BIOCHEMICAL PROPERTIES OF BRETYLIUM

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The effect of bretylium (10^{-3} M) on the respiration of brain slices and certain enzyme systems has been investigated. This concentration of drug is similar to that present in cat sympathetic ganglia after doses of bretylium sufficient to impair adrenergic nerve function. It was a weak inhibitor of monoamine oxidase, but had no effect on the other enzymes investigated. Prolonged treatment with bretylium (10 mg./kg.) did not affect the catecholamine levels of cat adrenals and sympathetic ganglia; larger doses depleted the amine from the ganglia. The noradrenaline content of rabbit spleen tended to rise after chronic dosage with the drug and this treatment appeared to render the amine refractory to the depleting action of reserpine.

BRETYLIUM (*N-o*-bromobenzyl-*N*-ethyl-*NN*-dimethylammonium) can impair the function of the peripheral adrenergic nervous system without affecting cholinergic nerves (Boura and Green, 1959). It accumulates in the adrenergic neurones of cats (Boura, Copp, Duncombe, Green and McCoubrey, 1960) and at the time when the adrenergic blocking action is at a peak, judged by the degree of relaxation of the nictitating membranes, the sympathetic ganglia have considerably higher concentrations of bretylium than the other tissues examined. If the bretylium in the sympathetic ganglia at the time of peak effect were uniformly distributed in the tissue fluids, the concentration there would be of the order 10^{-3}M. This high concentration could be inhibitory to neural biochemical reactions if the possibility is envisaged that bretylium is a drug with relatively non-specific biochemical properties but whose pharmacological effects are rendered specific by selective localisation. To test this hypothesis the effect of bretylium at about 10^{-3}M concentration was examined on a range of biochemical systems.

EXPERIMENTAL

Bretylium bromide and iodide were supplied by Dr. F. C. Copp.

Respiration of brain slices. Slices of guinea-pig cerebral cortex about 0·35 mm. thick were cut manually by template and razor blade. They were trimmed in cold oxygenated salines to about 70 mg. weight, drained on glass and weighed on a torsion balance. They were incubated under oxygen in salines (2 ml.) at 37° and the oxygen uptake followed manometrically for periods up to 2·5 hr. Drugs were added to the saline just before gassing the vessels with oxygen.

Salines. These were basically either a glucose-phosphate saline or a supplemented saline low in bicarbonate (medium II of Krebs, 1950). The potassium content of the latter was sometimes raised to 21 mM by

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using the calculated proportion of potassium bicarbonate to replace some of the sodium bicarbonate needed to neutralise the acidic additives. In some experiments additional potassium chloride equivalent to 20 or 30 mM, or ammonium chloride to 10 mM was added to the glucose phosphate saline. In a few experiments glucose was replaced by sodium L-glutamate, succinate, pyruvate or oxoglutarate (0·02M).

Assay of bretylium in brain slices. ^{14}C-*N*-Methyl labelled bretylium iodide was used in some experiments to facilitate determination of the amount of drug present in brain slices after completion of manometry. Slices from 5 flasks (about 400 mg. wet tissue) were pooled after rinsing for 2 sec. in saline containing unlabelled bretylium at the concentration used during manometry. They were drained on glass, dried at 100°, powdered and plated on polythene planchettes for counting to $\pm$10 per cent error at infinite thickness under an end window counter. The results were referred to known standards by combustion of a few samples in oxygen and counting the carbon dioxide as gas. The author is indebted to Dr. W. G. Duncombe for these standards and for counting the solid samples. The concentrations in the slices were calculated relative to the initial wet weight of the tissue and were not corrected for swelling. An increase in weight by one third due to swelling (Stern, Eggleston, Hems and Krebs, 1949) would reduce the stated concentrations by about one quarter.

Ammonia production by brain slices. The saline medium after incubation of brain slices (about 100 mg. in 2 ml.) was removed, rapidly cooled to 0° and centrifuged. An aliquot (1·5 ml.) was taken for determination of ammonia by the microdiffusion method of Conway (1947) using 0·002N HCl for the titration. The standard deviation in determination of ammonia from 20 μg. amounts of ammonium chloride was $\pm$3·7 per cent in 5 experiments.

