ABSTRACT

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Department of Physiology; Master of Science

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It was concluded that CO_2 potentiates the action of local anaesthetics by 1) a direct effect of CO_2 on the axon, 2) concentrating local anaesthetic inside the nerve trunk, 3) converting local anaesthetic to cation through its effect on H^+ and 4) summation of effects of CO_2 and local anaesthetics on the axon membrane.

CO_2 and Local Anaesthetics. R. Catchlove

THE INFLUENCE OF CARBON DIOXIDE AND pH ON LOCAL ANAESTHETIC ACTION

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

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Local anaesthetics with different combinations of CO_2 and pH were applied to sheathed and desheathed frog nerves during conventional stimulation and recording of the compound action potential. While CO_2 (1-50%) or local anaesthetic (1 mM) caused a small degree of block, together they had much greater effect, greater at a higher pH (7.3). The permeability of the nerve sheath to radioactive lidocaine and sucrose was measured by perfusing these solutions through a tube of isolated sheath. Samples were taken from a bath surrounding the sheath. The permeability to the anaesthetic was proportional to the fraction of non-ionized lidocaine. It was not changed by CO_2 or H^+.

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INTRODUCTION

History of Local Analgesia. (Davison 1965, De Jong 1970)

The application of cold was the first recorded form of local anaesthetic; about 1050 A.D. an unknown Anglo-Saxon monk described the use of cold to open an abscess. Subsequently, there were intermittent accounts, some quite practicable, of the use of cold for analgesic purposes. However, it was not used systematically, perhaps as suggested by Davison, because prior to the nineteenth century the abolition of pain was regarded as neither useful nor good.

After the introduction of a local anaesthetic drug, the use of local analgesia rapidly became widespread, suffering none of the difficulty of acceptance that had accompanied the introduction of general anaesthesia. Undoubtedly the approval eventually received by general anaesthesia over the previous thirty years and the difficulties experienced with ether and chloroform, made the concept of local anaesthetics acceptable and particularly attractive. The development in 1853 of a hollow needle by Alexander Wood and a glass syringe by Pravaz made local analgesia technically possible.

For centuries the natives of Peru had chewed the leaves of Erythroxyline coca for its stimulating properties, and it has been suggested that the natives may first have used saliva as an analgesic for wounds (De Jong, 1970). The first account of the tree was published in Europe by Pedro Ciezo de Leon in 1532. By the end of the eighteenth century, the leaf was available in Europe, and in 1855, Gaedicke isolated from the leaves an alkaloid "erythroxyline", which was later named "cocaine". The mydriatic effect of the new drug was reported in 1862,and in 1874 Bennett described an analgesic effect on mucous membranes. Moréno y Maiz, a Peruvian army surgeon, first suggested that cocaine might be useful as a local anaesthetic in a monograph on coca he published in 1868 (Von Oettingen, 1933). The monograph was largely concerned with disparaging the belief that the drug increased endurance and depressed appetite in the Peruvian peasants. It was later reported in 1889, that Fauvel, a laryngologist, had used a solution of cocaine for surface analgesia of the pharynx in 1869, but only for the relief of sore throat rather than to allow painless operation. Although suggestions were made that cocaine was an analgesic, its use became widespread only after Carl Koller demonstrated local

anaesthesia of the eye at the Congress of Ophthalmology at Heidleberg in 1884. It was probably first given by injection by H.J. Knapp about 1885, although Hirschfelder and Bieter (1932) state authoritatively that Halsted and Hall injected cocaine around their own nerves only two months after Koller's discovery. The first spinal (or epidural) injection of cocaine in a dog was done at about the same time by J.L. Corning. Regional analgesia was fully established by Braun, who realised that toxicity was related to the rate of absorption of the drug, and in 1897 advocated the addition of adrenaline to the solution.

Spinal analgesia in man was first described by Bier and by Tuffier in 1891, while epidural analgesia induced via the sacral opening, was described in 1901 by Secard and Cathelin. Epidural analgesia at a higher level was used by Pagès in Spain in 1920.

The search for new local anaesthetic drugs was stimulated by the toxicity of cocaine; for instance, in the first six years of its use,it caused ten reported deaths. The description of the chemical structure of cocaine in 1895 by Willstütter led to the synthesis of several new drugs, but the major advance was made by Einhorn

and Braun, who synthesized procaine in 1905. There were many modifications to the structure of procaine, none of which was remarkably better than the original for clinical purposes, until Löfgren synthesized the aniline derivative, lidocaine in 1943, the primary description of which was published in 1946 (Löfgren, 1946). Extensive chemical and clinical investigations were reported by him in 1948 (Löfgren, 1948).

Some Features of Local Anaesthetics

Active Form.

The mode of action of local anaesthetics has been extensively discussed since 1892, when Bignon noted that in the presence of alkali, a nerve block was obtained at a lower concentration of local anaesthetic, and concluded that the non-ionized base was the active form. This view was confirmed several times over the next half century by Gros (1910) who repeated Bignon's work with alkali, Trevan and Books (1927), and Gardner and Semb (1935) who showed an increased rate of block at high pH with many synthetic local anaesthetics when applied to frog nerve-muscle preparation; Löfgren (1948) and Ehrenberg (1948) also used the frog sciatic nerve and examined minimum blocking concentrations and rates of diffusion at different pHs. Similar findings were made by Skou (1954), Rud (1961) and Shanes (1963). Meanwhile, Krahl *et al* (1940) proposed the cation as the active form. They used CO_2 to vary pH inside and outside sea urchin eggs and established that the cation actively inhibited division of the eggs. Despite the accept-

ance of this evidence by Goodman and Gilman (1955), the work was ignored, perhaps because the experiments were not considered relevant to the peripheral nerve fibre. Krahl et al did not propose any role for CO_2 in their experiments save that of altering pH. It was not until Ritchie and Greengard (1961) produced evidence that the nerve sheath (epi- and peri-neurium) was a diffusion barrier to local anaesthetics and proposed that they diffused through the membrane as the base, and acted on the membrane as the cation, that any doubt was entertained about the active form.

Ritchie et al (1965 a, b) and Ritchie and Greengard (1966) obtained further support for these findings in subsequent experiments. Using single frog nerve fibres, Dettbarn (1962) also found the anaesthetic cation to be most active. A theoretical physico-chemical analysis of drug action, including local anaesthesia, by Arfens and Simonis (1963) came to a similar conclusion. However, Shanes (1963) had split the nerve sheath, and Skou used desheathed nerves, and both had concluded that procaine and other local anaesthetics were more active as the base. This difference of opinion was satisfactorily resolved by Strobel and Bianchi (1970 a, b). They applied procaine and lidocaine sepa-

rately to sheathed and desheathed nerves until partial block, and then exposed the nerves to Ringer's solution at different pHs. The block was relieved by high pH and reinstituted by low pH, showing that the cation blocked more effectively. Their studies on uptake established the base as the most diffusible form. They were not able to explain fully Shane's findings, but suggested that the desheathing may have been incomplete. It was also found (Camougis et al, 1967) that in frog nerve, tetrodotoxin is most active as the cation, rather than the zwitterion.

Mechanism of Action.

The first suggestion that local anaesthetics worked by interfering in some way with sodium mechanisms in the nerve was made by Lorento de Nó (1950), who found that nerve fibres were more susceptible to the action of cocaine in low Na^+ solutions. He also thought that cocaine could to a certain extent temporarily replace Na^+. An extensive analysis of the phenomenon on bullfrog A and C fibres followed (Lorente de Nó, 1951). The sensitizing effect of low Na^+ on cocaine block was confirmed by Crescitelli (1951)

for desheathed bullfrog nerves. Before this time, no such convincing relationships had been shown (Toman, 1952). The observations were substantiated by Condouris (1961), and extended to procaine and lidocaine by Shakalis and Condouris (1967), who used low Na^+ solutions and adrenalectomized bullfrogs. Their concept of the mode of action of cocaine was one of competitive inhibition between cocaine and Na^+. Meanwhile, Taylor (1959) showed that in the voltage clamped squid axon (Cole, 1949; Marmont, 1949; Hodgkin, Huxley and Katz, 1952), procaine caused a reduction in the amount and rate of development of the early transient (sodium), and late steady state (potassium) currents which occur during a depolarizing voltage step, (Hodgkin and Huxley, 1952 a,b,c), and he concluded that local anaesthetics block the action potential by reducing the permeability of the membrane to Na^+. Shanes et al (1959) showed similar effects of cocaine and procaine. They also demonstrated an interaction of Ca^{++} and local anaesthetic such that while the threshold was raised by both increased $[Ca^{++}]$ and local anaesthetic, the height and rate of rise of action potential was decreased by local anaesthetic, but was increased by raised $[Ca^{++}]$. With weak depolarization, high concentrations of Ca^{++} reduced Na^+ and K^+ currents, whereas

with strong depolarization the currents were greater with high Ca^{++}; this was the mechanism by which procaine block was overcome. Aceves and Machne (1963), in the frog spinal ganglion, and Blaustein and Goldman (1966), in the lobster axon, confirmed that local anaesthetics and Ca^{++} had similar but competitive actions on the nerve membrane. The latter authors suggested that procaine and Ca^{++} compete for certain sites on the membrane, and that whereas Ca^{++} has a facilitatory action on Na^{+} conductance (g_{Na}) increase during an action potential, local anaesthetics do not, and in fact prevent the increase in g_{Na}. Feinstein (1964) showed competitive binding of Ca^{++} and local anaesthetic to various lipids and suggested the binding is to $PO_3^{=}$ groups of two fatty acids, such as phosphatidyl serine and phosphatidyl ethanolamine. Hille (1968 a,b) has made similar studies on the single node of Ranvier of the frog nerve.

Site of Action.

In experiments on single frog myelinated fibres placed on a series of ridges to isolate a Ranvier node by air gaps, Kato (1936) and Tasaki (1939) showed that local anaesthetics acted only on the nodal membrane, and not on

the myelinated internodal regions. Apparently the local anaesthetic was assumed to act on the outside of the membrane (see model of Ritchie and Greengard (1966, p. 418)). Narahashi et al (1966), using the perfused squid axon, in fact showed that the large,highly ionized molecule of tetrodotoxin blocked the action potential and the increase in g_{Na} only when applied externally. An early disparity in results obtained by Nakamura (1965) in a similar preparation, was resolved in favour of the outside of the membrane. However they were not able to show a similar site of action for local anaesthetics; by applying lidocaine and two lidocaine analogues (with high and low pKs respectively) to the inside and outside of the membrane at different pHs, they very clearly located the site of action (in the squid axon) as the internal surface of the membrane; and at the same time they confirmed that the cation was the active form of the local anaesthetic (Narahashi et al, 1967, 1968, 1970; Frazier et al, 1970). If one examines in detail the previous observations, particularly by Dettbarn (1962) on single frog nerve fibres, Ritchie et al (1965 a) on the desheathed rabbit vagus, and Strobel and Bianchi (1970 a) on the desheathed frog sciatic, it is apparent that there must be some external action on

these fibres, contrary to the findings in the squid. Some of the results of the present investigation also suggest this and will be discussed later.

Barriers to Diffusion of Local Anaesthetics.

As mentioned above, Ritchie and Greengard considered the nerve sheath as a barrier to the diffusion of local anaesthetics, but the demonstration that the perineurium was a diffusion barrier to many substances had been difficult. Feng and Gerard (1930) found that splitting the sheath greatly accelerated block of conduction by increased concentrations of KCl, $CaCl_2$, etc. This idea was accepted by many authors, Feng and Liu (1949), Hodgkin (1949), Huxley (1949), but was very strongly opposed by Lorente de Nó, who wrote extensively on the subject (Lorente de Nó, 1947, 1950, 1951, 1952). Experiments on the perfused hindlimb of the frog by Krnjević (1954), and on the isolated sheath by Shanes (1954) and Crescitelli (1951) quite clearly demonstrated that the rate of diffusion of several cations through the sheath is less than that during free diffusion in water. While the sheath is a barrier to electrolytes

and to relatively large molecules, it is permeable to lipoid soluble substances (Feng and Gerard, 1930). The role of the sheath was further analysed by Sunderland (1965) and Shanthaveerappa and Bourne (1966) who stressed its mechanical properties as well as its role in isolating nerve fibres from the surrounding tissues.

Effects of CO_2 on Peripheral Nerve

Local anaesthetics and CO_2 were first tested in combination by Condouris and Shakalis (1963, 1964) because of the apparently similar mode of action of each. The effect of CO_2 alone on isolated nerves has been studied since the nineteenth century, and in fact J.Y. Simpson had even tried it as a local anaesthetic, without success (quoted by Davison, 1965). The work of Davis, Pascual and Rice (1928), Necheles and Gerard (1930) and Hettwer (1938) established that CO_2, when applied to the region stimulated, caused an increase in the threshold of excitation, related to the concentration of CO_2. There was also an increase in the amplitude of the compound action potential, quickly followed by depression as the concentration of CO_2 was raised. Straub (1956) using the sucrose gap method of Stämpfli (1954), found in frog nerves a hyperpolarization of 1.6 mV during the application of 5% CO_2 and a reduction in the depolarization caused by 160 mM NaCl. CO_2 had no effect on nerves in Na-free solutions. He concluded that CO_2 might act on the "sodium carrying

system", either directly or indirectly, by producing intracellular pH changes. Although Caldwell (1958) described quite marked depolarization of squid giant axons by 100% CO_2, this may have been caused by hypoxia since the effect became greater with time. Most reports indicate that CO_2 may have several effects on cells; Chalazonitis (1963) reported depolarization and increased activity of the neurones of Aplysia, but a decrease in firing rate if the cells were already active. Similarly, Walker and Brown (1969) and Brown et al (1970) found that some Aplysia cells were depolarized by CO_2 and others hyperpolarized. Papajewski et al (1969) discovered a similar variety of membrane potential changes in cat spinal motor neurones which were associated with an increase in membrane resistance (R_m). Krnjević et al (1965) found an increase in excitability of mammalian cortical neurones with small increase in P_{CO_2}, followed by decrease in excitability as P_{CO_2} rose further.

Walker and Brown (1969), Brown and Berman (1970), Brown et al (1970) inserted microelectrodes specific for Cl^- and K^+ into neurones of Aplysia while superfusing them with solutions with various concentrations of CO_2

and H^+. They also measured the membrane potential (E_m), and resistance (R_m). The changes in E_m and R_m did not occur when the pH of CO_2-containing solutions was maintained at the normal value. The changes found with CO_2 and lower pH were the same with solutions at the same pH without CO_2. Experiments in which the external [Cl^-] was halved, suggested that g_{Cl} was changed, but several other unexplained effects were also noted in these experiments. The slope of regression of E_m on outside [Cl^-] was increased by changing the external pH from 8·0 to 5·0. The change was not very clear and unfortunately no statistical confirmation was presented. With the chloride electrodes, two distinct groups of internal [Cl^-] activities were found, with the value for E_{Cl} either above or below the E_m of the cell. The response of the chloride electrode was very clearly related to the chloride concentration of test solutions, but its specificity was not high.

