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EFFECTS OF CENTRAL DEPRESSANT DRUGS UPON ACETYLCHOLINE RELEASE

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Several central depressant and other drugs have been examined for their effects upon acetylcholine release from the stimulated, perfused cat superior cervical ganglion and rat isolated phrenic nerve-diaphragm preparations. The acetylcholine released was assayed biologically. Amylobarbitone sodium, chloral hydrate, trichloroethanol, methylpentynol, methylpentynol carbamate, paraldehyde, procaine hydrochloride and troxidone reduced the presynaptic release of acetylcholine from the ganglion. They also exhibited a postsynaptic blocking action, this component of depressant activity being particularly prominent with paraldehyde and troxidone. Closely analogous findings were obtained at the neuromuscular junction with methylpentynol and its carbamate, paraldehyde, procaine hydrochloride, trichloroethanol and troxidone. At both sites the drug-induced depression, both of transmission and of acetylcholine output, was reversible. Whereas hexamethonium regularly blocked ganglionic transmission with no effect upon acetylcholine release, tetraethylammonium not only completely blocked ganglionic transmission but concomitantly augmented acetylcholine output. These results are discussed in relation to the electrophysiological and metabolic events associated with neuro-effector transmission.

Ever since the demonstration by Sherrington (1906) that the function of central synapses associated with reflex activity appeared to be more susceptible to general anaesthetic substances than was axonal conduction, it has become customary to regard central depressant drugs generally as having a selective blocking action upon transmission at the synapse. Nevertheless it was not until comparatively recently that evidence for a similar selective blocking action at a simple peripheral synapse was provided by the work of Larrabee & Posternak (1952) using the stellate ganglion in the cat. These authors have pointed out, however, that not only does the more detailed mechanism underlying the production of a selective block remain obscure but there is still doubt whether central depressant drugs act uniformly on all parts of a neurone, or whether specific synaptic regions, such as the presynaptic nerve terminals, cell somata or dendrites, are more readily and selectively depressed. Additional indirect evidence has now accumulated which suggests strongly that, at several cholinergic neuro-effector sites, central depressant drugs may inhibit the release of acetylcholine from prejunctional nerve terminals (Nicholls & Quilliam, 1956; Quilliam, 1959).

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The study described here was therefore undertaken to attempt a more precise definition of the mechanism by which central depressant drugs block junctional transmission, with special attention to prejunctional effects. The synapses at the superior cervical ganglion of the cat and the neuromuscular junction of the diaphragm of the rat were chosen for this evaluation since each preparation offers a comparatively simple cholinergic neuro-effector system amenable to study and about which much is already known of the characteristics of acetylcholine release and of the transmission processes generally. A preliminary report of part of this work was made to the Federation of American Societies for Experimental Biology (Matthews & Quilliam, 1962).

METHODS

Perfusion of the superior cervical ganglion of the cat

Anaesthesia was induced with ethyl chloride and diethyl ether, and maintained by the intravenous injection of α-chloralose (80 mg/kg). The right superior cervical ganglion was isolated for perfusion *in vivo*, essentially in the manner of Feldberg & Gaddum (1934), but with those modifications recommended by MacIntosh and cited by Perry (1953). A detailed description of the procedure and of the perfusion apparatus used has been made elsewhere (Matthews, 1961).

Perfusion fluids. The perfusion fluid was Locke solution of the original composition (Locke, 1901), as was employed by Emmelin & MacIntosh (1956) (g/l.): NaCl 9.0, KCl 0.42, $CaCl_2$ 0.24, $NaHCO_3$ 0.5 and glucose 2.0 g. This solution when bubbled with oxygen had a *p*H between 7.9 and 9.0 (mean 8.7), measured at 20° C with a glass electrode. No drug, in the concentrations employed, materially affected the *p*H of the perfusion fluid. In most experiments, Locke solution alone was perfused but, in some, this solution was supplemented by cat plasma (10% v/v, heparinized). In those experiments in which acetylcholine output was to be measured, physostigmine sulphate was added to the perfusion fluid in a concentration of 1×10^{-5} or 5×10^{-4} g/ml. Choline chloride (10^{-6}) was also included in the perfusion fluid in some experiments. Drugs under test were added to the perfusion fluid in one reservoir of the apparatus; the other reservoir contained perfusion fluid free from drug for use in the initial and final phases of perfusion. The perfusion pressure was adjusted to maintain the perfusion flow rate at as near the optimal level of 0.3 ml./min as possible. All perfusion fluids were filtered through a sintered glass filter of No. 2 porosity before use.

In a few of the earlier experiments, drugs dissolved in Locke solution were injected into the perfusion system through the side arm of a modified arterial cannula. All solutions so injected were warmed to 37° C before use and the total volume of each injection was 0.2 ml.

Nerve stimulation. An electronic stimulator provided rectangular pulses at a frequency of 10 shocks/sec, of duration 0.5 msec and at a voltage of 7 to 10 V, for excitation of the preganglionic cervical sympathetic nerve (see Bell, 1957). Supramaximal stimuli were applied through a fluid electrode (filled with Locke solution) to the nerve and an indifferent electrode was attached to the muscles of the neck. Transmission through the superior cervical ganglion upon stimulation of the preganglionic nerve was assessed by the retraction of the ipsilateral nictitating membrane, which was recorded upon a smoked paper by means of a frontal writing lever (tension 4 g; magnification ×7).

Collection and storage of samples. When the last visible traces of blood had been washed from the perfusion system (taking approximately 10 min), the blood-free effluent fluid was collected into glass tubes at 0° C during successive timed periods. The initial sample of perfusate was usually collected during a 4 min interval. All subsequent samples were then collected during successive 6 min periods. When employed, preganglionic stimulation was applied only for the first 3 min of a 6 min collection period, the remaining 3 min allowing wash-over into the collecting vessel of the acetylcholine released during stimulation; such 6 min periods incorporating nerve stimulation are hereafter called "stimulation periods."

The samples were rapidly frozen and stored at −14° C until required for assay. Estimation of the acetylcholine content of samples was carried out the next day, approximately 20 hr after collection, using the rat blood pressure or leech dorsal-muscle preparation. The standard acetylcholine solutions to be used for assay purposes were prepared in the Locke solution removed from the reservoirs at the cessation of a perfusion experiment and stored with the samples of collected perfusate, under identical conditions. This collection and storage procedure was found by previous experiment to cause little if any loss of acetylcholine-like activity.

The isolated phrenic-nerve diaphragm preparation of the rat

Female rats of the Wistar strain weighing between 180 and 280 g were used. A triangular segment of the right hemidiaphragm with attached phrenic nerve was rapidly removed from the bled-out decapitated animal. The segment was then set up in one of two different ways depending upon whether acetylcholine output or transmission alone was to be observed.

Transmission studies. In this instance acetylcholine release was not measured. The diaphragm preparation was attached to a suitable holder with combined platinum electrodes to which the phrenic nerve was applied. The whole assembly was then clamped firmly in a 50 ml. organ-bath containing Krebs solution maintained at 37° C and continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide (v/v, *p*H of solution about 7.4). Muscle contractions were recorded upon smoked paper by a semi-isotonic spring lever connected to the superior central muscle tendon. Stimulation of the phrenic nerve was by rectangular pulses, of 0.1 msec duration at a rate of 15 shocks/min and at a supramaximal voltage, delivered from an electronic stimulator assembly (Bell, 1957). So that the effect of drugs upon transmission of nerve impulses along the phrenic nerve alone could be studied, in some experiments the nerve was drawn into a separate nerve bath, of 0.5 ml. capacity, through a small perforation in the rubber dam closure at one end, and applied to bipolar platinum stimulating electrodes within this isolated chamber.

Acetylcholine release experiments. The method devised originally by Burgen, Dickens & Zatman (1949), but modified by Straughan (1959), was employed. The diaphragm was carefully trimmed of ribs and adherent tissue, especially at its intercostal margin. It was then fixed, central muscle tendon downwards, to a special glass holder incorporating platinum electrodes for phrenic nerve stimulation, and firmly supported in a small Perspex organ-bath (3.5 to 4.0 ml. capacity) containing Krebs solution at 37° C bubbled with a mixture of 95% oxygen and 5% carbon dioxide (v/v). Muscle contractions were recorded. Sampling of bath fluid was by complete withdrawal of the fluid from the bath with a syringe, the bath being refilled by a measured volume of fresh fluid at 37° C from a pipette.

Initially the diaphragm was soaked in Krebs solution containing physostigmine sulphate (5×10^{-6}) for 30 min. Towards the end of this period, the preparation was subjected for 2 min to a conditioning period of stimulation at a low frequency (15 shocks/min) to obviate a low initial acetylcholine output which was otherwise sometimes observed during the first period of tetanic stimulation (Straughan, 1959). The following cycle was then adopted:

(i) 3.5 ml. of fresh Krebs solution containing physostigmine (5×10^{-6}) was added to the bath and the diaphragm was stimulated through the phrenic nerve at 25 shocks/sec for the test period of 20 min. A pulse duration of 0.1 msec and supramaximal voltage were employed.

(ii) The sample was withdrawn 30 sec after the cessation of stimulation and was replaced by fresh Krebs solution containing physostigmine for a 10 min rest period.