Assay of catecholamines. Tissues from cats or rabbits were dissected, cooled in ice, weighed and homogenised in 6 per cent trichloroacetic acid. They were assayed the same day by the fluorimetric method of Euler and Floding (1956). The cats were exsanguinated from the aorta under ether anaesthesia. The author is indebted to Mr. A. L. A. Boura for supplying the tissue specimens and for dosing the animals. Recovery of 2·7 μg. amounts of noradrenaline added to rat liver homogenates averaged 52 per cent in 4 trials (range 41–65). Bretylium did not fluoresce under the conditions of assay and there was no evidence for the presence of fluorescent metabolites in the tissues or urines from treated animals (Duncombe and McCoubrey, 1960).

Enzymes

Adenosine triphosphatase. An acetone dried powder of rat liver was prepared and assayed as described by Lardy and Wellman (1953). The adenosine triphosphate was 75 per cent pure and contained no inorganic phosphate, using the analytical methods of Eggleston and Hems (1952). The inorganic phosphate liberated by the enzyme was determined by the method of Weil-Malherbe and Green (1951).

Carbonic anhydrase. The enzyme was provided by cat erythrocytes, prepared by the method of Roughton and Booth (1956) for assay by the veronal buffer method of Miller, Dessert and Roblin (1950).

Choline dehydrogenase. The enzyme was provided by acetone-dried rat liver mitochondria, solubilised by sodium desoxycholate and assayed as described by Williams and Sreenivasan (1953).

Dopa decarboxylase. The enzyme was obtained from rabbit kidney cortex and assayed manometrically (Bertler and Rosengren, 1959).

Glucose-6-phosphate dehydrogenase. The enzyme was provided by an extract of rat adrenals and was assayed spectrophotometrically (Glock and Maclean, 1954).

Glutamic decarboxylase. The enzyme was obtained from a homogenate of guinea-pig brain in 0·05M phosphate buffer, pH 5·9, fortified with pyridoxal phosphate (1 mg. for two brains in 15 ml. buffer). Activity was assayed manometrically (Roberts and Frankel, 1951).

Monoamine oxidase. The enzyme was provided by an acetone dried preparation of guinea-pig liver mitochondria and was assayed manometrically with oxygen gas phase at 37° in phosphate buffer pH 7·4. Substrates, all at 0·01M final concentration were tryptamine, tyramine, 5-hydroxytryptamine and 3-hydroxytyramine. A short pre-incubation period of 10 min. with the drugs preceded addition of substrate from the side arm.

Thiaminase. Fresh bracken was extracted and assayed as described by Kenten (1958) using piperidine as the acceptor amine.

Trypsin. A 1 per cent solution of commercial trypsin (1 ml.) was incubated at 37° with bovine albumen (10 mg.) in 0·05M phosphate buffer pH 7·5 (3 ml.) for 1 hr. Protein was assayed as described by Robinson and Hogben (1943).

RESULTS

Respiration of brain slices. Table I shows that bretylium at 5×10^{-4}M inhibited the resting respiration of guinea-pig cerebral cortex slices in glucose phosphate saline containing 6·7 mM potassium ion by 21 per

TABLE I

INHIBITION OF RESPIRATION OF BRAIN SLICES BY BRETYLIUM

Guinea-pig cerebral cortex slices were incubated in glucose phosphate saline containing 5×10^{-4} M bretylium

Figures express μmoles O_2/g. tissue/hr. with S.D.

