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THE EFFECTS OF INERT GASES AND OTHER GENERAL ANAESTHETICS ON THE RELEASE OF ACETYLCHOLINE FROM THE GUINEA-PIG ILEUM

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1 The actions of a range of general anaesthetic agents on the rates of release of acetylcholine from the guinea-pig ileum were tested, by means of a superfusion system designed to maintain the tissues under physiological conditions in a high pressure chamber.

2 Anaesthetic pressures of nitrous oxide, nitrogen, argon, sulphur hexafluoride and carbon tetrafluoride caused increases in acetylcholine ouput but the concentrations required did not parallel their general anaesthetic potencies. The changes were not altered by the application of a pressure of helium which reverses their general anaesthetic actions *in vivo*.

3 Urethane (50.5 mM and 101 mM, but not 16.8 mM) decreased acetylcholine release rates and this effect was not reversed by helium pressure.

4 Octanol (1.0 mM, but not 0.124 mM or 0.496 mM) decreased the acetylcholine output. This action was not reversed by helium pressure. The lack of effect on acetylcholine release from tetrodotoxin-treated tissues suggested that the changes were produced by blockade of action potential conduction.

5 Phenobarbitone (0.4 mM but not 0.2 mM) also decreased acetylcholine output. Although the concentrations required were lower than those which have been previously shown to block axonal conduction, no changes were seen in tetrodotoxin-treated tissues. The decreases were less when helium pressure was applied than at atmospheric pressure but full pressure reversal, as occurs *in vivo*, was not seen.

6 The effects on acetylcholine output exerted by the anaesthetics studied did not reflect their general anaesthetic action in the concentrations required, the direction of the changes produced or in the response to helium pressure. They represent specific actions which are likely to contribute to the individual differences which are seen between the physiological actions of the anaesthetics *in vivo*.

Introduction

The ability to produce general anaesthesia is shared by a very large number of agents. The variety of chemical structures which possess this property illustrates its non-specific nature. The correlation of anaesthetic potency with lipid solubility, originally due to Meyer (1899) and Overton (1901) has been amply substantiated and extended to fluorinated compounds for which other correlations fail. It is considered therefore that any substance will produce general anaesthesia if a sufficient concentration can be achieved in some critical hydrophobic region of cells, for instance in the cell membrane. Physical changes in natural and artificial membranes attributed to the presence of the anaesthetic in the lipid have been described but the physiological changes which underly general anaesthesia have not been elucidated. No specific chemical structure appears to be involved in producing anaesthesia but the results of specific interactions may complicate the study of the mechanisms involved; the inert gases therefore are particularly useful agents to use. In this work we were looking for common physiological actions among anaesthetic agents of different types, to gain more information about possible sites of action of general anaesthetics and the basic mechanism underlying anaesthesia.

General anaesthetics produce blockade both of axonal conduction and of synaptic transmission. It seems likely that interference with synaptic transmission is the more important because it occurs at anaesthetic concentrations which are lower than those affecting axonal conduction (Larrabee & Posternak, 1952). However, it is difficult to extrapolate from studies on peripheral nerves and synapses to the axons and neurones within the brain and the ratio between the concentrations required for axonal or for synaptic blockade is variable and can be quite low

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(e.g. for urethane). The recent demonstration of the frequency-dependence of the concentrations of local anaesthetics required to cause blockade of action potential conduction (Courtney, Kendig & Cohen, 1978) makes a re-evaluation of these data necessary. A major lack has been direct evidence of the effects of anaesthetics on the different components of synaptic transmission. The phenomenon of pressure reversal (Lever, Miller, Paton & Smith, 1971) provides an additional method of assessing which effects of anaesthetics are important in the production of anaesthesia. Pressure reversal of anaesthesia *in vivo* using both hydrostatic pressure and helium pressure has been clearly demonstrated with many different types of anaesthetics (Lever *et al.*, 1971; Halsey & Wardley Smith, 1975). Any valid theory of the basic mechanism of anaesthesia must incorporate its reversal by high pressure.