(iii) At the end of the rest period the washing fluid was withdrawn (and in some instances assayed), and the procedure indicated under (i) and (ii) repeated in cycle.

Each preparation released acetylcholine regularly for at least six 20 min periods of tetanic stimulation, with interpolated 10 min rest periods. Drugs, when tested, were included in the fluid (containing physostigmine) bathing the diaphragm only from the cessation of the second period of tetanic stimulation to the end of the fourth such period (as seen in Fig. 8). Samples, stored at room temperature (about 20° C), were assayed for acetylcholine content

upon the rat blood pressure within 20 min of withdrawal. Standard acetylcholine solutions for assay were made up in Krebs solution containing physostigmine and, when appropriate, the drug under test.

The Krebs solution used for all diaphragm experiments was of the following composition (g/l.): NaCl 6.92, KCl 0.354, $CaCl_2$ 0.282, $NaHCO_3$ 2.1, KH_2PO_4 0.162, $MgSO_4$ $7H_2O$ 0.294 and glucose 2.0. No drug, in the concentrations employed, materially affected the *p*H of this solution.

Estimation and identification of acetylcholine

Rat blood pressure preparation. The technique used was similar to that of Straughan (1958, 1959) with minor modifications (Matthews, 1961). Male rats of the Wistar strain weighing between 220 and 320 g were anaesthetized by intraperitoneal injection of a mixture of pentobarbitone sodium (4 mg/100 g) and urethane (40 mg/100 g). The blood pressure from a common carotid artery was recorded upon smoked paper by a small-bore mercury manometer (Condon, 1951). Injections were made through a cannula in a femoral vein and all injections were of a constant volume (0.3 ml.). The animal was sensitized to injected acetylcholine by several prior injections of the wash-in Krebs fluid which contained physostigmine sulphate (10^{-6}). Estimation of the acetylcholine contents of the samples from the ganglion or diaphragm was made by a comparison of the depressor effects produced by such samples with those produced by standard solutions of acetylcholine chloride: the assay method of bracketing responses was used. Where samples were collected during drug action the appropriate standard solution of acetylcholine for assay contained an identical concentration of the test drug. No drug (except amylobarbitone) present in a sample caused, alone, any material fall in blood pressure, nor did any interfere with the normal depressor responses to injected acetylcholine.

Leech dorsal muscle preparation. Amylobarbitone sodium, in contrast to its depressor effect upon the rat blood pressure preparation, did not interfere with the assay of acetylcholine upon the leech muscle preparation. The latter method was therefore used when samples containing amylobarbitone were to be analysed for their acetylcholine contents. Contralateral strips of the anterior part of the cleaned leech dorsal muscle were set up in separate tissue-baths, each of small capacity (1 ml.), containing "leech solution" (see below) at room temperature (about 20° C) bubbled with a mixture of 98% oxygen and 2% carbon dioxide (v/v, *p*H of solution 7.2). Muscle contractures in response to acetylcholine were recorded by a gimbal lever (1.5 to 2 g tension; × 10 magnification) writing upon smoked paper. The fluid used to bathe the muscle (leech solution) was Locke solution as used for ganglion perfusion experiments but diluted 1 to 1.4 with distilled water. Samples for assay were diluted similarly in the same ratio before use. The muscle was sensitized to acetylcholine by addition of physostigmine sulphate (10^{-5}) to the leech solution. Relaxation of the muscle after acetylcholine action was facilitated by the presence in the bathing fluid of morphine sulphate (2×10^{-5}) (after Murnaghan, 1958). A constant volume of fluid, whether containing a standard or unknown dose of acetylcholine, was added to the tissue-bath for each response. When samples for assay contained amylobarbitone an identical amount of this compound was added to the standard acetylcholine solution. The assay method was by bracketing responses.

The acetylcholine contents of all samples are expressed in terms of nanograms of acetylcholine chloride.

Identification of acetylcholine. The following criteria were satisfied to establish that the substance found in samples collected during stimulation, both from the ganglion and from the diaphragm preparations, was acetylcholine.

In the absence of an anticholinesterase agent (physostigmine) in the fluid perfusing the stimulated ganglion or bathing the stimulated diaphragm, no acetylcholine-like activity upon the test object was apparent in the samples.

The depressor effect of the sample upon the blood pressure of the rat, or the effect in producing contracture of the leech dorsal muscle, was abolished after treatment of the sample

with alkali (0.17 M-sodium hydroxide solution) at room temperature for 10 min but remained undiminished after similar treatment with acid (0.17 M-hydrochloric acid). The treated samples were neutralized before application to the test object.

The administration of atropine (200 μg/rat) abolished the depressor activity both of the samples and of the standard acetylcholine solutions used for comparison.

Drugs

The drugs used were: acetylcholine chloride, amylobarbitone sodium, chloral hydrate, choline chloride, physostigmine sulphate, heparin, hexamethonium chloride, methylpentynol,* methylpentynol carbamate, morphine sulphate, paraldehyde,* pentobarbitone sodium, procaine hydrochloride, tetraethylammonium bromide, trichloroethanol,* troxidone and urethane. The doses given refer to the appropriate salt, with the exception of those compounds (marked *) which are liquids.

Statistical methods

The means, standard deviations, and standard errors of the means were computed: the significance of difference between means was assessed on the basis of Student's *t*-test.

RESULTS

Superior cervical ganglion of the cat

The effect of drugs upon ganglionic transmission

As little information is available concerning the blocking activity of central depressant drugs in perfused autonomic ganglion preparations, some preliminary experiments were first made to examine the general nature of the synaptic blocking action of these substances.

When the superior cervical ganglion was perfused with Locke solution without physostigmine, periods of repetitive stimulation of the preganglionic nerve were followed by sustained synaptic transmission, as indicated by the retraction of the nictitating membrane to a steady level. The duration of nerve stimulation was either 4 or 5 min and between such periods were interposed rest periods of equal length. Doses of drug were injected into the perfusion stream to the ganglion through a modified arterial cannula 2 or 3 min after the start of nerve stimulation. Fig. 1 depicts block of ganglionic transmission after injection of tetraethylammonium, methylpentynol, its carbamate and paraldehyde. Similarly Fig. 2 illustrates the ganglion-blocking activity of troxidone, methylpentynol and chloral hydrate and demonstrates the dose-dependence of the magnitude of ganglion-block ; for example, compare the effects of 2 and 3 mg of troxidone (T) and of 2 and 1 mg of chloral hydrate (CH). Ganglion-block induced by all the drugs was rapid in onset and was generally brief, the nictitating membrane response returning quickly to its control size usually within 2 min. Control injections of Locke solution were without effect (Fig. 1).

In the experiment illustrated in Fig. 3, the perfused ganglion was also stimulated chemically by intra-arterial injection of acetylcholine into the perfusion stream, one or two such doses being alternated with periods of electrical stimulation of the preganglionic nerve. When performed 10 or 15 sec before excitation of the ganglion, intra-arterial injection of 1 mg each of methylpentynol, methylpentynol carbamate and paraldehyde reduced the retraction of the nictitating membrane, whether this

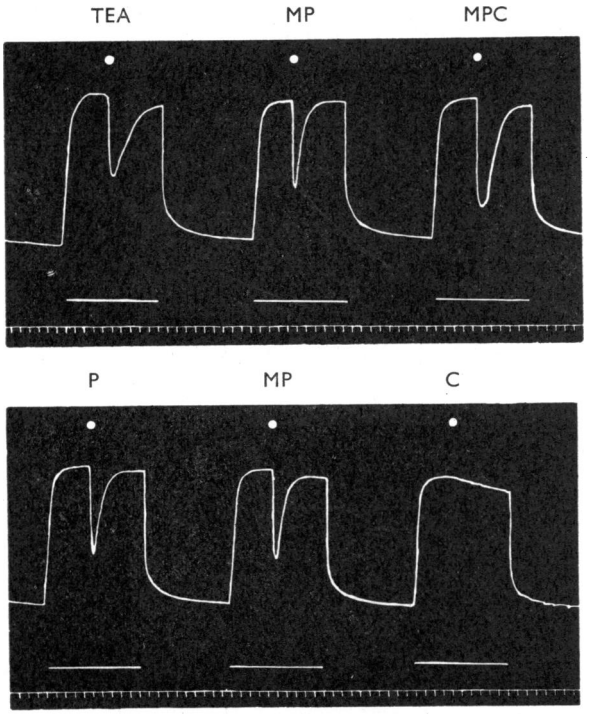

Fig. 1. Cat, 3.3 kg, chloralose anaesthesia, perfused superior cervical ganglion preparation. Effect of drugs, injected into the perfusion stream, upon the retraction of the nictitating membrane in response to stimulation of the preganglionic sympathetic nerve for 5 min at 10 shocks/sec. The lower record is continuous with the upper one. Stimulation (duration indicated by horizontal white bars) was alternated with rest periods of equal length. Doses of tetraethylammonium (20 μg at TEA), methylpentynol (1 mg at MP), methylpentynol carbamate (1 mg at MPC), paraldehyde (1 mg at P), and 0.2 ml. of Locke solution (at C) were injected intra-arterially at white dots, 2 min after start of a period of stimulation. Time marks, 30 sec.

was induced by injected acetylcholine or by preganglionic nerve stimulation. In all instances the response to preganglionic nerve stimulation appeared less affected than was that to a corresponding injection of acetylcholine. Paraldehyde was less potent than either methylpentynol or methylpentynol carbamate in blocking transmission and reducing the response to injected acetylcholine. Nevertheless the finding that all three compounds reduced the response to injected acetylcholine reveals their ability to exert a postsynaptic blocking effect. A similar effect has also been noted for methylpentynol and methylpentynol carbamate, in the perfused ganglion preparation, by Marley & Paton (1959). Since injections of methylpentynol carbamate evoked a brief retraction of the nictitating membrane (Fig. 3) this compound must possess some initial ganglion-stimulant activity in addition to its marked ganglion-blocking action (Fig. 1). This transient ganglion-stimulant action has been attributed (Marley & Paton, 1959) to the presence of a basic carbamino group in the structure of an otherwise depressant molecule, for methylpentynol itself entirely lacked this stimulant effect, a finding confirmed by us (Fig. 3).

