THE EFFECT OF CARBON DIOXIDE ON SNAIL NEURONS.

by R. W. Mathews, B.Sc.

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Department of Physiology and Biochemistry,
The University,
Southampton.

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ABSTRACT

FACULTY OF SCIENCE

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The effects of changes in pCO_2, pO_2, and pH have been investigated in the neurons of the isolated brain of the snail, Helix aspersa, using intracellular electrodes. Anoxia depolarised the membrane of all the cells tested and increased their firing frequency; oxygen reversed this. A few cells were hyperpolarised by levels of pO_2 above normal. Small increases in pCO_2 produced a response similar to that induced by anoxia; large increases in pCO_2 produced a more acute depolarisation and decreased both the frequency and the overshoot of the spikes, and this was not a result of the depolarisation. It is suggested that CO_2 acts by decreasing the membrane permeability to K^+ and/or Cl^- as an increase in membrane resistance was measured. Decreasing the external pH produced some depolarisation but was less potent than solutions of CO_2.

Some cells responded to increased pCO_2 with rhythmic activity and this may be relevant to the control of respiratory movements. These cells showed cyclic changes in their membrane permeability coherent with the rhythmic pattern of their activity.

CONTENTS.

INTRODUCTION.

The effects of carbon dioxide, hydrogen ions, and anoxia on nerve has been primarily and extensively investigated as a property of chemosensitivity in the mammalian respiratory neurons. Haldane (1905) reported that the respiratory mechanism was "exquisitely sensitive" to a rise in alveolar pCO_2 but relatively insensitive to hypoxia; a rise of 0.2% in pCO_2 would double the ventilation whereas the pO_2 could be reduced by 7% without effect. Winterstein in 1911 found that the respiratory mechanism was also sensitive to blood acidity and proposed his "Reaction Theory of Ventilatory Control" suggesting that the specific stimulant was not the pCO_2 of the blood but the decrease in blood pH produced from the dissociation of carbonic acid. However in 1912 Lacqueur and Verzar demonstrated that perfusions of carbonic acid into the rabbit aorta were sixty times more potent in stimulating ventilation than perfusions of hydrochloric acid of the same pH. The Reaction Theory was therefore modified to suggest a barrier between the pH receptors and the blood to allow CO_2 molecules to pass more easily than hydrogen ions.

In 1927 Heymans reported the existence of peripheral chemoreceptors and the existence of a respiratory reflex. These were located in the carotid and aortic bodies and were shown to be stimulated by the presence of CO_2, acidity, or hypoxia in the blood. Denervation showed that most of the total effect of blood acidity and anoxia on ventilation was mediated by this reflex.

The question as to whether these bodies contained any specific CO_2 receptors or only pH receptors with the application of the Reaction Theory was resolved by Hornbeim and Roos (1963). The pCO_2 : pH ratio of the blood of cats was increased by injecting sodium bicarbonate into the blood stream; no displacement of the curve relating carotid body activity to pH was observed indicating the absence of any specific effect of CO_2 and that CO_2 excited this organ by lowering the pH of the receptor cells which surround the ends of the sensory nerves.

The first demonstration of intracranial sensitivity was by Leusen (1954) who perfused the cerebral ventricles of dogs with an artificial CSF. He found that perfusion of the area of the respiratory centre with solutions made acid with a bicarbonate buffer (and therefore with a high pCO_2) produced an increase in ventilation whereas solutions acidified with a borate buffer were less effective per unit of pH. Loeschcke et al (1958) found the opposite in that only acidity increased the tidal volume and at a constant pH the effect of an increase in pCO_2 was to slightly decrease the tidal volume. The problem was defined by Winterstein (1961) to lie in the location and nature of the chemosensitivity. It had been assumed by Leusen and previous workers (e.g Pitts 1946) that CO_2 molecules or H ions had their effect by non-specifically increasing the excitability of the neurons in the respiratory centre facilitating the peripheral respiratory reflex and the central drives to cause a more rapid discharge in the

respiratory neurons. Von Euler and Söderberg (1952) first attempted to demonstrate specificity and the existence of special central chemoreceptive neurons. They found that chloralose depressed the central effects of CO_2 but not the peripheral effects indicating that central chemoreception lay outside the path of the respiratory reflex. Loeschcke et al (1958) found that perfusions of 10^{-3} N HCl onto the floor of the fourth ventricle, the region of the respiratory centre, produced no effect on ventilation. Finally in 1963 Mitchel, Loeschcke, et al discovered that chemosensitivity was specific to paired areas on the medulla away from the respiratory centre. Narcotizing or cooling this area prevented the normal response of the respiratory centre to hypercapnia and produced apnea. The application to this area of paper pledgets containing a solution of CO_2 was found to stimulate ventilation but analysis showed that only the hydrogen ion was stimulatory, the CO_2 molecule and the bicarbonate ion having a slight depressive effect. Mitchel (1966) has suggested that the selective permeability of the blood brain barrier normally hinders the diffusion of hydrogen ions from the blood to these intracranial pH receptors and that the pH of the CSF is almost exclusively determined by the pCO_2 of the blood.

Thus the work on respiration has demonstrated that in this portion of the mammalian CNS chemosensitivity is confined to a few neurons and then to pH and pO_2. There has been no demonstration of any excitatory effects of CO_2 per se. Little is known of

the effect of anoxia except the report by Von Euler (1952) that it had a nonspecific excitatory effect on all neurons tested in the isolated rhombencephalon. Winterstein (1961) has suggested that that intracranial chemoreceptors of anoxia might also exist.

It has not been possible to record the electrophysiology of the chemosensitivity outlined above and so no comparison can be made to the electrophysiology of other sense organs which have been analysed and shown to have a common mechanism, the different stimuli being transduced to a generator potential which promotes an increase in the frequency of steady activity (Gray 1959). The special case in the control of respiration in most animals is that activity in the efferent nerve to the lungs or gills must be expressed as a change in the pattern of the rhythmic bursts. Rhythms have been detected in the completely isolated systems of the goldfish brain (Adrian and Buytendijk 1931) and of the mammalian medulla (Hoff and Breckenridge 1959), and the theories explaining their generation have been of two types. The first presumes that neurons exist with a property of inherent rhythmicity and the suggestion was made by Adrian (1931) that rhythmic firing could be caused by periodic depolarisation of the membrane of the cell body: Gesell (1940) suggested that the nerve membrane might exhibit periodic changes in its excitability. The second type of theory presumes neurons to have no special properties but that the rhythm occurs from their organisation into two groups which fire alternately under the influence of

EPSPs and IPSPs. Pitts (1946) suggested that in mammals such a system operated with one group located in the "pneumotaxic centre" of the pons and the other in the respiratory centre of the medulla. Burns and Salmoiraghi suggested both groups were located in the medulla and their theory appears to be currently accepted to explain mammalian respiration (Salmoiraghi 1963). The system which has been proposed requires the organisation of the neurons to possess three properties to enable them to generate a rhythm by firing alternately :-

1. Inhibitory connections between neurons of opposing groups,
2. Self re-excitation among neurons of the same group,
3. Fatigue of the excitability of both groups of neurons during their activity.

However there is no evidence as to how chemoreceptors might operate in this mechanism to regulate the rhythm.

As in the case of stretch receptors the electrical events involved in the functioning of a sense organ can only be properly examined by intracellular recording. Experimental difficulties have precluded this in the respiratory chemoreceptors of mammals but the effects of pO_2, pCO_2, and pH, on nerve membrane have been studied in preparations where intracellular recording was easier. Most work has been carried out on the neurons of the isolated visceral ganglion of the mollusc, Aplysia, by Arvanitaki, Chalazonitis, and co-workers. It was found that the neurons of this preparation, identified from their

position, could be classified into three categories according to their activity (Arvanitaki and Chalazonitis, 1958) :-

1. Neurons whose activity is determined presynaptically e.g. the "B" and "pcr" neurons.
2. Neurons whose activity is spontaneous and regular, e.g. the "A" neuron.
3. Neurons which are inherently rhythmic (autorhythmic) e.g. the "Br" and "Gen" neurons. The membrane potential of such neurons shows regular alternating periods of depolarisation and hyperpolarisation and activity appears in bursts of spikes separated by periods of silence.

The response of all these neurons to an increase in the pCO_2 or a decrease in the pO_2 was found to be a depolarisation. In the simplest case, that of the "A" neuron, CO_2 depolarised the membrane from 60 mV to 40 mV in one minute, and the firing frequency was first stimulated then inhibited. The cause of the inhibition was suggested to be over-depolarisation (Chalazonitis, 1959). A similar effect was observed in the presynaptically activated neurons except that the pattern was temporarily interrupted by short periods of hyperpolarisation and silence which were considered to be the summated effects of a burst of IPSPs (Chalazonitis, 1961). Chalazonitis and Romey, (1964) have also reported that CO_2 increased the membrane resistance by 100%. However their method made no allowance for membrane rectification (Kandel and Tauc, 1966) and this value is probably inaccurate.

The effects of anoxia on the "A" and "B" neurons was similar to the effects of CO_2 except that the latency was greater. The membrane was depolarised (Chalazonitis and Takeuchi, 1964), the frequency of spikes first increased then decreased, and the membrane resistance of the "A" cell increased (Chalazonitis, 1964). The effects of anoxia were confined to the cell body and the axon was unaffected (Nahas, Cook, and Chalazonitis, 1964).

The most interesting effects of an increased pCO_2 or a decreased pO_2 were found in the autorhythmic "Br" and "Gen" cells. The depolarisation was accompanied not by a steady increase in the frequency of the spikes but an increase in the frequency of the slow wave-form variations of the membrane potential and of the bursts of activity (Chalazonitis, 1959, Arvanitaki and Chalazonitis 1965). The suggestion was made (Chalazonitis, 1963) that such activity could be considered to explain the origin of the respiratory rhythm as an alternative to explanations involving groups of neurons.

It was originally suggested that the effects observed with CO_2 might in fact be pH effects (Chalazonitis, 1959). Chalazonitis and Takeuchi H, (1966), have shown that acid will cause depolarisation but their graphs show that the effects of CO_2 and acid are different when both are measured in pH units. An opposite effect on membrane resistance, a reduction, was detected in acid by their method (Chalazonitis and Takeuchi 1966).

The effect of the three factors, pCO_2, pO_2, and pH has been investigated by intracellular recording in other preparations

but in less detail. Anoxia has been reported to depolarise the neurons of the cat spinal cord (Kolmodin and Skoglund 1959) and of the brain of Helix pomatia (Chalazonitis & Takeuchi 1964). The effects of carbon dioxide on the squid giant nerve appears similar to that of Aplysia neurons and in this case the use of an internal pH electrode demonstrated a decrease in intracellular pH of 1 - 1.5 units (Caldwell 1958). Decreasing the external pH about this preparation is reported to also depolarise membrane and reduce the spike amplitude (Spyropoulos 1960) but less effectively than with CO_2.

