# THE ABSORPTION OF BRETYLIUM AND RELATED QUATERNARY AMMONIUM SALTS FROM THE ALIMENTARY TRACT

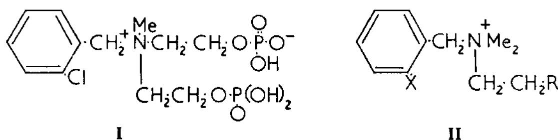
By A. L. A. Boura* and A. McCoubrey*

*From The Wellcome Research Laboratories, Beckenham, Kent*

Received April 30, 1962

*N*-Methyl-$^{14}$C-labelled bretylium was injected into the lumen of segments of the gastrointestinal tract of cats prepared so that blood draining from these segments could be collected for analysis. The drug was not absorbed from the stomach. The concentration of drug in the venous blood from the duodenum rose steadily during 1·5–2 hr. but then declined to a value slightly exceeding that in the systemic blood. The greater part of the dose was not absorbed. Bretylium slowly traversed rat duodenum (mucosa to serosa) *in vitro* and the rate was not affected by factors that can influence carbohydrate metabolism. Transfer proceeded equally well from serosa to mucosa. To investigate means of obtaining better absorption of quaternary ammonium anti-adrenergic drugs a close analogue of bretylium bearing a choline residue was phosphorylated to give a phosphoric ester betaine (III). This was about twice as active by the oral route in cats as the parent drug, in which form it was excreted in the urine.

Only a small proportion of oral doses of quaternary ammonium salts is absorbed by the intestine (Levine, Blair and Clark, 1955). The effective oral to subcutaneous dose ratio of bretylium (II; R = H, X = Br) was about 5:1 in cats (Boura and Green, 1959) but a suspected erratic absorption of oral doses in man has been a source of criticism in the therapeutic application to treatment of hypertensive disease (Dollery, Emslie-Smith and McMichael, 1960). The absorption of bretylium was therefore studied *in vitro*, using rat duodenum, and *in vivo*, using cats.




I II

An analogue of bretylium, *o*-chlorobenzyl-*NN*-dimethyl-*N*-2-hydroxyethylammonium iodide (BW 329C57; II; R = OH, X = Cl) had comparable adrenergic neurone blocking activity to bretylium in the Finkleman preparation and in intact cats (Boura and Green, unpublished). Since the rate of intestinal absorption of weak acids and bases has been related to their pK values (Hogben, Tocco, Brodie and Schachter, 1959), it was considered that the phosphoric ester betaine of BW 329C57 (II; R = O·PO(OH)O$^-$; X = Cl), a weak acid, might have more favourable ionisation characteristics for absorption from the intestine. It was further

* Present address: Research and Development Laboratories, Reckitt and Sons, Ltd., Hull.

surmised that body phosphatases could hydrolyse the ester after absorption to liberate the active quaternary ammonium salt. The concept was extended to a brief study of the more strongly acid betaine, BW 293C60 (I), and to the neutral α-glycerophosphate ester betaine BW 564C61 (II; R = $O{\cdot}PO(O^-){\cdot}O{\cdot}CH_2{\cdot}CHOH{\cdot}CH_2OH$, X = Cl).

## METHODS

New drugs are described in the preceding paper (Copp, Jones and McCoubrey, 1962). All chromatograms were run in s-butanol-acetic acid-water (12:5:3) unless stated otherwise, and drugs were visualised either by spraying with Dragendorff's reagent diluted to pale orange colour or by autoradiography.

*Assay of BW 329C57 (II).* Since this compound did not form complexes with sulphonic acid dyes soluble in halogenated solvents the method used for bretylium (Duncombe and McCoubrey, 1960) could not be applied. It was assayed nephelometrically by titrating Dragendorff's reagent with the drug eluted from paper chromatograms. Using a reagent (1 ml.) containing one molecular proportion of bismuth trichloride to four of potassium iodide a minimum of 25 μg. BW 329C57 could be assayed.