Alterations of the Na^+ concentration had no effect on the changes induced by H^+, and the rate of rise of the action potential was unaffected by pH. The authors therefore attributed the effects of CO_2 on Aplysia cells to changes in the external pH altering g_{Cl}, and to a lesser

extent, g_K. Thus while the effects of CO_2 on excitable tissue are well documented, the mechanism of this action is still far from clear. This subject will be examined further in the discussion.

Interaction of CO_2 and Local Anaesthetics.

From what has been said so far, it can be seen that one way in which the local anaesthetics could be made more effective would be to enhance diffusion through the perineurium and other tissue barriers. Alkalinization of the injected solution, by increasing the concentration of the base was an obvious step. For instance, 4% procaine when applied to the cornea is ineffective, but together with $NaHCO_3$, it causes potent anaesthesia (de Jong, 1970, p. 71). Unfortunately, alkalinization tends to precipitate the relatively insoluble base, making the local anaesthetic unsuitable for the storage and sterilization and so this technique is not employed.

The apparently similar actions of local anaesthetics and CO_2 on peripheral nerves prompted Condouris and Shakalis (1963, 1964) to try the experiment of applying

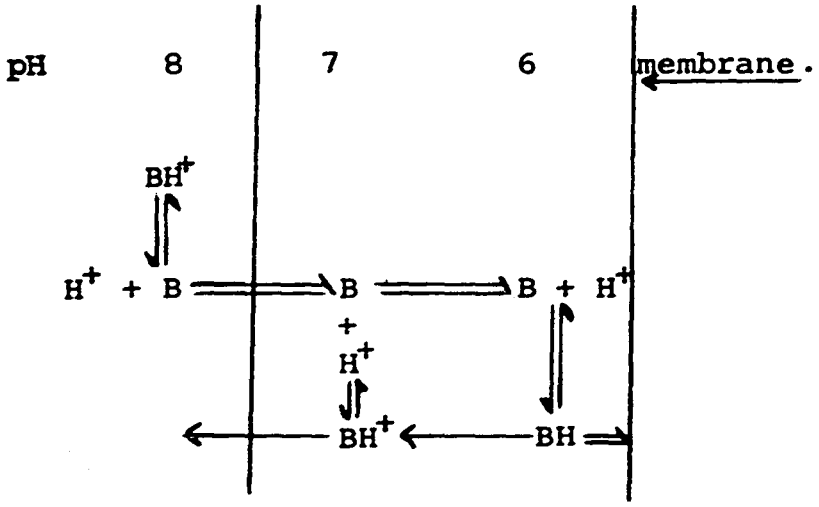
them simultaneously to rat sciatic nerves. At 35°C, the nerves were first equilibrated with Tyrode's solution, at a pH of 7·2 to 7·4, saturated with various CO_2 concentrations. This saturated solution also containing cocaine, procaine or lidocaine was then applied, resulting in much faster rate of block of the compound action potential than when the local anaesthetic was applied without CO_2. They were not able to explain their findings satisfactorily, and have not pursued them further.

Bromage first reported the results of epidural analgesia in humans with local anaesthetic solutions equilibrated with 100% CO_2 ("carbonated solutions") (Bromage, 1964). He used carbonated solutions at the suggestion of Dr. A.P. Truant (Bromage, 1971, personal communication), who had been told by an engineer testing various local anaesthetics in aerosols, that much faster numbing was obtained when CO_2 was the propellant, than with other propellants. The injection of the carbonated local anaesthetic into the epidural space resulted in a faster onset and a more profound and longer-lasting block than with normal solutions. These findings were extended in subsequent papers (Bromage, 1967; Bromage et al, 1967). Recently,

Schulte-Steinberg et al (1970) reported similar effects when carbonated solutions were used for brachial plexus block.

Bromage et al (1967) suggested that CO_2 acted largely by facilitating the diffusion of local anaesthetics across diffusion barriers. They envisaged a series of barriers which were freely permeable to CO_2, partly permeable to local anaesthetics, and impermeable to H^+. CO_2 would diffuse rapidly from the injected solution (previously buffered to pH 6·5) into the compartments of the whole nerve, lowering the pH as it passed to each compartment (see below).

Fig. 1



If the pH of the original solution were made high, or became higher because CO_2 diffused from the solution, there would be different pHs in the various compartments, and if these were in the order shown in the diagram, the highest concentration of base would be in the compartment with high pH, and the lowest in the low pH. This follows from the law of mass action and the pk_a of the drugs:

$$\text{Base} + H^+ \rightleftharpoons BH^+ \qquad 1.$$

$$B \times H^+ \rightleftharpoons k_a \times BH^+ \qquad 2.$$

taking negative logarithms and rearranging,

$$pH = pk_a + \log \frac{[B]}{[BH^+]} \qquad 3.$$

Consequently, if the diffusion barriers are permeable to the base, it will diffuse along the concentration gradient from the first compartment of high pH to the second with a low pH, where it will become ionized (eqn. 1). Diffusion of cation in the reverse direction is comparatively small because of the impermeability of the sheath, so the diffusion gradient is maintained. As a result, the cation accumulates in the second compartment. If the pH of this

compartment is higher than a third adjacent compartment, the process will be repeated. If the pH is lower, no such process can occur.

Bromage et al also observed that solutions containing CO_2 alone were able to reinstitute a waning local anaesthetic block, and so suggested that a direct action of CO_2 on nerves contributes to the efficacy of carbonated local anaesthetic solutions.

There has been no further development of the above model. The number of barriers has not been specified, nor have the diffusion characteristics of these barriers. The pH changes proposed in the compartments have also not been examined experimentally. The pHs indicated in the model seem highly unlikely.

Aims of the present work.

The purpose of the present work was to investigate by in vitro experiments the mechanism of the observations which had been made during the clinical use of carbonated local anaesthetics, and to try to establish the validity

and limitations of the model proposed by Bromage et al (1967). Furthermore, it was hoped that the interaction of local anaesthetic and CO_2 might help to explain the way in which each drug acted on the neurone, and give some indication of their site of action. It was hoped that the role of the perineurium as a barrier to local anaesthetics could also be more precisely defined. The initial electrophysiological experiments essentially mimicked the in vivo trials. They involved the recording of the compound action potential when the nerve was exposed to a variety of conditions. As these experiments progressed, it became obvious that more direct information about the perineurium and CO_2 and local anaesthetics was required. Therefore studies were made of the passage of radioactively labelled compounds through the nerve sheath. A model was constructed to explain the results of these experiments.

METHODS

Two quite different methods were employed, and for clarity it is necessary to describe each separately. Similarly, because the results of each are in different terms, the results will be reported separately and brought together in the discussion to produce a unified account of carbonated local anaesthetic.

Electrophysiology of Isolated Nerves

Preparation.

Frogs (Rana pipiens) were decerebrated and pithed and the lower half of the body stripped of skin. One sciatic plexus,nerve and its tibial and peroneal branches were exposed and tied at both ends. The nerves were removed with as little adventitious tissue as possible. The other nerve remained *in situ* until required.

Nerves were desheathed by removing their epineurium and perineurium for about 1·5 cm from just below

the start of the sciatic nerve. They were tied with silk at both ends and pinned to the wax floor of a bath of Ringer's solution. Under a binocular dissecting microscope, the perineurium was cut at a branch with fine forceps and scissors. A small amount of tension on the perineurium made it more visible. It was divided circumferentially and rolled distally and removed. After removal of the sheath the nerve fibres could be quite easily separated, and microscopical examination of stained sections of the removed sheath showed one or two layers of cells, indicating complete desheathing. The frog perineurium is known to be only one or two cells thick (Krnjević, 1954; Bourne, 1968).

In initial experiments, the various solutions were tested on one nerve with an intact sheath and on the desheathed nerve from the other side. In some later experiments the nerve was first tested intact, then, after soaking for several hours in Ringer's solution at a low temperature, was desheathed and retested.

Nerve Chamber.

For stimulating, recording and the application of solutions, the nerve was placed in a plexiglass nerve bath (Fig. 2). The nerve was carefully surrounded by vaseline where it passed through each partition.In this way, six well-separated compartments were formed. The first two compartments were 1·3 x 0·3 x 2 cm; each contained a platinum stimulating electrode, with the pair of wires 0·4 cm apart. The next chamber was 1·0 x 2 x 1·3 cm, with inflow and outflow holes in the side walls and was closed by a glass cover sealed with vaseline. This was the test chamber through which Ringer's or other solutions flowed continuously via a three-way tap. The solution was removed by siphon. The time taken to completely empty and refill the test chamber was five seconds.

The last three chambers were 0·7 x 2 x 1·3 cm. The one nearest the test chamber served to isolate the test solution further from the platinum bipolar recording electrodes. These electrodes were in the last two chambers and were 1 cm apart. A platinum ground electrode was placed in the proximal recording chamber. All compartments except the test chamber were filled with Ringer's solution.

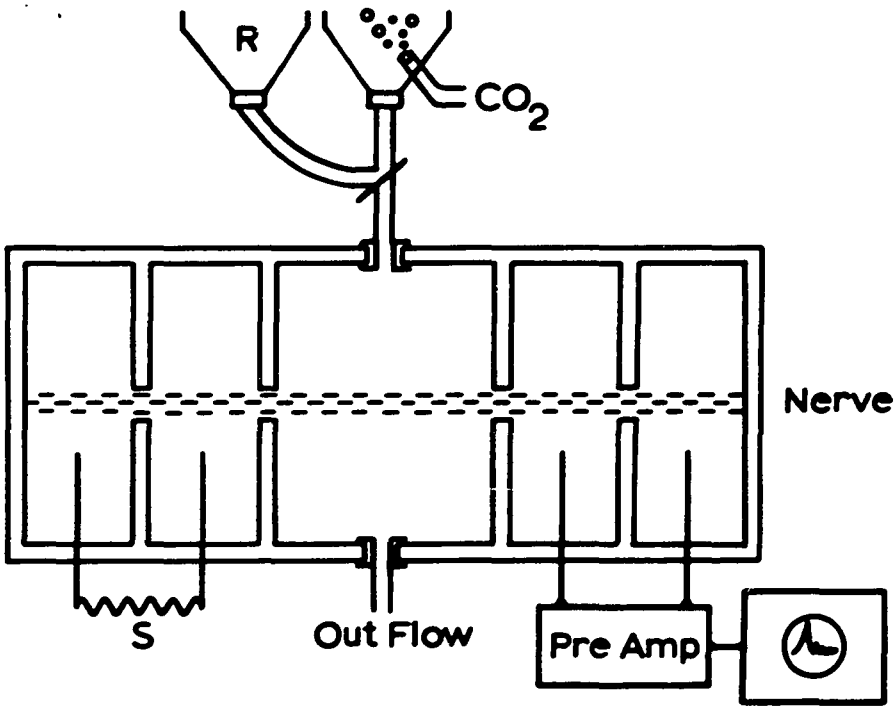


Fig. 2

Diagram of apparatus used to test effect of drugs on compound action potential of frog nerves. Ringer (R) or test solution was selected by three way tap. Test compartment closed by glass side. Outflow was by siphon. Other compartments were filled with Ringer. S, Grass SD 5 stimulator, 1/sec. Compartments completely sealed around nerve with vaseline.

When solutions were being applied randomly, the test chamber was flushed with Ringer's solution to allow complete recovery of the action potential between tests. In order to show that the result of any test was uninfluenced by a previous test, experiments were done in which the nerve was removed and soaked in Ringer's solution for two to three hours between tests. The results were the same as those obtained with the previous method.

The pH of the outflow solution was tested at intervals and was always within 0·05 pH units of the inflow pH. Tests on frog nerves were performed at room temperature (21° C).

Three Sprague Dawley rat sciatic nerves were obtained from anaesthetized rats and tested at 34·5° C with local anaesthetic and CO_2 in Tyrode's solution. They were tested first intact and then desheathed and tested again.

Nerve Stimulation.

Square wave stimulating pulses were delivered at one per second through the platinum electrodes by a Grass

S.D.5 stimulator. The characteristics of the supramaximal stimulus were: Duration 0·12 m sec amplitude 3 Volts. Maximum response with this duration was obtained with 0·5 Volts.

Recording.

The compound action potential was recorded by the other pair of platinum electrodes, amplified by a Tektronix RM 122 preamplifier and displayed on a Tektronix 502 dual beam oscilloscope. The oscilloscope gain was adjusted to give a deflection of exactly 10 cm for the $A\alpha$ component of the control compound action potential. The actual potential recorded was 7 - 20 mV, most commonly about 10 mV in amplitude.

The deflection was measured on the graticule at known intervals after the application of the test solution and in earlier experiments recovery was also recorded. The recovery was complete, but its rate varied with different test solutions. The test was usually applied until complete block, except where this would have taken a very long time.

Only partial block was sought when using low concentrations of local anaesthetic.

Solutions and Drugs.

The Ringer's solution was (Strobel and Bianchi, 1970a): NaCl 111 mM, KCl 1·6 mM, $CaCl_2$ 1·0 mM, Tris 10 mM, adjusted to pH 7·3 with HCl. The Tris buffer has the advantage over the phosphate buffer that with increased pH, it does not precipitate Ca^{++}.

Lidocaine HCl 1 mM (Astra Pharmaceuticals, Canada) in Ringer's solution was made each day from stock. In experiments with partial block 0·25 mM was used.

Procaine (Abbott Laboratories, Chicago) was used as 2 mM and 0·25 mM, diluted from a freshly made concentrated solution.

CO_2 in oxygen was delivered from pre-mixed cylinders, the correct concentrations of which were determined in duplicate on a micro-Scholander or Haldane apparatus. The gas was bubbled into the test solutions through a

sintered disc. As judged by measurement of pH, the time to full equilibration was about five minutes.

