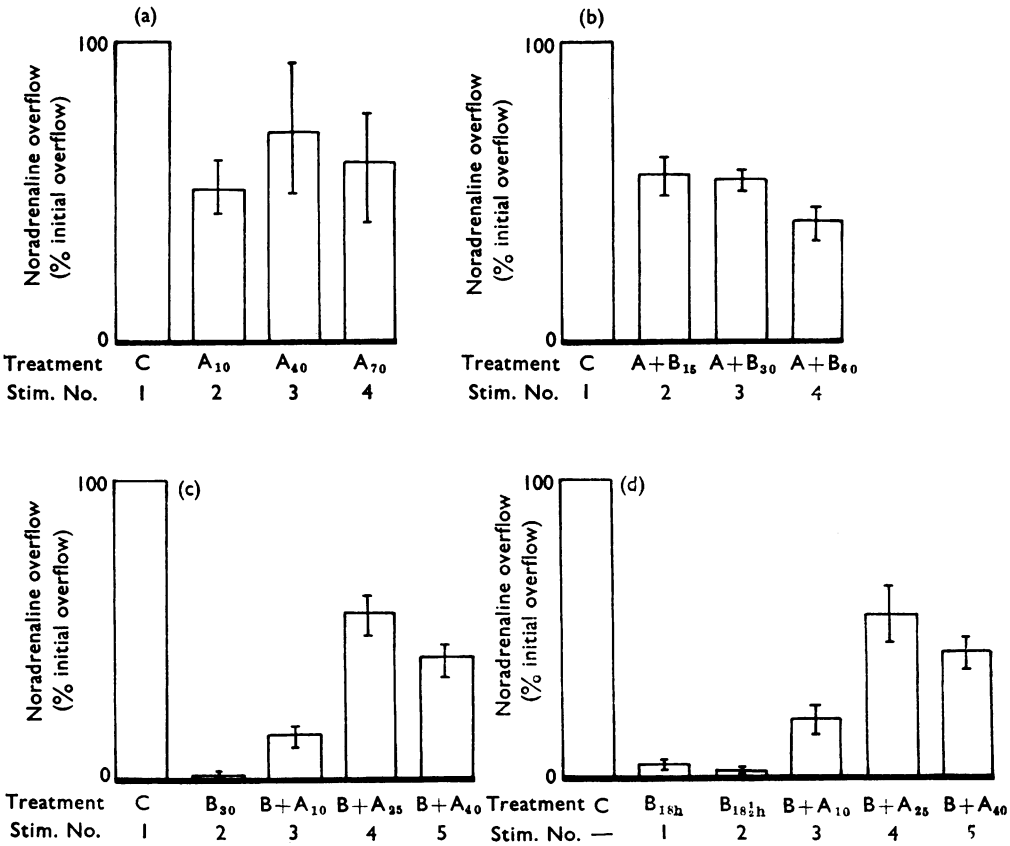

FIG. 6. Action of amphetamine and the interaction of bretylium and amphetamine on the overflow of noradrenaline after splenic nerve stimulation. Results are means ± S.E. (a), Effect of 2·5 mg/kg amphetamine (A) ; subscripts indicate time (min) after injection of amphetamine. (b), Effect of injection of 10 mg/kg bretylium (B) into cats injected 40 min previously with 2·5 mg/kg amphetamine ; subscripts show time (min) after injection of bretylium. (c), Effect of amphetamine (2·5 mg/kg) injected into cats injected 30 min previously with 10 mg/kg bretylium ; subscripts following B indicate time (min) after injection of bretylium ; subscripts following B+A indicate time (min) after the injection of amphetamine into the bretylium-pretreated cats. (d), Effect of 2·5 mg/kg amphetamine injected into cats which had been injected with bretylium (10 mg/kg) 18 h before the first stimulation ; mean results are here expressed as % of the mean results of thirty-six control experiments in which the initial stimulation was made before drug treatment ; subscripts following B indicate time (h) after injection of bretylium ; subscripts following B+A indicate time (min) after injection of amphetamine into bretylium-pretreated cats. C, Controls. Numbers of observations: (a) and (b)=4, (c)=5, for stimulations 1–5 in (d)=3.

was somewhat lower and that from the third stimulations was somewhat higher than that obtained in the corresponding control experiments (Fig. 6a). In particular there was no progressive decline in overflow after the second stimulation as there was in controls ; the blood pressure throughout these experiments remained above the levels found in animals treated with 0·9% NaCl.