Samples of urine from cats that had received doses of BW 329C47 or BW 171C60 were depleted of salts and protein by addition of 2 volumes of alcohol and cooling to 0°. The solutions were filtered and aliquots chromatographed on Whatman No. 1 paper. Marker spots were visualised by spraying with a dilute Dragendorff's reagent (sensitivity 1 μg.) and the indicated area of paper was eluted by not more than 0·2 ml. water. The volume of eluate was measured in a graduated pipette (0·2 ml.) adapted as a burette, the tip of which had been ground to minimum area and waxed. The eluate was added to the reagent (1 ml.) contained in a scratch free tube in approximately 0·01 ml. amounts until the Tyndall effect due to a suitable beam of light persisted. Frequent controls were necessary to compensate for temperature changes. The reagent was prepared fresh for each titration by mixing bismuth trichloride (4 per cent in 2N hydrochloric acid; 0·5 ml.) and potassium iodide (8·4 per cent in 0·2N sodium hydroxide. Use of N hydrochloric acid increased the sensitivity slightly but the reagent became too unstable. Results were calculated from the expression

$$\mu g.\ \text{BW 329C57 in eluate} = VS\left(\frac{1+y}{y}\right)$$

where V = eluate volume, $S = \frac{100v}{1+v}$, v = standard titration volume using 100 μg./ml. BW 329C57, y = titration volume.

Recovery of 100 μg. amounts of drug averaged 86 ± 7 per cent in 8 trials.

*Assay of BW 171C60.* A satisfactory chemical assay was not devised. Perchloric acid oxidation (Hanes and Isherwood, 1949) of eluates from paper chromatograms and assay of inorganic phosphate gave erratic

results. A minimum of 20 μg. drug could be visualised on paper chromatograms by Dragendorff's reagent. It was finally assayed by use of $^{14}$C-labelled material.

*Assay of radioactive drugs.* Blood samples were dried to constant weight (4 days) over phosphorus pentoxide under reduced pressure. The residues were powdered and plated on polythene planchettes for counting at infinite thickness under a mica end window counter. Urines, intestinal contents or salines were depleted of salts and protein if necessary by the addition of two volumes of ethanol and cooling to 0°. Aliquots (usually 40 μl.) of the filtrates, concentrated if necessary, were plated on lens paper for counting. Similar aliquots with added radioactive drug sufficient roughly to double the counting rate (usually about 200 counts/min.) were used for calculation of results.

### Enzymes

An alkaline phosphatase was prepared from rat intestinal mucosa (Long, 1953). An acid phosphatase was prepared from rat liver (Goodlad and Mills, 1957). In both instances the rate of hydrolysis of phosphoric ester betaines (60 μmoles) was followed by assaying aliquots of the incubated mixtures for inorganic phosphate at suitable time intervals. The activity of the enzyme preparations was assessed by measuring the rate of hydrolysis of β-glycerophosphate.

A phosphodiesterase was prepared from rat liver (Dawson, 1956). The rate of hydrolysis of BW 564C61 was assessed qualitatively by examination of visualised spots of BW 329C57 (Dragendorff's reagent) and α- and β-glycerophosphates (acid molybdate reagent) on paper chromatograms.

### Pharmacological Evaluations

*Experiments* in vitro. The preparation described by Finkleman (1930) was used to study the effects of drugs on the function of inhibitory postganglionic sympathetic nerves supplying rabbit intestine. The mesenteric nerve was stimulated from platinum electrodes with supramaximal shocks at 50 pulses/sec. for 15 sec. every 3 min. The effect of drugs on motor actions of postganglionic sympathetic nerves was studied, using the hypogastric nerve-ductus deferens preparation of the guinea-pig (Huković, 1961). Supramaximal stimulation was applied to the nerve, using saline electrodes at 50 pulses/sec. for 1 sec. every min.

*Experiments* in vivo. Changes in sympathetic tone in groups of 5 cats were observed by measuring with calipers the portion of the nictitating membranes exposed across the palpebral fissure. Before dosage with drugs the cats were restricted to milk and water for 24 hr. and for oral dosage were lightly anaesthetised with ether to facilitate passage of a stomach tube.

