MATERIALS AND METHODS

Isolated rat ileum preparation

Healthy albino rats of either sex weighing 150 - 200 g were used for obtaining ileum preparations. The animals were starved for 18 hr but had free access to water before being used for experiment. The animals were stunned by a blow on the head, bled by cutting the neck vessels. The abdominal wall was opened and the distal 15 - 20 cm of the ileum was removed and washed in Tyrode solution of the following composition: NaCl 8 g; KCl 0.2 g; $CaCl_2$ 0.2 g; $MgCl_2$ 0.1 g; $NaHCO_3$ 1 g; NaH_2PO_4 0.05 g and dextrose 1 g per 1 litre of distilled water. A piece of ileum 2 cm long was suspended in Tyrode solution (containing hyoscine 1 µg/ml) at 35°C and was bubbled with air in an organ bath of 35 ml capacity. The movements were recorded on smoked drum with isotonic frontal writing lever that placed the tissue under 250 mg of tension in the horizontal equilibrium position. The lever system employed gave an eightfold mangification. The preparation was allowed to stabilize for 15 min before testing was begun.

Nicotine, DMPP, ACh and NA (test drugs) were allowed to act till maximum response was elicited; for this nicotine and DMPP took about 20 sec, NA about 30 sec and ACh about 45 seconds. A 6 min response cycle was used.

After obtaining equipotent response to test drugs, the preparations were exposed to phentolamine, pronethalol, adrenergic neurone blockers, ganglion blockers, cocaine, procaine and DMI for specified periods of time and responses to test drugs were reelicited in the presence of these agents. In the cases of tetrodotoxin or blockers of choline transport or choline, after exposure of preparations to these agents for specified periods of time, the preparations were given washouts and responses to test drugs were reelicited. In some experiments, responses to test drugs were studied at low temperature. In such cases, after eliciting control responses to test drugs at a bath temperature of 35°C, the bath temperature was lowered to 18°C and the preparation was allowed to stabilize at this temperature for 20 min before adding test drugs. In experiments designed to study the

effect of Ca^{++} -deprivation on the dose-response curves to nicotine, DMPP and ACh, the preparations were equilibrated with Ca^{++} -free Tyrode solution containing sodium edetate (30 µg/ml) for 30 minutes. After this, responses to different doses of nicotine, DMPP and ACh were elicited in the manner outlined for normal Tyrode solution. The preparations were then equilibrated in the presence of normal Tyrode solution for 45 min before repeating dose-response curves to these drugs.

Cocaine, DMI, procaine and tetrodotoxin had no intrinsic effects of their own. The adrenergic blockers, the ganglion blockers and dexamphetamine produced slight decrease in the tone of the ileum. The baseline tone could be restored by one or two washes. After exposure of preparations to phentolamine or pronethalol separately or together, the height of the writing point was lowered by 1 - 2 cm. Partial to full restoration occured after 1 - 2 washes. In the presence of Ca^{++} -free Tyrode solution containing sodium edetate, the tone of the muscle decreased very gradually. When such muscle was exposed to normal Tyrode solution, the tissue

went into spasm which disappeared within 45 minutes. When preparations were exposed to high concentrations of HC-3 (60 µg/ml) or triethylcholine (110 µg/ml), they suffered a gradual but considerable loss of tone over a period of 90 min; in such preparations, it was difficult to elicit consistent and reproducible relaxant responses to nicotine, DMPP and ACh and even to NA. However, three consecutive exposures to the same concentrations of these blockers at intervals of 30 min did not result in the loss of tone or relaxant responses to NA. There was tremendous increase in tone during equilibration of the tissue at low temperature. In such cases, the writing point was adjusted at lower level. Choline also produced marked increase in the tone of ileum. The tone was restored after 2 - 3 washes.

Reserpine treatment. Rats were given 2 mg/kg of reserpine subcutaneously for eight successive days and were killed 24 hr after the last injection.

Release of ACh by nicotine and DMPP

The release of ACh by nicotine and DMPP from the rat ileum

was assayed on a 2 cm long piece of guinea pig terminal ileum superfused with oxygenated Tyrode solution containing hexamethonium 20 (μg/ml), mepyramine (0.5 μg/ml) and morphine (1 μg/ml) (Kosterlitz et al. 1970). Contractions were recorded on smoked paper with isotonic frontal writing lever under 0.5 g tension and at eightfold magnification.