The pH of the equilibrated solutions was measured with a Sargent macro glass electrode and Radiometer PHM 22 pH meter. The pH of the solutions, provided they were not agitated, remained constant for at least twenty minutes, even while CO_2 was not being bubbled through. The pH was adjusted to the required value with HCl, $NaHCO_3$, or NaOH, without replacement. With very high concentrations of CO_2, (50%), adjustment to pH 7·3 required so much $NaHCO_3$ that the osmolarity was more than doubled. Therefore, control experiments were done at other pH values with the osmolarity increased to the same extent with NaCl, and by using lower concentrations of CO_2 the pH was adjusted by replacement of NaCl with $NaHCO_3$ to maintain normal osmolarity. The concentrations of CO_2 used were 52%, 20%, 9·6%, 4·5%, 2·4%, 1·1%. Details of pH, CO_2 and local anaesthetic concentrations are listed in Table 1.

TABLE 1

LOCAL ANAESTHETIC AND CONCENTRATION	pH	% CO_2	OSMOLARITY in mM
None	7·3	52	2·4x
	5·5	52	N
	5·5	0	N
Lidocaine 1 mM	8·3	0	N
	7·3	0	N
	5·5	0	N
	7·3	52	2·4x
	5·5	52	N
	5·5	52	2·4x
	7·3	20	1·5x
	7·3	9·6	N
	7·3	4·5	N
	7·3	2·4	N
	7·3	1·1	N
Procaine 2 mM	7·3	0	N
	9·3	0	N
	7·3	52	2·4x
	5·5	52	N
Lidocaine 0·25 mM	8·3	0	N
	7·3	9·6	N
	5·5	9·6	N
Procaine 0·25 mM	9·3	0	N
	5·5	9·6	N

Composition of the solutions applied to sheathed and de-sheathed sciatic nerves. N is normal osmolarity.

Perfusion of the Isolated Sheath

The purpose of extending this investigation to an examination of the nerve sheath was to obtain direct information about the permeability of the sheath to local anaesthetics, and the influence of pH and CO_2 on this permeability.

Preparation of the Perfused Sheath.

The nerve was removed in a manner similar to that previously described. The nerve was carefully cleaned of all visible adventitia which otherwise might have obstructed the lumen of the tube formed. The sheath was circumferentially divided just above the main branch of the sciatic nerve, and rolled distally. The nerve was cut just above the division of the sciatic and the sheath rolled off. The ends of the sheath were slipped over thin walled twenty gauge needles, the distance between which could be varied to ensure that only sheath free of branches lay between them. The sheath was tied firmly

to the needles with fine silk strands. The tube thus formed was an inverted length of perineurium, typically 1 cm long; in effect, fluid flowing through the tube was in contact with the original external surface of the sheath. A small plexiglass bath containing Ringer's solution at pH 7·2 was placed around the sheath and stirred by a vibrating rod. (Fig. 3)

The proximal needle was connected via a three-way tap to two reservoirs containing ^{14}C and/or ^{3}H labelled drugs in Ringer's solution. The fluid level was an average of 9·5 cm (range 8 - 12·5 cm) above the sheath. This would be the pressure in the tube only when the fluid was not flowing, because the resistance of the needles and the tube during flow would reduce the pressure reaching the tube.

The apparatus was observed with a binocular microscope for leaks whilst washed particles of India ink and China ink flowed through it. No leakage was seen with normal perfusion pressures, but some was seen when the perfusion pressure was trebled or fluid was forced rapidly through by a syringe.

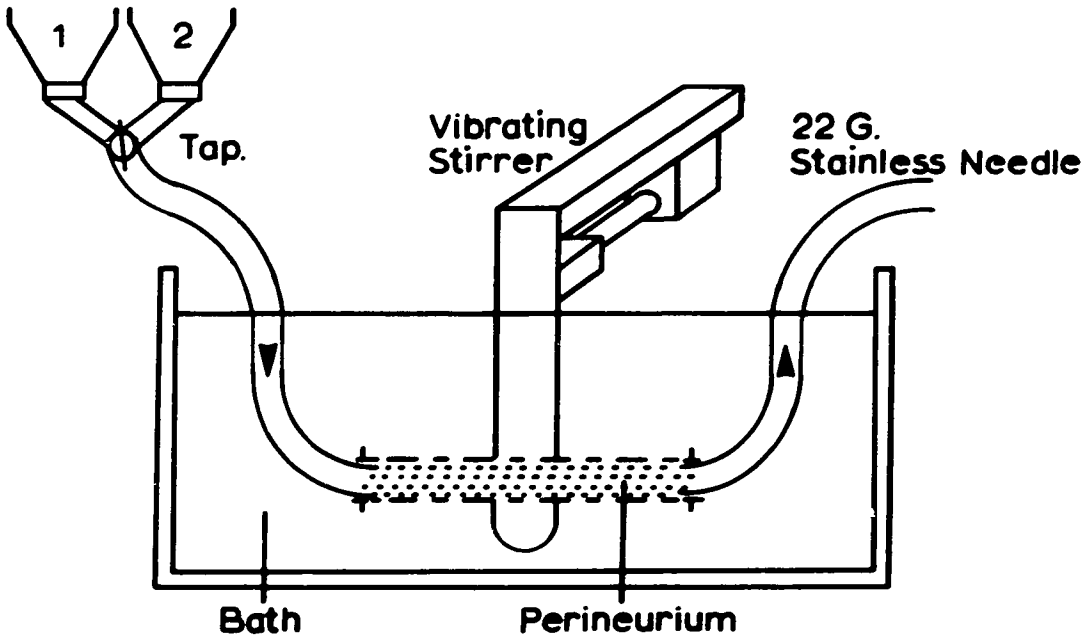


Fig. 3

Diagram of apparatus used to measure permeability of nerve sheath. Radio-active material flowed through sheath from reservoirs 1 or 2. 100ul samples were taken from bath. Average length of sheath 1 cm. External bath was continuously stirred by vibrating rod.

The length, diameter and wall thickness of each sheath were measured with a calibrated eyepiece in the binocular microscope at 40 x magnification. The thickness of the perineurium was also measured from histological sections of sheaths and intact nerves. The sheath was soaked in 10% formaldehyde for twelve hours, then perfused with and embedded in warm molten wax. A further group was perfused with agar after fixation, and frozen in embedding compound on a Reichert microtome, and sectioned. Similar procedures, except for the perfusion, were done on the whole nerve. Sections of 40 u were made on the microtome, stained with haematoxylin and eosin and examined microscopically.

Perfusing Solutions.

Ringer's solution containing ^{14}C lidocaine HCl (AB Astra, Södertalje, Sweden - specific activity, (sp.a.) 0·77 uC/mg), ^{14}C sucrose (New England Nuclear sp.a. 14·6 uC/mg), ^{3}H dextran (New England Nuclear sp.a. 0·625 uC/mg), at various H^{+} and CO_2 concentrations (see Table 2). The radioactivity in the solutions was approximately 5×10^5 DPM/ml.

In one type of experiment CO_2 was bubbled through both the perfusate and the bath. The pH was measured and adjusted as in the experiments on nerves. The different solutions were applied in random order, except that the solution first used was also the last used, thus serving as a check that there had been no radical change in the diffusion characteristics of the sheath.

Samples of 100 ul were taken from the bath at regular intervals with a lambda pipette, most commonly every three minutes, and immediately replaced with fresh Ringer's solution. Each test solution usually ran through the sheath for fifteen minutes and was then changed about 15 - 30 seconds before the end of the fifteen minute interval, to allow for the dead space of the system.

The effect of CO_2 on accelerating the diffusion of lidocaine into the lumen of the sheath was studied by placing a large bath containing ^{14}C lidocaine around the sheath, and flowing Ringer's solution through the sheath. The external solutions were: ^{14}C lidocaine (approximately 1 mM) at pH 7·3 and 0% CO_2, at pH 8·3 and 0% CO_2, and at pH 7·3 and 9·6% CO_2. The pH of the CO_2 solution was

adjusted by replacing NaCl with $NaHCO_3$. The pH of the solution was continuously monitored with the glass electrode and pH meter. The Ringer's solution was buffer-free and had a pH of 7·3. It flowed as slowly as possible from a very large reservoir. The outflow was collected directly into scintillation vials for a timed period. The actual flow rate was not measured, but the drip rate into the vials was kept constant throughout each experiment. On several occasions, the pH of the outflow was measured when the external bath was equilibrated with CO_2. These experiments were designed to determine whether in fact CO_2 could increase net flux across the sheath by creating a pH gradient.

Scintillation Counting.

A scintillation fluid was used which would accept up to 1·2 ml of water per 15 ml with minimal quenching. (Patterson and Greene, 1965). This was a 2/1 mixture of toluene (Fisher) and a detergent, Triton X-100 (Packard), with 2,5 Diphenyloxazole (PPO) 5·5 Gm./L, and 1,4 - bis - [2 - (4 dimethyl-5-phenyloxazolyl)] - Benzene (Dimethyl POPOP) 0·1 Gm./L. A quench curve was made by adding to

ten vials of fluid, a known fixed amount of ^{14}C toluene (N.E.N. sp.a. 2·6 x 10^{-4} uC/mg) with dilute picric acid to add colour quenching. These and all the other samples were counted for twenty minutes on a Packard Tricarb 3375 liquid scintillation spectrometer at 16·2% gain, with discriminators at 40 and 1000. The External Standard Ratio (ESR) and counts per minute (CPM) were obtained for each vial. CPM for each of the ten vials was divided by the known added disintegrations per minute (DPM) to derive the counting efficiency of each. The ESR was plotted against DPM for each vial and a regression line drawn through the points by the method of least squares, using a Focal programme on a Linc - PDP 8 computer. The regression equation CPM = 1·895 x DPM + 0·6256 was used to correct the CPM for quenching, and hence for any loss of counting efficiency in subsequent vials with unknown activity. In some experiments simultaneous counting of ^{14}C and ^{3}H was necessary. A correction for quenching was made as above. The fact that the mean energy levels of ^{14}C and ^{3}H are 0·05 Mev and 0·0055 Mev respectively, allows fairly reasonable separation of the emission of each by appropriate settings of the discriminators (Fig. 4). These were:

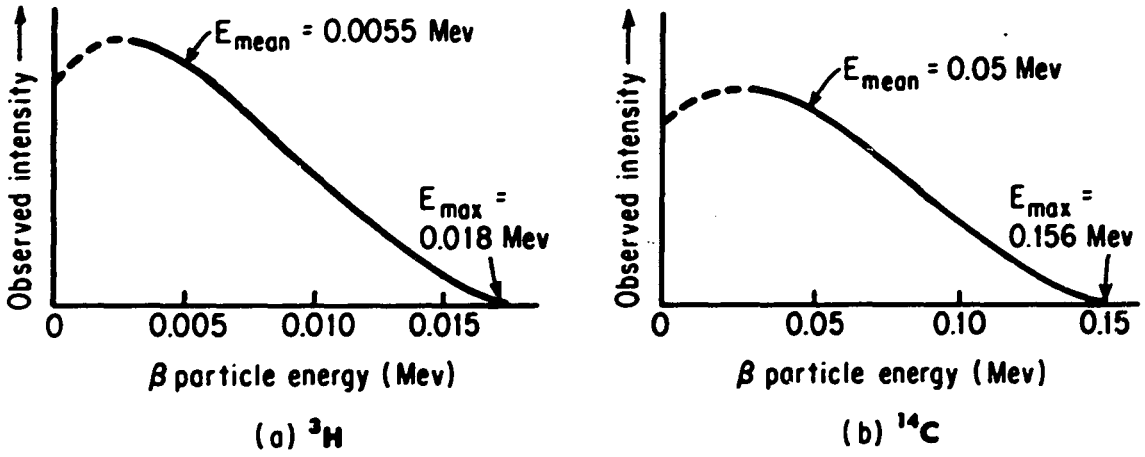


Fig. 4

Energy spectra of ^{14}Carbon and ^{3}Hydrogen (from Wang C.H. and Willis D.L., Radiotracer Methodology in Biological Science, Prentice-Hall; Englewood Cliffs, 1965).

^{14}C 16·2% gain, 220 - 1000, and for ^{3}H 40% gain, 45 - 160, resulting in a count of 63% of the total counts for the ^{14}C channel, and coincidentally 63% of the total counts in the ^{3}H channel. With these settings, there was spill of 9·7% of total CPM for ^{14}C into the ^{3}H counting range, but there is no detectable spill of ^{3}H into the ^{14}C range. Corrections have to be made for the overall counting efficiency (quenching), the fraction of the true total counted in each range, and in the case of ^{3}H, the spill of 9·7% of the total CPM of ^{14}C into the ^{3}H range. The values shown above were arrived at largely by trial and error to attain the optimum settings. The various percentages were calculated by counting six samples each of ^{3}H and ^{14}C with known DPM.

RESULTS

Calculations

The mean and standard error (S.E.) of the compound action potential height was calculated for each 0·5 or 1 minute interval for each form of treatment applied, and a curve fitted to the points by eye. In most cases, the effects were clearly indicated by these curves alone, and no other calculations were required. Nevertheless, in an attempt to have some statistical expression of differences in slope, the regression of the height and the logarithm of the height of the action potential on time was calculated for each experiment (approximately 150 experiments), as Ritchie *et al* (1965) had stated that the initial rate of block was approximately linear. However, the y - intercept (control height) which should have been 10 cm, was very often significantly different from this value. Furthermore, the S.E. of the slope was often very large, also indicating poor fit of the regression; that is, the initial rate of block was frequently non-linear. It was therefore decided that the analysis of covariance which had been planned, and for which a programme had been written, was not statistically

valid and could not be done. Because a measure of the rate of block was required for other calculations, the action potential height after 3 minutes of application of the test solution was calculated for each treatment. The action of the various agents could be most easily seen after about 3 minutes. Hereinafter this will be referred to as the T_3 and will be expressed as a percent i.e. 45% would indicate that the height of the action potential was 4·5 cm (or had been reduced to 45% of its initial value).

Permeability Constant.

A permeability constant for ^{14}C lidocaine and ^{14}C sucrose P, was calculated by the method of Davson (1959).

$$P = \frac{Q}{A\ (C_i - C_o)}$$

where Q = total efflux in DPM/sec.

A = surface area of the sheath in cm^2.

C_i = Internal activity in DPM/ml.

C_o = External activity in DPM/ml.

An apparent diffusion coefficient was obtained by multiplying P by the estimated sheath thickness (L).

$$D = P \times L$$

Statistical Analysis.

The regression analysis mentioned in previous sections was calculated by the method of least squares (Williams, 1959).

Student's 't' test was used for testing differences between permeability constants of sucrose and lidocaine with different test conditions, provided both sets of data were normally and similarly distributed. The similarity of distributions was checked by determining the F ratio for each set of data. Otherwise the Wilcoxon Rank Test was used (Campbell, 1967, p. 49).