### *Absorption from the Alimentary Tract* in vivo

Cats were fasted for 18 hr. and anaesthesia was induced by ether and maintained by chloralose (60 mg./kg. intravenously).

*Absorption from the stomach.* The abdomen was opened in the midline and the cardiac and pyloric sphincters ligated. The intestines and spleen were removed after tying all vascular connections including those between the stomach and omentum. A loose ligature was placed round the portal vein just caudad to its bifurcation before entering the liver. The peritoneal cavity was filled with warm liquid paraffin. Heparin (10 mg./kg.) was given intravenously and the stump of the superior mesenteric vein was cannulated retrogradely with polythene tubing. By pulling on the ligature the venous outflow from the stomach could be diverted into the cannula and the blood collected in tared tubes. The right carotid artery was cannulated for collection of systemic blood. The drug was injected into the lumen of the stomach and blood samples were collected during 30 sec. to 1 min., depending on flow rate, every 15 min., 1–2 ml. of blood was collected at each sampling.

*Absorption from the intestine.* The technique described for the stomach was applied to the study of intestinal absorption. A 10 cm. portion of the duodenum or colon was isolated between ligatures. The remaining portions of the intestine, the stomach and spleen were removed, preserving the vascular connections of the isolated segment. Blood draining from the intestine to the liver was collected as described above.

In other experiments cats were given oral doses of drugs and urine was collected, by suprapubic puncture if necessary, every 24 hr.

### *Absorption from the Intestine* in vitro

A portion of rat duodenum was everted, weighed and suspended in bicarbonate saline (50 ml.) at 37°. The tissue was cannulated at each end so that the contents from the serosal side could be washed out with warm saline (1 ml.) for analysis. In some experiments the tissue was not everted. Labelled bretylium was added to the bath to a final concentration, usually $10^{-3}$M. The serosal fluid was assayed for radioactivity by evaporation to dryness, dissolution in 0·2 ml. water and plating in 40 $\mu$l. aliquots on lens paper in planchettes. Samples were taken every 30 min. The results were calculated by reference to a standard calibration curve and expressed as mg. bretylium absorbed/g. tissue/hr.

## RESULTS

BW 171C60, LD50 approximately 80 mg./kg. intravenously, was less toxic in mice than its hydrolysis product BW 329C57, LD50 40 mg./kg. The betaine did not cause appreciable block of adrenergic neurones within 30 min. in the Finkleman and ductus deferens preparations at 100 $\mu$g./ml., whereas BW 329C57 was active at 3 $\mu$g./ml. within 10 min. Fig. 1 shows that BW 171C60 relaxed the nictitating membranes of cats after oral doses of 5 mg./kg., whereas BW 329C57 was inactive at 10 mg /kg. At higher dose levels, 10 mg./kg. BW 171C60 had a similar degree of activity to 20 mg./kg. BW 329C57.

BW 293C60 was inactive in 3 cats given oral doses of 20 mg./kg., but relaxed the nictitating membrane of 1 cat at 10 mg./kg., s.c.

BW 564C61 had a similar degree of activity to BW 171C60 after oral dosage in cats. There were no signs of any central effect to suggest that the drug had entered the brain.

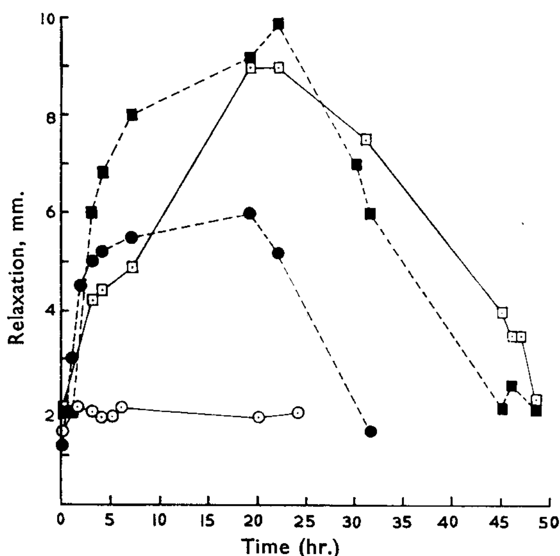



FIG. 1. Relaxation of the nictitating membranes of cats given oral doses of *N*-*o*-chlorobenzyl-*NN*-dimethyl-*N*-2-hydroxyethylammonium iodide (BW 329C57) and its phosphoric ester betaine (BW 171C60).