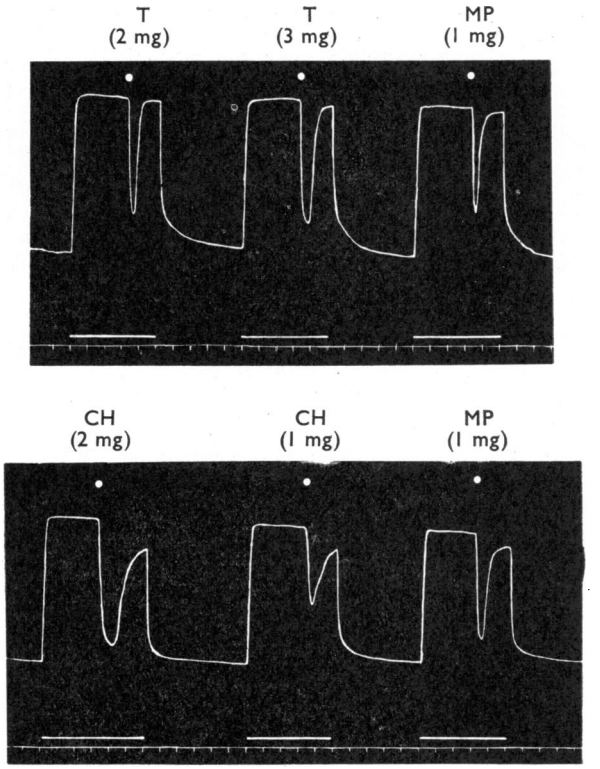

Fig. 2. Cat, 3.4 kg, chloralose anaesthesia, perfused superior cervical ganglion preparation. Effect of various central depressant drugs, injected into the perfusion stream, upon the response of the nictitating membrane to stimulation of the preganglionic sympathetic nerve for 5 min (6 min at CH 2 mg) at 10 shocks/sec. The lower record is continuous with the upper one. Stimulation (duration indicated by horizontal white bars) was alternated with rest periods of equal length. Doses of troxidone (2 mg and 3 mg at T), methylpentynol (1 mg at MP), and chloral hydrate (2 and 1 mg at CH) were injected intra-arterially at white dots, 3 min after start of a period of stimulation. Time marks, 1 min.

Acetylcholine release

Control experiments. The release of acetylcholine from the perfused ganglion during repetitive preganglionic nerve stimulation was detected only in the presence of an anticholinesterase agent, physostigmine. This result is in strict accord with the classical findings of Feldberg & Gaddum (1934), since confirmed by many workers (Feldberg & Vartiainen, 1934; MacIntosh, 1938; Emmelin & MacIntosh, 1956).

Control experiments were made with Locke solution, either alone or enriched with choline: both solutions contained physostigmine (5×10^{-6} g/ml.). During the short initial collection period of 4 min preceding nerve stimulation, usually no acetylcholine was detected in the perfusate, but occasionally there were traces too small for accurate assay. The procedure for all ganglionic acetylcholine release experiments was as follows. Two consecutive " stimulation periods " (6 min collec-

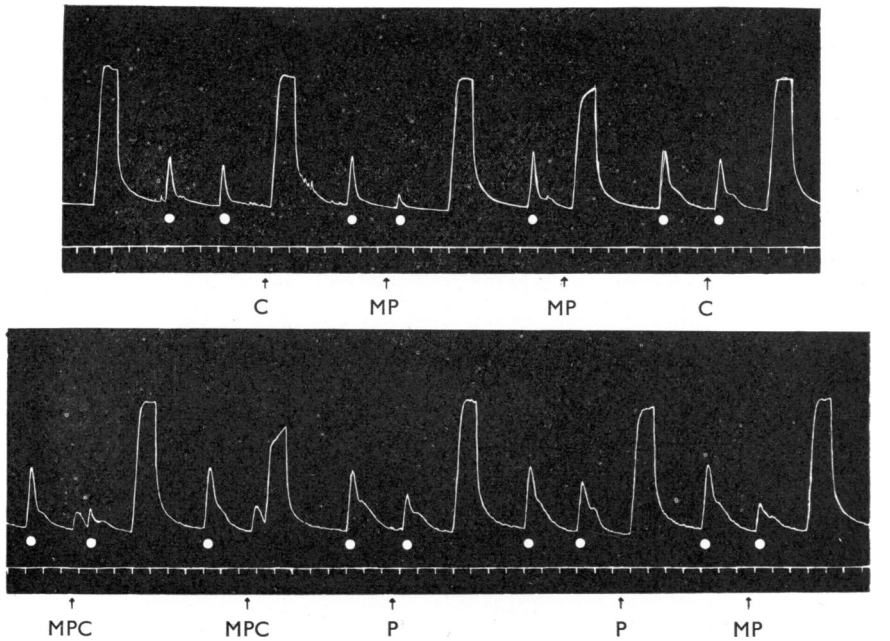

Fig. 3. Cat, 2.6 kg, chloralose anaesthesia, perfused superior cervical ganglion preparation. Effect of prior injections of central depressant drugs to the ganglion upon the response of the nictitating membrane to stimulation of the preganglionic nerve or to injection of acetylcholine. The lower record is continuous with the upper one. All injections were made into the perfusion stream through a modified arterial cannula. At white dots, 50 μg of acetylcholine were injected; the other retractions are the response to nerve stimulation for 1 min at 10 shocks/sec. Time marks, 1 min. Injections of methylpentynol (1 mg at MP) and paraldehyde (1 mg at P), and control injections of 0.2 ml. of Locke solution (at C) were made 10 sec before, and of methylpentynol carbamate (1 mg at MPC) 15 sec before, ganglion stimulation by acetylcholine or nerve excitation.

tion ; stimulation for first 3 min) were succeeded by a single 6 min rest period without nerve stimulation. This cycle was then repeated twice giving a total of six stimulation periods and three rest periods and this constituted one experiment, as described previously (Matthews, 1961, 1963). Use of this experimental pattern is illustrated in Fig. 4. If the mean outputs of acetylcholine from each two consecutive periods are expressed as percentages of those occurring in the first two stimulation periods, it is possible to compare acetylcholine outputs from different experiments (Table 1).

The results shown in Table 1 demonstrate that with Locke solution containing physostigmine there is a steady decrease in acetylcholine output with successive periods of preganglionic nerve stimulation, despite the interposition of rest periods. If choline chloride (10^{-6}) is added to the perfusion fluid then this decline in output is completely prevented (Table 1), as previously reported (Matthews, 1963). In some similar experiments with Locke solution containing physostigmine and plasma (10%) the acetylcholine output was then also better sustained (Matthews, 1961, and unpublished).

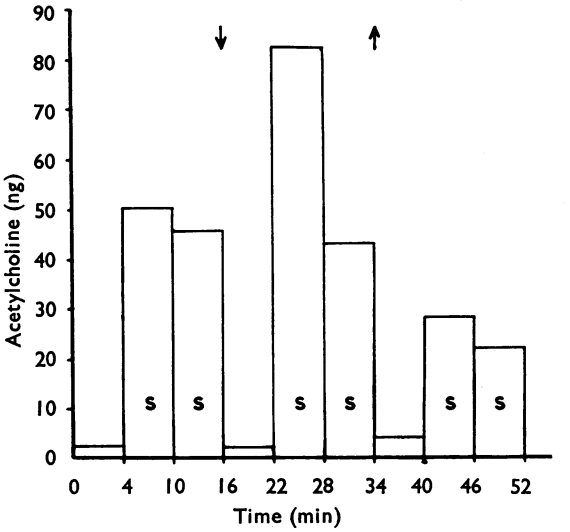

Fig. 4. The effect of tetraethylammonium on the acetylcholine output from ganglia (mean of two experiments). Perfusion was with Locke solution containing physostigmine and, between arrows, tetraethylammonium (10^{-4} g/ml.). Stimulation was applied to the preganglionic nerve for the first 3 min of each 6 min collection period marked S.

