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THE EFFECTS OF HIGH PRESSURE HELIUM AND NITROGEN ON THE RELEASE OF ACETYLCHOLINE FROM THE GUINEA-PIG ILEUM

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1 The effects of high pressures of helium and of nitrogen on acetylcholine release were tested using the guinea-pig ileum as a model preparation. A superfusion system was designed in which this tissue could be maintained under physiological conditions in a high pressure chamber.

2 Helium, at a pressure of 136 atm slightly increased the spontaneous output of acetylcholine but produced no significant changes at 68 atm (136 atm is close to the lethal pressure for small mammals).

3 The acetylcholine release evoked by electrical stimulation or by 55 mM potassium was not altered by 136 atm of helium. Effects on tetrodotoxin-treated tissues were not consistent.

4 Nitrogen, which in contrast to helium possesses general anaesthetic properties, caused considerable increases in spontaneous and in electrically evoked acetylcholine output at pressures which produce anaesthesia. These increases were not changed when helium was used to increase the total pressure to 136 atm, although this reverses the general anaesthetic actions of nitrogen *in vivo*.

5 The increases in rate of acetylcholine release produced by nitrogen were observed in tetrodotoxin-treated tissues and in tissues from reserpine-treated animals. In a calcium-free medium the increases were considerably smaller.

6 The conclusions from these results are that while high pressures of helium caused little or no change in acetylcholine release rates, nitrogen produced large changes, which were not due to effects on axonal conduction. The effect of nitrogen is not apparently related to its general anaesthetic actions. Differences such as these in transmitter release would be likely to contribute to the differing physiological effects of these two gases.

Introduction

The application of high pressure has been observed to produce characteristic behavioural changes in many species. The appearance of these effects depends on the compression rate used; generally at 70 atm tremors are seen, convulsions at 80 to 100 atm and respiratory distress, paralysis and death at about 150 atm. Symptoms analogous to the initial signs in animals have been reported in man at similar pressures. Tremors, EEG changes, 'microsleep' and other symptoms occur at approximately 20 atm (200 m.s.w.) (Hunter & Bennett, 1974).

The signs of the high pressure nervous syndrome (HPNS) suggest that the effect is to increase neuronal excitability, but the physiological basis of this has not been defined. Changes in action potentials have been observed at pressure but the reports are conflicting. Spyropoulos (1957) using toad nerve and Roth (1975) using frog nerve at 150 atm did not see any changes in amplitude. Kendig, Trudell & Cohen (1975) and Henderson & Gilbert (1975) showed decreases in amplitude in compound and single fibre action potentials, when the pressure was increased to over 136 atm or 204 atm respectively. An increase in duration of the compound action potential has been demonstrated more consistently (Grundfest 1936; Spyropoulos, 1957; Kendig *et al.*, 1975; Henderson, Lowenhaupt & Gilbert, 1977).

The above studies were carried out with either helium pressure or hydrostatic pressure. No anaesthetic effects of helium are seen at physiological temperatures, because its low (lipid) solubility means that the pressures required to produce a sufficient tissue concentration for anaesthesia are higher than those which cause death through the effects of pressure alone. As a result the effects of helium pressure are indistinguishable from those of hydrostatic pressure. The only pharmacological method of ameliorating the effects of high pressure so far identified is by the use of general anaesthetics. These postpone the appearance of the signs of the HPNS to higher pressures.

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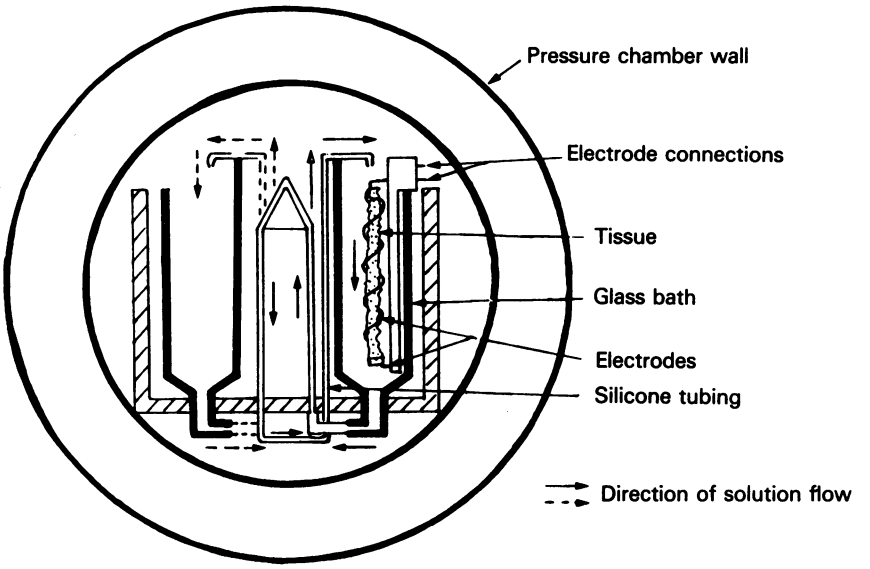