⊙——⊙ BW 329C57, 10 mg./kg. ●——● BW 171C60, 5 mg./kg.
⊡——⊡ BW 329C57, 20 mg./kg. ■——■ BW 171C60, 10 mg./kg.

*Absorption of Bretylium by the Gastrointestinal Tract of Cats*

*Stomach.* There was no detectable radioactivity in the blood draining from the stomach of a cat during one hour after injection into the lumen of 50 mg. $^{14}$C-labelled drug (approximately 42 $\mu$c).

*Duodenum.* Fig. 2 shows that the rate of absorption of $^{14}$C-labelled bretylium (50 mg.; approximately 21 $\mu$c) into blood draining from the duodenum was initially slow but that it began to rise fairly rapidly to reach a peak at 90–120 min. and then declined equally rapidly so that the concentration in the blood leaving the duodenum was scarcely higher than that in systemic blood. In this experiment 20·6 mg. of drug was absorbed during 210 min. In a second experiment, 6·0 mg. were absorbed during 165 min. leaving 32·0 mg. in the intestinal contents. A fairly high proportion (9·4 mg.) was found in the intestinal tissue. The amounts of drug absorbed were calculated by estimating the area enclosed by the curve relating $\mu$g./ml. in blood (corrected for systemic blood content) to time and multiplying by the average rate of blood flow from the duodenum.

*Colon.* A very slow rate of absorption was found during 3 hr. when the drug was injected into the lumen of the colon. The level in the blood draining from the tissue rose slowly during the experiment, finally

reaching 5·4 μg./ml. The level in systemic blood at this time was 0·6 μg./ml.

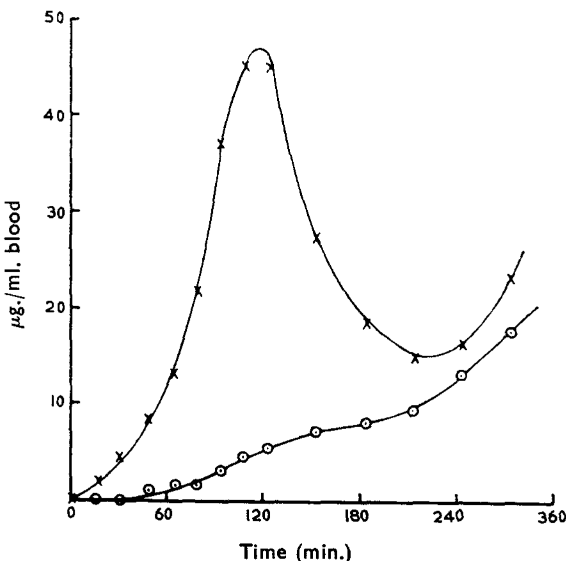



FIG. 2. The absorption of bretylium from the duodenum of cats. [14]C-*N*-Methylbretylium (50 mg.) was injected into the lumen of an isolated segment of cat duodenum. Samples of blood draining from the tissue and carotid blood were assayed for radioactivity. ×——× duodenal blood. ○——○ carotid blood.

*Absorption of BW 171C60 by the Gastrointestinal Tract of Cats*

*Stomach.* There was barely detectable radioactivity in the blood leaving the stomach during 200 min. after injection of 40 mg. of $^{14}$C-labelled drug into the lumen (approximately 60 μc). No radioactivity appeared in systemic blood. Radioassay of the stomach contents indicated a recovery of 38 mg. of the dose and autoradiographs of chromatograms showed one spot at $R_F$ 0·54, at the same position as a marker spot of BW 171C60.