In this work we wished to study neural transmission in an isolated tissue, in order to compare the effects of different types of anaesthetic agents at the various sites and the interactions between anaesthetics and pressure. The guinea-pig ileum was chosen for initial studies as the rates of spontaneous and evoked release of acetylcholine are large enough to be conveniently studied. In this work the effects of general anaesthetics, and their interaction with high pressure, have been studied on transmitter output from a peripheral nerve network, namely acetylcholine output from the nerve plexus in the wall of the guinea-pig ileum. Representatives of a variety of different types of anaesthetic agents have been compared. To apply high pressure, helium was used, since pressures produced by it have been found to be equivalent to hydrostatic pressure; it is believed that its lipid solubility is too low for it to exert any anaesthetic effect at the pressures used (Miller, Paton & Smith, 1972). In the preceding paper (Little & Paton, 1979) it was shown that helium at 136 atm, applied for 1 h, increased spontaneous acetylcholine output from this tissue by 20%. This pressure produces convulsions in small mammals and is close to the lethal pressure. It is also the pressure which causes reversal of general anaesthesia *in vivo* (Lever *et al.*, 1971; Hunter & Bennett, 1974).

Methods

A superfusion system was used for the study of the actions of the anaesthetics. A description of the apparatus is given in the preceding paper (Little & Paton, 1979). The tissues were immersed in Krebs solution containing 5 mg/ml of physostigmine sulphate for at least 90 min before use. Glass and silicone rubber were the only materials in contact with the bathing solution. Food was withheld from the guinea-pigs for 24 h before the tissues were removed. The tissues were tied to the tissue holders in such a way that the intestinal contents could not escape into the bathing solution. After each sample collection the contents were removed from the tissues and excess moisture removed with filter paper. The wet weights were used in the calculation of release rates.

The internal temperature of the pressure chamber was maintained at 37°C $\pm$ 0.5°C. The method of temperature control is described in the preceding paper, as are the details of compression and decompression procedures.

The results described are from sample collections lasting 1 h for the experiments on spontaneous release. Preliminary experiments showed that the control rates of spontaneous acetylcholine release did not alter with time over periods up to 2 h. Control release rates in the pressure chamber did not differ significantly from those obtained in concurrent collections, from tissues taken from the same animal, using a standard organ bath.

The results were expressed as acetylcholine release rates in ng g^{-1} min^{-1}. The figures show the mean values for each set of results with the standard errors, derived from pooled variance estimates. Student's t test was used as a measure of the level of significance of differences. Because of the variation in the control release rates from tissues from different animals the effects of the anaesthetics are always compared with control values obtained from tissues from the same animal. The order of the treatments was changed every day.

The samples of Krebs solution were bioassayed for acetylcholine content on the guinea-pig ileum (Paton, Vizi & Aboo Zar, 1971) and the results analysed after the method of Holton (1948). This analysis provided the dose-ratio compared with a standard solution and the confidence limits of this dose-ratio ($P = 0.05$). Acetylcholine was identified by (a) specific antagonism by hyoscine and (b) destruction by increase of pH to over 11.

Transmural electrodes were used in the experiments on electrically evoked release. Details of their arrangement and use are given in the preceding paper. The stimulation parameters used were a pulse width of 3 ms, a frequency of 2 Hz and a current of 150 mA. Stimulation was started after the gases had been added to the chamber and the final pressure reached. The time interval for this was 10 min; then stimulation was carried out for 30 min. The results are expressed as the mean rate of electrically evoked release of acetylcholine with the spontaneous release rate subtracted from each value. The spontaneous release rates were established every day, over the same time period as was used for the collection of acetylcholine release by electrical stimulation.

During every experiment, control samples of acetylcholine were run through the apparatus to ensure that the experimental conditions did not alter the recovery of acetylcholine. Control experiments also ensured that the anaesthetic agents did not affect the measurement of acetylcholine concentrations. The samples were stored in a deep freeze immediately after collection until just before assay. This was demonstrated not to alter the acetylcholine concentrations.