Effect of drugs. A technique of three-phase perfusion was employed when test drugs were assessed for activity upon acetylcholine release. A comparison of the outputs of acetylcholine in successive stimulation periods in the presence of drug (second phase) was made with the initial outputs in its absence (first phase) and with the appropriate controls (from Table 1). Restoration of perfusion with drug-free fluid (third phase) enabled recovery of output to be followed. The peak response of the nictitating membrane during each stimulation period served as an index of transmission through the ganglion. In the presence of physostigmine, there was some decline in the peak response of the membrane during the course of control experiments and this was allowed for in computing the absolute percentage block in transmission during test-drug action.

TABLE 1

CONTROL EXPERIMENTS SHOWING RELEASE OF ACETYLCHOLINE FROM PERFUSED GANGLIA

Each value for ganglionic acetylcholine output (mean with standard error) is for two successive stimulation periods and is expressed as a percentage of the output from the initial two periods. Values in parentheses indicate the corresponding decline (means and standard errors) of the nictitating membrane response expressed as a percentage of the mean of the two initial responses. All perfusion fluids contained physostigmine (1×10^{-6}) and were bubbled with pure oxygen

Perfusion fluid	No. of expts.	Acetylcholine output in stimulation periods		
		1 and 2	3 and 4	5 and 6
Locke solution	6	100 (0)	82±3 (11±3)	71±8 (19±7)
Locke solution +choline (10^{-6})	3	100 (0)	106±11 (4±2)	103±6 (9±2)

Central depressant drugs. (*i*) *Amylobarbitone sodium.* Amylobarbitone sodium reduced the release of acetylcholine from the stimulated ganglion but had a somewhat variable effect upon ganglionic transmission (Table 2). Perfusion with solution containing amylobarbitone (5×10^{-5}) led to a small but statistically significant reduction both of acetylcholine output (39%) and of ganglionic transmission (62%). There was no restoration of output in subsequent recovery periods yet there was, nevertheless, some recovery of transmission, which suggested the possibility that an additional postjunctional action of amylobarbitone may contribute to a given degree of ganglionic blockade.

An increase of amylobarbitone concentration to 10^{-4} ($n=2$) resulted in a somewhat greater reduction of acetylcholine release (52%) but no increase in the magnitude of ganglion-block (52%). Both acetylcholine output and ganglionic transmission showed considerable recovery in the subsequent recovery periods. Choline attenuated the action of amylobarbitone upon acetylcholine release. Thus in the presence of choline chloride (10^{-6}, $n=2$), the reduction of acetylcholine liberation by amylobarbitone (10^{-4}) was now much less (33% compared with 52%); the reduction was no longer significant ($P>0.05$), although ganglionic transmission was nevertheless severely and significantly impaired (73% block).

(*ii*) *Chloral hydrate.* Chloral hydrate (5×10^{-4}) caused no reduction in acetylcholine release when perfused through the stimulated ganglion but nevertheless induced a decrease in transmission of 20% (Table 2). On the other hand a twofold increase of concentration to 10^{-3} led invariably to a marked, and highly significant, reduction of acetylcholine liberation (77%) associated with a considerable transmission failure (75%). Some subsequent recovery of both acetylcholine output and transmission was observed. In the presence of choline chloride (10^{-6}), chloral hydrate (10^{-3}) again inhibited acetylcholine output (59%) and blocked transmission (86%), but the reduction of output was less than that induced by an identical concentration of chloral hydrate in the absence of choline. Surprisingly neither output nor transmission was restored in the ensuing recovery periods.

The central depressant activity of chloral hydrate may be due predominantly to its metabolic product trichloroethanol (Butler, 1949 ; Marshall & Owens, 1954), and many tissues are known to form trichloroethanol rapidly from chloral hydrate (Butler, 1949). When trichloroethanol (5×10^{-4}) was added to the perfusion fluid in one experiment, a marked and prolonged reduction of both acetylcholine liberation and of ganglionic transmission was observed (Table 2). Thus trichloroethanol appeared to be more potent than chloral hydrate both in terms of ganglion-blocking activity and the associated suppression of acetylcholine liberation, for an equivalent concentration of chloral hydrate (5×10^{-4}) had no effect upon acetylcholine release and only a minimal action upon ganglionic transmission.

(*iii*) *Methylpentynol.* As the result of an electrophysiological study, it was inferred by Quilliam (1959) that both methylpentynol and paraldehyde blocked ganglionic transmission by suppressing the release of acetylcholine from the presynaptic nerve terminals. This possibility was now tested directly. Nine experiments were made with methylpentynol and one with methylpentynol carbamate in concentrations ranging from 5×10^{-4} to 2×10^{-3} (Table 2). The lowest methylpentynol concentra-

tion (5×10^{-4}) induced, in one experiment, an appreciable reduction both in acetylcholine output (49%) and in ganglionic transmission (55%). An increase of concentration to 10^{-3} led to a more profound action, the output of acetylcholine being further reduced (73%) whilst the block of transmission became complete. Addition

TABLE 2

EFFECT OF CENTRAL DEPRESSANT DRUGS ON RELEASE OF ACETYLCHOLINE FROM PERFUSED GANGLIA

Each value for ganglionic acetylcholine output (mean with standard error) is for two successive stimulation periods and is expressed as a percentage of the output from the initial two periods. Values in parentheses indicate the corresponding decline (mean and standard error) of the nictitating membrane response as a percentage of the mean of the two initial responses. Perfusions were with Locke solution, or with Locke solution and choline (10^{-6})*, or with Locke solution and plasma (10%)**. † Values in this column were computed on a percentage basis by comparison of the outputs of acetylcholine (and the response of the nictitating membrane) during drug action with the values obtained in the corresponding periods of the appropriate control experiments (Table 1). ‡ Depression compared with controls

Drug	Drug concentration (g/ml.)	No. of expts.	Acetylcholine output (%) Initial (without drug)	During drug action	Final (without drug)	†% depression relative to appropriate controls	‡P
Amylobarbitone	5×10^{-5}	3	100	50±10	40±13	39	<0·05
			(0)	(70±22)	(53±22)	(62)	(<0·01)
	10^{-4}	2	100	39±14	67±15	52	<0·01
			(0)	(58±23)	(11±5)	(52)	(<0·02)
	10^{-4}	2*	100	71±21	78±29	33	>0·05
			(0)	(76±19)	(60±41)	(73)	(<0·01)
Chloral hydrate	5×10^{-4}	1	100	96	105	0	—
			(0)	(23)	(22)	(20)	
	10^{-3}	3	100	19±4	33±4	77	<0·001
			(0)	(84±8)	(31±6)	(75)	(<0·001)
	10^{-3}	2*	100	34±12	34±0	59	<0·05
			(0)	(90±11)	(100±0)	(86)	(<0·001)
Trichloroethanol	5×10^{-4}	1	100	28	24	54	—
			(0)	(94)	(83)	(84)	
Methylpentynol	5×10^{-4}	1	100	33	57	49	—
			(0)	(62)	(36)	(55)	
	10^{-3}	3	100	22±6	43±7	73	<0·001
			(0)	(100±0)	(39±14)	(100)	(<0·001)
	10^{-3}	2*	100	59±11	72±5	44	>0·05
			(0)	(53±1)	(13±13)	(51)	(<0·001)
	2×10^{-3}	1**	100	0	69	100	—
			(0)	(90)	(21)	(–)	
	2×10^{-3}	1*	100	25	75	76	—
			(0)	(100)	(40)	(100)	
Methylpentynol carbamate	10^{-3}	1	100	0	54	100	—
			(0)	(100)	(17)	(100)	
Taraldehyde	2×10^{-3}	1	100	42	55	49	—
			(0)	(91)	(28)	(81)	
	4×10^{-3}	4	100	57±11	63±10	31	<0·05
			(0)	(90±9)	(19±4)	(80)	(<0·001)
	2×10^{-3}	1*	100	95	87	10	—
			(0)	(92)	(28)	(88)	
	4×10^{-3}	1*,**	100	88	84	—	—
			(0)	(91)	(34)		
Troxidone	5×10^{-3}	4	100	32±15	59±13	61	<0·01
			(0)	(97±5)	(25±6)	(86)	(<0·001)
	5×10^{-3}	1*	100	76	66	28	—
			(0)	(52)	(40)	(50)	
	3×10^{-3}	1*	100	102	81	4	—
			(0)	(33)	(12)	(32)	

of choline chloride (10^{-6}) to the perfusion fluid modified considerably this ganglion-depressant activity of methylpentynol (10^{-3}), for acetylcholine output was then reduced by only 44%, a value which did not differ statistically from the controls ($P>0.05$). The action of methylpentynol upon transmission was also attenuated by choline so that the degree of depression of transmission (and of output) was similar to that observed with the *lowest* methylpentynol concentration of 5×10^{-4}, in the *absence* of choline. In view of this antagonism to the depressant action of methylpentynol (10^{-3}) by choline, the effect of increasing the methylpentynol concentration to 2×10^{-3} was investigated. Acetylcholine output was then abolished in one preparation perfused with plasma-supplemented Locke solution. But in the presence of choline chloride (10^{-6}), acetylcholine output in another preparation was reduced by 76%, an effect similar to that obtained with methylpentynol (10^{-3}) in the absence of choline. In both these experiments there was again a complete or almost complete failure of transmission. These experiments suggest that choline decreased the ganglion-depressant potency of a given concentration of methylpentynol (such as 10^{-3}) upon acetylcholine release by approximately one-half.