*Duodenum.* No BW 329C57 could be detected by nephelometric assay in the blood draining from the duodenum after injection of 15 mg. BW 171C60 into the lumen (<2·5 μg./ml.). Chromatography of the intestinal contents after 180 min. showed that both BW 329C57 and BW 171C60 were present. Nephelometric assay indicated that 30 per cent of the dose had been hydrolysed.

There was very little absorption during 90 min. of 40 mg. of $^{14}$C-labelled drug (approximately 60 μc). The maximum concentration of drug in the blood draining from the tissue was 4·4 μg./ml. at a time when the level in systemic blood was 1·2 μg./ml. Assay of the lumen contents showed that 35·8 mg. of the dose remained unabsorbed and this was accounted for on autoradiographs by BW 329C57 ($R_F$ 0·71). There was no trace of BW 171C60. Extraction of the pooled dried blood samples coming

from the intestine with ethanol and autoradiography of the extract revealed a weak spot at $R_F$ 0·55 due to BW 171C60. Incubation of heparinised cat blood (1 ml.) with BW 171C60 (1 mmole) at 37° for 2 hr. caused an increase of inorganic phosphate equivalent to 5·5 per cent of the added drug.

*After oral dosage in conscious cats.* A cat, deprived of food for 24 hr. received 52 mg. of drug orally. Urine was collected by suprapubic puncture at 0, 8, 24 and 32 hr. after the dose. The urine was collected in a 6 per cent trichloroacetic acid, centrifuged and assayed for BW 329C57 nephelometrically. Recoveries were respectively 0, 12·6, 10·8 and 3·6 mg., equivalent to a total of 24·6 mg. of BW 171C60. Chromatograms revealed no trace of BW 171C60.

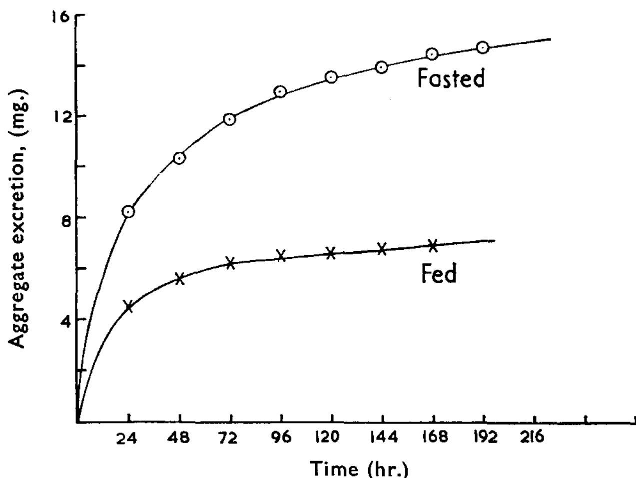



FIG. 3. Excretion of radioactivity in the urine by cats after oral doses (30 mg.) of *N*-*o*-chlorobenzyl-*N*-methyl-*N*-$^{14}$C-methyl-*N*-2-phosphatoethyl ammonium betaine (BW 171C60)

In a similar experiment, using a cat fed its normal diet before receiving 46 mg. of drug orally, the respective recoveries were 0, 2·8, 3·3 and 0 mg. BW 329C57, equivalent to 5·5 mg. BW 171C60 absorbed.

These experiments were repeated, using the $^{14}$C-labelled material. 30 mg. was given to a cat, fed normally before the dose, and to a cat whose food had been withdrawn for 24 hr. Urine was collected every 24 hr.

Fig. 3 shows that excretion of radioactivity persisted for 7 days and that a greater proportion of the drug was absorbed by the fasted cat. These figures may not represent the total absorption since a small proportion of all similar drugs examined so far has been excreted into the bile and is presumably voided in the faeces. The nictitating membranes of all four cats were relaxed.