Previous work (Little & Paton, 1979) demonstrated that the decompression procedure did not alter the acetylcholine content of the samples.

The anaesthetics used were chosen to include a range of lipid and water solubilities, namely, nitrogen (N_2), argon (Ar), nitrous oxide (N_2O), carbon tetrafluoride (CF_4), sulphur hexafluoride (SF_6), octanol, urethane and phenobarbitone. The latter two were dissolved in the Krebs solution before the tissues were put into the apparatus. The octanol was dissolved in distilled water, and the gases were introduced to the chamber at the beginning of each collection period.

The doses of anaesthetics chosen initially were derived from the ED_{50} values for loss of righting reflex in mice, or, where this was unobtainable, from the concentrations required to produce narcosis in tadpoles. This was because the work was intended to study the effects of concentrations similar to those which would be found in tissues during light anaesthesia. These concentrations are less than those required to block axonal conduction in peripheral nerves.

The following drugs were used: acetylcholine chloride (Sigma), argon (British Oxygen Company), carbon tetrafluoride (British Oxygen Company), helium (British Oxygen Company), hyoscine hydrobromide (Sigma), morphine sulphate (May & Baker), nitrogen (British Oxygen Company), nitrous oxide (British Oxygen Company), *n*-octanol (BDH), phenobarbitone sodium (BDH), physostigmine sulphate (Sigma), sulphur hexafluoride (British Oxygen Company), tetrodotoxin (Sankyo), urethane (BDH).

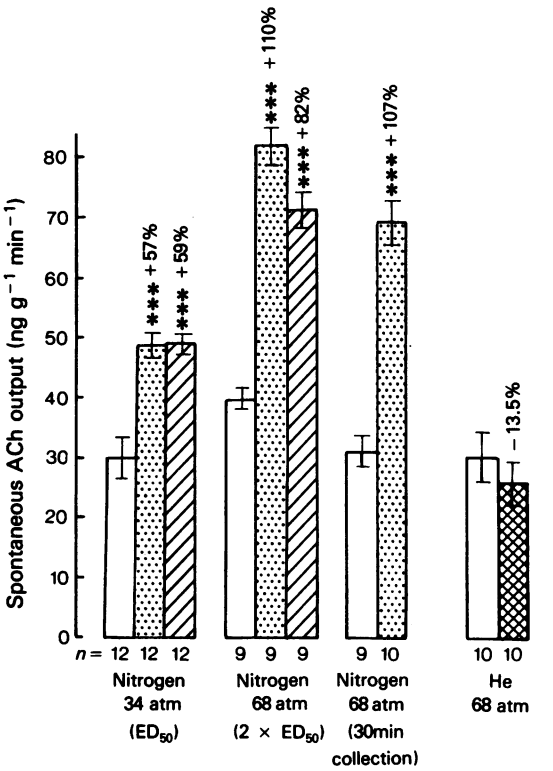

Figure 1 The effects of nitrogen, alone and with helium, on spontaneous acetylcholine (ACh) output. Mean values are shown; vertical lines indicate s.e. mean. Open columns = controls; stippled columns = effects of nitrogen; hatched columns = effects of nitrogen plus helium up to 136 atm; cross-hatched columns = helium alone. ***$P < 0.01$.

Results

Two inert gases, nitrogen and argon were tested: both increased the rates of spontaneous release of acetylcholine (Figures 1 and 2). Nitrogen, at 34 atm and 68 atm, increased the release rates by 57% and 110% respectively. Argon, at 15 atm and 30 atm, caused increases of 38% and 101%. The ED_{50} values for loss of righting reflex in mice are 34 atm for nitrogen and 15 atm for argon. These increases were not reversed by the application of helium pressure up to 136 atm. Figures 1 and 2 show that the increases in release rates were very similar whether or not helium pressure was applied. The differences between the mean release rates in the presence and absence of helium were not significant ($P > 0.1$) for either nitrogen or argon. This pressure of helium is sufficient to reverse completely the effects of the ED_{50} concentrations of the anaesthetics *in vivo* (Lever *et al.*, 1971).