Using external recording electrodes, Lorente de Nó (1947) made detailed studies in the sciatic nerve of Bullfrogs. Anoxia was found to reduce the demarcation potential 10 mV in five hours; recovery occurred rapidly after ten minutes in oxygen. Carbon dioxide was found to increase the membrane potential by 5 mV, decrease the excitability, and improve the ability to conduct, i.e. generally opposite to that of the preparations reviewed so far. Moderate reductions in the external pH (down to 5.65) had little effect on the membrane potential or conductibility. Similar effects for CO_2 in mammalian axons are reported by Carpenter (1963).

The experiments reported below extend the investigations of the effects of pCO_2, pO_2, and pH to the neurons of the snail, Helix aspersa. Some properties of autorhythmic neurons, considered by Chalazonitis as possible chemoreceptors, have also been reported.

METHODS

Experiments were performed on the larger neurons of the abdominal ganglion of the isolated snail brain. The membrane potentials were recorded by intracellular glass electrodes inserted into the cell bodies.

The Isolated Snail Brain Preparation.

Generally the snails were freshly collected whilst feeding. Snails in hibernation were activated by a warm damp environment. The brain was removed from the animal and secured to a glass plate with elastic bands. The outer connective tissue was removed before transferring the brain to the perspex bath where the inner connective tissue was torn with a glass hook, the preparation being viewed from the front through a Prior microscope and illuminated from the rear with a Beck microscope-lamp.

Recording and Display.

The electrodes were pulled on a Palmer machine from cleaned, graded, Pyrex glass tubing of 1.5 - 2.0 mm O.D. They were filled with molar potassium acetate and had a low resistance (5 - 8 megohms). They were clamped to a Prior micromanipulator and lowered into the cell by eye. Potentials were displayed on a Tektronix 502A oscilloscope being received from an M.E.1400 valve cathode follower connected to the electrode solution by a chlorided silver wire. The trace was recorded with a Cossor camera or an A.E.I. pen-recorder. A number of cathode followers were defective, emitting a

grid current which hyperpolarised the cell and prevented the normal discharge of spikes. However it was also possible to measure the membrane potential by connecting a Vibron voltmeter directly to the electrode. This was more reliable as this instrument has a high impedance input, but its poor time response caused most of the spike to escape the record. The cathode follower used in the experiments described was tested by alternately switching a neuron from the cathode follower to the Vibron and affirming that the membrane potential and frequency of activity appeared the same.

Measurement of Membrane Resistance.

A bridge similar to that described by Koketsu et al. (1959) was incorporated with the cathode follower. This allowed a current to be passed through the single recording electrode and across the cell membrane without affecting the ability to record the membrane potential. The membrane was swept by a linearly increasing current from a bootstrap circuit (Kerkut, Thomas, and Venning, 1963). The current was measured by the voltage drop across a 100 megohm resistor and displayed on the horizontal axis of the oscilloscope; the membrane potential was displayed on the vertical axis, and the resulting xy plot photographed to obtain the resistance from the gradient. A slow sweep was chosen with a current change of $0-7.10^{-9}$ amps in one second to minimise the effect of the capacitance of the membrane.

Changing The Gas Phase.

The bath was covered by a Perspex chamber (fig. 1) which was placed in position after the dissection. The side of the chamber which sealed around the electrode support was of thin polythene to enable subsequent implantation. Gases were admitted after passage through Ringer to standardise their temperature and humidity.

Oxygen Sensitivity.

The silver chloride of the bath earth and of the wire in the electrode was found to be sensitive to the concentration of dissolved oxygen and this was apparent as an anomalous potential of up to 20 mV. Hence it was necessary to shield both from a changing pO_2. The earth was placed in a separate bath connected to the main chamber by an agar/KCl bridge. The surface of the electrode solution was connected to the open atmosphere by a length of rubber tubing around the electrode wire and mounted on the open end of the electrode.

Ringer.

The Ringer used was that described by Thomas and Kerkut (1963) except that the Tris buffer was omitted; this left the pH unchanged at 7.9.

The Oxygen Electrode.

The pO_2 of the bath solution was measured polarographically by a platinum cathode and a silver/silver chloride anode. At a steady applied potential of 0.6 volts the current flowing across

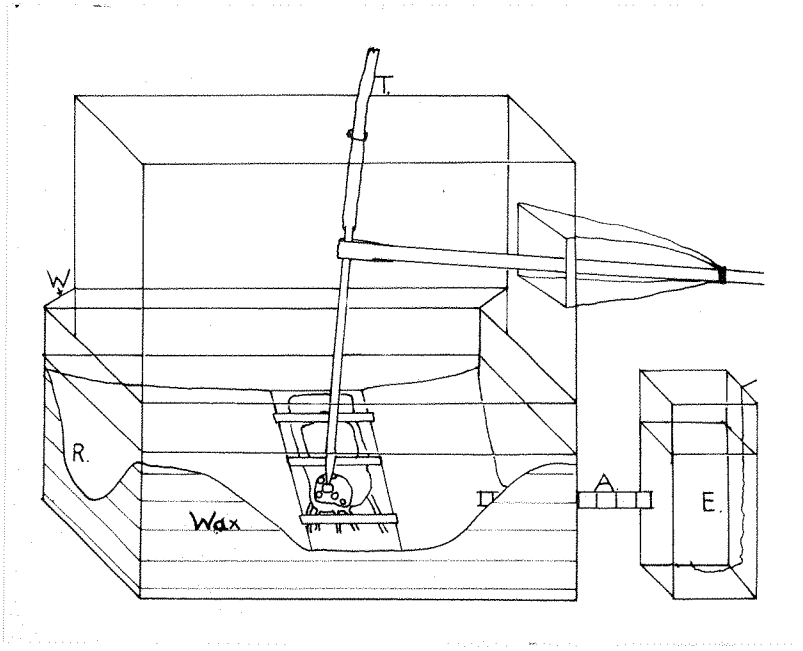


Fig.1 The experimental bath with the isolated snail brain, glass electrode, and overlying gas chamber in position.

A...Agar bridge connecting earth to main bath,
E...Separate bath containing Ringer and the Ag/AgCl earth,
R...Ringer,
T...Rubber tubing surrounding and protecting the electrode wire from a changing pO_2.
W...Glass window through which the brain was illuminated.
Not shown are:- The inflow and overflow tubes for the Ringer,
The gas input and exit tubes,
The oxygen electrodes which lay in front of, but at the same depth as the brain.

between the electrodes was taken as a measure of the oxygen concentration. The two electrodes were embedded into the Perspex chamber and insulated as far as the tips with epoxy resin. The extremes of anoxia and oxygen saturation were also measured by an Astrup oximeter to provide a calibration.

RESULTS.

A. THE EFFECTS OF A CHANGE IN pO_2 ON SNAIL NEURONS.

The bath was fitted with the overlying gas chamber. The cell was impaled by the electrode and a period of ten minutes allowed to elapse with the chamber ventilated with air. When stability had been established the pO_2 was rapidly changed from 250 to 1100 mm Hg by passing oxygen saturated Ringer into the bath and filling the chamber with oxygen (figs. 2 & 3). This caused a hyperpolarisation of the membrane and a decrease in the firing frequency. There was some variation amongst the cells in their sensitivity to this change and some cells were unaffected.

To lower the pO_2 below normal the chamber was filled with nitrogen. Fig. 2 records the effect on a cell which was initially silent; the 'oxygen' electrodes lying in the Ringer at the same depth as the brain provided the measurement of the pO_2. The effect of anoxia was found to be a depolarisation to a sub-threshold level, and this reversed rapidly on returning to a normal pO_2. The graph describing the onset of anoxia (fig.5)

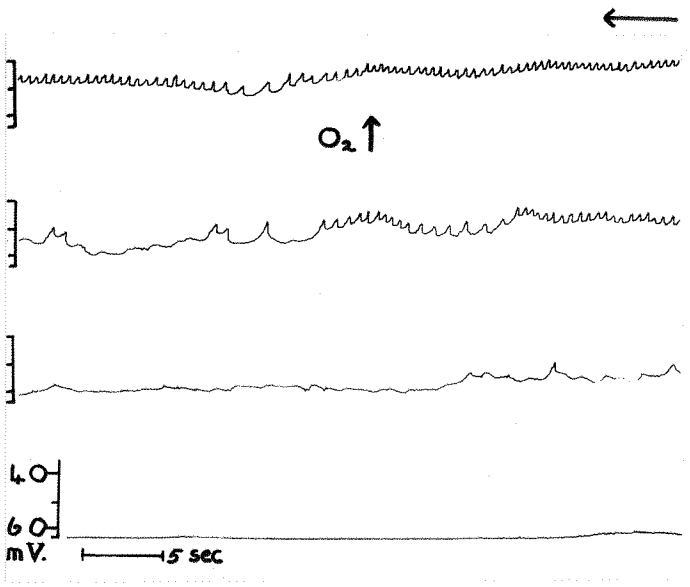


Fig.2 The effect of a high pO_2 on the membrane potential. At the arrow, the pO_2 was increased from 250 - 1100 mmHg. The record was made with a Vibron voltmeter printing out on the pen-recorder. The slow time response masked most of the spike. Traces were continuous and run from right to left.

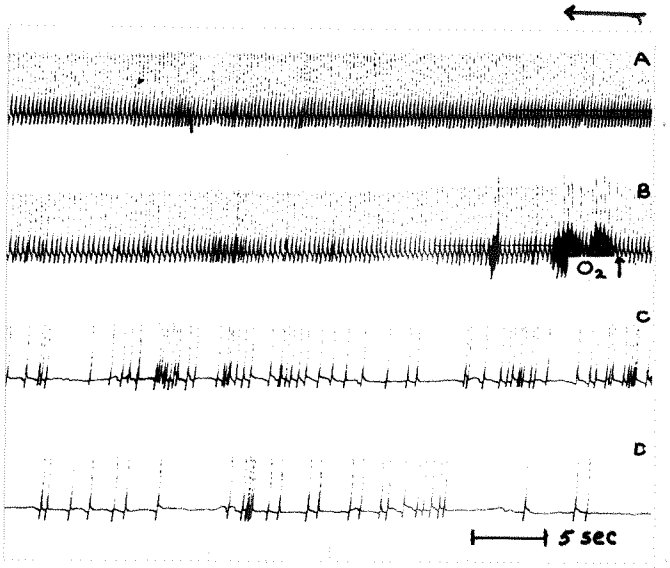


Fig.3 The effect of a high pO_2 on the frequency of activity. Conditions were as above except that this record was made with a cathode follower.