In one experiment, methylpentynol carbamate (10^{-3}) exerted a depressant action similar to but more powerful than that of methylpentynol, both acetylcholine liberation and ganglionic transmission being abolished.

In all experiments with methylpentynol and its carbamate, substantial recovery of acetylcholine output and ganglionic transmission ensued upon resumption of perfusion with drug-free fluid, pointing to the readily reversible action of these substances.

Methylpentynol seems to possess some vasodilator activity since, in its presence, a rise of perfusate flow rate was sometimes observed with the perfusion pressure constant throughout. This effect was particularly evident in one experiment in which the flow rate rose from 0.37 to 0.63 ml./min during the perfusion with methylpentynol (2×10^{-3}); when perfusion with drug-free fluid was resumed the flow returned to 0.35 ml./min.

(*iv*) *Paraldehyde.* Paraldehyde reduced acetylcholine release to a variable extent although, in the concentrations used, it consistently induced a block, often total, of tranmission through the ganglion. Thus in four experiments with paraldehyde (4×10^{-3}), the mean reduction of acetylcholine output induced, although statistically significant ($P<0.05$) when compared with control experiments, amounted to but 31%, whereas the mean transmission block was 80%. Indeed, in two of these four experiments, notwithstanding the fact that block of ganglionic transmission was almost complete, the pattern of acetylcholine output closely followed that seen in control experiments. This moderate activity of paraldehyde upon acetylcholine release was reflected in two further experiments, in one of which the paraldehyde concentration was 2×10^{-3} in Locke solution containing physostigmine and modified by the addition of choline chloride (10^{-6}), and in the other of which paraldehyde (4×10^{-3}) in Locke solution containing physostigmine, plasma (10%) and choline chloride (10^{-6}) was employed. Both experiments yielded similar results, namely 90% block of ganglionic transmission with little if any concomitant reduction of acetylcholine liberation.

(*v*) *Troxidone.* Perfusion of troxidone (5×10^{-3}, n=4) was followed by a significant reduction in mean acetylcholine output (61%) with a concurrent, and almost complete, block of transmission (86%). In one of these experiments, the output of acetylcholine was little affected, falling by only 6% in the presence of troxidone although a 90% block of transmission was observed. This effect resembled that often seen with paraldehyde, and the similarity is underlined by a further experiment in which a lower concentration of troxidone (3×10^{-3}) in Locke solution containing choline was perfused. Here there was a diminution of but 4% in acetylcholine output associated with a block of transmission of 32%. Addition of choline chloride (10^{-6}) considerably decreased the depressant action of troxidone (5×10^{-3}) both upon acetylcholine liberation and ganglionic transmission, as did the substitution of plasma (10%) for choline in the perfusion fluid.

Reference drugs. Hexamethonium, tetraethylammonium and procaine were included in this study as reference drugs (Table 3). The methonium compounds

TABLE 3

EFFECT OF REFERENCE DRUGS ON RELEASE OF ACETYLCHOLINE FROM PERFUSED GANGLIA

Each value for ganglionic acetylcholine output (mean with standard error) is for two successive stimulation periods and is expressed as a percentage of the output from the initial two periods. Values in parentheses indicate the corresponding decline (mean and standard error) of the nictitating membrane response as a percentage of the mean of the two initial responses. Perfusion was with Locke solution containing physostigmine. *See Table 2

Drug	Drug concentration (g/ml.)	No. of expts.	Acetylcholine output (%) Initial (without drug)	During drug action	Final (without drug)	*% depression relative to appropriate controls	*P**
Hexamethonium	10^{-5}	1					
	3×10^{-5}	1	100	85±2	59±3	0	
	5×10^{-5}	1					
Tetraethyl-ammonium	10^{-4}	2	100 (0)	131±5 (100±0)	53±5 (63±37)	0 (100)	(<0·001)
Procaine	5×10^{-5}	1	100 (0)	19 (97)	27 (100)	77 (86)	
	10^{-4}	1	100 (0)	10 (100)	11 (100)	88 (100)	

appear to block transmission of nerve impulses in autonomic ganglia competitely at the postsynaptic membrane (Paton & Perry, 1953) without impairing the release of acetylcholine from the presynaptic terminals (Paton & Zaimis, 1951). Our results with hexamethonium (1 to 5×10^{-5}) accord strictly with this general concept, since a marked, but readily reversible, block of ganglionic tranmission was induced by this compound, associated with an undiminished output of acetylcholine. Table 3 shows that, in the presence of hexamethonium, the acetylcholine output (mean and standard error, 85±2% of initial value) in the three experiments was not statistically different ($P>0.05$) from that (82±3%) in the corresponding periods of control experiments (Table 1).

It was anticipated that qualitatively tetraethylammonium would mimic faithfully the activity of hexamethonium, since both compounds have been classified as com-

petitive ganglion-blocking agents, acting solely at the postsynaptic site (Paton & Perry, 1953). The results shown in Table 3 reveal, however, a notable disparity between the two drugs, at least in regard to their presynaptic activity, for tetraethylammonium, in distinct contrast to hexamethonium, markedly augmented the release of acetylcholine upon preganglionic nerve stimulation. Following the addition of tetraethylammonium (10^{-4}, n=2) to the perfusion fluid there was a subsequent *increase* in output of approximately 60% compared with control levels (Table 1), although ganglionic tranmission was concurrently completely blocked. This considerable and statistically significant ($P<0.001$) increase of mean acetylcholine liberation was due entirely to a striking and approximately twofold increase of output which occurred in the first stimulation period in the presence of tetraethylammonium. The patterns of release in all subsequent periods resembled closely that of control experiments and that seen with hexamethonium. No increased liberation of acetylcholine was observed upon perfusion with tetraethylammonium in Locke solution in the absence of stimulation. This initial augmentation of acetylcholine release is thus apparent only upon stimulation ; it is illustrated in Fig. 4.

Harvey (1939) established that procaine interferes with the ganglionic discharge of acetylcholine, a finding confirmed by us. For example, procaine hydrochloride (5×10^{-5}) induced a powerful and prolonged suppression of both acetylcholine release and ganglionic transmission, effects which were intensified by a twofold increase of procaine concentration (Table 3). In subsequent recovery periods there was no restoration of transmission, and acetylcholine output showed only a small, almost negligible, degree of recovery. However, it is possible that after these concentrations of procaine, which seemed to cause an almost maximal effect, the total recovery time allowed (12 to 15 min) was insufficient for any appreciable recovery to become apparent.

Rat isolated phrenic-nerve diaphragm preparation

Neuromuscular transmission

An estimate was made of the relative neuromuscular blocking potency of some of those drugs previously examined for activity upon the perfused ganglion. The dose/response relationships are depicted in Fig. 5, in which percentage reduction of response to indirect stimulation through the phrenic nerve is plotted against the log of the concentration of the test drug present in the bath for 5 min. The equipotent molar ratios derived for the concentrations producing 50% block (ED50) appear in Table 4. Procaine was the most, and troxidone the least, potent neuromuscular blocking agent. Chloral hydrate caused no neuromuscular block ; in low doses it augmented the twitch of the indirectly stimulated diaphragm while in higher doses it elicited a muscle contracture. Trichloroethanol, however, blocked neuromuscular transmission, but a small increase in concentration induced a muscle contracture, an action also elicited by higher concentrations of amylobarbitone and methylpentynol carbamate.

As it was necessary to exclude an effect on conduction of impulses along the phrenic nerve, the nerve was mounted in a separate bath in some experiments. The concentrations of drug used for bathing the nerve were in each instance greater

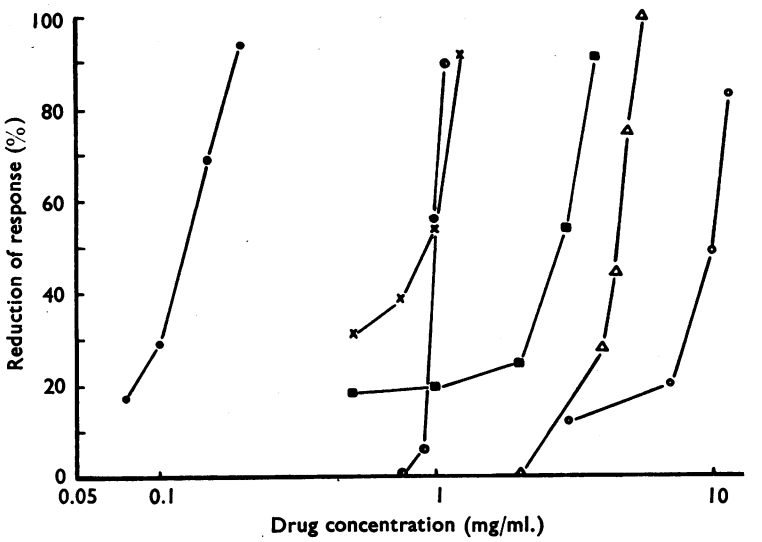

Fig. 5. Dose/response relationships for central depressant drugs, with procaine included for comparison, on the rat isolated phrenic nerve-diaphragm preparation. Ordinate: percentage reduction of the twitch height of the indirectly stimulated diaphragm. Abscissa: log bath concentration of drug (mg/ml.). Each point plotted is the mean of two determinations in different experiments. Each drug at each concentration was allowed to act for 5 min before measuring the percentage block. ●—●, procaine hydrochloride; ×—×, methylpentynol carbamate; ◑—◑, trichlorethanol; ■—■, methylpentynol; △—△, paraldehyde; and ○—○, troxidone.