*Absorption of BW* 564C61

A cat received 14 mg. of the drug orally and urine was collected for 24 hr. About 50 per cent of maximal relaxation of the nictitating

membranes occurred. Chromatography of aliquots of the urine in propanol-15 per cent ammonia (6:4) and spraying with Dragendorff's reagent showed 2 spots. That at $R_F$ 0·77 was identified as BW 329C57 by comparison with a marker spot. A spot at $R_F$ 0·65 may have been unchanged drug but the appropriate marker spot was double ($R_F$ 0·57 and 0·67). By rough assessment of the size of spots given by varying amounts of urine it was estimated that about 2·5 mg. of BW 329C57 had been excreted, probably with about the same amount of unchanged drug.

When the above dose (5 mg./kg.) was doubled there was a greater relaxation of the nictitating membranes and chromatograms of urine again revealed two spots, $R_F$ 0·74 and $R_F$ 0·64. The recovery was much smaller in this instance but the urine sample was also very small (47 ml.) and had to be collected by suprapubic puncture.

*Absorption of Bretylium by Rat Duodenum* in vitro

Fig. 4 shows that the rate of absorption of bretylium by everted rat duodenum (mucosa to serosa) rose to a peak at about 2·5 hr. and then

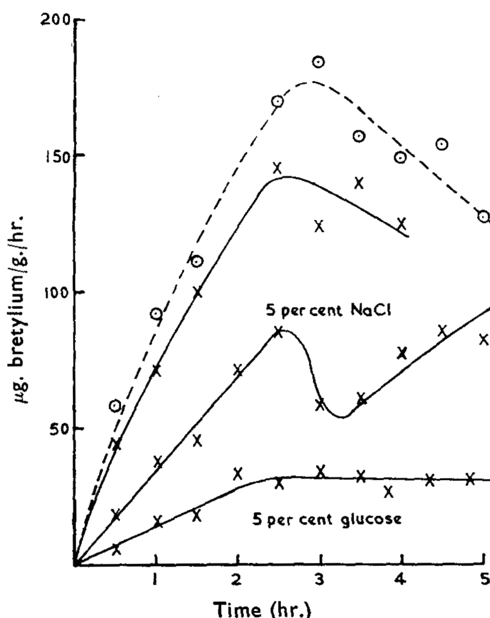



FIG. 4. The diffusion of $^{14}$C-*N*-methylbretylium across rat duodenum *in vitro*. ×——× Everted sacs of duodenum were suspended in bicarbonate buffer containing labelled bretylium ($10^{-3}$M). The serosal fluid was assayed for radioactivity. ⊙ - - - ⊙ Duodenal sac not everted.

began to decline, similar to the results found *in vivo* in cats. At the peak rate the concentration of bretylium on the serosal side was approximately half that of the bathing solution. The total amount of drug removed from the bath during the experiments had negligible influence on the

initial concentration present. Fig. 4 also shows that there was no noticeable difference in the rate of passage of drug from serosa to mucosa.

Some attempts were made to influence the rate of transfer by modifying the bathing solution at 90 min. Increasing the glucose concentration five times, replacing glucose by glutamate, omission of glucose, or addition of iodoacetate ($10^{-4}$M) had no appreciable influence. Placing $5 \times 10^{-4}$M bretylium on the serosal side caused a temporary slackening of the appearance of labelled bretylium on the serosal side during 60 min., followed by restoration of the original rate. Adrenaline ($10^{-5}$M) caused a temporary slackening during 60 min. Lowering the pH of the bath to a value of 5 had little effect. The only agents to cause notable change were 5 per cent glycine and 5 per cent sodium chloride which appeared to prevent further rise in absorption rate. This maintained the value attained before the addition.

The rate of absorption of bretylium from $5 \times 10^{-4}$M solution was about twenty times faster than from $5 \times 10^{-5}$M solution.

*Hydrolysis of BW* 171C60, *BW* 293C60 *and BW* 564C61 in vitro

No inorganic phosphate was liberated by heating BW 171C60 (3·3 mg.) for 30 min. on a steam bath in either 5N hydrochloric acid or 5N sodium hydroxide. Chromatograms of aliquots revealed only unchanged material after spraying with Dragendorff's reagent.