	6·7 mM K^+	26·7 mM K^+	36·7 mM K^+
No bretylium	56 ± 8	70 ± 12	82 ± 11
With bretylium	44 ± 6	60 ± 8	80 ± 9

cent. It almost suppressed the 25 per cent increase in respiratory rate due to additional 20 mM potassium ion but had no effect on the larger increment in rate due to 30 mM potassium ion. Bretylium was inactive at 10^{-4}M in similar experiments. The drug (10^{-3}M) had no influence on

the rate of respiration of rat liver slices in glucose phosphate saline. The rate of respiration of brain slices in the potassium enriched saline with added bretylium sometimes slackened progressively after 1 hr. incubation. The effect was not consistent even with slices from the same animal and the degree of slackening varied considerably. Of the results from 113 slices there was no slackening observed in those without added bretylium (56) whether the saline was potassium enriched or not, or in those with bretylium but without additional potassium (14). Slackening however occurred in 20 of 36 experiments with both bretylium and additional potassium. Attempts to make this effect reproducible for further study by replacing glucose with other substrates, L-glutamate, pyruvate, succinate or oxoglutarate (0·02M), or by reducing endogenous substrate by incubation of the slices for 30 min. before adding glucose, were all unsuccessful. In a short series of experiments, histamine phosphate 10^{-4}M, a component of adrenergic nerves (Rexed and Euler, 1951), also prevented the increment in respiratory rate of brain slices in glucose phosphate saline due to added potassium salts but there was no slackening of rate as observed for bretylium. Histamine did not enhance the inhibitory effect of bretylium on respiration.

TABLE II

INHIBITION OF RESPIRATION OF BRAIN SLICES BY BRETYLIUM

Guinea-pig cerebral cortex slices were incubated in the supplemented medium II of Krebs (1950) with the stated K^+ concentration. The average rates of oxygen uptake during the first and second hours are expressed as μmoles O_2/g. tissue/hr. $\pm$ S.D.

	5·9 mM K^+		21·1 mM K^+	
	A	B	A	B
1st hr.	89 $\pm$ 9	92 $\pm$ 7	105 $\pm$ 16	93 $\pm$ 18
2nd hr.	66 $\pm$ 15	59 $\pm$ 12	80 $\pm$ 14	53 $\pm$ 14

A. No bretylium. B. With bretylium (10^{-3} M)

Brain slices incubated in the supplemented medium low in bicarbonate (Krebs, 1950) respired at higher rates compared to those in glucose phosphate saline and these progressively slackened during 2·5 hr. During the second hr. the average rate was 80 per cent of that during the first hr. The initial respiratory rate was slightly increased by addition of potassium salts to a final concentration of 21 mM and fell by an average of 24 per cent during the second hr. In this saline without additional potassium salts the fall in respiratory rate during the second hr. was slightly increased by bretylium (10^{-3}M) to 36 per cent. With additional potassium salts the rate fell by 43 per cent. Bretylium had no influence on the initial rate of respiration in this saline without additional potassium. Bromide ion (10^{-3}M) introduced by the bretylium, had no influence when added as sodium bromide. Results are summarised in Table II.

Brain slices respiring in glucose phosphate saline with added ammonium chloride (10 mM) showed a slightly enhanced rate of oxygen uptake (60 $\pm$ 5 μmoles/g. tissue/hr. which was reduced to 45 $\pm$ 9 μmoles/

g./hr. by addition of bretylium (10^{-3}M). The flasks without bretylium showed a 15 per cent reduction in rate during the second hr. of incubation whereas those with bretylium showed a 50 per cent reduction.

Ammonia production by brain slices. Bretylium (10^{-3}M) had no influence on the amount of ammonia released into glucose phosphate saline by brain slices incubated anaerobically for 1 hr. ($3{\cdot}3 \pm 1{\cdot}0$ μmoles/g./hr.). There was a 30 per cent fall in ammonia output due to added bretylium by slices incubated anaerobically in the Krebs supplemented saline during 2·5 hr. but the difference was not significant in 6 paired results ($1{\cdot}52 \pm 0{\cdot}68$ compared to $1{\cdot}07 \pm 0{\cdot}59$ μmoles/g./hr.). Table III shows that the ammonia liberated into glucose-free phosphate saline under aerobic conditions was reduced by bretylium at 1 hr. but there was no difference at 3 hr. The drug did not inhibit tryptic digestion of albumen.