The noradrenaline concentrations (ng/ml) in resting samples whose collections were started at 9 min, 39 min and 69 min after the administration of 2·5 mg/kg amphetamine were as follows:

Results (means and standard errors) were, at 9 min, $3{\cdot}7 \pm 0{\cdot}8$; at 39 min, $7{\cdot}1 \pm 2{\cdot}1$; at 69 min, $7{\cdot}2 \pm 3{\cdot}1$. Number of observations=5.

A statistically significant difference ($P<0{\cdot}02$) from the resting levels in animals injected with 0·9% NaCl was demonstrable only in the 9 min sample.

Bretylium treatment in amphetamine-pretreated animals

The reduction in noradrenaline overflow, normally produced by 10 mg/kg bretylium, was prevented by an injection of 2·5 mg/kg amphetamine given 40 min before the injection of bretylium (Fig. 6b).

The mean overflows of noradrenaline were similar to those found in animals which had received amphetamine alone.

Amphetamine treatment in bretylium-pretreated animals

Two series of experiments were performed. In the first, the initial stimulation was made in the usual way and bretylium (10 mg/kg) was injected 15 min later. The overflow of noradrenaline was determined 30 min after the injection of bretylium, and amphetamine (2·5 mg/kg) was then administered ; the overflow of noradrenaline was measured 10 min, 25 min and 40 min after the injection of amphetamine (Fig. 6c).

Bretylium produced the usual marked reduction in overflow. Ten minutes after the injection of amphetamine there was a marked increase in overflow which became maximal 25 min after amphetamine. The overflow was similarly restored 40 min after the injection of amphetamine.

In the second series of experiments bretylium (10 mg/kg) was administered intravenously during light anaesthesia ; the first nerve stimulation was applied 18 h later under full anaesthesia. The overflow of noradrenaline was only about 5% (Fig. 6d) of the level of that in thirty-six experiments in which the first nerve stimulation was applied before the injection of any drug. The overflow was similarly reduced 30 min later. Amphetamine was injected and the overflow was determined 10 min, 25 min and 40 min after its administration. The overflows of noradrenaline, after amphetamine treatment, in these cats showed a pattern which was quantitatively very similar to that obtained in experiments in which the effects of bretylium were antagonized 30 min after its administration.

Discussion

Bretylium, when injected intravenously, produces a marked sympathomimesis (Boura & Green, 1959) caused by release of noradrenaline into the circulation (Abbs, 1966). In the present work elevated levels of noradrenaline were detected in plasma

for at least 30 min after the administration of bretylium. The noradrenaline which is released probably comes from storage sites in adrenergic nerves, for when subcellular levels of noradrenaline are examined 15 min after the administration of bretylium, there is a depletion of noradrenaline; the decrease in noradrenaline content is statistically significant only in the supernatant fraction. Furthermore, the noradrenaline which is released may be from the "compartment" of the "noradrenaline store" which is essential for the proper functioning of adrenergic nerves, because when it has been released the adrenergic nerves are blocked; the overflow of noradrenaline after nerve stimulation being reduced by approximately 100%.

The release of noradrenaline *per se* is unlikely to be the cause of the blockade because amphetamine, which is known to prevent or to reverse the blocking action of the adrenergic neurone-blocking agents (Day, 1962; Day & Rand, 1962), in a dose which produces a more powerful sympathomimesis than that produced by bretylium, neither causes depletion of noradrenaline nor produces adrenergic neurone blockade. Bretylium must therefore be producing some additional effect to account for its blocking activity. In order to produce adrenergic neurone blockade bretylium must not only remove noradrenaline from the "store" from which it is normally released on nerve stimulation, but it must also either prevent the "store" from refilling or, if the "store" refills, prevent the release of amine on nerve stimulation. The former mechanism seems the more likely, for while blockade exists the S fraction remains depleted of its noradrenaline and if the noradrenaline in this fraction is repleted—either by antagonizing the effects of bretylium with amphetamine or by allowing the effects of bretylium to wear off—this is accompanied by restoration of nerve function.