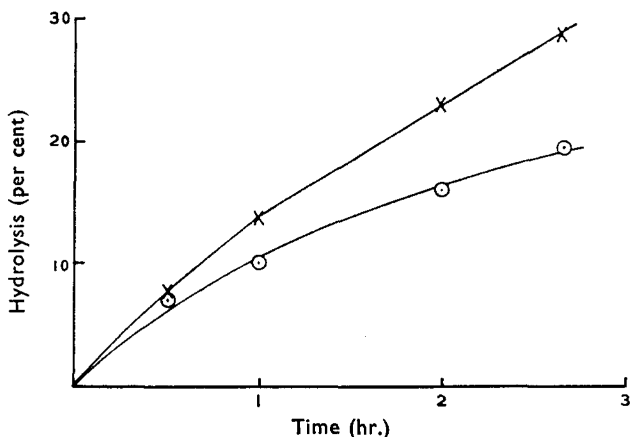



FIG. 5. The rate of hydrolysis of phosphate ester betaines by the alkaline phosphatase of rat intestinal mucosa. Aliquots of the incubated mixture of enzyme plus drug were analysed at intervals for inorganic phosphate. Each point is the mean of 2–3 experiments. ×——× BW 293C60. ○——○ BW 171C60.

BW 564C61 was completely hydrolysed during 15 min. heating on a steam bath in either N hydrochloric acid or sodium hydroxide. Paper chromatograms and suitable sprays revealed α- and β-glycerophosphate, inorganic phosphate and BW 329C57. It was stable to 0·1N acid or alkali at 37° during 2 hr.

Fig. 5 shows the rate of hydrolysis, calculated by analysis for inorganic phosphate, when BW 171C60 and BW 293C60 were incubated with the

alkaline phosphatase of rat intestine, equivalent to 2 per cent of the total extracted activity of the mucosa of one rat. β-Glycerophosphate (50 μmoles) was almost completely hydrolysed during 2 hr. under the same conditions. BW 171C60 (6 μmoles) was 10 per cent hydrolysed during 2 hr. at 37° by the acid phosphatase of rat liver whereas β-glycerophosphate (10 μmoles) was 10 per cent hydrolysed during 1 hr. under the same conditions.

BW 564C61 was unaffected by either alkaline or acid phosphatase from rat intestine and liver respectively but it was appreciably hydrolysed by the phosphodiesterase of rat liver. No attempt at accurate measurement was made but paper chromatograms revealed BW 329C57 and α- and β-glycerophosphate.

## Discussion

The rate of absorption of bretylium from the intestine of the cat *in vivo* showed the same peculiarity observed for other quaternary ammonium salts (Levine, Blair and Clark, 1955), that is, a steady rise followed by a steady fall, though most of the dose remained in the lumen. There were indications for similar behaviour *in vitro* using rat duodenum. At peak absorption rate *in vivo* the dose of 50 mg. could have been absorbed within 3 hr. It seems clear that the intestine is capable of absorbing bretylium at an appreciable rate but that the process is subject to self limitation. No notable irregularity in absorption rate was observed in any of these experiments but the occasional erratic control of blood pressure described in clinical use of bretylium (Dollery, Emslie-Smith and McMichael, 1960) could conceivably be due to rapid clearance of blocked absorption channels. Duncombe and McCoubrey (1960) obtained some evidence for erratic excretion of bretylium. From the experiments with rat duodenum *in vitro* the mechanism of absorption seems to be one of simple diffusion. Transfer of drug occurred equally well from serosa to mucosa as vice versa. Absorption was not affected by addition of iodoacetate and transfer of $^{14}C$-labelled drug from mucosa to serosa was only temporarily delayed by increasing the serosal concentration of unlabelled drug. It seems reasonable to conclude that the absorption channels become obliterated by the drug during transfer but the nature of the channels remains undefined.