TABLE III

PARTIAL INHIBITION OF AMMONIA PRODUCTION IN BRAIN SLICES BY BRETYLIUM

Guinea-pig cerebral cortex slices were incubated aerobically with bretylium (10^{-3} M) in a glucose-free phosphate saline

Figures expressed in μmoles NH_3/g. tissue are means of 4 results

	0·5 hr.	1 hr.	2 hr.	3 hr.
Control	$5{\cdot}5 \pm 0{\cdot}5$	$8{\cdot}6 \pm 1{\cdot}3$	$11{\cdot}6 \pm 1{\cdot}8$	$13{\cdot}4 \pm 2{\cdot}0$
Bretylium	—	$4{\cdot}7 \pm 0{\cdot}7$	$8{\cdot}6 \pm 2{\cdot}1$	$11{\cdot}8 \pm 1{\cdot}3$

Uptake of bretylium by brain slices. The mean concentration of bretylium in brain slices after respiring aerobically for 1 hr. in glucose phosphate saline containing 0·5 μmoles/ml. drug was $1{\cdot}44 \pm 0{\cdot}71$ μmoles/g./wet tissue. In saline with additional potassium ion (20 mM) this concentration was reduced to $0{\cdot}67 \pm 0{\cdot}33$ μmoles/g. ($P = 0{\cdot}1$). The bretylium was washed out of the slices fairly readily, one wash in saline reducing the concentration to about one quarter. After washing the respiratory rate returned to normal control value.

Influence of bretylium on enzymes. Of the nine enzymes tested, bretylium (10^{-3}M) inhibited only monoamine oxidase. Using tryptamine or 3-hydroxytyramine as substrate, inhibitions were 39 ± 4 and 38 ± 2 per cent respectively (means of 4 results). With tyramine or 5-hydroxytryptamine as substrate the inhibitions were 73 ± 8 and 69 ± 11 per cent respectively. The inhibition was readily reversed by washing. These results are comparable in so far as they were obtained with one batch of enzyme. Bretylium was almost inactive at 10^{-4}M.

Influence of bretylium on the catecholamine content of tissues. Table IV shows that the levels of noradrenaline in representative cat tissues after one dose of saline were reasonably constant. Cats receiving bretylium (10 mg./kg. s.c.) showed more variation in tissue amine levels with a tendency to increased values in the sympathetic ganglia. The levels in spleen and heart were little affected though there may have been some reduction after two weeks of daily dosage. The high values found in sympathetic ganglia could not be accounted for by an increased proportion of adrenaline which, in the assay used, gives a higher fluorescence intensity

compared to noradrenaline. The levels of adrenaline remained barely detectable. A larger dose of bretylium (30 mg./kg.) daily for 14 days caused about 50 per cent fall in the noradrenaline content of the superior cervical and stellate ganglia ($P = 0{\cdot}01$). A similar fall was found in spleen and heart, but the adrenal amine levels remained unaffected. The loss of noradrenaline in sympathetic ganglia, spleen and heart due to the larger dose of bretylium was not enhanced by reserpine (1 mg./kg.) given with the last dose of bretylium about 12 hr. before killing the animal but the adrenals showed a marked depletion of their catecholamines. By contrast the spleens of 4 rabbits given bretylium (30 mg./kg.) daily for one month contained $0{\cdot}62 \pm 0{\cdot}43$ μg./g. noradrenaline compared to $0{\cdot}27 \pm 0{\cdot}11$ μg./g. in 4 controls. The adrenals were not affected.

TABLE IV

THE CATECHOLAMINE CONTENT OF CAT TISSUES AFTER PROLONGED DOSAGE WITH BRETYLIUM

Bretylium bromide given daily to cats, s.c. The figures are μg./g. wet tissue by fluorometric assay. Control values are the means from 3–5 cats receiving one dose of saline.