than those producing complete neuromuscular block when present in the diaphragm-bath. Procaine in concentrations (300 μg/ml.) slightly in excess of those necessary for a total neuromuscular block had little or no action in blocking transmission in the motor nerve but, if the concentration was raised to 10^{-2}, there was a small decline, of approximately 7%, in the height of the twitch response to nerve stimulation. The small magnitude of this depression emphasizes the resistance of large diameter motor nerves to block by local anaesthetic agents (Matthews & Rushworth, 1957). However, the duration of exposure, 5 min, may have been too short for maximal nerve block to develop. No conduction block was seen with any of the

TABLE 4

NEUROMUSCULAR BLOCKING ACTIVITY OF CENTRAL DEPRESSANT DRUGS RELATIVE TO PROCAINE

Equipotent molar ratios were derived from doses producing 50% block of the twitches, elicited indirectly, of the rat phrenic-nerve diaphragm preparation

Drug	ED50 (mg/ml.)	*M*
Procaine hydrochloride	0·13	1
Methylpentynol carbamate	0·94	13
Trichloroethanol	1·00	13
Methylpentynol	2·90	60
Paraldehyde	4·60	71
Troxidone	10·00	143

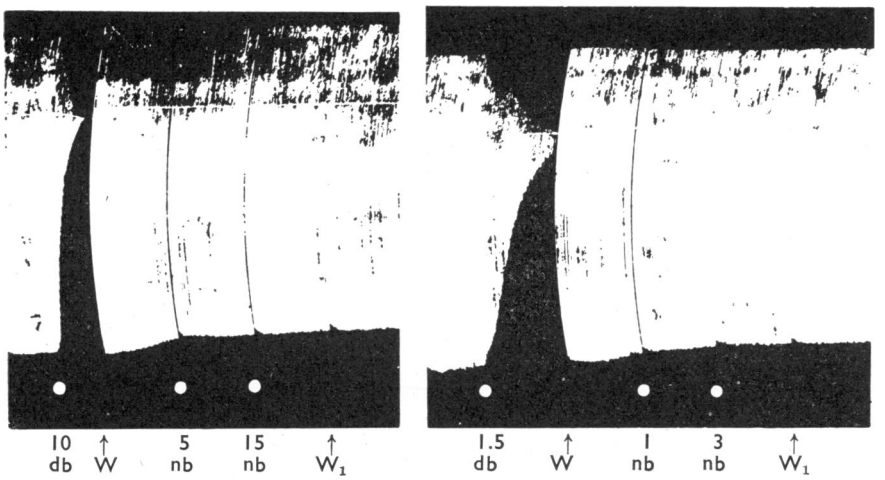

Fig. 6. Records of the semi-isotonic twitches of the rat isolated phrenic nerve-diaphragm preparation to single supramaximal stimuli applied to the phrenic nerve every 4 sec. Separate baths were provided for the muscle and for the phrenic nerve. Left and right records are from the same preparation.

Left record: Paraldehyde (10 mg/ml.) in the diaphragm bath fluid (at 10db) during 3 min caused a complete neuromuscular block. Transmission was fully restored by washing (at W) with fresh Krebs solution during 5 min; stimulation withheld and drum stopped. Paraldehyde (5 and 15 mg/ml.) in the nerve bath fluid (at 5 nb and 15 nb, respectively; contact time, 5 min) was without effect upon the response of the muscle to stimulation of the phrenic nerve. Nerve washed with fresh Krebs solution at W_1.

Right record: Methylpentynol carbamate (1.5 mg/ml.) in the diaphragm bath fluid (at 1.5 db) during 5 min, and 1 and 3 mg/ml. in the nerve bath fluid (at 1 nb and 3 nb, respectively; contact time, 5 min). Details as in left record.

central depressant drugs tested. Thus, as none of the drugs in the concentrations used to produce neuromuscular block impaired impulse conduction in the phrenic nerve, the observed block must depend upon an action either at, or beyond, the neuromuscular junction. The lack of effect of paraldehyde and methylpentynol carbamate upon nerve conduction is illustrated in Fig. 6.

Following the addition to the diaphragm-bath of methylpentynol carbamate, a small transient muscle contracture developed in the early phase of neuromuscular block. This contracture is reminiscent of the stimulant effect of methylpentynol carbamate at the ganglion (Fig. 3), and the similarity suggests that both effects may arise through a common mechanism.

Acetylcholine release experiments

Control experiments. In experiments of analogous pattern to those with the perfused ganglion, acetylcholine was found in the fluid bathing the isolated diaphragm following repetitive stimulation of the motor nerve only if an anticholinesterase agent, such as physostigmine, was present in the bath fluid (Fig. 7, lower part). A small resting release of acetylcholine was sometimes found in the absence of nerve stimulation but this rarely exceeded 0.4 to 0.7 ng/ml./20 min. The release was

generally so small in comparison with the output upon nerve stimulation that no correction factor was applied for this resting release.

The results of four control experiments measuring acetylcholine release from the stimulated diaphragm are summarized in Table 5. A total of six successive 20 min stimulation periods (interpolated by 10 min rest periods) constituted a single experiment. The mean acetylcholine outputs of the two initial periods of stimulation, of the following two and the final two periods appear in successive columns. The

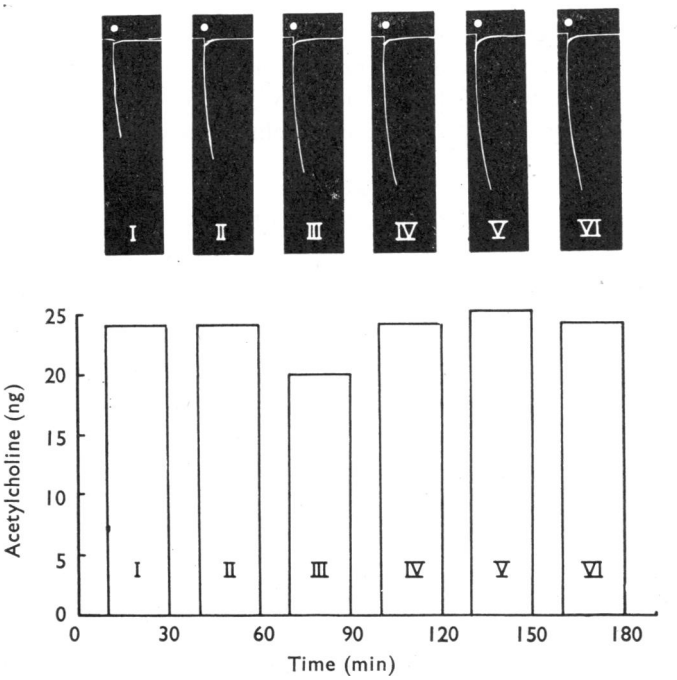

Fig. 7. Control experiment (Expt. no. 4, Table 5) with the rat isolated phrenic nerve-diaphragm preparation.

Upper: Records of muscle responses to supramaximal repetitive stimulation of the phrenic nerve at 25 shocks/sec for 20 min, commencing at the white dots in records I to VI. The period between the end of one record and the onset of stimulation in the next represents a rest period of 10 min (no stimulation) during which the preparation was washed with fresh solution. Krebs solution with physostigmine (5×10^{-6} g/ml.) was present throughout.

Lower: Acetylcholine output corresponding to upper record. Output is expressed in ng/20 min stimulation period. Stimulation periods I to VI alternated with rest periods of 10 min (no stimulation).

release of acetylcholine was well maintained (see Fig. 7, lower part, which illustrates experiment 4 of Table 5), and a small rise in output was often apparent in successive stimulation periods. This result was in direct contrast to the effect seen with the perfused superior cervical ganglion preparation, in which there was a progressive decrease of acetylcholine output in response to nerve stimulation unless choline (or plasma) was present in the Locke perfusion fluid.

In the presence of physostigmine, the mechanical response of the muscle to tetanic nerve stimulation was not sustained. It rapidly attained a maximum and thereafter soon declined to the baseline during each 20 min period of nerve stimulation. A similar effect has been reported by previous workers (Briscoe, 1936; Bacq & Brown, 1937). In control experiments, however, the peak response of the muscle increased in magnitude with successive stimulation periods; this is particularly evident in Fig. 7 (upper part). On the other hand, in the presence of a depressant drug the muscle response was reduced or abolished (Fig. 8 upper part). Central depressant drugs also attenuated the more sustained tetanic response of the muscle observed in the *absence* of physostigmine.