The effects of nitrogen on electrically stimulated acetylcholine output and on tetrodotoxin-treated tissues were described in an earlier paper (Little & Paton, 1979) and will be referred to in the discussion.

Nitrous oxide, at 1.5 atm (the mouse ED_{50}) increased the rates of spontaneous release of acetylcholine by 91% (Figure 3). When helium was used to raise the total pressure to 136 atm, this concentration of nitrous oxide increased the mean release rate by 86%. The difference between this and the release rates without helium was not significant. Half of the ED_{50}

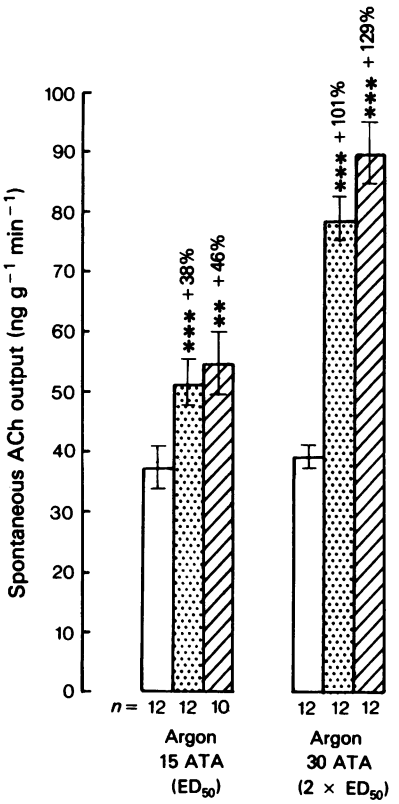

Figure 2 The effects of argon, alone and with helium, on spontaneous acetylcholine (ACh) output. Mean values are shown; vertical lines indicate s.e. mean. Open columns = controls; stippled columns = effects of argon; hatched columns = effects of argon plus helium up to 136 atm. $^{**}P$ 0.01–0.02; $^{***}P < 0.01$.

concentration of nitrous oxide also increased the release rates but the differences from control release rates were not significant ($P > 0.05$).

The fluorinated gases SF_6 and CF_4 caused similar changes to those described for the three previous anaesthetics in relation to their ED_{50} values but the increases seen were not as great. At 13.2 atm of SF_6 the mean release rate was 40% higher than the control value, and at 36 atm of CF_4 it was 71% higher (Figure 4). These doses are double the ED_{50} values for the loss of righting reflex in mice. The ED_{50} values themselves did not cause any significant changes. The differences seen with higher concentrations were significant ($P < 0.05$) in each case, but as seen with the other gases, the application of helium pressure caused no significant changes in the actions of the anaesthetics.

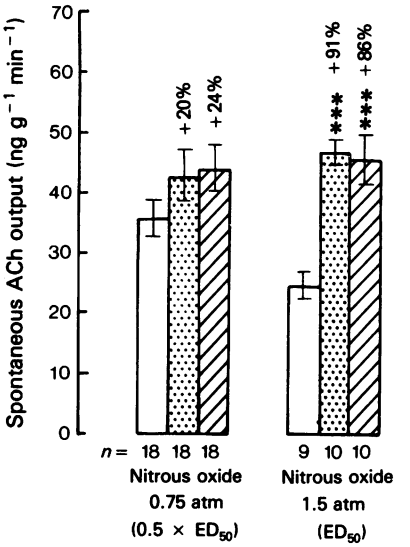

Figure 3 The effects of nitrous oxide, alone and with helium, on spontaneous acetylcholine (ACh) output. Mean values are shown; vertical lines indicate s.e. mean. Open columns = controls; stippled columns = effects of nitrous oxide; hatched columns = effects of nitrous oxide plus helium up to 136 atm. $^{***}P < 0.01$.