Figure 1 Multiple superfusion apparatus, T.S.

Both helium pressure and hydrostatic pressure have been shown to reverse general anaesthesia in a variety of species with many different types of general anaesthetic agents (Lever, Miller, Paton & Smith, 1971; Halsey & Wardley-Smith, 1975).

Possible sites of production of the effects of both general anaesthetics and pressure include axonal conduction, initiation of transmitter release by propagated activity, transmitter release mechanisms, postjunctional changes and production of postsynaptic responses. In neither case however has any of these been identified as the major site of action.

Synaptic transmission has been considered to be a more important site of action in the production of anaesthesia than axonal conduction since higher concentrations of anaesthetics are required to block the latter than the former (e.g. Larrabee & Posternak, 1952). However, there is considerable variation between different compounds with regard to this potency ratio. Because of the consistency of the pressure reversal of anaesthesia it may be of use as a criterion to judge whether or not certain actions of anaesthetics on model preparations are relevant to their production of anaesthesia *in vivo*.

In this work we have designed apparatus for the study of the effects of high pressure and of anaesthetics on synaptic transmission in isolated tissue preparations. The guinea-pig ileum was used for initial studies as its cholinergic nerve plexus is a good model on which to distinguish effects on axonal conduction and on synaptic transmission. Both the spontaneous and the evoked release of acetylcholine can be conveniently studied.

High pressures were applied to this preparation using helium gas. For comparison another relatively inert gas, nitrogen, which possesses anaesthetic properties was studied. The effects of pressure alone and the effects related to anaesthesia could therefore be compared. Both these gases are used routinely in deep sea diving as components of breathing mixtures, nitrogen for its actions in counteracting the effects of high pressure and helium as a non-anaesthetic diluent gas.

Methods

The main problems involved in the study of isolated tissues at pressure were those of the control of oxygenation, temperature and pH.

A superfusion system was chosen so that adequate oxygenation could be maintained without using the standard organ bath system. This also ensured rapid access of the gaseous anaesthetics to the tissue and fast equilibration. Furthermore this system can be used for gaseous, liquid and solid anaesthetic agents.

At the beginning of each experiment the pressure chamber was flushed with 95% O_2 and 5% CO_2. The gas pressures quoted below were applied on top of this initial one atmosphere. Standard Krebs solution was used except where alterations in calcium or potassium concentrations. were made. Alternative

buffer solutions (e.g. Tris, phosphate) were found to decrease the resting release of acetylcholine. Kendig *et al.* (1975) showed that on compression to 200 atm (using helium with P_{O_2} = 0.95 atm, P_{CO_2} = 0.05 atm) the pH of Krebs solution fell from 7.4 to 7.2.

The superfusion apparatus is illustrated in Figure 1. Six individual superfusion systems were mounted on a dural frame, which also carried the peristaltic pump motor and a fan. Glass and silicon rubber tubing were the only materials in contact with the bathing solutions. The latter contained 5 μg/ml physostigmine sulphate. Tissues were equilibrated in this solution for at least 90 min before use. 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; 5 ml of solution was continuously circulated over each tissue during the collection periods, at a rate of 1.25 ml/min.

The temperature inside the pressure chamber was controlled by copper coils round the outside. These were automatically fed by either hot (40°C) or cold water (0°C) from circulating pumps, in response to the internal chamber temperature measured by two thermistor probes. This system, combined with the use of an air thermostat to ensure that the starting temperature of the apparatus was 37°C, maintained the temperature during experiments at 37°C ± 0.5°C.