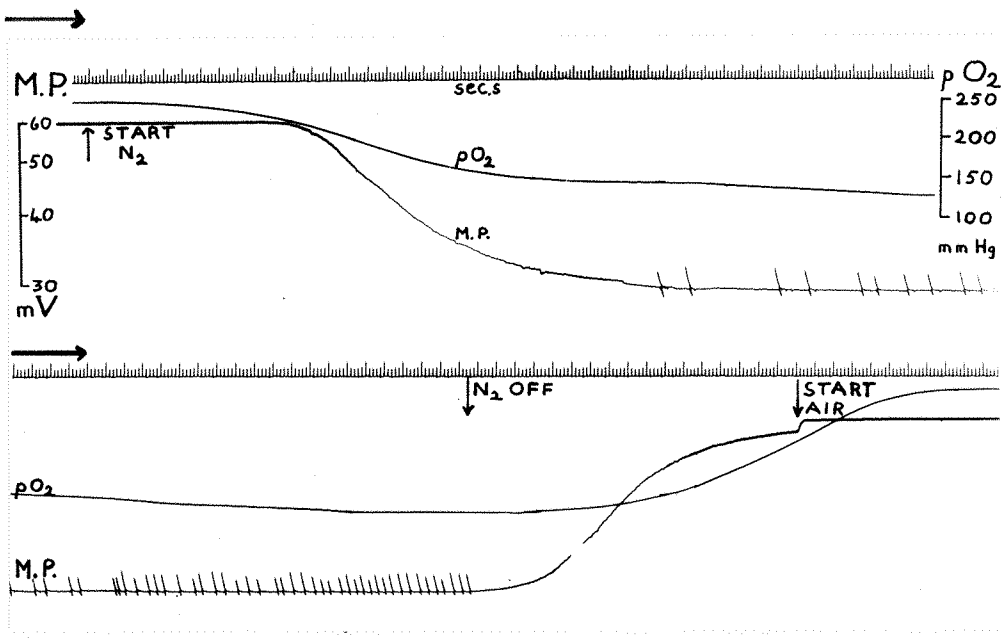


Fig.4 The effect of a low pO_2.
The upper line in each case records the time, the middle, the pO_2, and the lower, the membrane potential$_{\lambda}^{(M.P.)}$. The records run L.- R. and are continuous. Most of the spike is off the record.

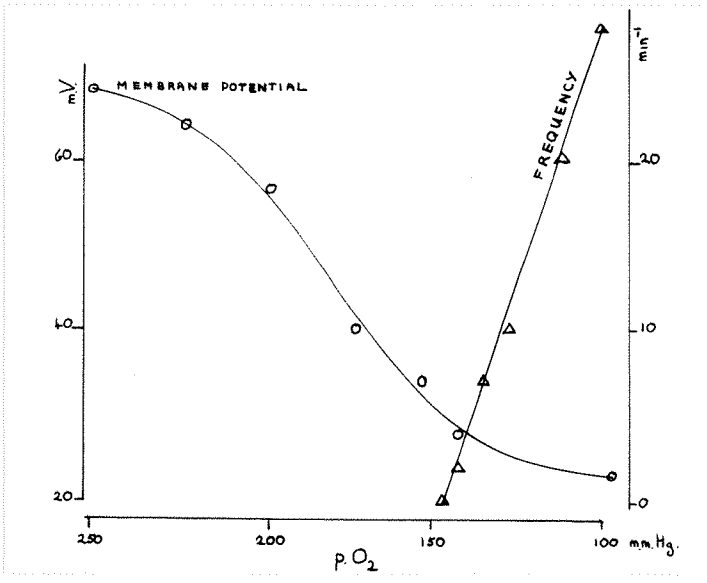


Fig.5 The results of the above experiment for the onset of anoxia expressed graphically.

shows how the membrane potential of the neuron was related to the pO_2 of the Ringer. When a sub-threshold level was reached the graph shows that the firing frequency was a linear function of the pO_2. These results are similar to those reported by Chalazonitis and Takeuchi, (1964) for the neurons of Helix pomatia and Aplysia.

B. THE EFFECTS OF AN INCREASE IN pCO_2 ON SNAIL NEURONS.

Chalazonitis (1959) reported that the neurons of Aplysia were depolarised by increasing the pCO_2 and that the effect was similar to that of anoxia. To investigate the effect of CO_2 on snail neurons the chamber was filled with 100% CO_2 gas. Fig. 6 shows that the effects of this procedure on a firing cell were :-

a. An increasing depolarisation of the membrane,

b. An initial increase in the frequency of spikes,

c. A subsequent reduction in the firing frequency associated with a progressive change in the shape of the spike with a decline in the overshoot and a slower rise and fall.

d. A final disappearance of the spike.

On replacing the Ringer in the bath with normal Ringer free from CO_2 the recovery was slow but found to be somewhat aided by the presence of a high pO_2.

To investigate the CO_2 effect in greater detail it was necessary to be able to measure the level of CO_2 in the Ringer. A simple method was to measure the pH change produced in the

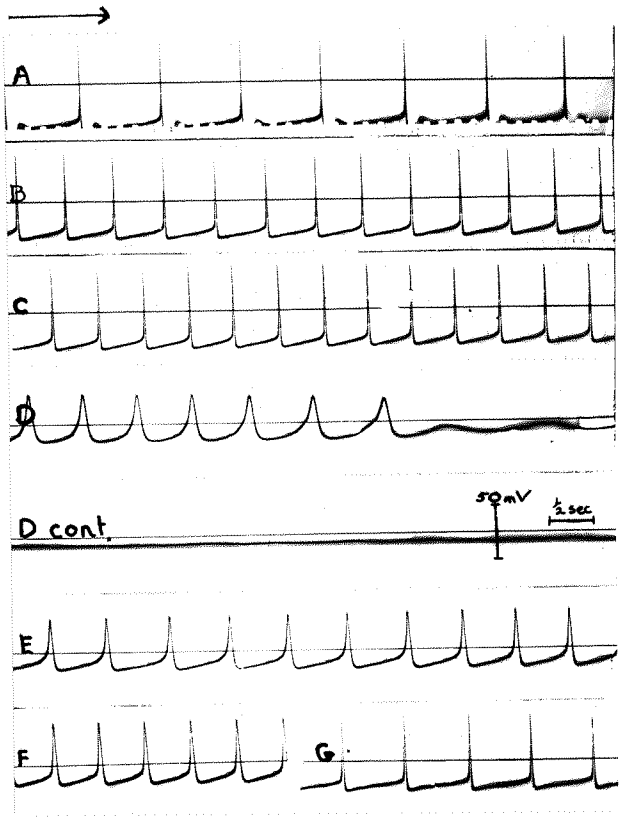


Fig.6 Photographs from the oscilloscope showing the effect of CO_2 on the shape of the spike.
'A' records the normal discharge of the cell, 'B', 'C', & 'D', were taken 2, 4, & 5 mins after ventilation with CO_2. 'E', 'F', & 'G' were taken at 6, 8, & 10 mins to illustrate the slow partial recovery induced by flushing the chamber with oxygen at 5 mins.

unbuffered Ringer by the solution and dissociation of the gas, in accord with the following equilibria :-

$$CO_{2(gas)} + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

In practice it was found that saturation produced a change in pH ("Δ pH") of 2.6 units. A less concentrated solution of Δ pH 2.2 held the gas sufficiently long for the experiments to be conducted and this was adopted as a standard solution. Successive 50% dilutions of this standard provided a range of concentrations for dose response investigations. The particular concentration of CO_2 in each solution is always expressed as the number of pH units it differs from that of normal Ringer (which has a pH of 7.9).

The Effect Of Carbon Dioxide On The "Resting" Membrane Potential.

The membrane potential of active cells was measured on the oscilloscope whilst the range of CO_2 enriched Ringers was run through the bath; between each the cell was allowed to recover in normal Ringer. The maximum depolarisation in each level of CO_2 was noted and plotted against the pH of the CO_2 Ringers (fig.7). From the equation above it can be seen that the pH of a solution of CO_2 is related to the log. of the concentration of the gas dissolved. The graph shows that up to ΔpH of 1.3 the relationship between depolarisation and Δ pH i.e log. $[CO_2]$, follows a straight line. Above a ΔpH of 1.3 the depolarisation becomes more severe and was less easily reversed. There were no cells that were not depolarised by CO_2.

The Effect Of Carbon Dioxide On The Frequency Of Activity.

The range of carbonic acid Ringers was run through the bath

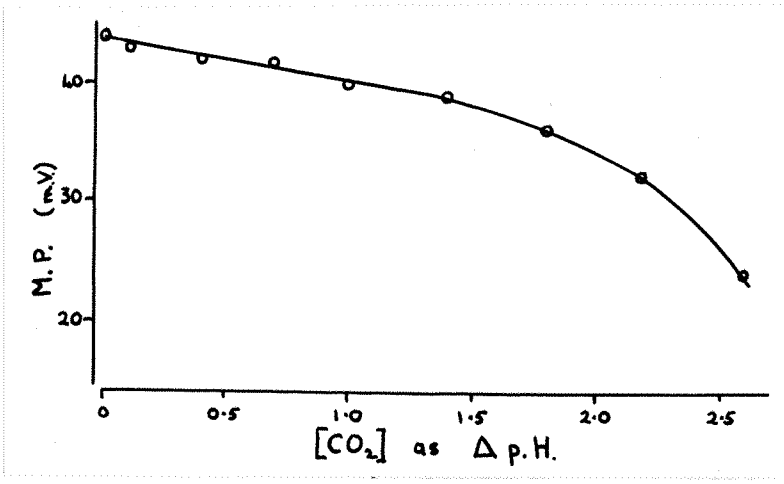


Fig.7 Initial effect on the membrane potential of increasing levels of CO_2 (mean of three experiments). The concentration of CO_2 was expressed as the pH change produced by the gas in the unbuffered Ringer.