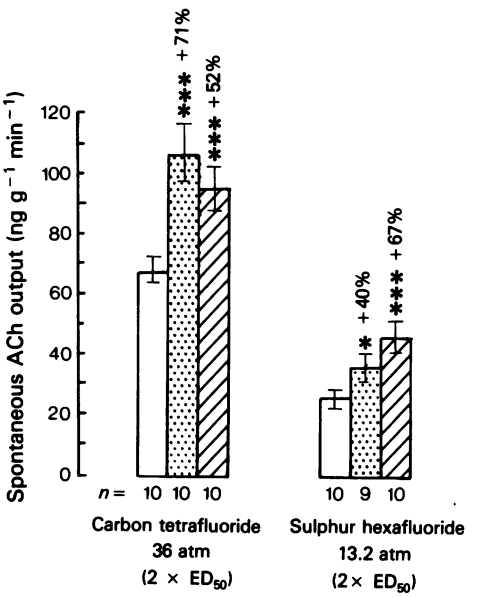

Figure 4 The effects of carbon tetrafluoride and of sulphur hexafluoride, alone and combined with helium, on spontaneous acetylcholine (ACh) output. Mean values are shown; vertical lines indicate s.e. mean. Open columns = controls; stippled columns = effects of anaesthetic; hatched columns = effects of anaesthetic plus helium up to 136 atm. $^{*}P = 0.02$–0.05; $^{***}P < 0.01$.

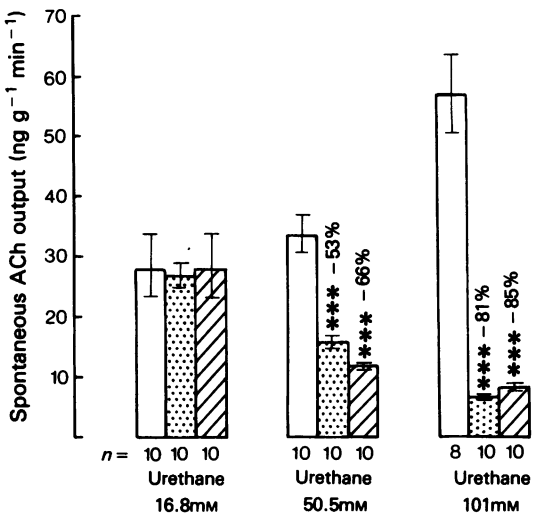

Figure 5 The effects of urethane, at atmospheric pressure and under helium pressure, on spontaneous acetylcholine (ACh) output. Mean values are shown; vertical lines indicate s.e. mean. Open columns = controls; stippled columns = effects of urethane; hatched columns = effects of urethane plus helium up to 136 atm. $***P < 0.01$.

Urethane, in contrast to the gaseous anaesthetics caused decreases in spontaneous acetylcholine output, at concentrations of 50.5 mM and 101 mM, but not at 16.8 mM (Figure 5). In comparison, Johnson & Flagler (1950) showed that 80 mM caused narcosis in tadpoles. Urethane is unusual in that similar concentrations are required to block both axonal conduction and synaptic transmission. For instance, Larrabee, Ramos & Bülbring (1952) showed that 140 mM decreased the postsynaptic compound action potential in rabbit isolated ganglia and 110 mM decreased the amplitude of the compound action potential in the cervical sympathetic nerve trunk in the same species.

When the pressure was raised to 136 atm with helium the decreases caused by the 50.5 mM and 101 mM concentrations of urethane were 66% and 85% respectively, as compared with 53% and 81% respectively at atmospheric pressure. There was no significant difference in either instance between the effects of urethane at atmospheric or at high pressure.

The effects of octanol on spontaneous and evoked acetylcholine output (Figure 6) may be compared with the effects of these concentrations in other systems. A concentration of 0.127 mM was found to decrease spontaneous movement in tadpoles (Vernon, 1913), 0.12 mM to decrease the synaptic transmission