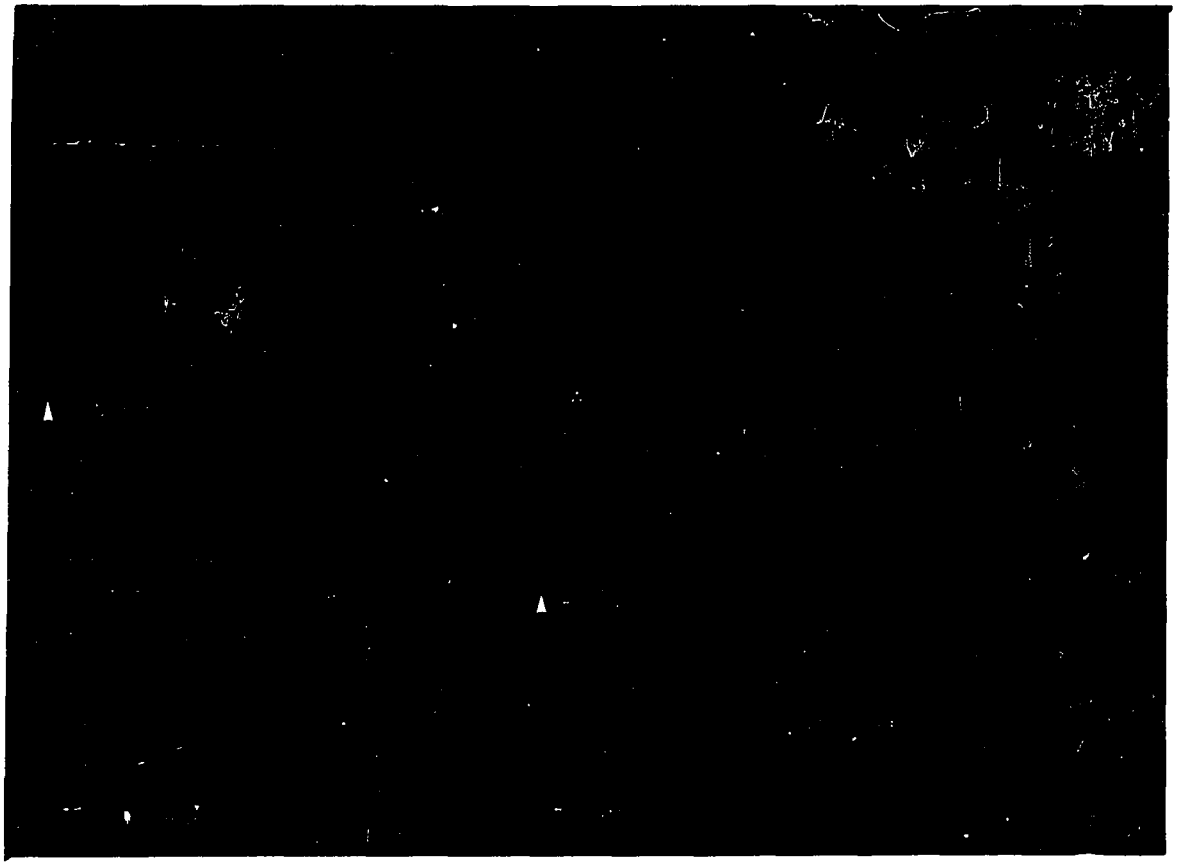


Fig. 5

An example of effect of 1 mM lidocaine and 9·6% CO_2 at pH 7·3 on supra maximal compound action potential of sheathed frog sciatic nerve. Top line, control. Second line, test solution, applied at beginning of line and changed to Ringer at arrow on third line. Time base and stimulating frequency changed at point indicated by time marker on third line.

Experiments on Nerves

A typical experiment in which local anaesthetic plus CO_2 was applied to a nerve is shown in Fig. 5. Serial photographs of the oscilloscope display of the A spike of the compound action potential show the way in which the spike decreased after the drug, and the recovery when the drug was withdrawn.

The control responses of intact and desheathed nerves to changes in pH and CO_2 concentration are shown in Figs. 6 and 7. The intact nerves were little affected by low pH or elevated CO_2 concentration, but desheathed nerves, while also little affected by low pH, were significantly blocked by 50% CO_2 (T_3 approx. 65%), especially when the pH was low. When the nerves were exposed to 1 mM lidocaine (Fig. 8), sheathed nerves were hardly affected at pH 7·3, but desheathed nerves were moderately blocked with a mean T_3 of 60% both at pH 7·3 and 5·5.

The effects of combining 50% CO_2 and 1 mM lidocaine are illustrated in Fig. 9. The desheathed nerves were

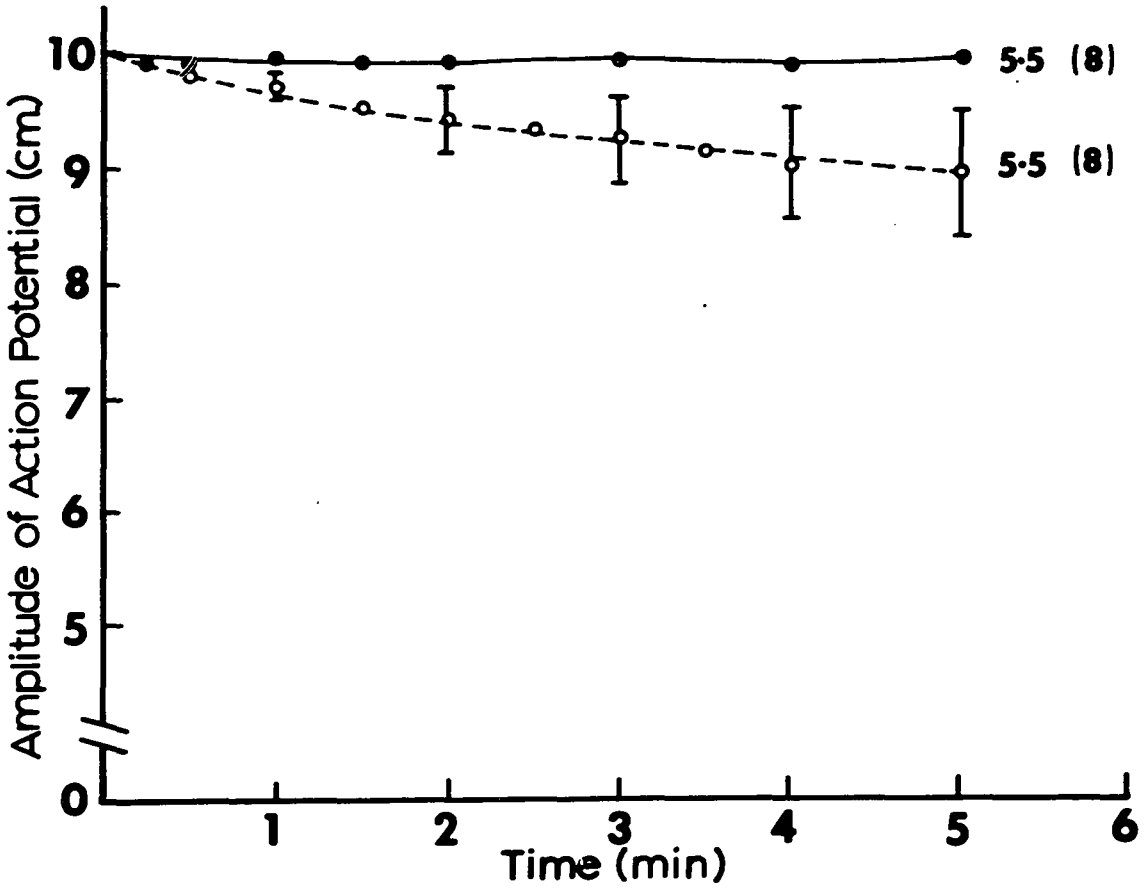


Fig. 6.

Effect of changing pH of Ringer's solution on height of compound action potential of frog sciatic nerves. Test solution applied at zero time. Closed circles with solid line, sheathed nerves. Open circles with dashed line, desheathed nerves. Points are mean ± 1 S.E. First number at end of each line is pH of solution; number of nerves tested is in brackets.

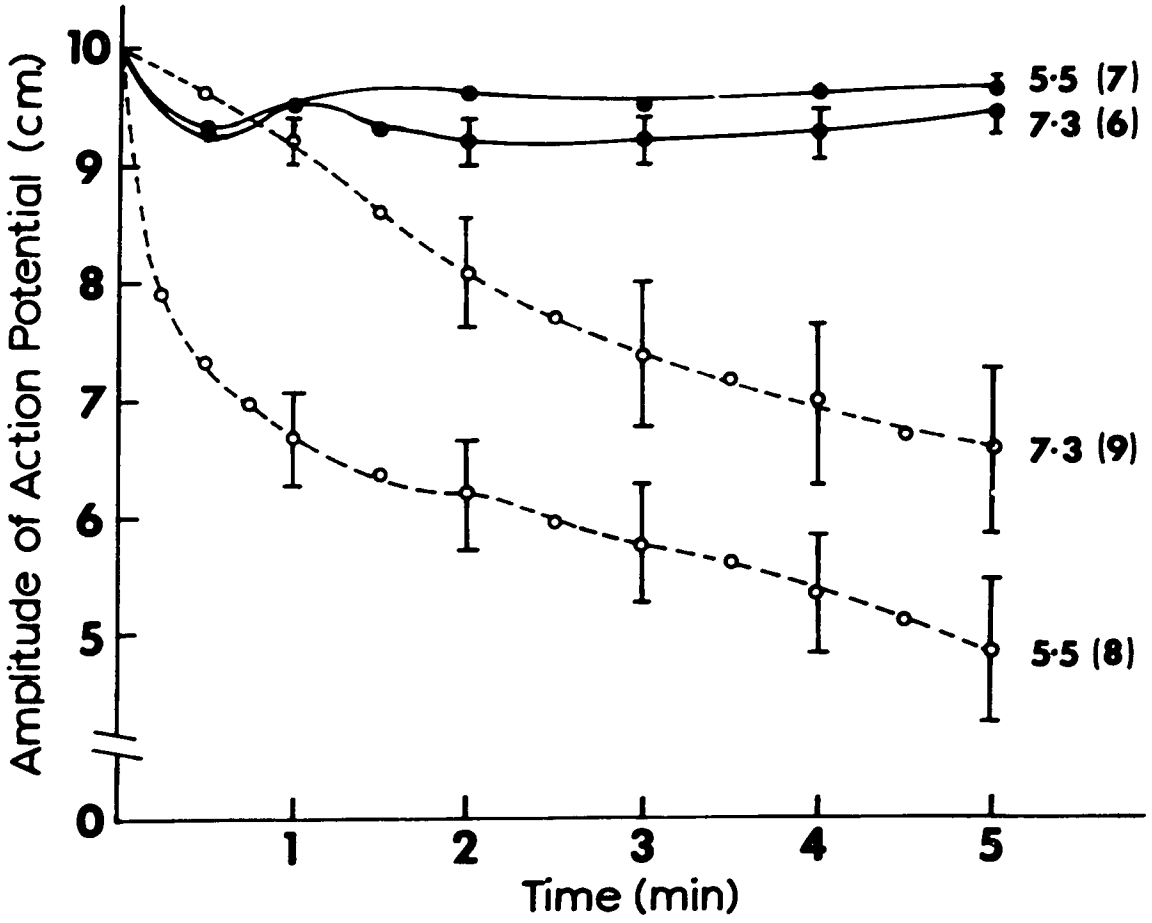


Fig. 7

Effect of 50% CO_2 at different pHs on compound action potential of frog nerves. Test solution applied at zero time. Closed circles with solid line, sheathed nerves. Open circles with dashed line, desheathed nerves. Points are mean ± 1 S.E. First number on each line is pH of solution; number of nerves tested is in brackets.

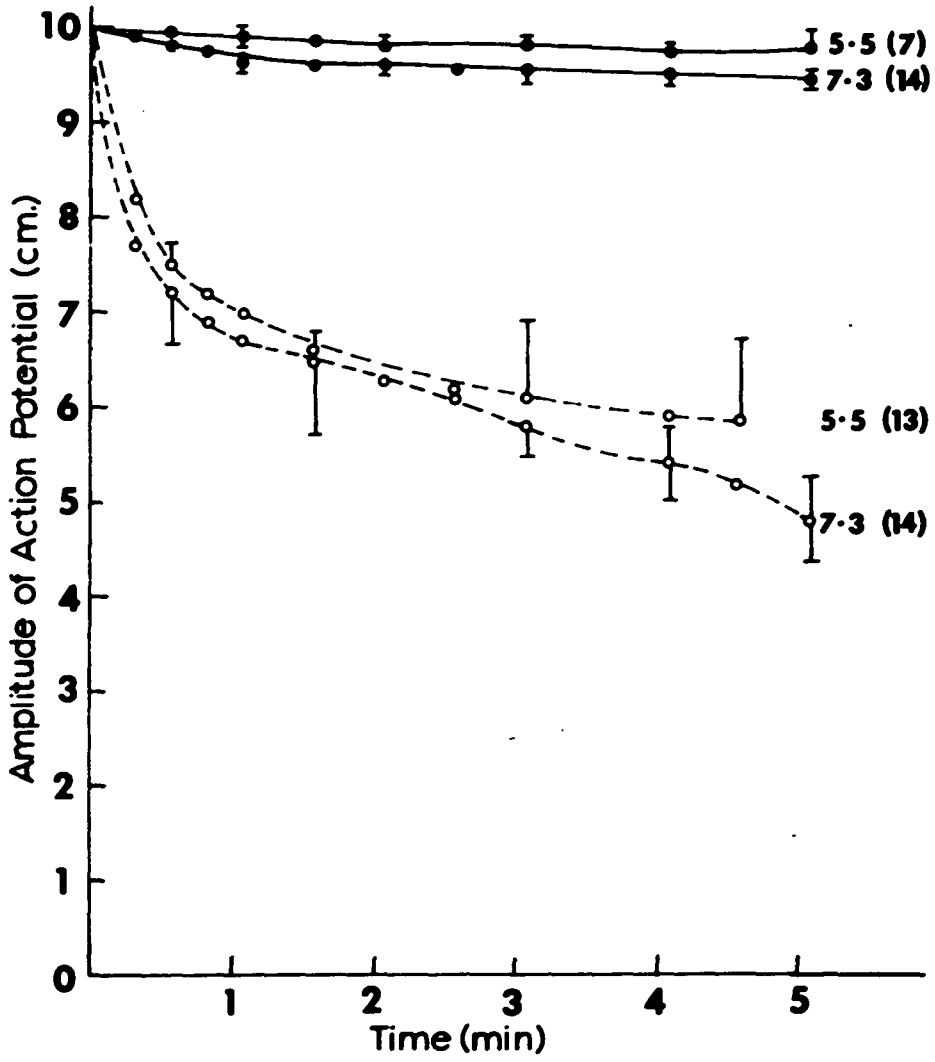


Fig. 8

Effect of 1 mM lidocaine at different pHs on compound action potential of frog nerve.