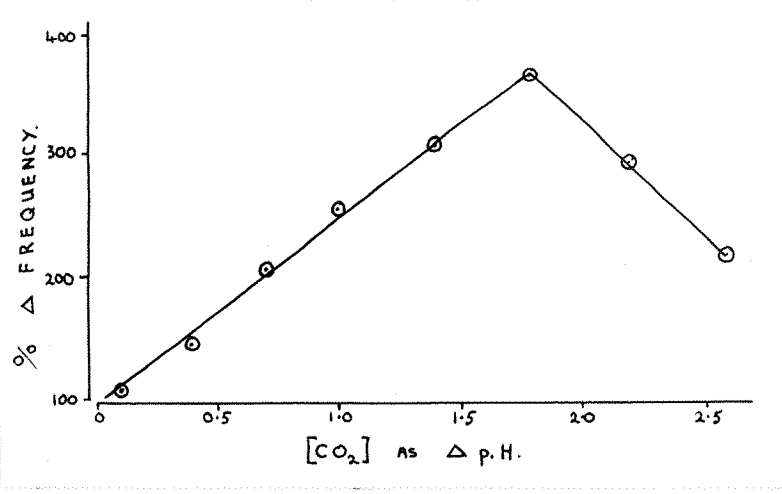


Fig.8 Maximum frequency of activity in the different levels of CO_2, (mean of six experiments). In each experiment the frequency in normal Ringer was taken as 100%. The concentration of CO_2 was again expressed as the pH change.

and the frequency change counted from the pen recorder. The dose response pattern for this parameter was found to be complicated and the results must be related to the mechanism which normally generates activity in the cells. As in Aplysia three categories of activity in snail neurons may be defined:-

1. Spontaneously Active Neurons.

These cells discharge with great regularity but show no sign of post synaptic potentials. They resemble the 'A' cells of Aplysia and the pacemaker cells of Tauc (reviewed 1966).

2. Synaptically Controlled Neurons.

These cells discharge with an irregular activity which is clearly the result of competition between EPSPs and IPSPs.

3. Autorhythmic Neurons.

These cells show their activity in regular bursts of spikes similar to autorhythmic neurons of Aplysia. They are described at the end of this section.

Fig.9 is a record of a typical dose response experiment on a spontaneously active cell. Up to Δ pH 1.4 stimulation alone occured, above this level inhibition followed the stimulation. In the most concentrated CO_2 Ringer the inhibition so predominated as to rapidly silence the cell. This was followed by a peculiar long lasting hyperpolarisation of the membrane. Washing off the CO_2 with normal ringer first removed this hyperpolarisation, then high frequency activity returned, and finally the cell repolarised to normal with a slow fall in the

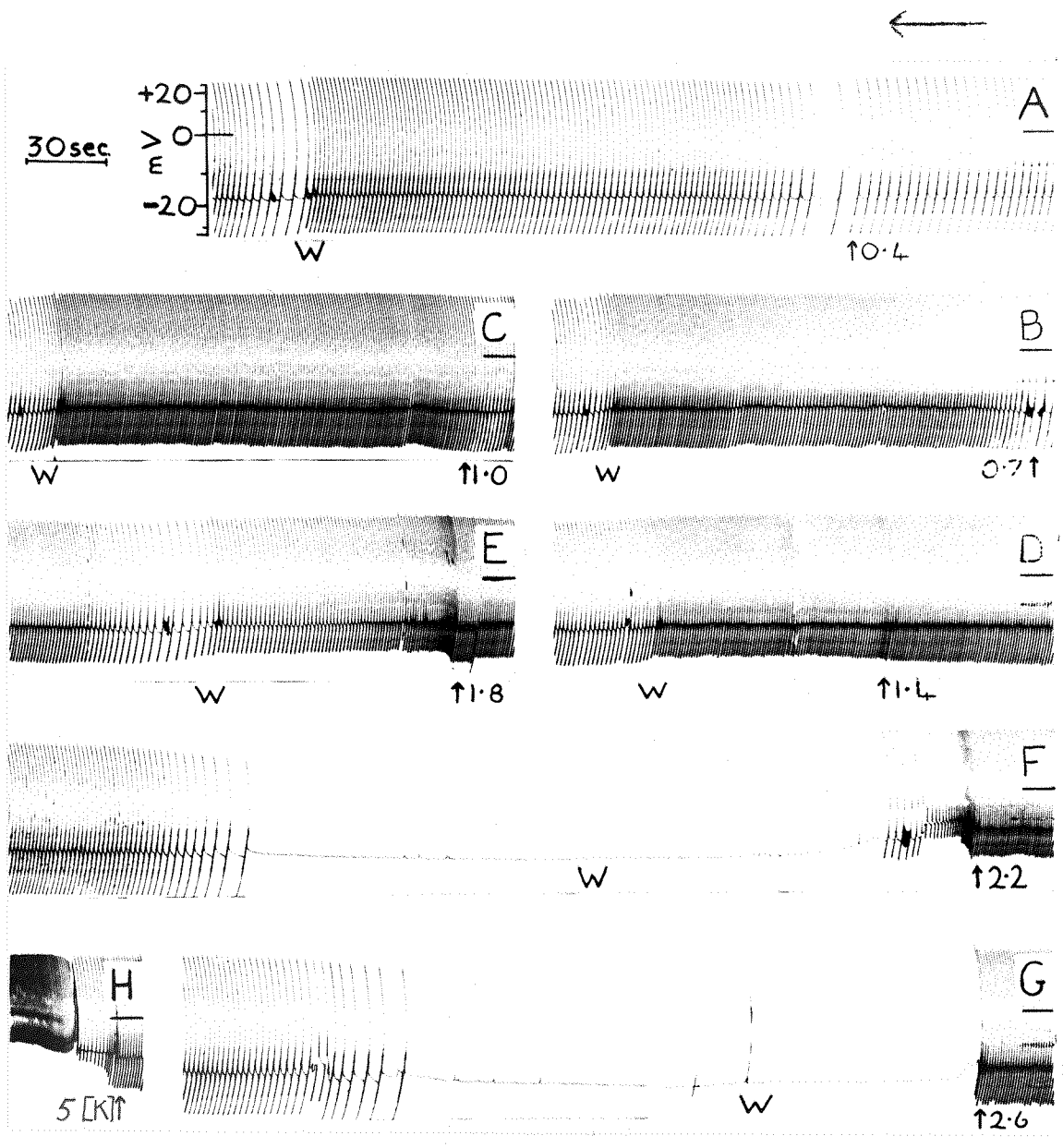


Fig.9 Pen recording of the effect of different levels of CO_2 on a spontaneously, regularly fast active cell.
Traces read from right to left, figures denote the pH of the CO_2 solution applied as the number of units below that of normal Ringer. W denotes washing with normal Ringer. The gap in G = 2mins. In H, Ringer containing five times the normal concentration of potassium was applied.

frequency to normal. In Aplysia the inhibition of the "A" neuron discharge in high pCO_2 was reported and the cause was suggested as over-depolarisation. In fig.9H this neuron was subjected to a strong depolarisation by washing with Ringer containing five times the normal concentration of potassium; the result was found to be not inhibition but strong excitation producing a higher rate of discharge than any of the levels of CO_2.

Fig.10 is a record from a slow active cell. These cells particularly illustrated the phenomenon of adaptation. To again illustrate that inhibition was not caused by over-depolarisation the cell was further depolarised whilst it lay inhibited in strong CO_2 Ringer by passing a current across the membrane and rapid activity was the result,(fig.10G).

Fig. 11 is a record from a cell whose activity was clearly the result of integration between EPSPs and IPSPs. The effect of CO_2 was to depolarise the cell and increase the frequency of discharge as in the cells apparently spontaneously activated. However in the more concentrated solutions of CO_2 there were temporary interruptions caused by bursts of IPSPs which could summate to cause a hyperpolarisation and inhibit the activity for 15 sec. The effect was temporary perhaps due to the exhaustion of the transmitter or adaptation in the post synaptic membrane and the pattern again resembled the response of the spontaneous cells. It may be noted that IPSPs did not reappear when the cell began recovery in normal Ringer.

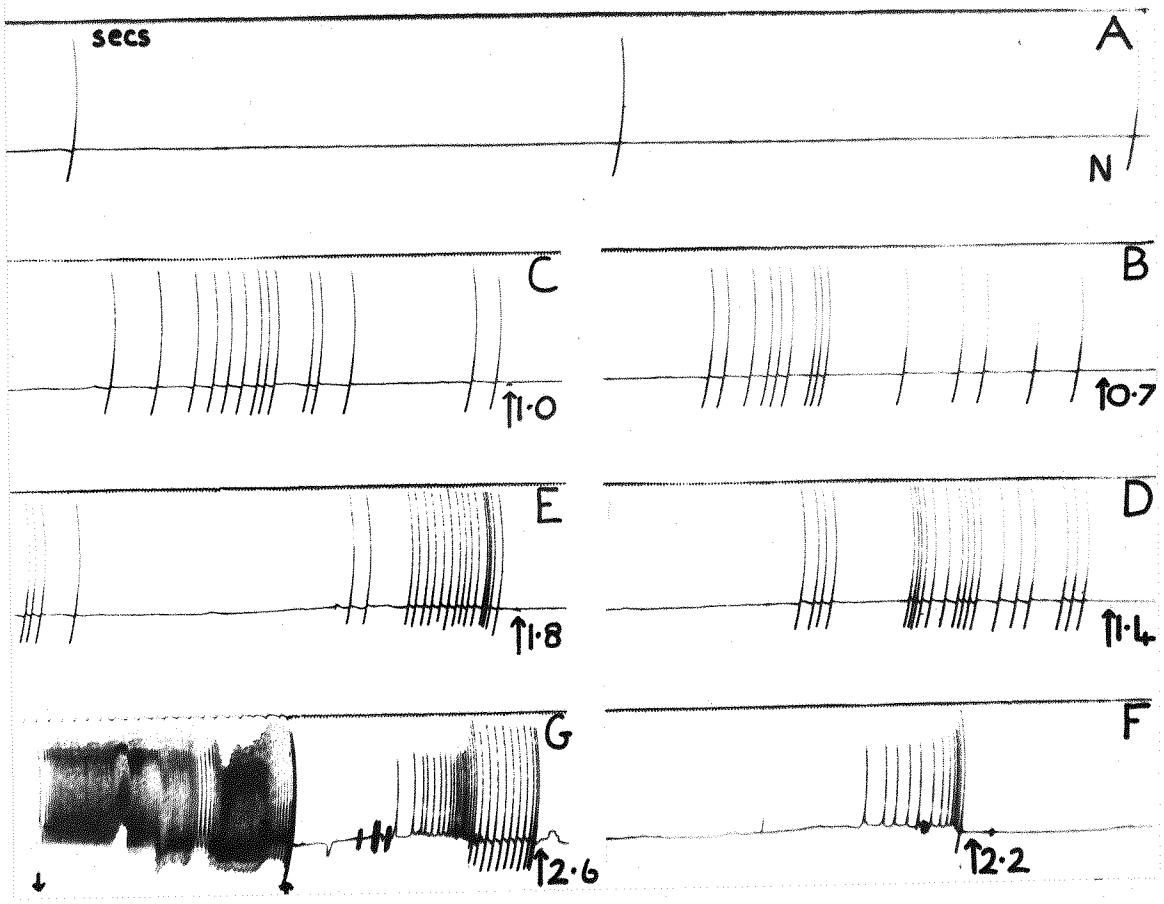


Fig.10 Pen recording showing adaptation to CO_2, and the effect of an inward current on a slow active cell. (between the small arrows) In G, the cell was depolarised by a current of 5×10^{-9} amps whilst in the most concentrated CO_2 Ringer. Trace runs from right to left and the figures denote the a.p.d.