Effect of drugs. The test drug was present only during the third and fourth consecutive stimulation periods of the six successive 20 min stimulation periods forming one experiment. A mean output of acetylcholine was calculated from the outputs

TABLE 5

CONTROL EXPERIMENTS SHOWING ACETYLCHOLINE RELEASE FROM THE RAT DIAPHRAGM

Each value for acetylcholine output is for two successive stimulation periods and is expressed as a percentage of the output from the initial two periods. The lowest line gives means and standard errors for the columns. Krebs solution containing physostigmine (5×10^{-6}) was used throughout

Expt. No.	Acetylcholine output in stimulation periods		
	1 and 2	3 and 4	5 and 6
1	100	105	113
2	100	100	100
3	100	117	146
4	100	92	104
Mean	100	104±6	116±10

of these two periods. This mean output was then expressed as a percentage of the mean acetylcholine output of the two initial stimulation periods in the absence of the drug, to give evidence of any depression of acetylcholine output. The mean output from the two stimulation periods following drug action gave evidence of any subsequent recovery. Similarly the mean maximal height of the muscle response during drug action was compared with the mean during the two initial periods on a percentage basis to give a measure of neuromuscular block. Unfortunately it proved impracticable to examine the action of amylobarbitone upon acetylcholine release from the diaphragm since the concentrations of the barbiturate required for action at the neuromuscular junction interfered seriously with the assay preparations.

At the neuromuscular junction, the activity of the central depressant drugs examined displayed a high degree of congruence with their action at the ganglionic synapse (Table 6). Methylpentynol, and its more potent carbamate, suppressed the release of acetylcholine, at the same time blocking neuromuscular transmission; both effects were intensified by an increase in drug concentration until finally each was completely, though reversibly, abolished. As at the ganglion, paraldehyde was characterized by its ability to induce a considerable or even complete block of transmission in concentrations which, at the same time, left acetylcholine release

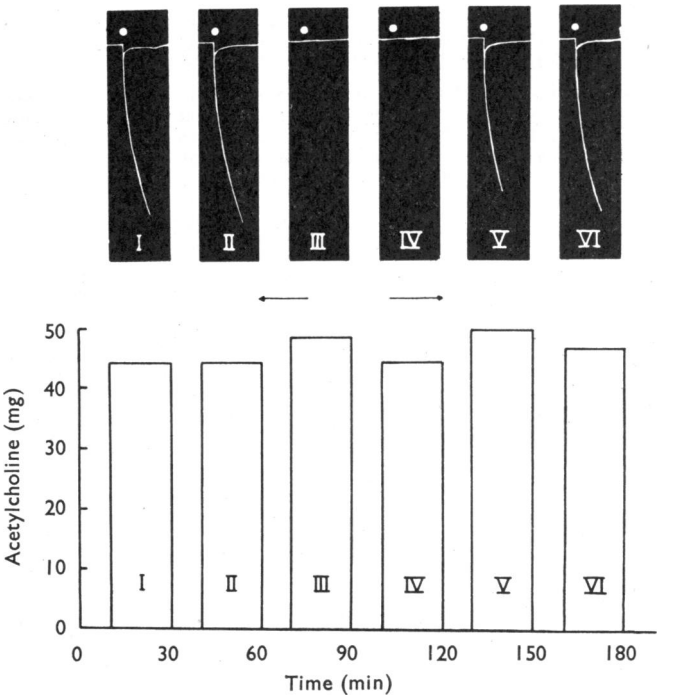

Fig. 8. Effect of paraldehyde on the rat isolated phrenic nerve-diaphragm preparation.

Upper: Records of muscle responses to repetitive supramaximal stimulation of the phrenic nerve at 25 shocks/sec for 20 min, commencing at white dots in records I to VI. The period between the end of one record and the onset of stimulation in the next represents a rest period of 10 min (no stimulation) during which the preparation was washed with fresh solution. Krebs solution with physostigmine (5×10^{-6} g/ml.) was present throughout with, between the arrows, the addition of paraldehyde (5×10^{-3} g/ml.).

Lower: Acetylcholine output corresponding to upper records. Output is expressed in ng/20 min stimulation period. Stimulation periods I to VI interpolated by rest periods of 10 min (no stimulation).

totally unaffected. Although higher concentrations of paraldehyde did abolish transmission with some reduction in acetylcholine output, the recovery of acetylcholine liberation in subsequent periods in the absence of drug was rapid. Procaine and trichlorethanol seemed very similar in their ability to inhibit acetylcholine release and depress junctional transmission, both effects being accentuated with an increase of concentration but readily reversible in subsequent recovery periods. The observations with procaine support those of Straughan (1961), who likewise found acetylcholine liberation to be reduced in the presence of this substance. High concentrations of troxidone were required to impair neuromuscular transmission. This drug could, nevertheless, produce a neuromuscular block but with relatively little effect upon acetylcholine output. Moreover the latter effect, unlike that of paraldehyde, was not intensified by an increase of drug concentration. The recovery periods after troxidone were typified by an apparent increase of acetylcholine output above the initial normal values, an effect which was peculiar to this drug.

TABLE 6

EFFECT OF CENTRAL DEPRESSANT DRUGS AND PROCAINE ON ACETYLCHOLINE RELEASE FROM THE RAT DIAPHRAGM

Each value for acetylcholine output is for two successive stimulation periods and is expressed as a percentage of the output from the initial two periods. Numerals in parentheses indicate percentage depression of muscle response by drug; each value is derived from a comparison of the mean contraction height from two successive periods of stimulation before and during drug action.
*Mean of two experiments

Drug	Drug concentration (g/ml.)	No. of expts.	Acetylcholine output in stimulation periods: 1 and 2 (Krebs solution)	3 and 4 (Krebs solution and drug)	5 and 6 (Krebs solution)	% reduction of acetylcholine output during drug action compared with initial value
Methylpentynol	10^{-3}	1	100	100 (34)	63	0
	3×10^{-3}	1	100	59 (91)	82	41
	5×10^{-3}	1	100	0 (100)	100	100
Methylpentynol carbamate	10^{-3}	1	100	34 (100)	103	66
	3×10^{-3}	1	100	0 (100)	17	100
Paraldehyde	3×10^{-3}	1	100	100 (68)	114	0
	5×10^{-3}	1	100	105 (100)	109	0
	6×10^{-3}	2	100	65 (100)	83	35*
	8×10^{-3}	1	100	33 (100)	105	67
Procaine	10^{-4}	2	100	31 (66)	93	69*
	2×10^{-4}	1	100	0 (100)	66	100
Trichloroethanol	5×10^{-4}	2	100	45 (49)	93	55*
	$7{\cdot}5\times10^{-4}$	1	100	4 (74)	75	96
Troxidone	5×10^{-3}	2	100	43 (55)	115	57*
	$7{\cdot}5\times10^{-3}$	1	100	73 (100)	119	27
	10^{-2}	1	100	70 (100)	150	30

DISCUSSION

In his studies of the ganglion-blocking action of barbiturates, Exley (1954) detected no reduction of acetylcholine release from the cat superior cervical ganglion perfused with Locke solution (containing physostigmine) upon addition of amylobarbitone sodium, except in concentrations (about 400 mg/kg) far in excess of those believed necessary for general anaesthesia in the intact animal. On the other hand, we found that a small but definite impairment of the release of acetylcholine from preganglionic nerve endings can occur with a concentration of amylobarbitone as low as 5×10^{-5}, and that this effect may be accentuated by an increase of concentration to 10^{-4}. With each concentration ganglionic transmission was simul-

taneously reduced. It is interesting to note that Goldbaum (1948) found that the blood concentration of amylobarbitone during anaesthesia in rabbits, induced by an intravenous dose of 50 mg/kg, was approximately 5×10^{-5}. The failure of Exley (1954) to observe suppression of prejunctional release of acetylcholine, unless massive doses of amylobarbitone were used, is puzzling, but might have arisen from his use of single intra-arterial injections of the drug directly to the perfused ganglion, which contrasts with the more precise application of a controlled concentration of drug by perfusion for a given time used in our experiments. Furthermore, the latter technique would be expected to reveal small inhibitory effects and especially those which might develop progressively during the sustained presence of a low concentration of drug, a situation closely analogous to that arising in the tissues of the intact animal during barbiturate anaesthesia. Our results do, however, indicate that an effect other than a simple reduction of prejunctional acetylcholine output, and possibly of postsynaptic origin, may contribute to the block of ganglionic transmission by amylobarbitone, the latter effect according with the finding of Exley (1954).

A close relationship existed between the ganglion-blocking activity of chloral hydrate and its ability to suppress powerfully acetylcholine release. The reduction in acetylcholine output and in postganglionic response after trichloroethanol afforded indirect support for the view that chloral hydrate may exert its effect, at least in part, by its metabolite trichloroethanol. The rapid effect of chloral hydrate in producing ganglion-block, however, suggested that chloral hydrate must itself be responsible for a substantial proportion of the block, as seen in Fig. 2.