Closed circles with solid line, sheathed nerves. Open circles with dashed line, desheathed nerves. Points are mean ± 1 S.E. First number on each line is pH of solution; number of nerves tested is in brackets.

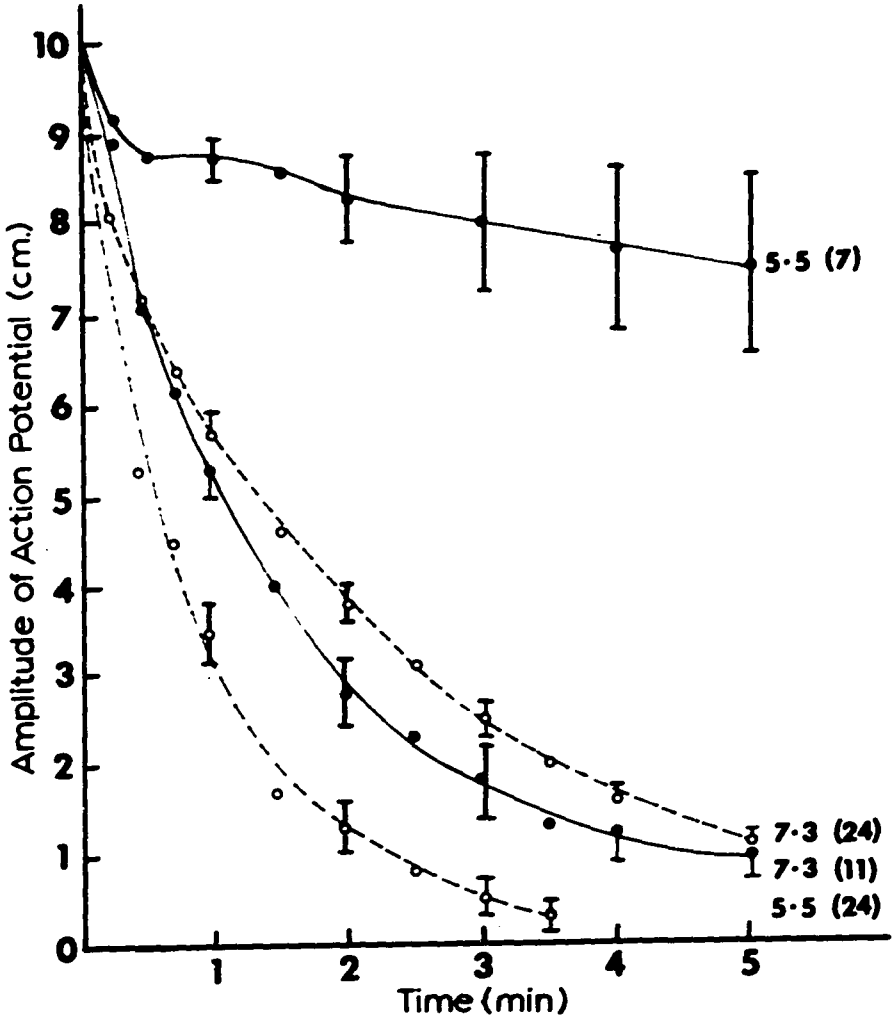


Fig. 9

Effect of 50% CO_2 and 1 mM lidocaine at different pHs on compound action potential. pH was adjusted by adding $NaHCO_3$. No correction was made for change in osmolarity (but see Fig. 13).

Closed circles with solid line, sheathed nerves. Open circles with dashed line, desheathed nerves. Points are mean $\pm$ 1 S.E. First number on each line is pH of solution; number of nerves tested is in brackets.

blocked rapidly with pH 5·5, T_3 was 5%. This was significantly different from the block at pH 7·3 when T_3 was 25%. The feature of outstanding interest in this figure was the block of intact nerves when the pH of the test solution was maintained at 7·3. The result was not significantly different from that seen when the same test solution was applied to the desheathed nerve. In contrast, at a low pH there was very little block of the sheathed nerve (T_3 80%). Fig. 10 shows blocking and recovery of a nerve during and after the application of several test solutions, indicating that the effects are not due to progressively irreversible block. The effect of changing the concentration of CO_2 in the lidocaine solution over a range from 1·1% to 50% at constant pH can be seen in Fig. 11. There was a progressive decrease in T_3 from 95% at 1·1% to 15% at 20% CO_2. The T_3 at 50% CO_2 is not significantly different from that at 20% CO_2.

Several combinations of pH and CO_2 in lidocaine solutions were tested on three sheathed and desheathed rat sciatic nerves, with effects qualitatively similar to those obtained from frog nerves (Fig. 12). Lidocaine 1 mM at pH 7·3 partially blocked intact and desheathed nerves

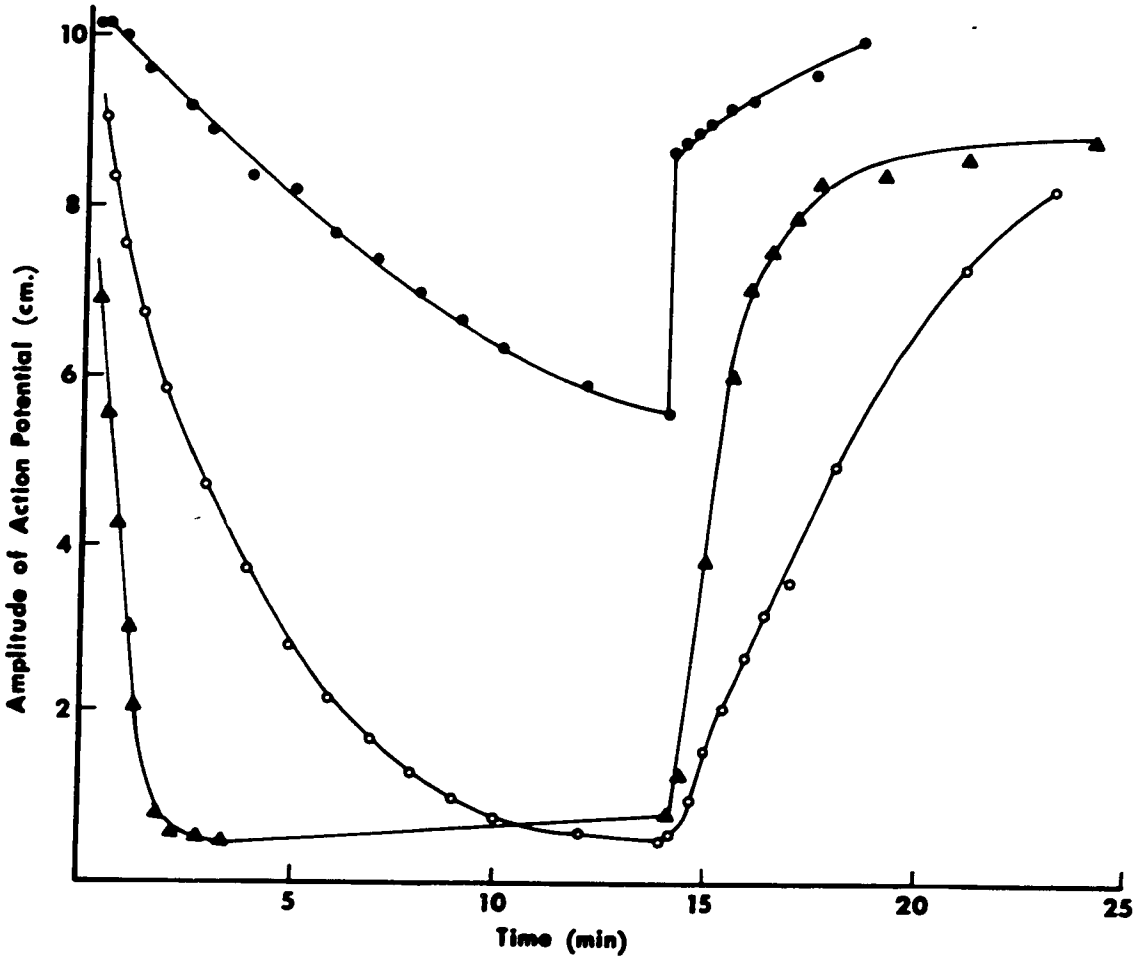


Fig. 10

An example of block by 1 mM lidocaine with several concentrations of CO_2 and recovery in sheathed frog sciatic nerve.

Closed circle, 2·5% CO_2; open circle, 5%; triangle, 50%. Test solution applied at zero time.

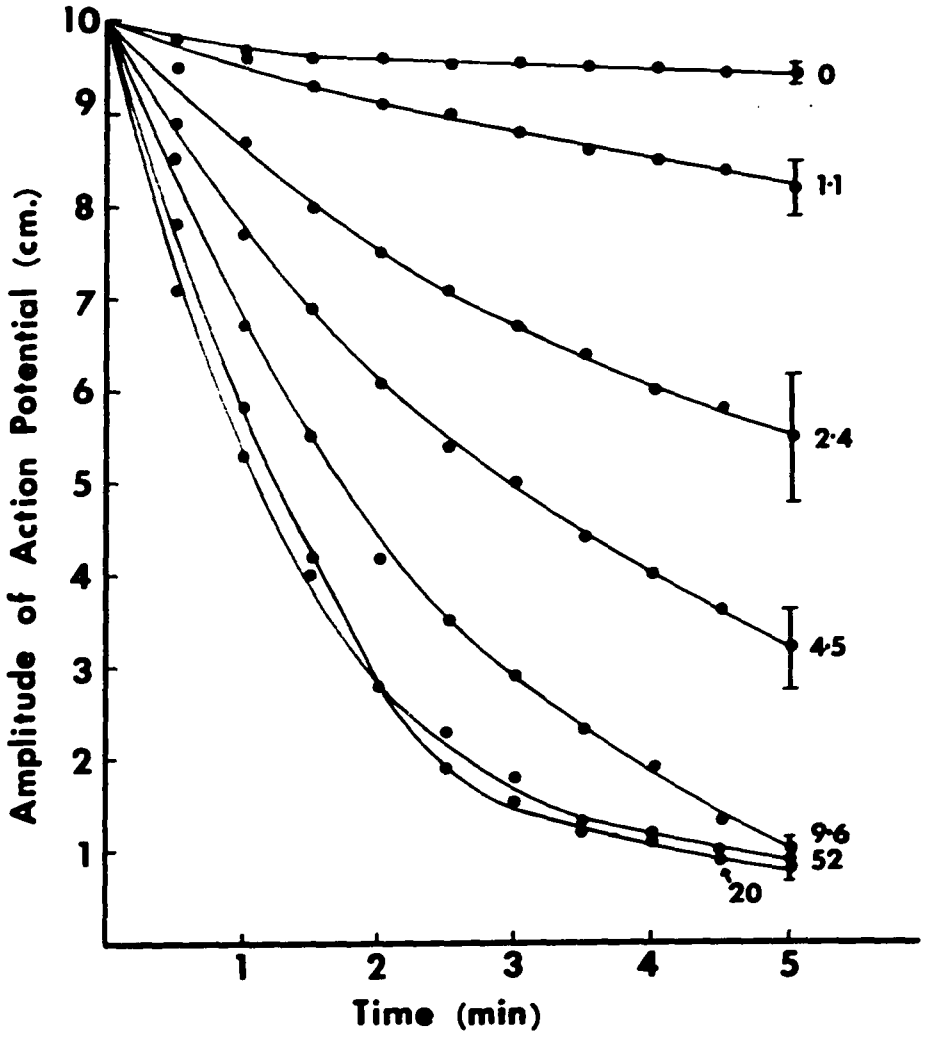


Fig. 11

Effect of different concentrations of CO_2 in 1 mM lidocaine at pH 7·3 on compound action potential of sheathed frog sciatics.

Points are means and bar on last point is $\pm$ 1 S.E. (This was largest S.E.). Number at end of each line is percent CO_2 in test solution.

(T_3 67%). Addition of 50% CO_2 to the lidocaine greatly increased the block, giving a T_3 of about 25%; and as for frog nerves, there was little difference between the intact and desheathed nerves.

Effect of pH on Local Anaesthetic Applied to Desheathed Nerves.