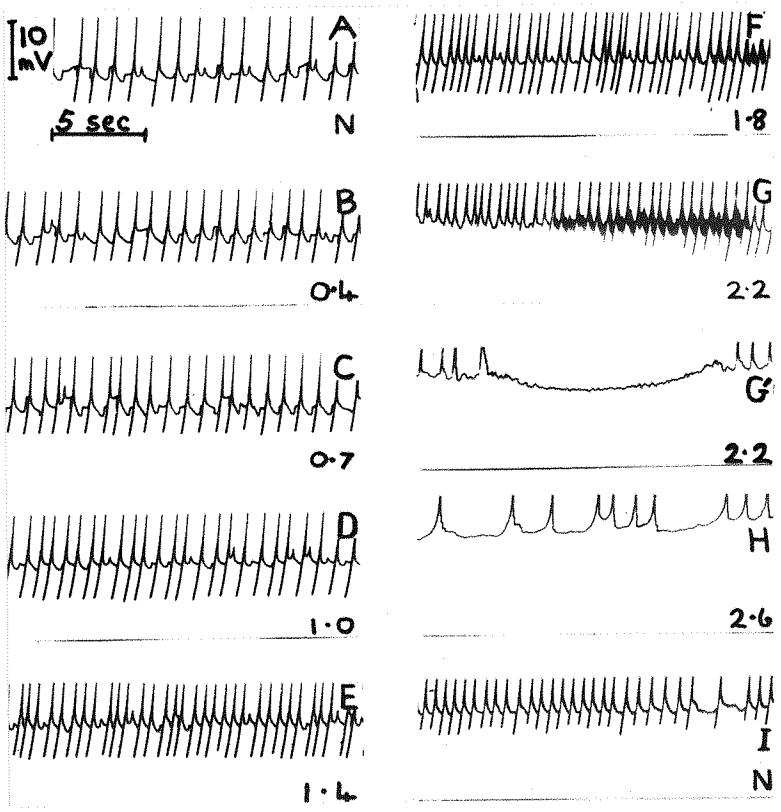


Fig.11 Pen recording of the effect of increasing levels of CO_2 on a cell whose activity was the result of post synaptic potentials. EPSPs and IPSPs may be seen in trace A taken in normal Ringer (N), In the stronger concentrations of carbon dioxide eg. trace G' which is a continuation of trace G, the activity was interrupted by a train of IPSPs summating to produce a transient hyper-polarisation and inhibition of activity, this can be compared to the different character of the permanent inhibition in strong CO_2 Ringer (eg trace H). Trace I is a record of the activity when returned to normal Ringer; the paucity of IPSPs may be noted.

The general effect of the different levels of CO_2 on the frequency of these two categories of cells is shown by the graph (fig.8), where the parameter of the maximum frequency in each CO_2 Ringer, expressed as the % change above normal, was plotted against the log. of the concentration of CO_2, expressed as the change in pH. The graph has a maximum with the frequency being increased by CO_2 Ringers of a ΔpH below 1.8 and reduced by CO_2 Ringers of a ΔpH 1.8 to 2.6.

The Effect Of CO_2 On The Membrane Resistance.

Membrane resistance was measured by passing a hyperpolarising current across the cell membrane and recording the ensuing current : voltage relationship by photographing the xy plot on the oscilloscope as described in the Methods section. Because the resistance of molluscan neurons has been shown to be a function of the membrane potential the resistance was always measured at 75 mv by taking the gradient of the xy plot at that point. Fig.12 shows such determinations of resistance in four different levels of CO_2. Three plots were taken in each level, the value of the mean is given in the table and shown graphically in fig.13. It was found that CO_2 giving a ΔpH of 2.6 increased the membrane resistance to three times the normal. The change in this level of CO_2 took over two minutes to develop fully and was slower to reverse. The xy plot in the final washing (fig.12,I) showed how unstable the membrane had become.

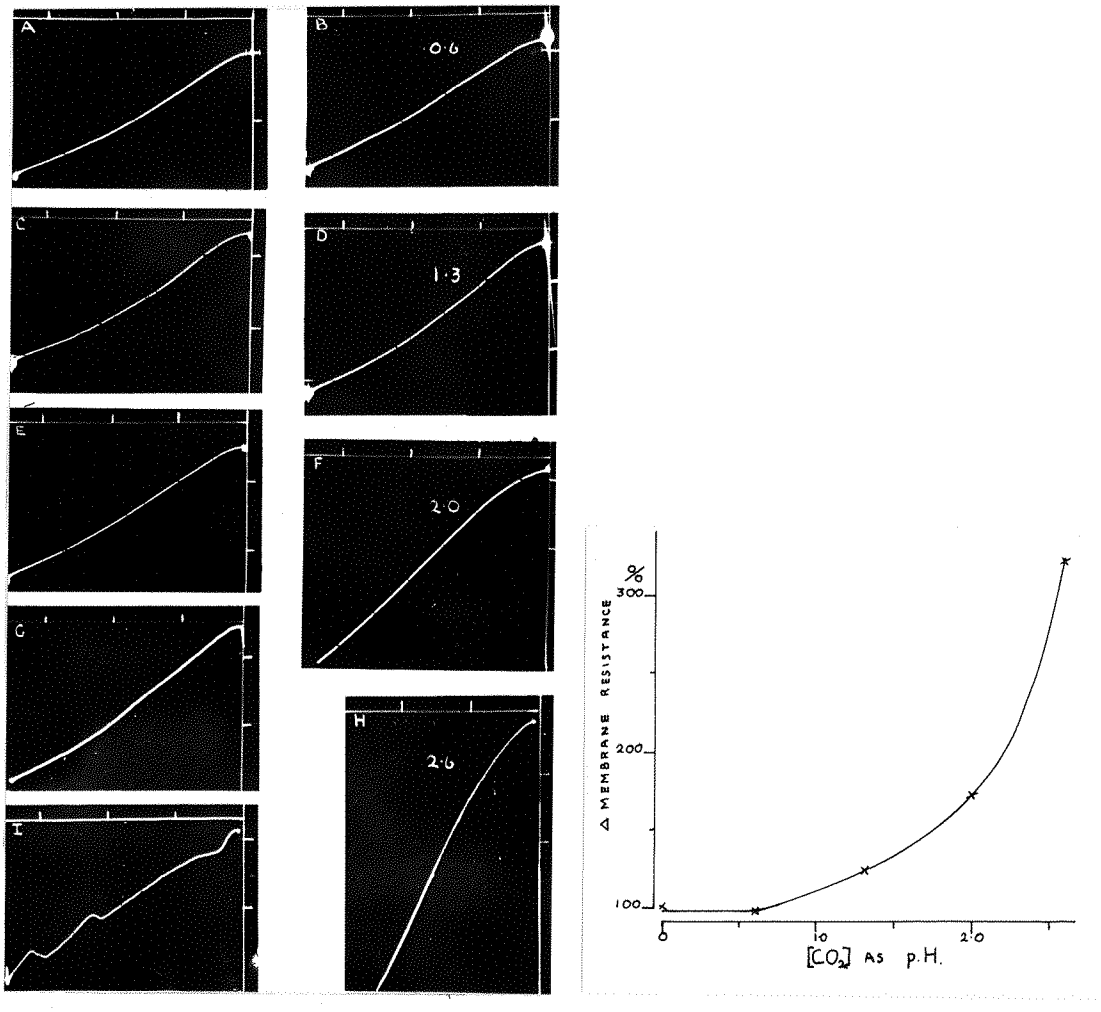


Fig.12 The effect of CO_2 on the membrane resistance.
In each photo of the oscilloscope xy plot the horizontal axis measured the current (divisions R - L represent 0, 2, 4, 6, $x10^{-9}$ amps outward), and the vertical axis measured the membrane pot. (upper division ≡ -60 mV, lower division ≡ -80 mV). Photos in the R.H. column were taken in the CO_2 Ringers, the △pH being given beside the trace; photos in the L.H. column were taken in normal Ringer. Changes in membrane resistance altered the gradients.

Fig.13 (right) Graph of membrane resistance at 75 mV plotted against the CO_2 concentration (results of fig.12).

Results Table For The Effect Of CO_2 On The Membrane Resistance Of A Neuron.

	RINGER	MEMBRANE RESISTANCE AT 75 mv.	
		Megohms	% change
A.	Normal	6.3	(=100)
B.	CO_2(Δ pH 0.6)	6.1	97
C.	Normal	6.5	
D.	CO_2(Δ pH 1.3)	7.7	123
E.	Normal	6.1	
F.	CO_2(Δ pH 2.0)	10.6	170
G.	Normal	8.0	
H.	CO_2(Δ pH 2.6)	20.0	320
I.	Normal	6.7	

Fig.17 displays on one graph the mean changes in membrane potential (mv), maximum frequency (%), and membrane resistance (%), previously described, plotted against the log. of the concentration of CO_2 (as ΔpH). It was observed that the increase in membrane resistance correlates with the severe phase of membrane depolarisation and the inhibition of the activity.