The results from conscious cats given BW 171C60 fulfilled the expectations mentioned in the introduction. The drug was more readily absorbed than was BW 329C57, judging by the effect on the nictitating membranes. Though not proven, there is little reason to doubt that the pharmacological effects of BW 171C60 at the doses used are those of its hydrolysis product, BW 329C57, in which form it was excreted in urine. BW 171C60 was inactive *in vitro*. Nevertheless, assuming that urinary excretion accounts for the greater part of the dose given, the proportion of BW 171C60 absorbed is still relatively low. Several points of interest deserve mention. The drug was not hydrolysed during passage across the intestinal wall and was not actively hydrolysed by blood. Since it was excreted in urine as BW 329C57, the hydrolysis must have occurred in other tissues,

presumably mainly in the liver. Though the drug is a weak acid it was not absorbed appreciably by the stomach. Brodie and Hogben (1957) consider that all weak acids are, at least theoretically, capable of traversing the stomach wall. The drug was not hydrolysed during its stay in the stomach though it was rapidly hydrolysed in the intestine. The failure to observe appreciable absorption by the isolated intestinal loop may be accounted for by the liberation of phosphatase into the lumen during surgical preparation though the point was not investigated experimentally. This may also account for the smaller degree of absorption during digestion, though in unpublished work we have noted a smaller degree of absorption of a similar drug given after feeding and in this instance phosphatase can play no direct part.

The results with BW 564C61 were disappointing in so far as this drug was synthesised with a view to studying the influence on the brain of an anti-adrenergic drug conveyed by a natural as opposed to a parenteral route. Being a neutral molecule it was considered that it might traverse the blood brain barrier to suffer hydrolysis within cerebral tissue by phosphodiesterase. Since it was not feasible to make $^{14}$C-labelled material and chemical analysis of brain tissue for small amounts of BW 329C57 is virtually impossible, the brains of animals receiving the drug were not analysed. It is interesting however that the drug appeared to be much more stable to the tissues than BW 171C60. This could be due to its stability to phosphomonoesterases, a property shared by $\alpha$-glycerophosphorylcholine. Conversely the product was a racemic mixture and one isomer may be less susceptible to enzymic hydrolysis. Further work with this substance was abandoned because of its intractable nature.

*Acknowledgements.* The authors gratefully acknowledge the counting of our $^{14}$C-labelled specimens by Dr. W. G. Duncombe, and the technical assistance of Messrs. J. Harry, A. Lakeman and R. Palmer.

## REFERENCES

Boura, A. L. A. and Green, A. F. (1959). *Brit. J. Pharmacol.*, **15**, 536–548.
Brodie, B. B. and Hogben, A. M. (1957). *J. Pharm. Pharmacol.*, **9**, 345–380.
Copp, F. C., T. S. G. Jones and A. McCoubrey, (1962). *Ibid.*, **14**, 641–646.
Dawson, R. M. C. (1956). *Biochem. J.*, **62**, 689–693.
Dollery, C. T., Emslie-Smith, D. and McMichael, J. (1960). *Lancet*, **1**, 296–302.
Duncombe, W. G. and McCoubrey, A. (1960). *Brit. J. Pharmacol.*, **15**, 260–264.
Finkleman, B. (1930). *J. Physiol. (Lond.)*, **70**, 145–157.
Goodlad, G. A. J., and Mills, G. T. (1957). *Biochem. J.*, **66**, 346–354.
Hanes, C. S. and Isherwood, F. A. (1949). *Nature Lond.*, **164**, 1107–1112.
Hogben, C. A. M., Tocco, D. J., Brodie, B. B. and Schachter, L. S. (1959). *J. Pharmacol.*, **125**, 275–282.
Huković, S. (1961). *Brit. J. Pharmacol.*, **16**, 188–194.
Levine, R. M., Blair, M. B. and Clark, B. B. (1955). *J. Pharmacol.*, **114**, 78–86.
Long, C. (1953). *Biochem. J.*, **53**, 7–11.