The pressure inside the chamber was increased slowly (13.6 atm/min) so that the temperature remained within the above range. Decompression was carried out in 2 min.

The results for spontaneous release described below are from sample collections lasting 1 h (with the exception of the study of the effects of nitrogen over 30 min). 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 did not differ significantly from those obtained in concurrent collections from tissues taken from the same animal, using a standard organ bath.

The experiments on electrically stimulated release were carried out using transmural stimulation with platinum electrodes. The arrangement of the electrodes is illustrated in Figure 1. One electrode was in the lumen of the tissue and the other was coiled round the outside, just in contact with the tissue surface. The electrodes were connected in series within the pressure chamber and the stimulation pulses were monitored continuously on an oscilloscope during every experiment. Preliminary experiments showed that a current of 150 mA was just supramaximal for the stimulation of acetylcholine release under these conditions and this value was maintained throughout the experiments, by means of a constant current device. The pulse width was 3 ms. In each experiment stimulation was begun after the gases had been added to the chamber and the final pressure had been reached. The time to add all the gases was made as short as possible, so that the stimulation samples should not contain too large a component of resting output, but too rapid addition of gases caused temperature increases. This time period was 10 min for the 1 Hz and 2 Hz experiments and 5 min for the 10 Hz experiments. The duration of stimulation was 30 min for the former experiments and 2 min for the latter ones. The control release rates, which were used to correct the stimulated release rates, were established over the same total period as the latter in each case.

The samples of Krebs solution were bioassayed for acetylcholine content on the guinea-pig ileum. A 4 by 4 latin square arrangement was used for administration to the assay preparation and the results analysed after the method of Holton (1948). This analysis provided a potency ratio in relation to a standard solution with 95% confidence limits. Acetylcholine was identified by (a) specific antagonism by hyoscine and (b) destruction by increase of pH to over 11.

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. The samples were stored in a deep freeze from immediately after collection to just before assay. This was demonstrated not to alter the acetylcholine concentrations.

The results are expressed as the mean release rates, with the standard errors derived from pooled variance estimates, in ng g^{-1} min^{-1}. After each experiment the tissues were cleaned of their contents and excess moisture removed with filter paper; they were weighed and the wet weight used for the calculations. Student's *t* test was used to calculate the significance of the differences.

The control rate of acetylcholine release varies considerably with tissues from different animals but experiments showed that there were no significant differences between repeated sample collections using tissues from the same animal. Control values for release were therefore established every day and the effects of the treatments were always compared with control values for tissues from the same animal. The treatments were carried out in a different order each day. In the experiments on electrically stimulated release, five estimates of the spontaneous release rates under atmospheric pressure with no anaesthetic were obtained for each animal and the mean of these subtracted from the corresponding values for stimulated release.

The following drugs were used acetylcholine chloride (Sigma), helium (British Oxygen Company), hyoscine hydrobromide (Sigma), morphine sulphate (May & Baker), nitrogen (British Oxygen Company),

physostigmine sulphate (Sigma), reserpine (Sigma), tetrodotoxin (Sankyo).

Results

The first study compared the spontaneous acetylcholine release by the preparations exposed to helium at 136 atm (the maximum working pressure of the chamber) with that at normal atmospheric pressure (Table 1). The release rates at 136 atm were 21% higher than the corresponding control values. Analysis of variance was performed in order to eliminate the variation between sets of samples and compare the variation between treatments taking into account the within sample variation. The P value obtained was 0.05.

To determine whether or not the fast decompression might have affected the acetylcholine release by causing tissue damage, a further series of experiments was carried out with helium. In these the pressure was increased to 136 atm, only during the final 10 min of the 1 h collection period. Decompression was carried out at the end of the hour as described previously. No significant differences were seen between release rates with this compression schedule and those at atmospheric pressure.

The effects of compression to 136 atm were further examined with Krebs solution containing 1 μg/ml choline chloride. This was to ensure that the release of acetylcholine was not limited by the supply of choline. The control acetylcholine release rates with and without choline did not differ significantly, although the increase in release at 136 atm was higher than seen in the absence of choline (Table 1).