C. THE EFFECT OF A DECREASE IN pH ON SNAIL NEURONS.

A pH control was designed using Ringers containing small quantities of hydrochloric acid. This produced a significant change only in the concentration of hydrogen ions but was less

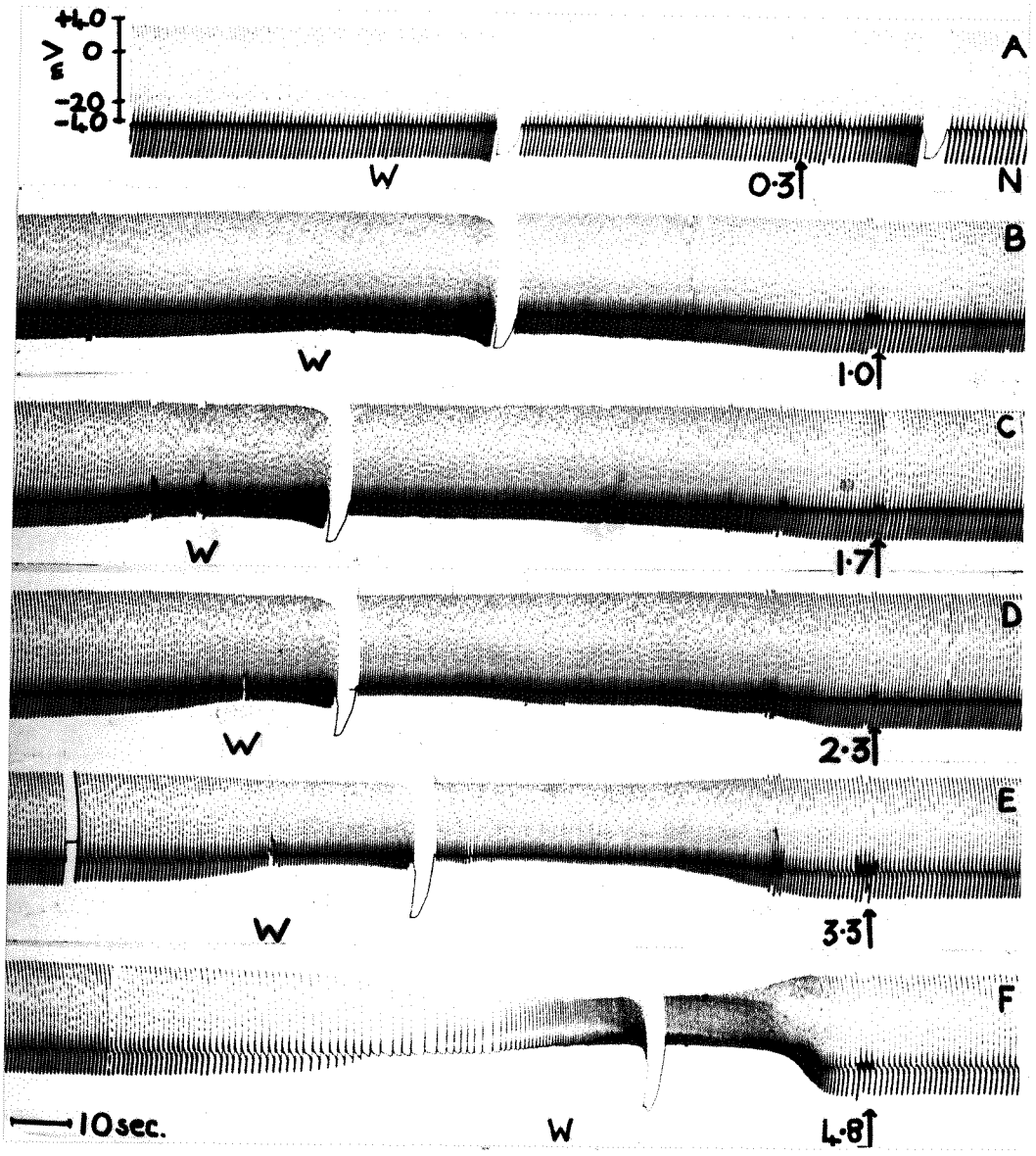


Fig.14 Effect of pH on a sensitive neuron.
The pH of the Ringer was lowered by small amounts of HCl to the values shown. W denotes washing with normal Ringer. Each gap represents a determination of membrane resistance, and the photos are shown in fig.15.

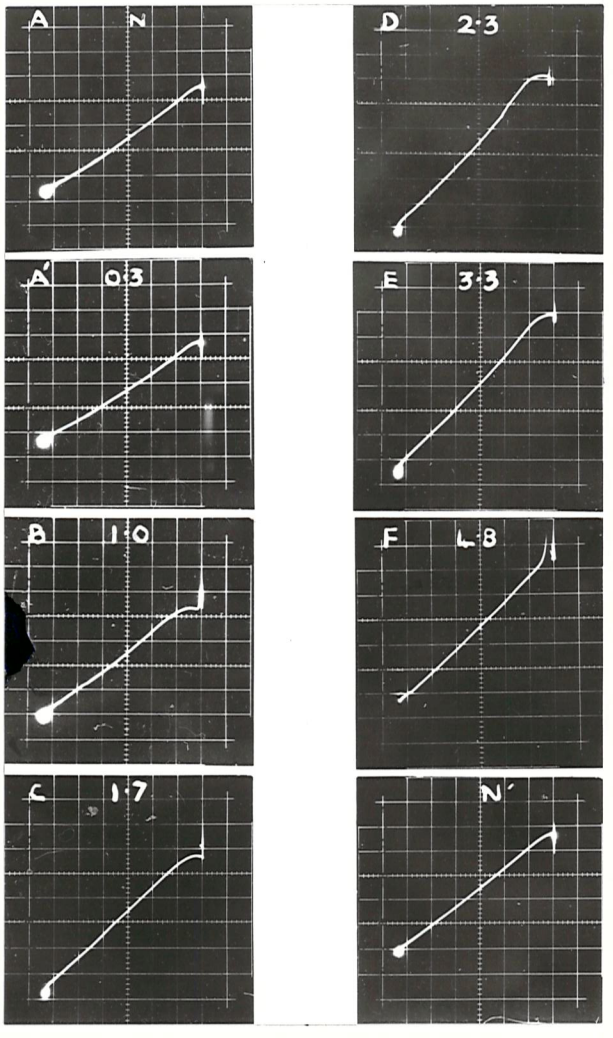


Fig.15 Effect of pH on the membrane resistance.
These photos of the oscilloscope xy plots were taken in the same experiment as fig.14, and the same labelling has been used. 'N'' was taken in normal Ringer at the end of the experiment.
The larger divisions of the horizontal scale $\equiv 10^{-9}$ amps.
the upper and lower calibrated lines cut the vertical scale at -60 and -80 mV respectively. The resistance was measured at 75 mV from the gradient and plotted in fig.16.

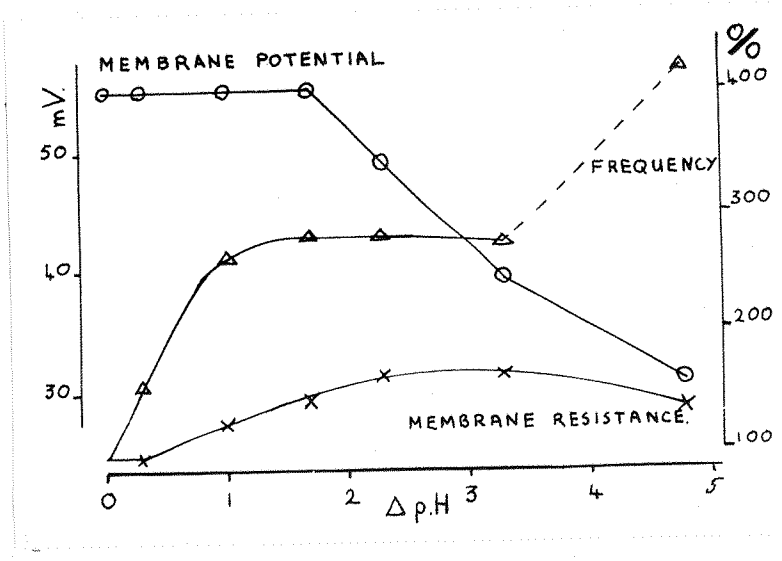


Fig.16. Graph showing the relationship of membrane potential,(mV), membrane resistance(%), & maximum frequency of activity(%) to pH in the cell found most sensitive, (from figs.15 & 14).

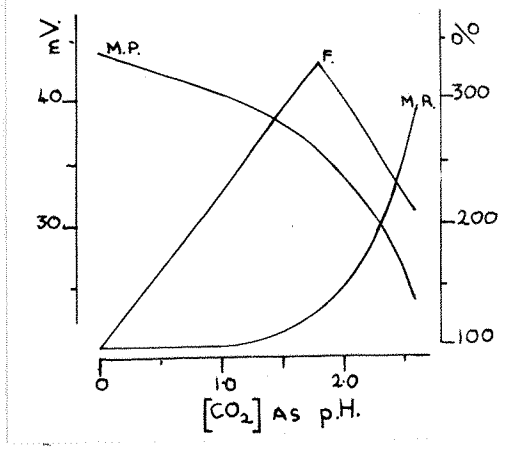


Fig.17. Graph showing the relationship of the three parameters to the concentration of CO_2 as the general effect found in all cells. (Membrane potential curve from fig7, frequency curve from fig.8, membrane resistance curve mean of three.)

stable than a buffered change in pH. The pH of the Ringers was adjusted immediately before use and then applied in a continuous flow to overcome the buffering capacity of the brain.

In contrast to the results from CO_2 not all cells were affected by acidity per se ($\triangle$pH $<$2.6). Figs.14,15,& 16, record the effects of acidity on a cell which showed the greatest sensitivity to this factor. Figs.16&17 enable the comparison of this with the consistent effects of CO_2. It was apparent from the two graphs that the lowering of the extracellular pH can only be a partial explanation for the effects of CO_2 on the frequency of activity, the spike overshoot, or the membrane potential. To produce the same changes in these parameters much larger decreases in pH were required than present in the CO_2 Ringers. The increase in membrane resistance with HCl was only about one third that with carbonic acid and there was no inhibition of the frequency stimulation or long lasting hyperpolarisation as noted with strong CO_2 Ringers (cf. fig.9G).

D. PROPERTIES OF AUTORHYTHMIC NEURONS IN THE SNAIL BRAIN.

On page 15 one type of activity was categorised as autorhythmic. This has also been recorded in Aplysia and Chalazonitis (1963) has considered these neurons in the role of respiratory chemo-receptors. Such autorhythmic neurons were identified in the snail's lower abdominal ganglion and right parietal ganglion.