For the collection of samples, a 2 cm long piece of rat ileum was suspended in an organ bath of 10 ml capacity containing Tyrode solution (hyoscine free) maintained at 35°C and bubbled with air. The entire 10 ml sample was withdrawn with the help of a syringe and collected in chilled glass test tubes. The contact period for nicotine or DMPP was 20 seconds. The volume of the collected sample used for ACh assay at a given time did not exceed 0.2 ml. Different concentrations of ACh were prepared immediately before use.

Assays were carried out by matching responses of standard and test solutions. A 3 min response cycle was used. The limit of sensitivity was 10 ng/ml. In most experiments,

the following tests were made to check that the substance present was ACh : (i) abolition of both test and standard responses after 0.1 ml of 0.5 NaOH had been added to 1.0 ml of each solution, which were then allowed to stand for 30 min before being neutralized by 1 N HCl. (ii) block of standard and test responses by superfusion with Tyrode solution containing 0.1 µg/ml of hyoscine and (iii) potentiation of standard and test responses by superfusion with Tyrode solution containing 0.1 µg/ml of physostigmine.

Three consecutive control samples were collected after three additions of 1 µg/ml of nicotine or DMPP. The preparations were then treated with HC-3 or triethylcholine or bretylium or guanethidine in the manner already described; this was followed by washout and three consecutive samples collected again after the additions of nicotine or DMPP. The values of release obtained for the three consecutive additions were averaged for further analysis of the data. The release of ACh was expressed as ng/g/min.

Finkleman preparations from young rabbits

Newborn rabbits of either sex and 1 -12 days old were sacrificed and about 3 cm long piece of the distal ileum together with adjacent mesentery and arteries running in it was dissected by the method of Finkleman (1930). The contents of the ileum were washed out by gentle blowing of McEwen's solution through the lumen. The ileum was placed in a Petri dish and a ligature was applied at the end of the artery. This end was drawn into a pair of platinum ring electrodes of the pattern described by Burn and Rand (1960 a). The preparation was then set up in an isolated organ bath of 35 ml capacity, containing McEwen's solution (1956) of the following composition: NaCl 7.6 g; KCl 0.2 g; $CaCl_2$ 0.24 g; NaH_2PO_4 0.143 g; $NaHCO_3$ 2.0 g; dextrose 2.0 g and sucrose 4.5 g per 1 litre of distilled water. The solution was maintained at 35°C ± 1.0°C and bubbled with a mixture of 95% oxygen and 5% carbon dioxide. The movements of the ileum were recorded on a smoked drum with isotonic frontal writing lever that placed the tissue under 300 mg of tension in the horizontal

equilibrium position. The lever system employed gave an eightfold magnification. The preparation was allowed to stabilize for 30 minutes.

The periarterial nerves were stimulated by square wave stimulator (Toshniwal, Type EM01) at supramaximal voltages at frequencies of 1, 2, 5, 10 and 20 Hz with pulses of 2.5 msec duration for 30 - 40 sec every 5 minutes.

In all experiments, after eliciting control responses at different frequencies the preparations were exposed to NA (1 µg/ml) for 20 min, washed and restimulated.

Estimation of NA

The NA content to ileum of rabbits was estimated by the method of Anton and Sayre (1962). The lumen of the ileum was cut open and washed with chilled McEwen's solution. It was then divided into three approximately equal pieces. One piece was kept in deep freeze to be used later for estimating endogenous NA content. The other two pieces were used for studying the uptake of exogenously added NA. The two pieces

were incubated separately at 35°C in 35 ml of McEwen's solution containing sodium edetate (10 µg/ml) and bubbled with 95% oxygen and 5% carbon dioxide for 10 minutes. The medium incubating one of the pieces also contained cocaine (3.4 µg/ml). After 10 min, the cocaine medium was replaced with one containing cocaine (3.4 µg/ml) and NA (1 µg/ml). The medium incubating the other piece was replaced with one containing NA (1 µg/ml) only. After incubating for a further period of 20 min, each tissue was washed three times with normal McEwen's solution and dried by gently pressing between filter paper. All the three pieces were weighed and homogenized with a pestle in 3 ml of 0.4 N percholoric acid containing glass powder in chilled porcelain mortar. The homogenate was transferred in chilled glass test tubes. The mortar was rinsed with 5 ml of 0.4 N perchloric acid and this was added to the homogenate in the test tube. The homogenate was stored in deep freeze for one hr, after which it was thawed and centrifuged in cold centrifuge (Janetzki, Model K 23) at 8000 R.P.M. (8000 g) for 15 min at 4°C. The supernatant was saved and the pellet was resuspended in 5 ml