It has previously been shown (Paton, Vizi & Aboo Zar, 1971) that 60% of the spontaneous release of acetylcholine from the guinea-pig ileum is due to action potential propagation as shown by sensitivity to tetrodotoxin (TTX). (The longitudinal strip preparation of the ileum was used). In this study of the effects of pressure therefore, tetrodotoxin was used to eliminate this component of release. When the Krebs solution contained 100 ng/ml of tetrodotoxin, which reduced control output, the release rates were 54% higher at 136 atm than at atmospheric pressure. However when tetrodotoxin concentrations of 10 μg/ml or 10 ng/ml were used there were no significant differences between release rates at pressure and control values (Table 1).

Outside the pressure chamber, 100 ng/ml tetrodotoxin was shown to decrease the contractile responses to electrical stimulation of this tissue by 80% and 10 μg/ml by 100%, but 10 ng/ml did not cause any significant change. Throughout these experiments the control release rates inside the chamber were comparable with those obtained outside, using the same solutions.

When the spontaneous release of acetylcholine was increased by use of a bathing solution with a potassium concentration of 55 mM there were no significant changes in the release rates at 136 atm (Table 1). (In this solution the sodium concentration was correspondingly reduced).

Nitrogen was then used, as an example of an inert gas with anaesthetic properties for comparison with

Table 1 The effects of helium on spontaneous acetylcholine (ACh) release

Helium pressure (atm)	*Tissue pre-treatment*	*% change in ACh release due to He*	*Significance P value (t-test)*	n_1	n_2	*Control ACh output ± s.e. ($ng\ g^{-1}\ min^{-1}$)*
136	—	+21	0.05	22	22	46.7 ± 3.6
68	—	−13.5	Greater than 0.1	10	10	30.4 ± 4.0
136	Choline chloride 1 μg/ml	+35	0.01–0.02	16	18	31.6 ± 2.8
136	TTX 100 ng/ml	+54	0.02–0.05	11	11	19.3 ± 2.2
136	TTX 10 μg/ml	+21	Greater than 0.1	12	12	11.4 ± 3.0
136	TTX 10 ng/ml	−24	Greater than 0.1	11	11	40.0 ± 6.8
136	High (K^+) Krebs	−2	Greater than 0.1	10	10	70.0 ± 9.2
136 (last 10 min only)	—	−32	Greater than 0.1	9	9	41.4 ± 7.8

n_1 = number of control values; n_2 = number of test values.

helium. Two different concentrations, 34 atm and 68 atm were used, the former being the ED_{50} for loss of righting reflex in mice (Lever *et al.*, 1971); 68 atm of nitrogen was shown to depress the amplitude of the action potential in peripheral nerves by 20% (Roth, 1975). These concentrations were used so that tissue concentrations approximating to those during light anaesthesia *in vivo* would be produced.

Both pressures of nitrogen caused significant increases in spontaneous acetylcholine release (Table 2). The increases were 57% at 34 atm ($0.02 < P < 0.05$) and 110% at 68 atm ($P < 0.01$). Table 2 also compares the effects of these two pressures of nitrogen alone with their effects when the total pressure was increased to 136 atm with helium, immediately after the introduction of nitrogen into the chamber. At neither concentration of nitrogen did the increase of pressure with helium significantly affect the changes in acetylcholine release. In the presence of helium the increases were 58% with 34 atm nitrogen and 82% with 68 atm nitrogen. All these comparisons are made between results obtained from tissues removed from the same animals.

The experiments using 68 atm nitrogen were repeated with tissues from reserpine-treated animals in order to determine whether reduction of sympathetic tone contributed to the effects of the anaesthetic. Reserpine, 2 mg/kg was administered intraperitoneally to the guinea-pigs 24 h before the tissues were used. The increases in release rate seen in tissues from untreated animals were also seen in these results: 95% increase with 68 atm nitrogen and 96% increase with 68 atm nitrogen plus helium pressure to 136 atm ($P < 0.01$).

Table 2 also shows the effects of 68 atm of nitrogen on tissues treated with 100 ng/ml tetrodotoxin. Similar increases with nitrogen (108%) and nitrogen and helium (131%) were seen as were found with normal tissues. The difference between the results with nitrogen and those with nitrogen and helium was not significant ($P > 0.1$).

When calcium ions were omitted from the Krebs solution the acetylcholine output was greatly depressed and the changes observed with nitrogen were greatly reduced. Although slight increases were seen with both nitrogen (68 atm) and nitrogen and helium these were only significant in the case of nitrogen alone, and the nitrogen alone results did not differ significantly from the nitrogen plus helium results.