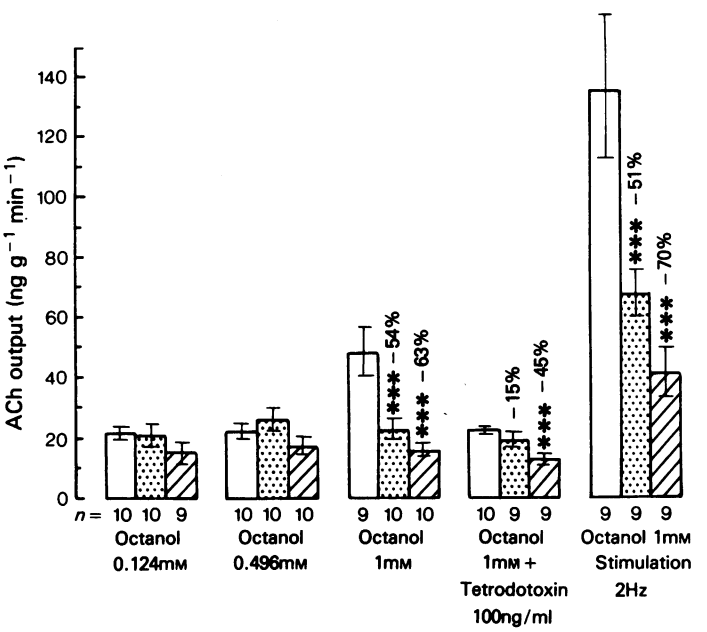

Figure 6 The effects of octanol, at atmospheric pressure and under helium pressure, on spontaneous and evoked acetylcholine (ACh) output. Mean values are shown; vertical lines indicate s.e. mean. Open columns = controls; stippled columns = effects of octanol; hatched columns = effects of octanol plus helium up to 136 atm. $***P < 0.01$.

in the stellate ganglion of the cat while 0.35 mM was needed to affect axonal conduction in the preganglionic nerve in the latter preparation (Brink & Posternak, 1948). In the present study neither 0.124 mM nor 0.496 mM caused any significant changes in the spontaneous acetylcholine release at atmospheric pressure or at 136 atm of helium. However, a concentration of 1.0 mM decreased the spontaneous release of acetylcholine and the stimulated release, by 54% and 51% respectively at atmospheric pressure and by 63% and 70% at 136 atm. In tissues treated with tetrodotoxin there were no significant differences between the control release rates of acetylcholine and those in the presence of 1.0 mM. However, the rates were significantly lowered by the combination of 1.0 mM octanol and 136 atm of helium. It can be seen from Figure 6 that the release rates when octanol and pressure were combined were lower in each case than with octanol alone.

The effects of phenobarbitone, 0.4 mM, on spontaneous and evoked acetylcholine output are shown in Figure 7. A concentration of 0.2 mM did not cause any significant changes in spontaneous acetylcholine release. The 0.4 mM concentration decreased the spontaneous acetylcholine release by 31% and the evoked release by 65% but did not affect the release from tetrodotoxin-treated tissue. The difference between the release rates with 0.4 mM at 136 atm and control values was not significant, and the decrease in evoked release at 136 atm was only 33%. The concentrations used were chosen from those found effective by other workers, for example Thompson & Turkanis (1973) showed changes in miniature endplate potentials at the skeletal neuromuscular junction of the frog at between 20 μM and 200 μM, while Waddell & Butler (1957), showed that the concentration in the brain of a dog during anaesthesia was approximately 0.6 mM.