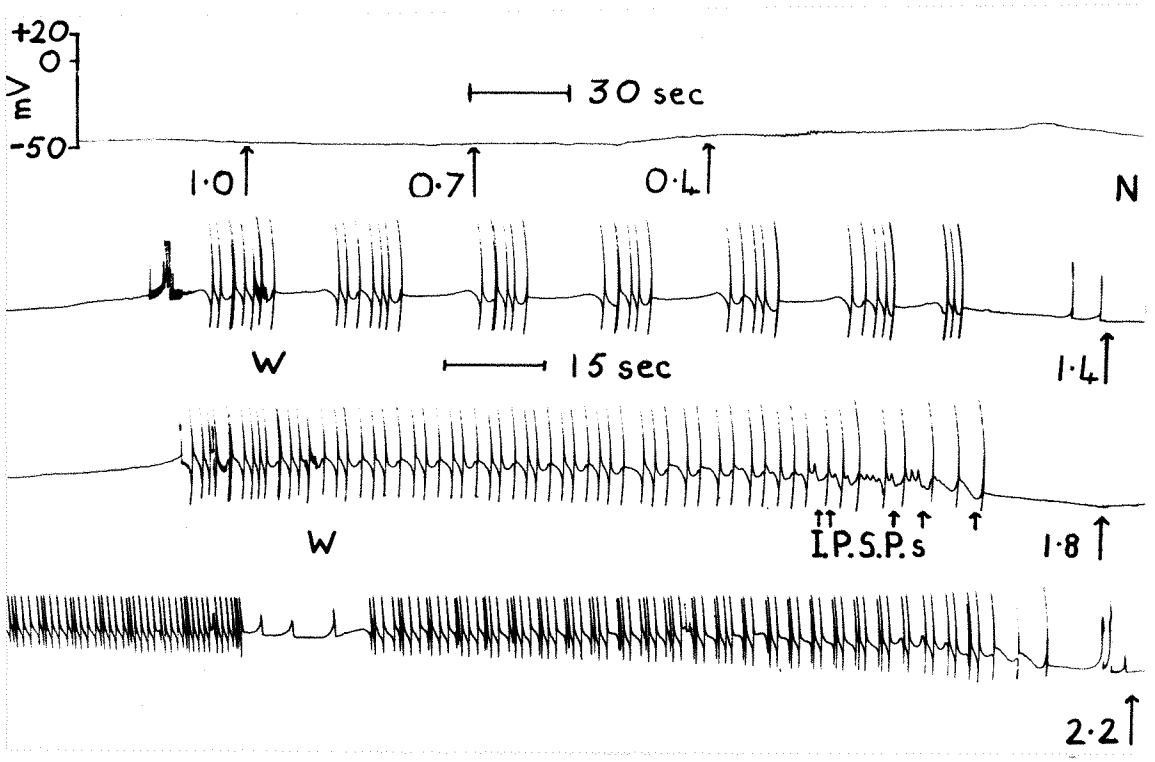


Fig.18 Pen recording of a cell responding to increasing levels of CO_2 with an autorhythmic discharge.
Traces are continuous and run R.- L. with the first taken at the slower speed. A spontaneous ripple of depolarisation may be observed in normal Ringer, (N), before CO_2 was applied. At soln. ΔpH = 1.4 , activity appeared in waves of spikes. In higher levels of CO_2 the frequency of the waves increased.

The effect of CO_2 on such a neuron is shown in fig.18. In normal Ringer this cell was silent showing a slow variation in its membrane potential as the only indication of its autorhythmicity. The cell responded to CO_2 with the usual depolarisation but with the activity in waves of spikes. As the level of CO_2 increased the frequency of the waves increased.

Resistance Changes In Autorhythmic Activity.

One possible cause of the waveform variations in the membrane potential of an autorhythmic cell is a change in the membrane permeabilities to K^+, Cl^-, or Na^+, and this might be reflected as a change in membrane resistance. To measure any such changes an autorhythmic neuron was selected which had a slow period (5 mins) in normal Ringer (fig.19). The membrane resistance (at a standard membrane potential of 80 mv) was measured by xy plotting at various points in the cycle over a number of cycles. A spontaneous variation in the resistance parallel to the wave of activity was found (fig.20), such that :-

$$\frac{\text{Resistance in Active Phase}}{\text{Resistance in Silent Phase}} = \frac{9}{7}$$

Two representative xy plots are shown in fig.22 A & B; the maximum membrane resistance occured at the end of the period of maximum activity.

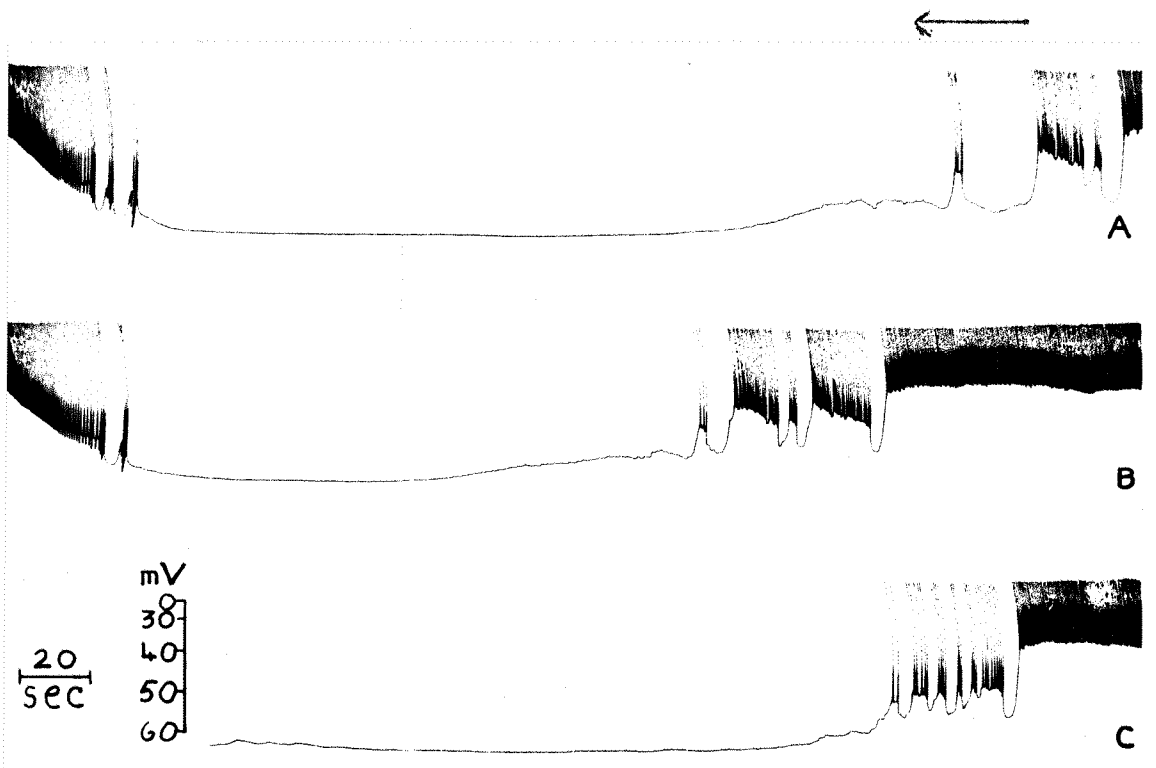


Fig.19 Activity from an autorhythmic cell.The traces run R.→ L. and are continuous.

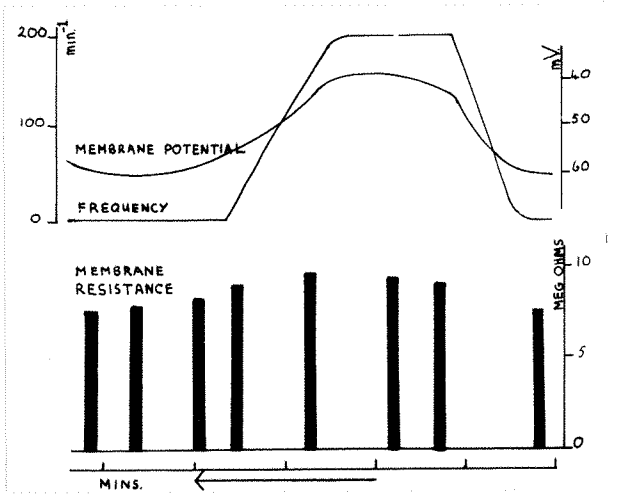


Fig.20 Changes occuring through one cycle in the above cell. The membrane resistance was measured at a M.P. of 80 mV by making xy plots at different points through a number of cycles, subsequent to those shown in fig.19.

The effect of an increase in extracellular $[K^+]$ on autorhythmicity.

With the aim of relating the CO_2 effect on autorhythmicity to the depolarising action of the gas, an attempt was made to otherwise depolarise the cell by the application of Ringer containing five times the concentration of potassium. This is shown in fig.21 and the result was not the normal depolarisation to be expected from a membrane potential dependent on a potassium diffusion potential but a small hyperpolarisation and inhibition, (c.f. fig.9E from a normal cell). This was maintained until the cell was returned to normal Ringer upon which the cell further hyperpolarised. Activity then returned with a depolarisation, some oscillation of the membrane potential, and a change in the rhythm with shorter periods of activity and much shorter periods of silence. Large changes in membrane resistance accompanied these changes (fig.22 C & D) i.e.:-

Phase of activity in normal Ringer ..... 9.5 Megohms
During mild hyperpol. of 5. $[K^+]$ Ringer ..... 3.3 "
During large hyperpol. on washing ..... 5.3 "
During depol. and high activity following ..... 10.4 "

A possible explanation for these observations and a comparison to similar properties which have been reported in the autorhythmic neurons of Aplysia will be given in the following section.