Table 3 shows the effects of helium and of nitrogen on electrically stimulated acetylcholine release. At frequencies of 1 Hz, 2 Hz or 10 Hz a pressure of 136 atm of helium did not cause any significant changes in stimulated release (P values all greater than 0.1). The control values at atmospheric pressure, obtained at each frequency were not significantly different from those obtained by use of a standard organ bath outside the pressure chamber, with tissues from the same animal.

Nitrogen, at 68 atm caused increases in the stimulated release rates at both the 1 Hz and 2 Hz frequency of 38% and 72% respectively (P values 0.05 to 0.1 and 0.05 respectively). Increases were also seen

Table 2 The effects of nitrogen on spontaneous acetylcholine release

Nitrogen pressure (atm)	*Helium pressure (atm)*	*Tissue pretreatment*	*% change in ACh release due to He and/or N_2*	*Significance P value (t test)*	n_1	n_2	*Control ACh output ± s.e. (ng g^{-1} min^{-1})*
34	—	—	+57	0.02–0.05	12	12	30.3 ± 6.5
34	102	—	+58	0.02–0.05	12	12	30.3 ± 6.5
68	—	—	+110	Less than 0.01	9	9	40.1 ± 3.3
68	68	—	+82	Less than 0.01	9	9	40.1 ± 3.3
68	—	TTX 100 ng/ml	+108	Less than 0.01	10	10	19.8 ± 1.6
68	68	TTX 100 ng/ml	+131	Less than 0.01	10	10	19.8 ± 1.6
68	—	Reserpine (see text)	+95	Less than 0.01	10	10	47.4 ± 9.2
68	68	Reserpine	+96	Less than 0.01	10	10	47.4 ± 9.2
68	—	No Ca^{2+}	+36	Less than 0.01	15	15	6.9 ± 0.3
68	68	No Ca^{2+}	+11	Greater than 0.1	15	15	6.9 ± 0.3
68	—	30 min collection time	+107	Less than 0.01	9	10	33.5 ± 4.5

when the effects of 68 atm nitrogen plus 68 atm helium were studied, but the increase at the 1 Hz frequency was not significant ($P > 0.1$). These changes may be compared with the effects of nitrogen on spontaneous activity when applied for 30 min, which are shown in Table 2. During the 30 min collection time nitrogen increased the spontaneous release rates by 107%, a change which was very similar to that seen over the 1 h collection period.

Discussion

The apparatus designed for the measurement of transmitter release appears to be suitable for the maintenance of the tissues under physiological conditions at very high pressures as the resting and stimulated outputs recorded did not differ significantly from those obtained in the usual way in an organ bath. The variations in output under changing conditions (e.g. tetrodotoxin, high potassium) were similar in the two situations and there was no loss of the acetylcholine added to the recirculating system. The possibility that the increase in release after exposure to 136 atm helium was due to tissue damage caused by the final decompression procedure was excluded by the fact that it did not occur when pressure (and subsequent decompression) was applied only at the end of the collection period. The presence of choline did not appear to be essential for the maintenance of acetylcholine release.

The effect of helium pressure on spontaneous acetylcholine release, an increase of 21%, was small, especially when compared with its *in vivo* effects. This pressure is close to the lethal pressure in small mammals and produces gross behavioural signs, including convulsions. Sixty-eight atm, having no effect on spontaneous acetylcholine release, causes coarse tremors and ataxia *in vivo*. The effect on acetylcholine release, such as it is, cannot be attributed to changes in the activity of acetylcholinesterase, or its inhibition by physostigmine. The concentration used of the latter inhibits the enzyme completely at 1 atm (Silver, 1974) so that an increase in the affinity for the enzyme would have no further effect. Wilson (1975) reported an increase in acetylcholinesterase activity at high pressures of helium and of nitrogen. Such an action would lead to a fall in the measured output, as would a reduction in the affinity of physostigmine for the enzyme. Since the pattern of the high pressure nervous syndrome suggests 'increased excitability' of the nervous system another possibility is that pressure causes an increase in the frequency of propagated action potentials in the nerve fibres of Auerbach's plexus. Paton *et al.* (1971) showed that 60% of the resting acetylcholine release by the guinea-pig ileum was sensitive to TTX and was therefore presumably due to propagated activity. However, the results of experiments on the effect of TTX on the response to pressure were conflicting. At TTX 100 ng/ml, helium at 136 atm still increased output, suggesting an effect, not on propagated activity, but on the release process itself at the nerve terminal. However, with 10 fold lower and 100 fold higher concentrations, no significant effects were obtained. A sensitivity of the TTX-nerve membrane interaction to pressure may well complicate the issue. The lack of effect of high