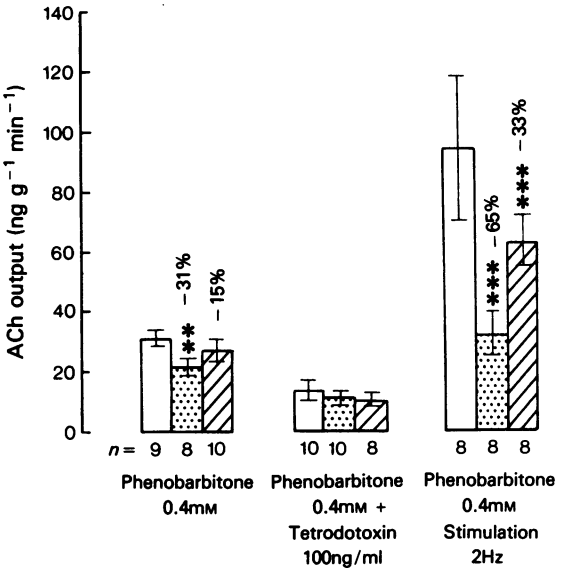

Figure 7 The effects of phenobarbitone, at atmospheric pressure and under helium pressure, on spontaneous and on evoked acetylcholine (ACh) output. Mean values are shown; vertical lines indicate s.e. mean. Open columns = controls; stippled columns = effects of phenobarbitone; hatched columns = effects of phenobarbitone plus helium up to 136 atm. $^{**}P$ 0.01–0.02; $^{***}P < 0.01$.

Discussion

The gases nitrous oxide, nitrogen, argon, carbon tetrafluoride (CF_4) and sulphur hexafluoride (SF_6) caused increases in spontaneous acetylcholine output at concentrations which produce anaesthesia *in vivo*. Urethane, octanol and phenobarbitone however decreased the release rates.

A comparison of the magnitude of the effects of the gaseous anaesthetics shows that nitrous oxide was the most effective, nitrogen and argon slightly less so and CF_4 and SF_6 caused the smallest changes, when equianaesthetic doses were compared. The effects of the gases varied directly with concentration, over the small range studied. The action of these gases in increasing acetylcholine release therefore does not correlate with the general anaesthetic actions or the lipid solubility of the compounds. It is possible that the effects of the gases may have been limited by their distribution through the Krebs solution and the tissue during the 1 h collection period. However, the fact that nitrogen caused the same percentage increase over 30 min as over 60 min (see previous paper) suggests that this was not a determining factor in this case, but it may have affected the results obtained with the fluorinated compounds, because of their low aqueous solubilities.

The concentrations of these gaseous anaesthetics used were considerably below those required to affect axonal conduction, for instance, a pressure of 4.5 atm of nitrous oxide was needed to decrease the compound action potential amplitude of frog sciatic nerve by 50% (Roth, 1975).

These results clearly show that there was little effect on the changes produced by the gases when helium was used to increase the total pressure to 136 atm. Taking all the gaseous anaesthetics together, the effect of helium was to alter the magnitude of the percentage increase in output by 2.0 ± 7.0%. The slight increase in acetylcholine output observed with this pressure of helium alone (Little & Paton, 1979) might

have masked to some extent an antagonistic effect of this pressure but the magnitude of the actions of the gases were too great for this to have been the whole explanation for the lack of pressure reversal. This pressure of helium reverses completely the anaesthesia produced by these gases *in vivo* (Lever *et al.*, 1971).

Urethane, in contrast to the gaseous anaesthetics, decreased acetylcholine output. The concentrations required were similar to those reported to be necessary to block ganglionic transmission and axonal conduction but were greater than those suggested to be found during anaesthesia, the latter being approximately 10 mmol per kg body weight. The results also showed that there was no reversal of the effects of urethane on acetylcholine release by the ileum when 136 atm of helium was applied. This, and the concentrations required suggest that the effects of urethane on the CNS may be by a different mechanism from those seen in peripheral preparations.

The effects of octanol on acetylcholine release rates were not apparent at general anaesthetic concentrations or those affecting synaptic transmission in other preparations. A comparison of the effective concentrations (see Results) and the lack of effect of 1 mM octanol on acetylcholine output from tetrodotoxin-treated tissues, suggests that the decreases in spontaneous and stimulated release produced by this concentration were due to blockade of action potentials. Armstrong & Binstock (1964) showed that 0.5 to 1.0 mM blocked conduction in the squid axon. The effect on the ileum was not reversed by helium pressure. This result is in accord with those of Roth, Smith & Paton (1976) who showed that blockade of compound action potentials in the peripheral nerve of the frog by ethanol and by butanol was not affected by pressure, although the action of gaseous anaesthetics on this preparation was reversed. Rang (1960) showed that 0.25 mM of octanol caused a 50% decrease in the contractions of the electrically stimulated guinea-pig ileum. The lack of effect of this concentration on acetylcholine release suggests a postsynaptic site for the basis of this action. Throughout the results of the effects of octanol on the ileum it can be seen that the mean release rates in the presence of octanol and helium were lower in each case than those with octanol alone. It is unlikely that this was due to an effect on solubility, as although the 1.0 mM concentration dissolved in water only with difficulty, pressure would be expected to decrease rather than increase the solubility.