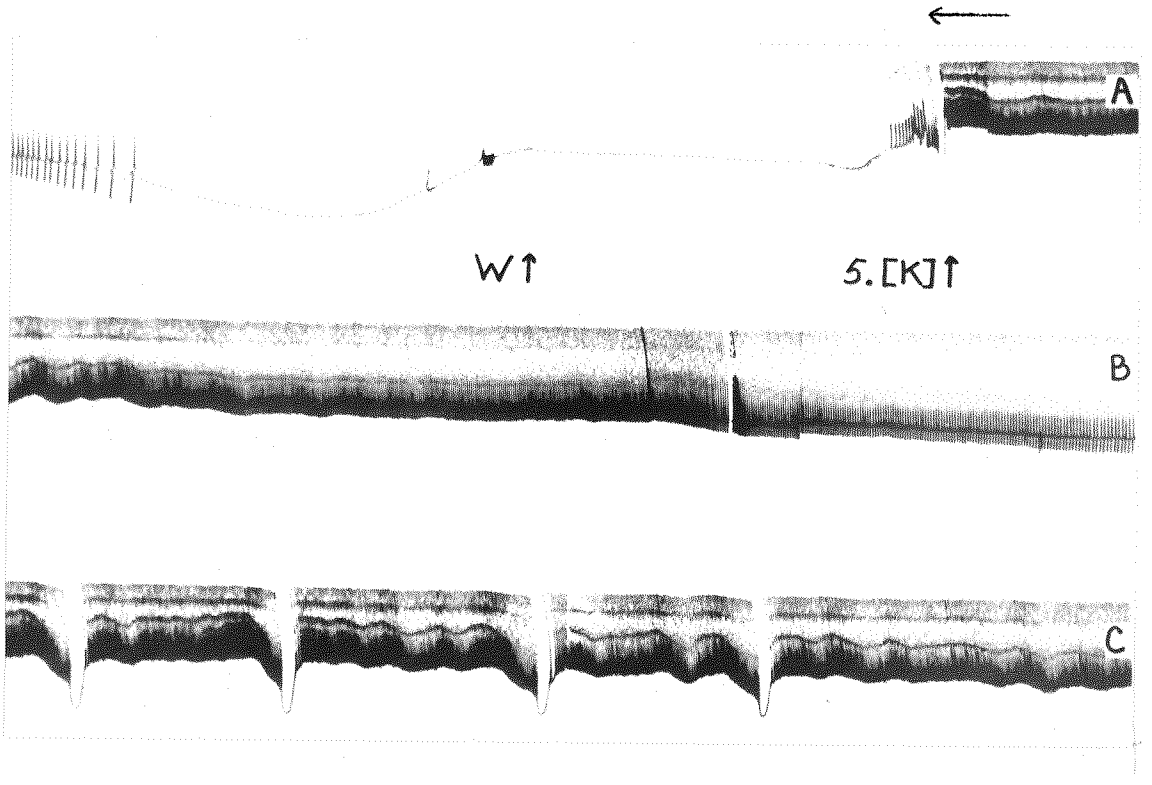
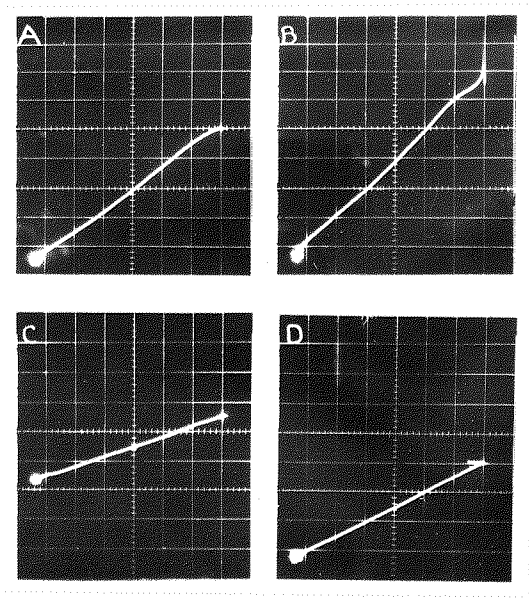


Fig.21 The effect of an increased $[K^+]$ concentration on autorhymicity. Normal activity is shown in fig.19. During the phase of depolarisation Ringer containing five times the normal conc. of potassium was applied (A); at 'W' it was washed off with normal Ringer. Traces run R.- L. and are continuous.

Fig.22 Sample xy plots to show the changes in membrane resistance during normal auto rhythmicity and with $5[K^+]$. 'A' was taken in the normal hyperpol., 'B' in the depol. 'C' was taken when hyperpol. in $5[K^+]$ Ringer, 'D' in the strong hyperpol. of washing. Horizontal divisions $\triangleq 10^{-8}$ amps Vertical divisions $\triangleq 10$ mV.



DISCUSSION

ANOXIA.

The results reported here for the neurons of Helix aspersa show that anoxia produces the same sort of depolarising effect as has been reported for the neurons of Helix pomatia, Aplysia, and mammals, and for the nerve fibres of frogs. A number of explanations have been advanced :-

1. Lorente de Nó (1947) concluded that the normal membrane potential was not directly dependent upon the ratios of the concentrations of potassium ions inside and outside the nerve fibres but on the respiratory metabolism which separated oppositely charged particles in the membrane to form electric double layers.
2. Chalazonitis (1961) suggested that in Aplysia neurons anoxia stimulated anaerobic respiration and it was the products of this (CO_2 or acidity) which caused the depolarisation.
3. Chalazonitis (1964) suggested that the membrane might be analagous to a semiconductor in which the polarisation of the surface barrier can be modified by the absorption of oxygen molecules.
4. Arvanitaki and Chalazonitis (1965) have suggested the involvement of an intracellular heme protein whose state was a function of the pO_2. This state was suggested to control "intrinsic generator currents" upon which depended the cells activity.

Experiments with intracellular recording techniques support the view that the membrane potential of a neuron is "passive" and derived from the concentration gradients of potassium or chloride ions across the nerve membrane (Davson 1964). The cell metabolism contributes energy by maintaining the concentration gradients. Baker, Hodgkin, and Shaw (1962) have demonstrated that a nerve membrane has the necessary permeability properties for this theory when they perfused the squid giant axon with various solutions and found that the intracellularly recorded membrane potential agreed closely to the theoretical values. In comparison there is little evidence to support explanation 1 and there are no experimental justifications for explanations 3 or 4. In the results reported here on the neurons of the snail the resemblance of the effect of anoxia to that of an increasing pCO_2 would suggest the second explanation to be the most likely particularly as it does not conflict with the accepted theory of membrane function.

CARBON DIOXIDE.

The method of using set concentrations of CO_2 rather than a steadily increasing value as used on Aplysia neurons showed that the CO_2 response can have a number of sequential phases:- depolarisation, increased frequency of spiking, decreased frequency of spiking, and delayed, longlasting hyperpolarisation. In the weaker concentrations of CO_2 (of pH $<$1.8) the maximum frequency and the depolarisation which generated it were proportional to the log. of the concentration of CO_2. It could

therefore be said that all snail neurons have some properties of chemoreception. In the stronger CO_2 concentrations the inhibitory phase became more pronounced and overcame the stimulatory phase with a latency which shortened as the concentration increased. Chalazonitis (1961) suggested that in the neurons of Aplysia this inhibition was a result of overdepolarisation producing a cathodal-block type of effect as suggested by Eyzaguirre and Kuffler (1955) to explain inhibition in the crayfish stretch receptor. This cannot be the case here in the snail as externally applied depolarisations failed to produce any inhibition. In the highest levels of CO_2 the inhibition was often followed by a delayed hyperpolarisation exceeding the original resting potential. Some form of chemical narcosis is probably indicated.

CO_2 AND MEMBRANE RESISTANCE.

If the membrane potential of the snail neuron depends on the selective permeability of the membrane to Na^+, K^+, and Cl^-, and can therefore be described by the Goldman equation then a depolarisation can originate from an increase in the permeability to Na^+ or a decrease in the permeability to K^+ and/or Cl^-. That a decrease in total membrane permeability (increase in membrane resistance) was measured under CO_2 indicates that the depolarisation was caused by a decrease in the membrane permeability to K^+ and/or Cl^-. A similar correlation has been found with the effect of temperature (Murray 1966) and specific illuminations (Chalazonitis 1964) on Aplysia neurons. This is in contrast with the usual mechanism of a generator potential in the

specialised sense organs which is an increase in the permeability to Na^+ (Gray 1959, Terzuolo and Washizu 1962, and Diamond, Gray and Inman 1958).

THE EFFECT OF pH.

The failure of changes in extracellular pH (using HCl) to reproduce the effects of CO_2 with the same magnitude suggests that the latter are caused by either the bicarbonate ion or the dissolved CO_2 molecule (the equilibria result in a low concentration of carbonic acid molecules). The finding that acidity produced a mild increase in the membrane resistance is opposite to that reported for Aplysia neurons (Chalazonitis and Takeuchi 1966). These workers always measured the resistance at the resting potential; as acidity reduces the resting potential and as membrane resistance has been found to be a function of membrane potential (Kandel and Tauc 1966) the decrease in resistance they report was probably attributable to the depolarisation.

Two explanations for the effect of CO_2 on membrane permeabilities may be suggested. That acidity partially reproduces the effects of CO_2 might indicate that it acts by lowering the intracellular pH. The nerve membrane of the squid has been shown to be fairly impermeable to hydrogen ions but permeable to CO_2 molecules which can diffuse through and lower the intracellular pH (Caldwell 1958). This might denature the membrane proteins or affect the dissociation of free amino or carboxyl groups near the pores. An alternative explanation for the CO_2 effect might be the precipitation of membrane calcium as the carbonate.

AUTORHYTHMIC NEURONS.

It is reported here that CO_2 increases the frequency of the rhythm of snail autorhythmic neurons and this is similar to the result reported for the corresponding neurons of Aplysia (Arvanitaki and Chalazonitis 1958). The origin of the rhythm has not been elucidated but there is evidence that it is generated intracellularly (Strumwasser 1966). The fact reported here that there is a parallel variation in membrane resistance and therefore membrane structure such that the phase of depolarisation was associated with a decrease in conductance indicates a variation in K^+ or Cl^- permeability. Strumwasser has reported that in Aplysia the hyperpolarising phase was abolished by Cl^- injection.

The hyperpolarisations that were induced here in a snail autorhythmic neuron by applying then washing off a high (K^+) Ringer were very similar to :-

a. the "on" and "off" effects of an antidromic barrage in the "Br" autorhythmic neuron of Aplysia (Arvanitaki and Chalazonitis 1964),

b. the "on" and "off" effects of a train of EPSPs on the same neuron (idem. 1965),

c. the "inhibition of long duration" which can be evoked orthodromically on certain normal snail neurons (Tauc 1959).

The effect of the high concentration of K^+ was therefore probably indirect and brought about through the stimulation of an interneuron or through antidromic stimulation. The large

increase in membrane conductance during the hyperpolarisations cannot reflect increases in K^+ or Na^+ permeability (as in the presence of the increased concentration of K^+ and the normal concentration of Na^+ in the ringer both would cause depolarisations) and therefore probably reflects an increase in Cl^- conductance.

With respect to the function of these neurons they may be first considered to confirm Adrians (1930) prediction of autorhythmicity. At present mammalian neurophysiologists have only made eight intracellular recordings from respiratory neurons (Salmoiraghi and Von Baumgarten 1960) and there is no evidence to suggest that a theory of single cell genesis of respiratory rhythm should not have equal merit to multicell theories. Finally whatever the function of these neurons may be they illustrate how important intracellular factors are in the molluscan nervous system and that it is probably a gross oversimplification to consider integration only in terms of EPSPs and IPSPs.

CONCLUSIONS.

1. The membrane of the snail neuron is reversibly depolarised by anoxia.
2. Carbon dioxide has initially the same effect as anoxia but high concentrations later produce narcosis causing the spike train to regress to a damped oscillation. These effects are slowly reversible.
3. A decrease in membrane permeability accompanies the CO_2 depolarisation.
4. A decrease in pH also produces depolarisation and some decrease in membrane permeability but is less effective than CO_2.
5. CO_2 changes the period of the rhythm of autorhythmic neurons.
6. Autorhythmic neurons show increases in membrane permeability during the phases of hyperpolarisation in their normal rhythm and during an "inhibition of long duration" induced by increasing the external K^+ concentration.

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