Table 3 The effects of helium and of nitrogen on electrically stimulated acetylcholine release (corrected for spontaneous release)

Helium pressure (atm)	*Nitrogen pressure (atm)*	*Stimulation frequency*	*% change in ACh release due to He and/or N_2*	*Significance P value (t test)*	n_1	n_2	*Control ACh output ± s.e. (ng g^{-1} min^{-1})*
136	—	1 Hz	+20	Greater than 0.1	10	10	31.6 ± 5.4
136	—	2 Hz	+34	Greater than 0.1	8	7	80.3 ± 10.1
136 (last 10 mins only)	—	2 Hz	−37	Greater than 0.1	8	8	94.0 ± 14.9
136	—	10 Hz	+12	Greater than 0.1	15	16	297.0 ± 37.0
—	68	1 Hz	+38	0.05–0.1	5	5	69.0 ± 10.8
68	68	1 Hz	+28	Greater than 0.1	5	6	69.0 ± 10.8
—	68	2 Hz	+72	0.05	9	9	72.1 ± 12.5
68	68	2 Hz	+49	0.05–0.1	9	8	72.1 ± 12.5

pressure on potassium-stimulated output possibly points away from any effect on the release process itself but its main significance perhaps is to emphasise the slightness of the effect of pressure *per se* on acetylcholine output. The lack of increase in stimulated acetylcholine release at pressure may be of relevance to the greater synaptic fatigue at these pressures observed by several workers (e.g. Henderson *et al.*, 1977) although the release in response to 10 Hz stimulation did not decrease at pressure as might have been predicted from this observation.

In contrast, raised pressures of nitrogen had a considerable and consistent effect in increasing acetylcholine output. Although it is known that this pressure of helium antagonizes nitrogen narcosis *in vivo* the addition of helium to a total pressure of 136 atm had no effect on the actions of either pressure of nitrogen used. There was, therefore, no sign of 'pressure reversal'. Analysis of the effect of nitrogen showed that it remained after treatment with TTX (which reduced the control output by approx. 50%) so that it was not due to a facilitation of propagated activity. It was also still there after reserpine treatment, and so cannot be attributed to a selective inhibition by nitrogen of endogenous sympathetic activity. The effect appeared to be at least partly calcium-dependent, since when calcium was removed from the bathing fluid, 68 atm of nitrogen increased the output by only 36% (against 110% in normal solution) and 68 atm nitrogen plus 68 atm helium only by 11% (against 82%).

The increase in acetylcholine release by nitrogen recalls the observation by Speden (1965), that trichlorethylene increased spontaneous output from the guinea-pig ileum at 'general anaesthetic' concentrations, but he also found that volatile anaesthetics, in contrast to our finding, decreased the electrically evoked acetylcholine release. Increases in spontaneous output with anaesthetics have been reported at the skeletal neuromuscular junction as shown by an increase in the frequency of minature endplate potentials (eg. Quastel, Hackett & Okamoto, 1972). This effect was shown to be independent of the presence of calcium ions: m.e.p.p. frequency is not as dependent on calcium as is spontaneous release from the ileum. No very satisfactory mechanism offers itself to account for our results. An effect by nitrogen on passive permeability as shown for instance with artificial membranes (Johnson, Miller & Bangham, 1973) or an effect on fluidity of the nerve membrane, both seem unlikely since in each case anaesthetics and pressure had mutually opposed effects. Possibly very high pressure helium and nitrogen at narcotic pressures each increase calcium availability.

The most important conclusion from this work is, however, that neither in the effects of high pressure *per se* nor in the production of anaesthesia by nitrogen are presynaptic effects on acetylcholine release a major factor. Although the size of the effect of nitrogen on acetylcholine output is large enough to suggest that it would contribute to its action *in vivo*, it appears that for the pressure-sensitive effects, directly or of anaesthesia, either one should look postsynaptically, or at presynaptic release mechanisms with characteristics distinct from those of the cholinergic release mechanism.

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