of 0.4 N perchloric acid and recentrifuged. The two supernatants were pooled and the volume was made upto 20 ml by additional 0.4 N perchloric acid. The supernatant was transferred to a 50 ml beaker containing 500 mg of activated alumina, 200 mg of sodium edetate and 10 mg of sodium metabisulphite. The mixture was continuously stirred by the glass rod of a Stirrer (B.T.L.) for 5 min and its pH was adjusted to 8.6 with 5.0 N sodium hydroxide using direct reading pH meter (E.I.L. Model 23 A). After pH adjustment, the stirring was stopped and alumina was allowed to settle down. The supernatant was decanted and discarded.

The alumina was transferred to a glass column whose tip was plugged with glass wool. The column of alumina so formed was washed with 30 ml of double distilled water. The column was then eluted with 6.0 ml of 0.2 N perchloric acid.

The pH of the elute was adjusted with pH paper (BDH 5570) to 6.5 with 5 N potassium carbonate. The precipitate of potassium perchlorate was allowed to settle down for 30 min and was discarded. The supernatant was used for NA estimation by trihydroxyindole procedure.

1.0 ml of the eluted material was used for the development of fluorescence and an equal volume was used for preparing blank. To both the blank and the sample were added 1.0 ml of 0.1 M phosphate buffer (pH 6.5), 1.6 ml of distilled water and 0.1 ml of zinc sulphate (0.025%) The blank tube was prepared first by adding to it 0.1 ml of potassium ferricyanide (0.25%) followed by 0.9 ml of 5.0 N sodium hydoxide. The blank tube was allowed to stand for 15 min. At the end of this period, fluorescence developed in the blank tube was completely destroyed in the strongly alkaline medium. The sample was then developed by adding to it 0.1 ml of potassium ferricyanide (0. 25%) followed after 2 min by 1 ml of freshly prepared mixture of 5 N sodium hydroxide and 2% ascorbic acid (ratio, 9:1). After this 0.1 ml of ascorbic acid (2%) was added to the blank tube.

The fluorescence was estimated by G.K. Turner fluorimeter (Model 110), using primary filters (2A and 47B) and a secondary filter 24-15 A. The blank was adjusted to zero and fluorescence of the sample was read.

In separate experiments, it was determined that the recovery of NA added to the mixture of alumina, sodium edetate and sodium metabisulphite was 70%. The fluorimetric readings obtained were, therefore, corrected for 70% recovery. The values of NA were expressed as µg/g of wet weight of the tissue.

Drugs

Acetylcholine chloride (ACh) (E. Merck); bretylium tosylate (Wellcome Research Laboratories); choline chloride (Chemo Puro); xylocholine (choline 2:6 xylyl ether bromide) (Smith Kline and French), cocaine hydrochloride (May and Baker); desmethylimipramine hydrochloride (DMI) (Geigy); dexamphetamine sulphate (Smith, Kline and French); 1-dimethyl-4-phenylpiperazinium iodide (DMPP) (Parke Davis); guanethidine sulphate (Ciba); hemicholinium bromide (HC-3) (Aldrich Chemicals); hexamethonium chloride (Sarabhai Chemicals); hyoscine hydrobromide (E. Merck); mecamylamine hydrochloride (Merck, Sharp and Dohme); mepyramine maleate (May and Baker); morphine sulphate (May and Baker);

nicotine (B.D.H.); (-) noradrenaline (NA) (Rhone Poulenc); pempidine tartrate (May and Baker); phentolamine methanesulphonate (Ciba); procaine hydrochloride (May and Baker); pronethalol hydrochloride(I.C.I.); reserpine (Serpasil) (Ciba); sodium edetate (B.D.H.); tetrodotoxin (Calbiochem); triethlcholine iodide (Ward Blenkinsop).

Stock solutions of NA were prepared in 0.01 N HCl containing 0.05% sodium metabisulphite. Stock solutions of ACh were prepared in 0.1 M phosphate buffer (pH 5). Stock solutions of tetrodotoxin were prepared in distilled water. Dilutions of stock solutions and solutions of other drugs were made in Tyrode medium before the start of experiment. Concentrations of NA and nicotine are in terms of base and those of other drugs are in terms of respective salts.