One week after a single injection of bretylium there is no depletion of noradrenaline and the overflow of noradrenaline after nerve stimulation is normal.

The results thus indicate that noradrenaline from the "store" which is essential for the proper functioning of adrenergic nerves appears in the S fraction.

Further support for this view comes from experiments in which the interactions of bretylium and amphetamine were studied. In amphetamine-pretreated cats both the adrenergic neurone-blocking and the noradrenaline-depleting actions of bretylium were prevented. Repletion studies, in which the effects of bretylium were antagonized by amphetamine, provide perhaps the most convincing evidence that it is noradrenaline in the supernatant fraction which is concerned with the integrity of nerve function. Ten minutes after the injection of amphetamine into bretylium-treated cats there was a small, but not statistically significant, increase in the noradrenaline content of the S fraction; this was accompanied by an increase in the overflow of noradrenaline after nerve stimulation. When overflow became maximal, 25 min after the injection of amphetamine, the noradrenaline content of the S fraction was significantly repleted; there was no such increase in the noradrenaline content of the P2 fraction. The noradrenaline content of the P2 fraction only returned to control levels 40 min after the administration of amphetamine.

The "store" which is essential for the proper functioning of adrenergic nerves is thus unlikely to reside in the P2 fraction because at a time when adrenergic neurone blockade occurs there is no demonstrable depletion of noradrenaline from this fraction. Furthermore the results of those studies in which the bretylium-

induced adrenergic neurone blockade was reversed by amphetamine and other studies with debrisoquine (Abbs & Robertson, to be published) demonstrate even more convincingly that the noradrenaline in the P2 fraction is unconnected with adrenergic neurone blockade.

The further reduction of splenic noradrenaline 30 min, 60 min and 18 h after the administration of bretylium and the depletion of noradrenaline from other subcellular fractions—particularly from the P2 fraction—requires further explanation. This further depletion of noradrenaline cannot be the cause of the adrenergic neurone blockade because it occurs after blockade has been established. If an equilibrium exists *in vivo* between the " stores " of noradrenaline which give rise to the P2 and S fractions on homogenization then the initial localized depletion of noradrenaline in the S fraction might trigger an attempted redistribution in order to refill this " store ". Depletion of noradrenaline from the S fraction would therefore lead to a depletion of noradrenaline in the P2 fraction in an attempt to restore the equilibrium. Under the influence of bretylium, however, it appears that the S fraction is unable to retain noradrenaline. Alternatively, the noradrenaline released from the P2 fraction could be the result of a direct action of bretylium on this fraction—an action unconnected with its adrenergic neurone-blocking activity. Whatever the ultimate mechanism the noradrenaline mobilized from the P2 fraction together with additional amine from the S fraction may slowly escape as free amine into the circulation. This would agree with the elevated plasma levels of noradrenaline found in our experiments and also with the findings of Burnstock & Holman (1964) who demonstrated the return of spontaneous miniature end-plate potentials, which have been associated with transmitter release, shortly after the administration of bretylium even though adrenergic neurone blockade was still present.

Repletion of noradrenaline in the P2 fraction in experiments in which the adrenergic neurone blockade was reversed by amphetamine may be explained on the basis that once the " store " essential for the proper functioning of adrenergic nerves—the S fraction—is refilled with noradrenaline then the suggested *in vivo* equilibrium is no longer disturbed.

No assertion can yet be made as to the precise physiological nature or function of the noradrenaline " stores " which, on homogenization, give rise to the P2 and S fractions. It seems probable, however, that the noradrenaline which is essential for the proper functioning of adrenergic nerves appears in the supernatant fraction, for when it is depleted the nerves fail to function.

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