The greater blocking of desheathed nerves by lidocaine 1 mM at pH 5·5 seen in Fig. 9, was examined in more detail by applying lidocaine 0·25 mM plus 9·6% CO_2, alternately at pH 7·3 and 5·5. The pH was adjusted by replacing NaCl with sodium bicarbonate to maintain a constant osmolarity, to check for any effect of differences in tonicity in the previous experiments (Fig. 9). The test solutions were changed from pH 7·3 to 5·5 on eight occasions in three desheathed nerves, and from 5·5 to 7·3 on a similar number of occasions. Fig. 13 shows the results of an experiment on one nerve. At this concentration the amount of block was limited; changing from pH 5·5 or 6 to 7·3 caused a rapid transient (0·5 - 1·0 min) reversal of block by about 12% of control height, followed by a slow increase in block

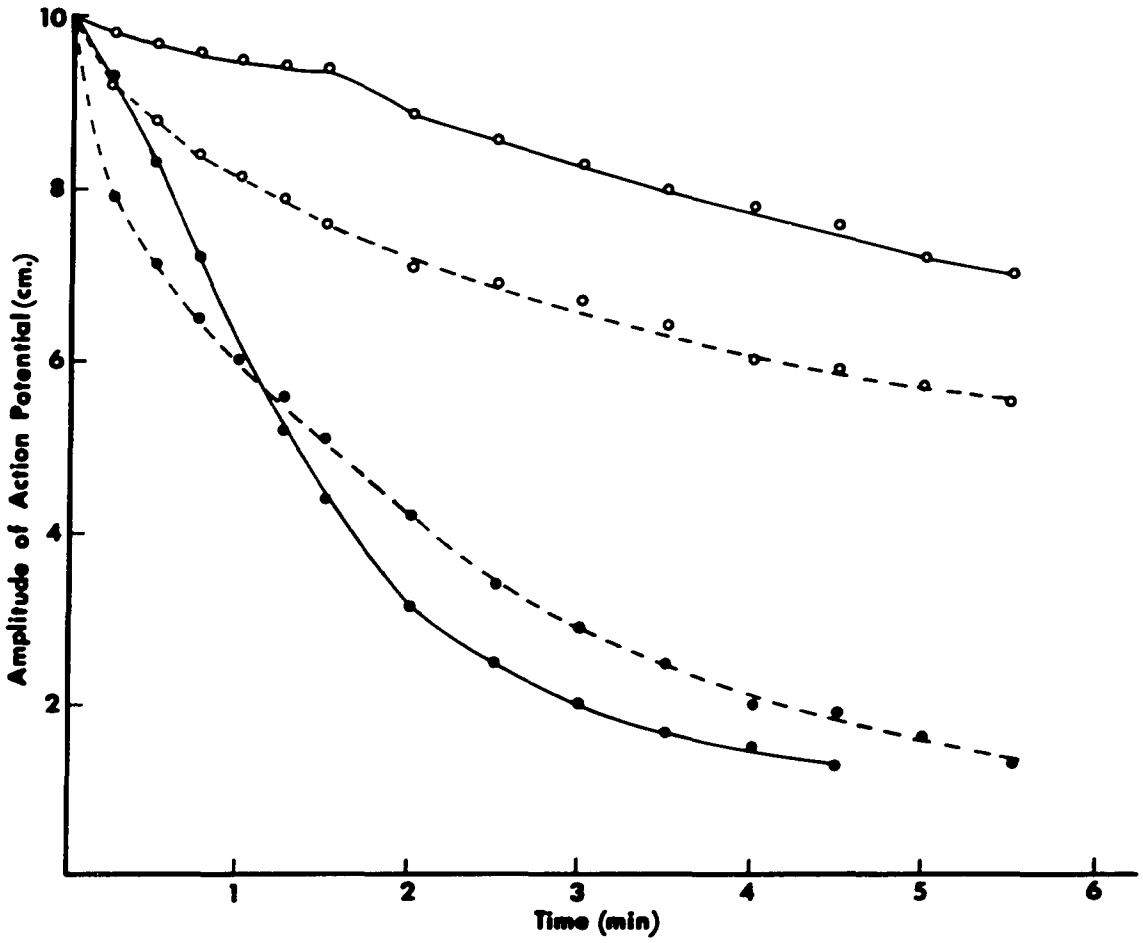


Fig. 12

Effect of 1 mM lidocaine and 50% CO_2 at pH 7·3 on compound action potential of rat sciatic nerves. Mean data from three nerves, S.E. not calculated.

Open circles 0% CO_2, closed circles 50%. Solid lines indicate sheathed nerves, dashed lines desheathed nerves.

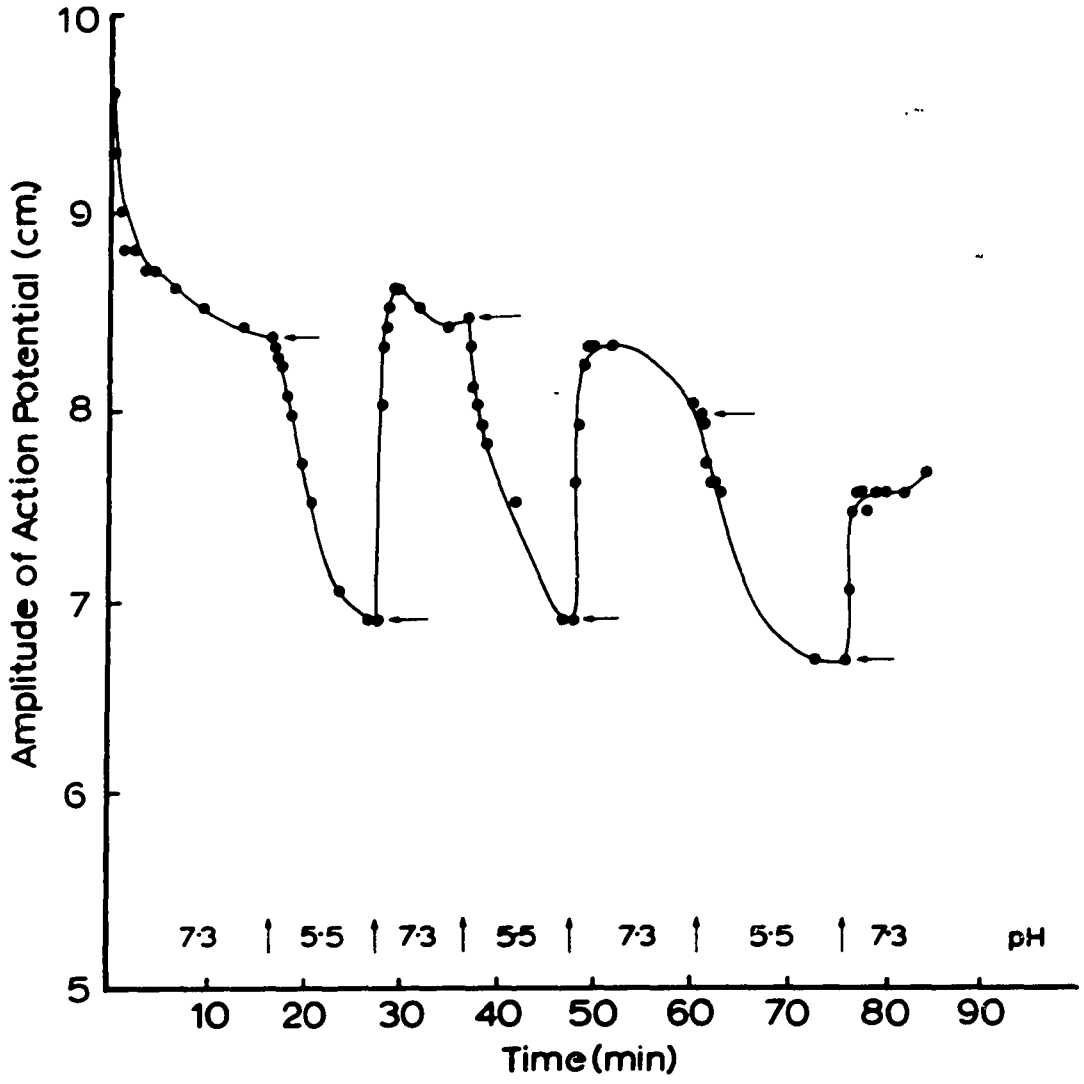


Fig. 13

Effect of changing pH on action of lidocaine 0·25 mM with 9·6% CO_2 on a desheathed frog nerve. pH was adjusted with $NaHCO_3$ by replacement of NaCl to maintain constant osmolarity. Figure shows that results seen in Fig. 9 were not due to differences in tonicity. Horizontal and vertical arrows indicate time at which solutions were changed. Complete change of solution took about 5 sec.

over the following 10 - 12 minutes, usually to just below the initial level of block. Returning to the pH 5·5 solution caused a rapid, exponential increase in block over the following 7 - 12 minutes of about 15%. There was usually a small very rapid phase at the beginning of this increase in block.

Direct Action of CO_2 on Nerve Conduction.

When 50% CO_2 alone was applied to intact and desheathed nerves, some block was produced which was greater in the desheathed nerves (Fig. 7). To examine this effect further and to distinguish between the different possible actions of CO_2 — viz. (i) creating a pH gradient across diffusion barriers, and (ii) a direct effect on the axon membrane — nerves were tested by repeated changes of solution without return to control between the tests. Lidocaine 0·25 mM, pH 8·3 without CO_2 was applied to a sheathed nerve to cause block to about 90% in 30 minutes. The solution was then changed to lidocaine 0·25 mM, pH 5·5 and 9·6% CO_2, causing a rapid increase in block, (40% in 2 minutes),

but this reversed over the next 5 minutes to about 30% block (Fig. 14). Allowing the pH to fall to 5·5 almost completely prevents any further increase in block by the local anaesthetic (see effect of lidocaine at pH 5·5 on sheathed nerve Fig. 8). Returning to the first solution allowed more rapid continuation of the reversal of block for several more minutes, after which a slight increase in block occurred during the following 7 - 10 minutes. The process could be repeated at will. Finally, Ringer's solution allowed full recovery of the action potential. The experiments were repeated using procaine 0·25 mM, pH 9·1, with generally very similar results, except that procaine without CO_2 caused faster block than lidocaine at the same concentration (Fig. 15). The reason for this may be that procaine is more potent than lidocaine.

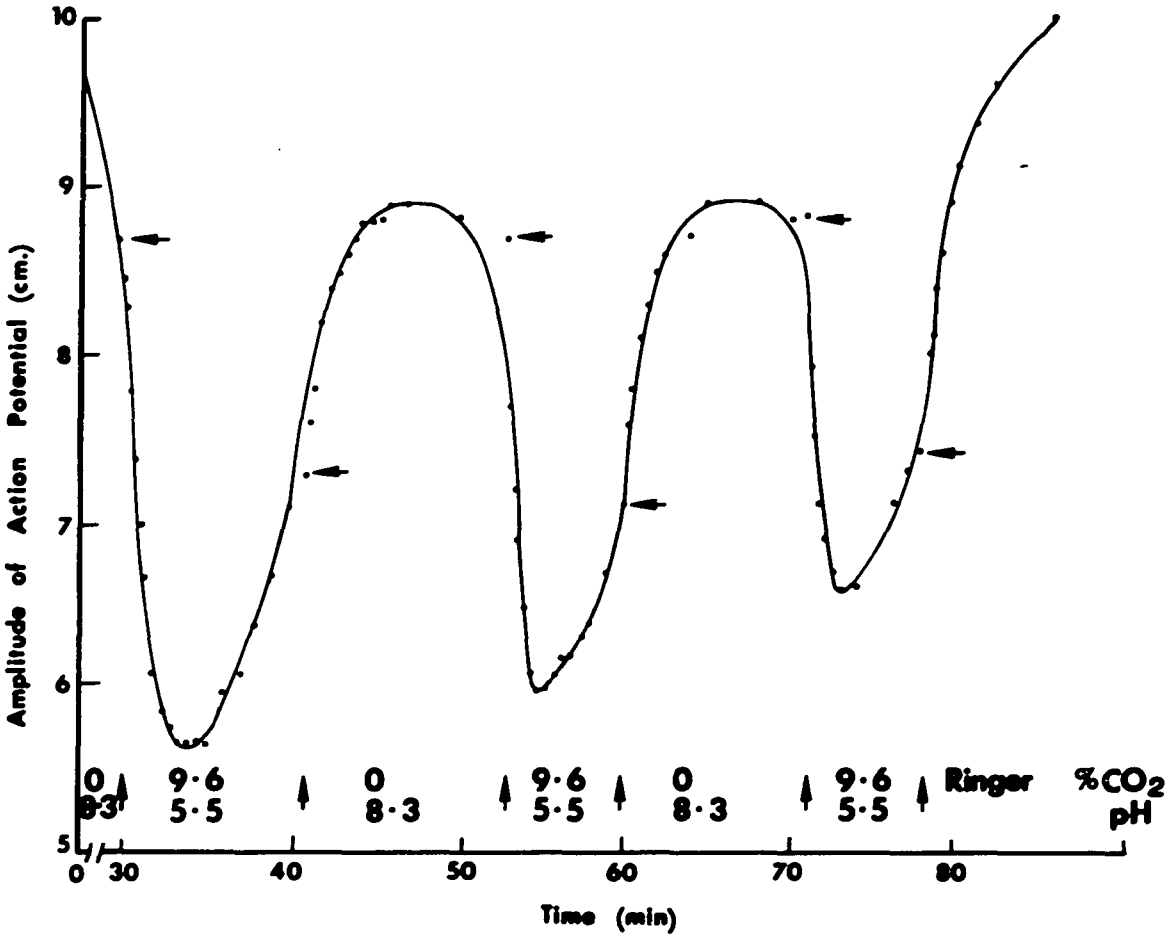


Fig. 14

Response of one sheathed frog sciatic nerve to alternate application of 0·25 mM lidocaine solutions with 9·6% CO_2 and pH 5·5, or 0% CO_2 at pH 8·3.

Horizontal and vertical arrows indicate time at which solutions were changed.

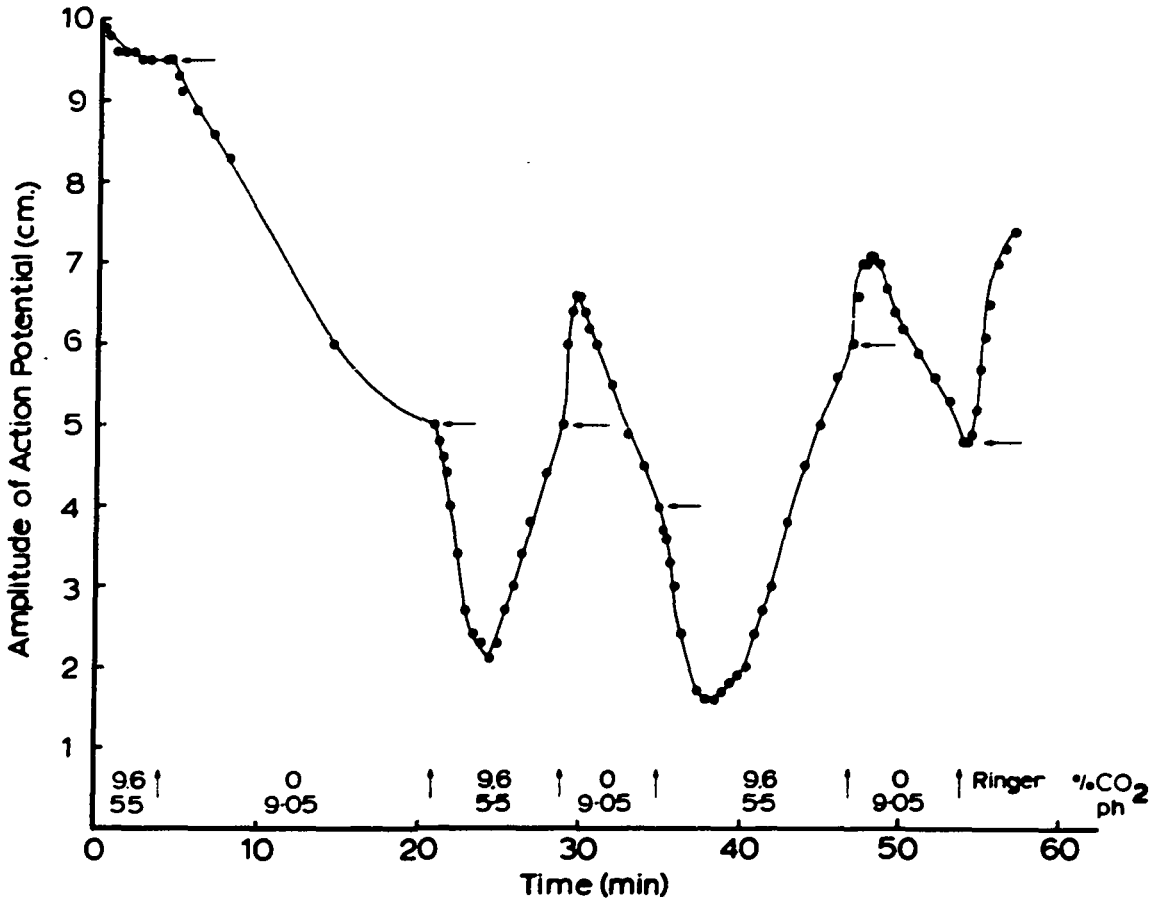


Fig. 15

Responses of one sheathed frog sciatic to alternate application of 0·25 mM procaine solutions with 9·6% CO_2 and pH 5·5 or 0% CO_2 at pH 9·05.