Previous work has suggested that the failure of junctional transmission induced by methylpentynol and paraldehyde could be accounted for if these compounds reduced the prejunctional release of acetylcholine (Nicholls & Quilliam, 1956; Quilliam, 1959). Our experiments afford direct evidence that methylpentynol and paraldehyde can indeed inhibit acetylcholine liberation, but also demonstrate that a postsynaptic depression of effector cell excitability is exerted by both these compounds. With paraldehyde the postjunctional effect was more striking while its prejunctional action upon acetylcholine liberation was capricious. Similarly the results from the experiments with troxidone point to a postjunctional component, in addition to the inhibition of acetylcholine liberation from the prejunctional nerve terminals, to account completely for the observed junctional depression by this drug.

Hitherto it appears to have been tacitly assumed that the ganglion-blocking activity of tetraethylammonium is identical qualitatively in all respects with that displayed by similar competitive blocking agents such as hexamethonium. However, we found that tetraethylammonium invariably augmented acetylcholine release in the initial period of stimulation following its addition to the perfusion fluid, transmission being concurrently completely blocked, as reported by Matthews (1961). A similar observation was made independently by Douglas & Lywood (1961). This unique feature distinguishes tetraethylammonium pharmacologically from the related ganglion-blocking agent hexamethonium which is devoid of such activity. It seems likely that the observed effect may stem from the action of tetraethylammonium in prolonging the nerve action potential (Koketsu, 1958) rather

than from initiating repetitive discharge of the nerve axon in response to a single shock (Cowan & Walter, 1937), for the concentrations of tetraethylammonium necessary for the latter effect are high, some twenty-times or more above the concentrations which we have employed (about 0.5 mM). There is electrophysiological evidence (Castillo & Katz, 1956) that, when the action potential at the neuromuscular junction is extended in duration (and increased in amplitude), as for instance by the passage of hyperpolarizing currents through the nerve terminals, an increased output of transmitter ensues. A longer-lasting action potential implies a greater or more prolonged influx of sodium into the nerve terminals and there is now firm evidence that the inward flux of sodium ions may be important in mobilizing cellular acetylcholine for release (Quastel & Birks, 1962; Birks, 1963). Thus prolongation of the action potential in the nerve terminals by tetraethylammonium might ultimately bring about a more rapid mobilization and release of the acetylcholine available from intracellular stores. It is interesting that an effect at the neuromuscular junction with tetraethylammonium, similar to that reported here for the ganglion, has been reported by Collier & Exley (1963) who measured the release of acetylcholine from the isolated rat diaphragm. Their findings provide direct evidence supporting the suggestion, made by Koketsu (1958) on the basis of interpretation of an electrophysiological finding, that tetraethylammonium increases the output of acetylcholine from motor nerve terminals.

It has long been contended that the ganglionic synapse and the skeletal neuromuscular junction show many similarities in their transmission processes and in their reactivity to drugs. Such differences as have been noted are generally ascribed to a variation in anatomical complexity (Paton, 1954b) or in membrane environmental state (Perry, 1954) rather than to any more fundamental difference. The remarkably close analogy found between the effects of central depressant drugs on ganglionic and on neuromuscular transmission strongly supports this contention. It seems noteworthy however, that, whereas preganglionic nerve endings require, in addition to glucose, a supply of exogenous choline and a labile plasma factor for the maintenance of optimal synthesis and release of acetylcholine (Birks & MacIntosh, 1961), the absence of choline from the fluid bathing an isolated stimulated diaphragm appears to place no restraint upon the amount of acetylcholine available for release at motor nerve endings. Hence the terminals of the motor nerve would seem to maintain a sufficiently high intracellular concentration of acetylcholine precursors to require only the provision of glucose in the saline medium for a sustained and efficient release of acetylcholine (Straughan, 1960; Matthews, 1961). It seems likely, therefore, that the apparent difference between acetylcholine release at the preganglionic and at the motor nerve terminals stems largely from the greater viability of acetylcholine synthesis in the latter structures, rather than from any more major distinction between the actual mechanisms of acetylcholine release, although it cannot be assumed tacitly that these mechanisms are necessarily identical.

In contrast to those quaternary ammonium compounds, typified by hexamethonium, which block junctional transmission by a selective action, competing with acetylcholine for receptor sites at the postjunctional membrane (Paton & Perry, 1953), central depressant drugs are characterized in our results by a wider field

of action upon both pre- and postjunctional structures, although the relative degree of activity at either site varied with the individual drug. The diversity in the molecular configurations of the central depressant drugs, and their lack of resemblance to hexamethonium or to acetylcholine, make it most unlikely that these drugs depress the excitability of the postjunctional effector cell competitively, as do many methonium compounds. Their action resembles more closely that of procaine and of procainamide which depress acetylcholine release from the prejunctional nerve terminals and also reduce the excitability of the postjunctional cell (Harvey, 1939 ; Paton & Thompson, 1953a ; Straughan, 1961).

The question thus now arises as to whether a common mechanism operates in the production of a junctional block by the local anaesthetic procaine on the one hand, and central depressant drugs on the other. Indeed, if a similar mode of action could be assumed then there would seem little reason to suppose that the observed pre- and postjunctional effects of central depressant drugs arise as the result of a fundamentally different mechanism at each region of the junction: for it has been suggested that the junctional blocking activity of procaine depends upon a common effect, that is a stabilization of the neuronal membranes, to which the fine prejunctional nerve endings as well as the adjacent effector cell membrane are particularly sensitive (Paton, 1954a). There is also evidence that, like procaine (and adrenaline), various central depressant drugs block junctional transmission at the ganglion with no ganglionic depolarization or hyperpolarization (Paton & Thompson, 1953b ; Exley, 1954 ; Quilliam, 1959). Although the possibility cannot be discounted entirely that some degree of conduction failure in the presynaptic nerve trunk of the perfused ganglion may have resulted from central depressant drug action, conduction in the motor nerve trunk to the isolated diaphragm was unimpaired both by central depressant drugs and by procaine in concentrations above those required for neuromuscular block.

The actions of both procaine and central depressant drugs might, then, devolve upon their comparatively ill-defined " stabilization " of the neuronal membranes for, since normal excitation of nerve involves changes in these membranes including an increased selective permeability to ions, such an effect might well be sufficient to impair the normal transmission of nerve impulses, especially at synaptic regions. The suggestion of Thesleff (1956), that central depressant drugs act by inhibition of the neuronal membrane " sodium carrying mechanism " responsible for the production of a normal nerve action potential, may therefore be of a particular importance, especially when viewed from the standpoint of prejunctional acetylcholine release. For the mobilization of acetylcholine and its liberation from its retaining structures within the prejunctional nerve terminals is known to occur normally as a consequence of the invasion and depolarization of the terminal by the nerve action potential and this, in turn, depends upon the inward flux of sodium ions. Any interference by central depressant drugs with the ionic flux of sodium, whether the result of a direct or an indirect effect, would be of supreme importance, for recent evidence suggests strongly that the normal influx of sodium ions at nerve terminals may play a role in mobilizing cellular acetylcholine for release and in triggering the synthesis of acetylcholine (Quastel & Birks, 1962 ; Birks, 1963).

An alternative possibility is that central depressant drugs might more directly interfere with acetylcholine synthesis and this would lead, ultimately, to a reduced release of acetylcholine. The enzyme choline acetylase is responsible for the biological production of acetylcholine in nervous tissue (Nachmansohn & Machado, 1943). There is, however, considerable experimental evidence which militates against the direct action of many central depressant drugs upon the choline acetylase system isolated *in vitro* (Johnson & Quastel, 1953 ; Kumagai, Ebashi & Takeda, 1954 ; Morris, 1961). By contrast it is well established that inhibition of acetylcholine synthesis can occur in *intact* tissue as the result of drug action (McLennan & Elliot, 1951 ; MacIntosh, Birks & Sastry, 1956). More recent evidence points to the intracellular availability of choline as an essential rate-limiting factor governing acetylcholine synthesis (Birks & MacIntosh, 1961) ; and further work has disclosed that the limited path for the entry of choline into the subcellular particles containing choline acetylase is more susceptible to drug action than is the acetylating mechanism itself (Gardiner, 1961). The prevention of access of choline to its site of acetylation therefore presents a plausible locus of central depressant drug action, for any restraint upon synthesis would result ultimately in a decreased acetylcholine liberation. Our findings that choline can antagonize the reduction of acetylcholine output caused by central depressant drugs, as exemplified in experiments with methylpentynol (Table 2), would accord with this view, although this does not exclude other explanations.

Although we have found that there may be a postjunctional action of the centrally active drugs studied, the prejunctional effect in reducing release of acetylcholine by the nerve terminals is of especial and far reaching importance ; for there is a steadily increasing body of evidence (Larrabee & Bronk, 1947 ; Lloyd, 1949 ; Eccles & Rall, 1951) which points to the phenomenon of facilitation at various junctions in the nervous system, both cholinergic and noncholinergic, central and peripheral, being dependent upon some change resident in the prejunctional pathway, probably the result of an increased liberation of the transmitter substance from the nerve terminals. Whilst our study has been confined to central depressant drugs, it may be of some interest to note here that Birks (personal communication) has found that caffeine, a central stimulant drug, augments the release of acetylcholine by potassium ions in the perfused ganglion. Any alteration in the normal level of prejunctional activity by centrally active drugs suggests, therefore the potential ability of modifying the phenomenon of facilitation. At least in the central nervous system such an effect must have a considerable bearing on the function of the brain.

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