The decrease in spontaneous and in stimulated acetylcholine output produced by phenobarbitone at 0.4 mM may also have been due to an effect on action potential conduction as there were no significant changes in release rates from tetrodotoxin-treated tissues. However, the concentrations required to affect axonal conduction in peripheral nerves in other species are considerably higher than this (e.g. Rosenburg & Bartells, 1967).

The actions of phenobarbitone in decreasing both spontaneous and stimulated output of acetylcholine were smaller in magnitude under helium pressure than at atmospheric pressure. This was the only instance where pressure consistently and significantly decreased the effects of any of the anaesthetics tested. However, the reversal was not complete and in view of the small increase in spontaneous acetylcholine release seen with helium pressure alone it might be rather premature to correlate this with pressure reversal *in vivo*.

It is possible that the changes seen may have been affected by action of helium or of nitrogen in increasing the activity of acetylcholinesterase as reported by Wilson (1974). However, the concentration of physostigmine used was considerably above that required to inhibit the enzyme under normal conditions (e.g. Silver, 1974). The changes reported by Wilson would cause decreases in the acetylcholine content of the samples if they made any contribution and these were not seen with these gases. Experiments are in progress to determine the effects of helium and of nitrogen on the activity of the enzyme in this tissue and its inhibition by physostigmine.

The effects of general anaesthetics on transmitter release in other preparations have been found to vary. An increase in the spontaneous release of acetylcholine from the guinea-pig ileum with an anaesthetic was also shown by Speden (1965), using trichlorethylene. He also found that ether and chloroform decreased the electrically evoked output from this tissue. The concentration of ether required to produce this effect was higher than that found in blood during surgical anaesthesia, but the concentrations of the other two compounds correspond to their anaesthetic doses. Changes in evoked acetylcholine release at the skeletal neuromuscular junction with anaesthetics have been reported but are not consistent (e.g. Matthews & Quilliam, 1964; Gage, 1965; Gergis, Sokoll, Cronnelly, Dretchen & Long, 1974). An increase in miniature endplate potential frequency, which was not calcium-dependent, was described by Quastel, Hackett & Ojamoto (1972). It is tempting to contrast these results with those in the present study as the drugs used by these authors included urethane and the alcohols. However, the mechanism of spontaneous transmitter release may differ at the two sites, as m.e.p.ps are not as dependent on calcium ions as is the spontaneous release in the ileum.

The results in the present study correlate with those of Kendig, Trudell & Cohen (1975) and Kendig & Cohen (1976), although a different preparation was used. These authors demonstrated that the action of volatile anaesthetics on ganglionic transmission and

on skeletal neuromuscular junction was not reversed by helium pressure, although their effects on conduction of compound action potentials by peripheral nerves were antagonized.

It is clear from the results presented here and the work of other authors that general anaesthetics have multiple actions at peripheral synapses. Effects on pre- and postsynaptic mechanisms need not act in the same direction and the final outcome will be the result of the balance between these. The differences between the actions of the anaesthetics tested on the ileum illustrate the importance of studying a variety of different types of agent when searching for common mechanisms of action.

Provisionally it must be concluded that the mechanism by which the anaesthetics altered acetylcholine release differs from that by which they cause anaesthesia *in vivo*, for three reasons; the variation in potency when expressed in terms of the anaesthetic ED_{50}, the variation of qualitative effect and the lack of effect of pressure at a level sufficient to annul anaesthesia in mice. In this work we are therefore looking at specific actions of anaesthetics rather than non-specific. The demonstration of considerable changes in rates of acetylcholine release at low concentrations of the gaseous anaesthetics suggests that this may contribute to the physiological changes seen during the production of anaesthesia.

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