Horizontal and vertical arrows indicate time at which solutions were changed.

Results From Experiments on the Perfused Sheath

Validity of the Technique.

The following evidence suggests that the perfused isolated sheath fairly well maintained its normal properties as a diffusion barrier.

1. The usual perfusion pressures did not cause visible leakage of large particles. (The diameter of China ink particles measured with a microscope ranged from 4 - 8u).

2. Tritiated large molecular weight dextran (approx. 90,000) had an average permeability constant, P, of $1{\cdot}04 \times 10^{-6}$ cm/sec. This was about one fortieth of the maximum value for lidocaine.

3. The rates of efflux of the radioactive tracers through the sheath remained constant despite variations in perfusion pressure. Fig. 16 shows the increase in DPM in the bath with time in several experiments, when lidocaine and sucrose at various

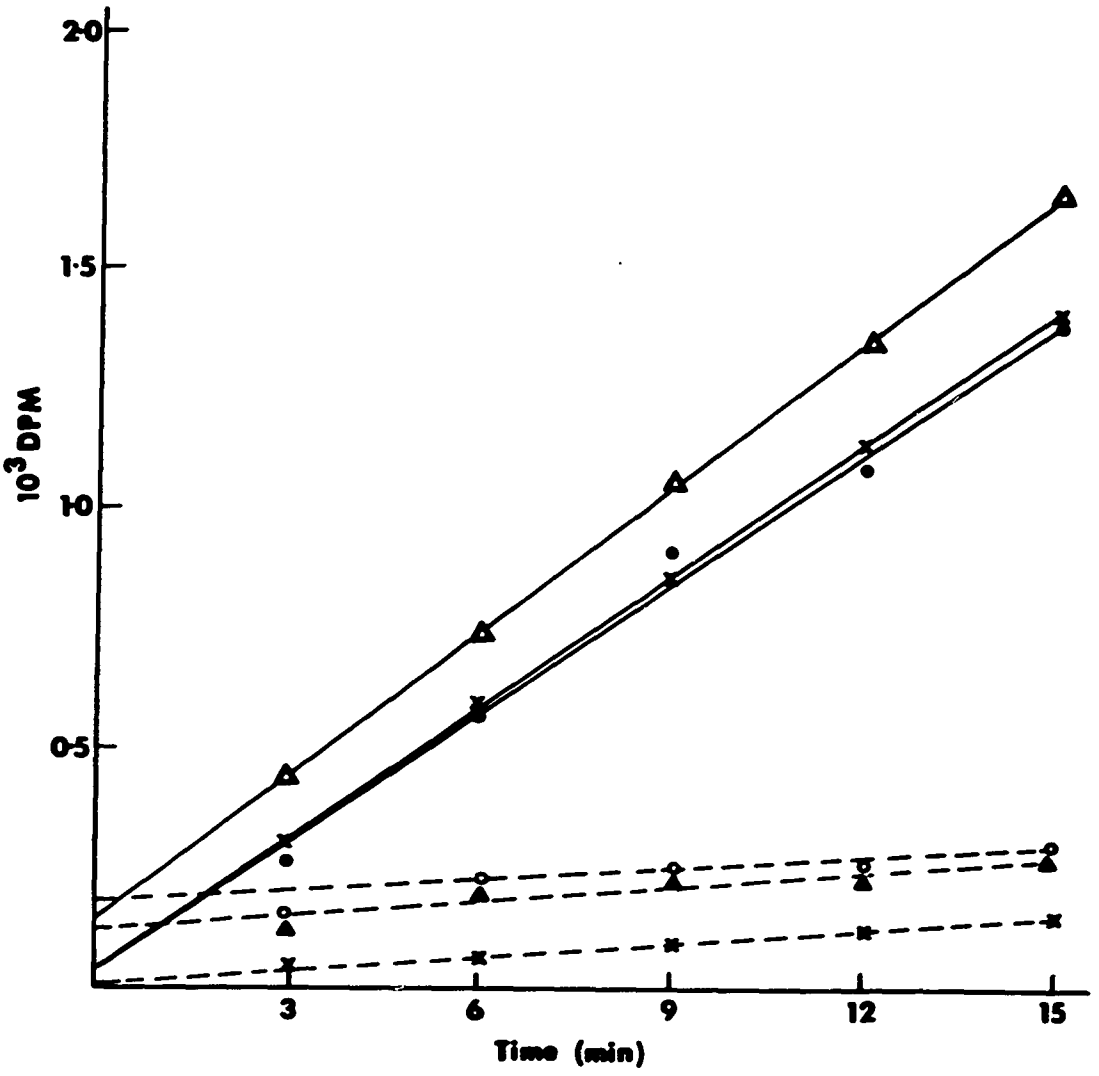


Fig. 16

Relation between efflux of radioactivity from perfused sheath and time. Activity was measured in external bath. Ordinate is total activity in bath corrected to hypothetical perfusate concentration of 500 x 10^3 DPM/ml. Dashed lines, ^{14}C sucrose, pH 7·3; solid line ^{14}C lidocaine, pH 8·3; several examples of each are shown from different sheaths. The slope of the line is proportional to flux through the sheath.

pHs were perfused through the lumen of the sheath. The points fall on a straight line with no tendency to plateau after long periods of perfusion, indicating that the efflux remained constant, and that the concentration in the bath did not rise sufficiently to affect diffusion.

4. The permeability remained constant during the course of the experiment (see Fig. 17).

In all, thirty-five nerve sheaths were used, and with each sheath approximately twenty-five samples were taken from the bath. A number of sheaths were rejected for several reasons. If the permeability constants of a solution tested at the beginning and end of an experiment were markedly different, it was considered that the permeability had changed at some time during the experiment, and the results could not be used. Most of the early experiments were of this kind. The reason for this was probably the effect of changes of pressure on the sheath, an effect which was not appreciated initially. In the early experiments when the perfusate was changed, the flow was stopped and then restarted by compressing the rubber inflow tubing;

whenever the tubing was compressed in this way during continuous flow of perfusate in a control run, the counts in the external bath were always raised following this manoeuvre. Subsequently, the technique was modified to allow a change of fluid without raising the pressure. When blockage of the tube by adventitial tissue occasionally caused inadequate flow, the results were rejected.

Dimensions of the Sheath.

The mean length of sheaths was 10·2 mm (± 0·76 SD, n = 14), and the diameter 0·679 mm (± 0·05 SD, n = 14). The calculated mean surface area was 0·218 cm^2 ± 0·018 SD, n = 14). An estimation of wall thickness was attempted with a binocular microscope, but as it was actually the edge of a translucent cylinder which was being viewed, the estimates, which varied between 70 - 100u were only approximate. Furthermore, on occasions when coloured fluids were used, there appeared to be a much thicker wall. Similarly, when air bubbles flowed through the sheath, they did not appear to extend to the supposed boundary of the sheath. It seems that there was a layer with the same refractive index

as water lining the internal aspect of the sheath. Whether it had the same permeability as water is of course unknown, but the difficulty was further compounded by the measurements from histological sections of sheath. The values varied between 7u and 40u and it was often difficult to define precisely the sheath edges. Consequently little reliance can be placed on these measurements, or on the consequent estimates of diffusion coefficients.

The flow was measured through a number of sheaths by weighing the fluid collected over a timed interval and assuming a specific gravity of 1·0. The contents of the sheath were replaced by fresh fluid 100 - 250 times per minute. This was more than adequate to maintain the internal concentration to within 1·5% of the original concentration with the slowest flow rate and highest rate of efflux.

Scintillation Counting.

In preliminary counting of a sample of vials, the background activity was on the average of 22 CPM. In all subsequent experiments, this background was automatically subtracted.

Permeability of the Nerve Sheath to Lidocaine

Effect of pH.

The isolated sheath was perfused with ^{14}C lidocaine, approximately 1 mM. The efflux through the walls of the sheath was measured at regular intervals. The mean surface area of the sheath was 0.218 cm^2.

Figure 17 illustrates the effect of changing the pH of the perfusate from 5·5 to 8·3. The accumulation of radioactivity (as DPM) in the bath during the three minutes between sampling was small when pH was 5·5, but rose dramatically when the pH of the perfusate was changed to 8·3, and fell again when the pH returned to 5·5. In this case, the fall was not quite as rapid because the flow from the pH 5·5 reservoir was slower than from the other, owing to an obstruction in the three-way tap (this was later corrected). Fifty-four measurements at pH 5·5 and forty-eight at pH 8·3 were made on four nerve sheaths; the results were significantly different at the 1% level. The apparent permeability constants for all tracers, calculated by the method of Davson (1959), are listed in Table 2.

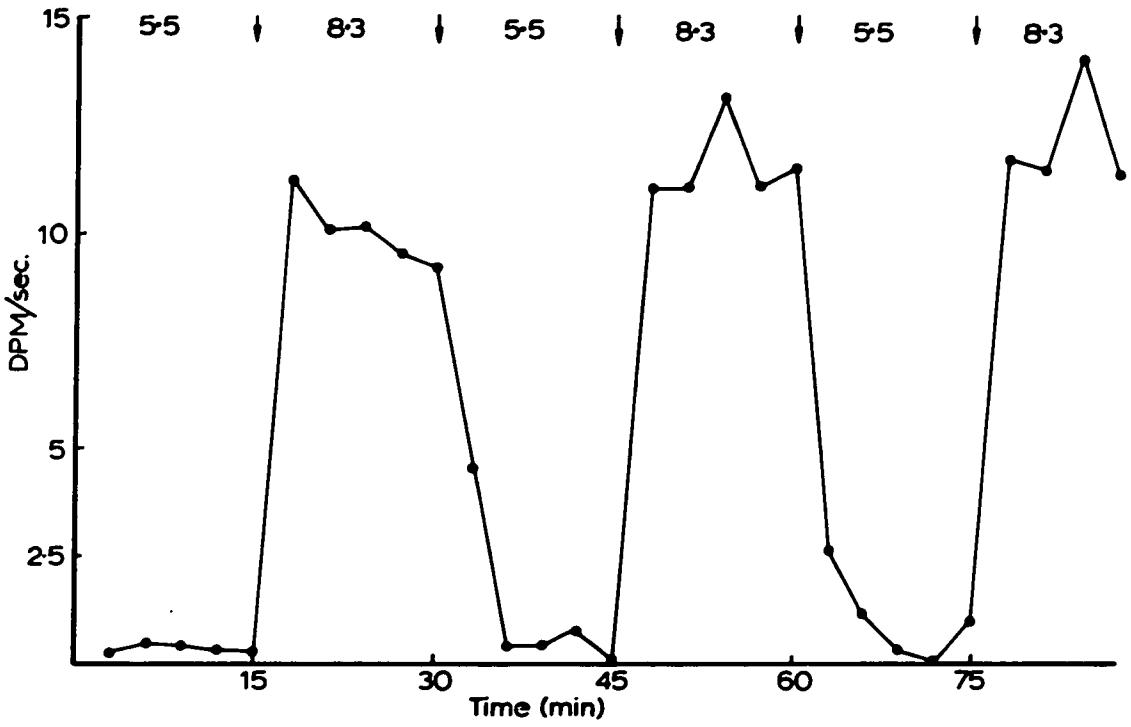


Fig. 17

An example of the effects of changing pH of the perfusing fluid on efflux of ^{14}D lidocaine through frog nerve sheath. Vertical arrows at the top of the figure indicate time at which solution was changed. Numbers between arrows are the pHs of the perfusing solution. pH of the bath was 7·3. Ordinate is efflux from sheath corrected to hypothetical perfusate concentration of 500 x 10^3 DPM/ml.

TABLE 2

pH	Lidocaine			Sucrose		
	Mean	S.E.	N	Mean	S.E.	N
5·5	4·5	0·57	55	1·5	0·45	15*
6·3	5·4	0·53	31			
7·0	9·2	1·08	16			
7·3	14·5	0·99	55	3·2	0·58	56
				1·5	0·28	23*
7·6	12·2	0·86	8			
7·8	20·9	0·75	30			
8·3	27·9	0·97	55			
9·3	28·0	0·93	31	1·9	0·51	15*
7·3+ 9·6% CO_2	15·8	0·78				
7·3+ 2·4% CO_2				3·2	1·08	60

Average apparent permeability coefficients, P, of ^{14}C lidocaine and ^{14}C sucrose for the frog sciatic nerve sheath. Effect of pH and concentration of CO_2.

*Asterisk indicates a small group of experiments with low perfusion flow rates.

Units of apparent permeability constants for Lidocaine and Sucrose are x 10^{-5} cm/sec.

Relationship of Ionization of Lidocaine to its Permeability.

To determine the nature of the exact relationship of pH to lidocaine diffusion, perfusates with a range of pHs centred on the pK_a of lidocaine (7·85) were tested. The apparent permeabilities from 250 tests on 10 sheaths were grouped according to pH, and the mean and S.D. calculated and plotted against pH in Fig. 18. The figure shows a sigmoid type of curve with the greatest rate of change at about 7·6. The largest permeability was at high pH, but at low pH there was still an appreciable permeability of the sheath. This causes the curve to be different from a typical titration curve, which it otherwise resembles.

Direct Effect of CO_2 on the Sheath.