Figures given for the 30 mg./kg. dose are the mean of 3 results

Duration of dosage (days)	10 mg./kg. dose							
	Noradrenaline							Adrenaline
	Superior cervical ganglion	Stellate ganglion	Coeliac ganglion	Splenic and gastric nerves	Spleen	Heart	Adrenal	Adrenal
Controls	5·5 ± 0·7	5·7 ± 0·5	9·4 ± 4·9	5·7 ± 2·7	0·56 ± 0·15	0·50 ± 0·07	355 ± 171	342 ± 54
1	4·9	2·7	10·5	12·5	—	—	436	598
3	10·7	17·1	1·9	8·8	0·6	0·6	—	—
7	5·0	14·6	33·2	20·2	—	0·3	—	—
10	7·4	6·4	18·7	4·2	0·8	0·4	173	358
15	—	11·6	21·7	0·7	0·2	0·1	340	149
22	—	11·0	—	0·8	0·6	0·6	427	366
31	3·0	4·5	11·9	—	0·3	0·1	414	1005
	30 mg./kg. dose							
14	2·2 ± 1·1	1·8 ± 1·6	—	—	0·28 ± 0·23	0·07 ± 0·04	709 ± 286	509 ± 128
14*	2·6 ± 1·2	2·2 ± 1·3	—	—	0·11 ± 0·00	0·20 ± 0·20	102, 231	149, 270

* Reserpine (1 mg./kg.) given on the 13th day.

When reserpine (1 mg./kg.) was given to rabbits followed at daily intervals by either saline or bretylium (30 mg./kg.), the noradrenaline content of the spleen fell more slowly in the bretylium group (Table V). The long delay in the return of noradrenaline content in the spleens of these rabbits. is notable. They were sensitive to reserpine and 2 mg./kg. caused delayed fatalities.

DISCUSSION

Since a pharmacologically effective dose of bretylium (10 mg./kg.) given daily for as long as 31 days did not deplete the catecholamine content of sympathetic ganglia or adrenals, it seems reasonable to exclude the inhibition of biosynthesis of noradrenaline as a mode of action of the drug, unless the biosynthesis is linked firmly to the processes of release. The tendency to higher values of noradrenaline in sympathetic ganglia after prolonged dosage at this level suggests rather that the catecholamine

release mechanism is impaired and accumulation of the amine occurs by continued, though possibly slower, biosynthesis. Conversely, the depletion of the amine from the ganglia by larger doses suggests that the noradrenaline storage mechanism becomes deranged, indicating an affinity of bretylium with guanethidine or reserpine, both of which deplete tissue noradrenaline stores. The noradrenaline content of the spleen of rabbits increased, though the wide scatter of the results suggest that the effect is inconsistent under the experimental conditions described. The result however is consistent with the finding that the release of noradrenaline from rabbit spleen by reserpine appeared to be slower when bretylium was given subsequent to the dose. The results obtained with

TABLE V

NORADRENALINE CONTENT OF RABBIT SPLEEN AFTER RESERPINE FOLLOWED BY DAILY DOSES OF BRETYLIUM

Reserpine (1 mg./kg.) was followed after 12 hr. by bretylium (30 mg./kg.) or saline at daily intervals. Figures are μg./g. noradrenaline of single rabbits. Normal controls 0·27 $\pm$ 0·11 μg./g.

	Days after reserpine									
	1	4	8	9	11	15	17	19	22	26
Saline	0·084	0·057	0·063	0·015	0·044	—	0·050	0·061	0·027	0·166
Bretylium ..	—	0·037	0·185	0·136	0·133	0·114	0·050	0·160	0·074	0·058

rabbits suggest that bretylium may limit the egress of noradrenaline from its site of biosynthesis or its binding site, possibly by affecting membrane permeability. These alternative hypotheses seem preferable to the hypothesis that bretylium has non-specific biochemical properties rendered pharmacologically specific by localisation at selected sites. On the contrary, the drug has no influence on a number of enzymes that attack substrates with formal resemblance to bretylium. The evidence presented here suggests that bretylium may produce its effects by specifically affecting catechol amine storage and release mechanisms.

The partial inhibition of ammonia output by brain slices deprived of glucose derives interest from observations (Larrabee, Horwicz, Stekiel and Dolivo, 1957) that stimulated sympathetic ganglia can oxidise nitrogenous substrates (see, however, Borowicz and Larrabee, 1962), but further work on this topic was deferred since major sources of ammonia in nervous tissue are conjectural and probably composite. Unlike a related drug, benzethonium (Beck, Pinter and McKenna, 1960), bretylium did not inhibit trypsin.

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