To see whether CO_2 directly increased the general permeability of the sheath, solutions of ^{14}C sucrose (1 mM) with 0% CO_2 at pH 7·3 and 2·4% CO_2 at pH 7·3 were perfused through the sheath. In Fig. 19, it can be seen clearly that changing from one to the other did not cause any significant change in the efflux of ^{14}C sucrose. The mean P for

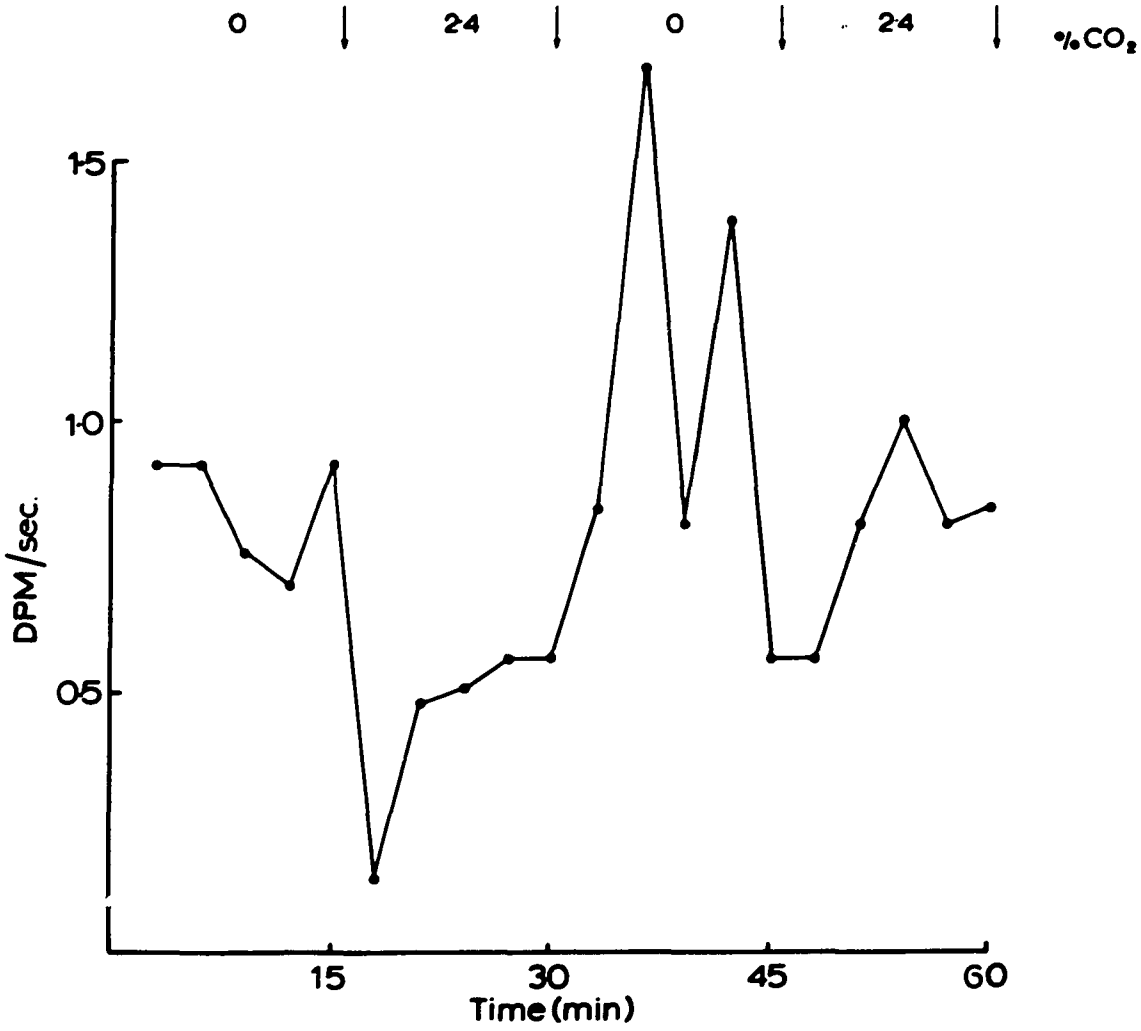


Fig. 19

Effect of changing concentration of CO_2 in bath and perfusate on permeability of a frog nerve sheath to ^{14}C sucrose. Numbers at top of figure are concentration of CO_2 (Note that scale of ordinate is different from Fig. 17).

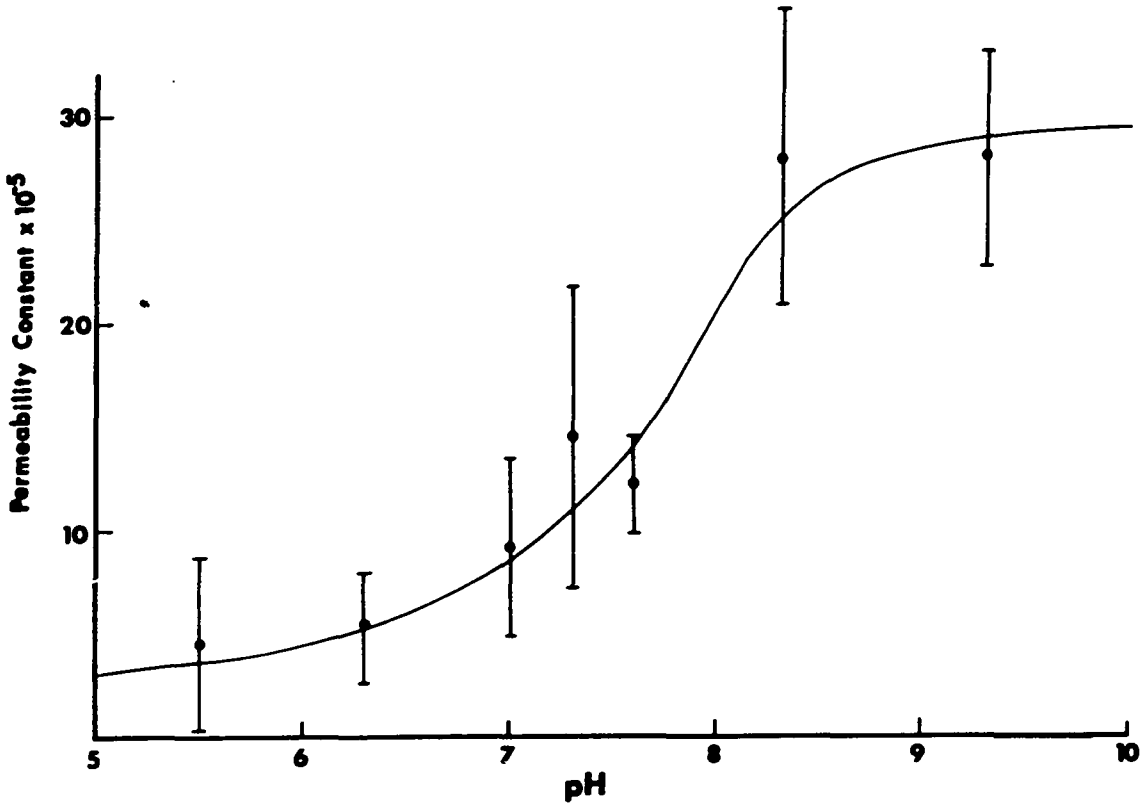


Fig. 18

The relation between pH of perfusing solution and permeability constant for lidocaine in frog nerve sheath.
Each point is mean ± 1 Standard Deviation. Line fitted by eye. Figure is drawn from data in Table 4. Perfusate was ^{14}C lidocaine 1 mM (Sp.a. 0·77 uC/mg). Permeability constant is CM/sec.

sucrose without CO_2 was $3 \cdot 2 \times 10^{-5}$ cm/sec ($\pm$ 0·58, n = 12) and with 2·4% CO_2 was $3 \cdot 21 \times 10^{-5}$ cm/sec ($\pm$1·08, n = 12).

The same tests using lidocaine with a higher CO_2 (9·6%) in both bath and perfusate also did not show a significant difference ($P > 0 \cdot 05$). The permeability constant with 9·6% CO_2 was $15 \cdot 8 \times 10^{-5}$ cm/sec ($\pm$ 0·78 SD, 23), and that without CO_2 was $14 \cdot 5 \times 10^{-5}$ cm/sec ($\pm$ 0·99 SD, 55).

Apparent Diffusion Coefficient.

Estimates of the thickness of the sheath varied from 7u to 100u. Therefore possible values of D for lidocaine at pH 7·3 would range between 0·92 and $14 \cdot 5 \times 10^{-7}$ cm^2/sec.

Acceleration of the Diffusion of Lidocaine Through the Nerve Sheath by a Gradient of CO_2.

Seventy-four observations were made on five nerve sheaths. The perfusion rate of CO_2 free Ringer inside the sheath was kept constant during each experiment, but it

varied in different experiments, so that the results from each of the experiments were not grouped. Therefore Student's 't' tests were done on the data from each sheath (Table 3). Forty observations were made on four sheaths comparing the fluxes of ^{14}C lidocaine at pH 7·3 and 0% CO_2, and at pH 7·3, 9·6% CO_2. In all four cases, the net flux of lidocaine into the lumen of the sheath was significantly greater with the 9·6% CO_2 solution ($P < 0·02$). On the average, the influx from the CO_2 solution was 2·1 ($\pm$0·09 SD, n = 4) times greater than that from the non-CO_2 solution, although the external pH was the same.

Thirty-five observations were made on two sheaths comparing the net influx with 0% CO_2 at pH 8·3 to that with 9·6% CO_2 at pH 7·3. In one sheath, the influx with 9·6% CO_2 was significantly greater (1·2 times) ($P < 0·02$). In the other case, there was no significant difference. The average influx with 9·6% CO_2 at pH 7·3 was 1·1 times that with 0% CO_2 at pH 8·3. When a CO_2 containing solution was in the external bath, the pH of the outflow of perfusate from two sheaths was 6·9 and 7·0. The initial pH of this unbuffered solution was 7·3.

TABLE 3

pH	7·3		7·3		8·3		Probability
CO_2 %	0		9·6		0		
Experiment	Mean	S.E.	Mean	S.E.	Mean	S.E.	
1	8·5	0·48	16·8	0·49			<·001
2	5·1	1·7	10·6	0·42			<·02
3	1·16	0·03	2·47	0·45			<·02
4	5·4	0·32	10·3	0·23			<·001
5			11·9	0·47	11·4	0·29	<·5
6			14·0	0·45	12·0	0·49	<·02
Mean	4·79		11·03		11·70		

Results of six paired experiments on five nerve sheaths to compare net flux of ^{14}C lidocaine into the nerve sheath from solutions with and without CO_2. Values include an unknown constant different for each sheath. Units are arbitrary but constant for each sheath. Probability is derived from Student's 't' statistic for each sheath. Mean of all results is included for comparison only.

DISCUSSION

The results of these studies which bear directly on the potentiating effect of CO_2 on local anaesthetic action will be stated briefly to summarise the relevant points for discussion.

Sheathed nerves were blocked as effectively as desheathed nerves by local anaesthetic solutions with 50% CO_2 at pH 7·3 (Fig. 9).

The sheathed nerve was blocked very much less when the pH of the solution was changed from 7·3 to 5·5 (Fig. 9).

Desheathed nerves were blocked by local anaesthetic and CO_2 most effectively by solutions of low pH (Fig. 9).

Fifty per cent CO_2 in Ringer's solution partially blocked the action potential. Desheathed nerves were blocked much more than intact nerves (Fig. 7).

CO_2 dramatically increased the amount of block in an intact nerve already partially blocked by local anaesthetic (Fig. 14).

Partial local anaesthetic block in desheathed nerves was increased by a fall in pH (Fig. 13).

Within the range of pH 5 - 10, the movement of lidocaine through the nerve sheath increased with the pH of the local anaesthetic (Fig. 18), but pH did not change the flux of sucrose.

The permeability of the sheath to lidocaine and sucrose was not altered directly by CO_2 (Fig. 19).

CO_2 caused a greater net flux of lidocaine through the sheath from solutions at pH 7·3 than when no CO_2 was present, owing to a fall of pH in the unbuffered solution within the lumen of the sheath (Table 3).

Permeability of the Nerve Sheath

The present experiments have shown that the sheath is a substantial barrier to lidocaine and sucrose. The permeability constant, P, of the sheath for sucrose was $3 \cdot 2 \times 10^{-5}$ cm/sec. ($\pm$ 0·58 SE, n = 56). Since other authors have reported apparent diffusion coefficients, D, an estimate of this coefficient will be made for the present results.

The calculation of the apparent D, from P requires an estimate of sheath thickness (D = P x L, where L = thickness). The measurements of thickness in these experiments were technically unsatisfactory. Values found with the binocular microscope ranged from 70 to 100u, which probably overestimates the thickness because the distance measured was the edge of a translucent cylinder. The range from the histological sections was 7u to 40u, partly owing to the difficulty of cutting transverse sections. Thus the difference between the results obtained with the two methods was large, as was the variability in each. The results

from histological sections are probably nearer the true value in view of the estimate of 40u for the bullfrog sheath given by Lorente de Nó (1952); 30u was accepted as the thickness of the frog sheath.

Therefore, the D_{app} for sucrose was approximately 1 x 10^{-7} cm^2/sec. (range 0·22 to 3·2 x 10^{-7} cm^2/sec.). The D for sucrose in water is 5 x 10^{-6} cm^2/sec. (Gosting and Morris, 1949), some 50 times greater than that for the sheath. The D_{app} for sucrose for bullfrog sheaths was 1·1 x 10^{-8} cm^2/sec. (De Feudis, 1970), one ninth of that in the present experiments. This difference may be due in the present experiments to errors inherent in the measurement of the sampled volume, the radioactivity of the samples, the surface area and particularly the sheath thickness. Similar variability is likely in the experiments by De Feudis, particularly in the measurement of the surface area of the sheath 'bags'.

Another difference between the two types of experiment is that the hydrostatic pressure in the frog sheath may have caused some bulk flow of sucrose through the sheath. This effect would not have occurred in the bags.

Although there may be a true species difference for D_{app} between bullfrog and frog sheaths, this is not proven for the reasons given above.

The effect of pH on the permeability of the sheath to lidocaine is clearly seen in Fig. 18. This figure suggests that the P for lidocaine is proportional to the fraction of non-ionized lidocaine. This relationship is more clearly seen by superimposing the titration curve of lidocaine on Fig. 18. However, the experimental curve is appreciably different from the predicted curve (Fig. 20). One reason for this is that the sheath is to some extent permeable to cationic local anaesthetic; that is, the P for lidocaine at pH 5·5 when 99·6% ionized is $4{\cdot}5 \times 10^{-5}$ cm/sec, while the predicted curve assumes that the sheath is totally impermeable to the cation. The curve corrected for the fraction of cation present at each pH (large dashed line in Fig. 20) closely follows the one predicted. The relationship of P to ionization is again shown in Fig. 21 in which each value of P was corrected for the proportion of cation and was plotted against the ratio of the fraction of base. A regression line was

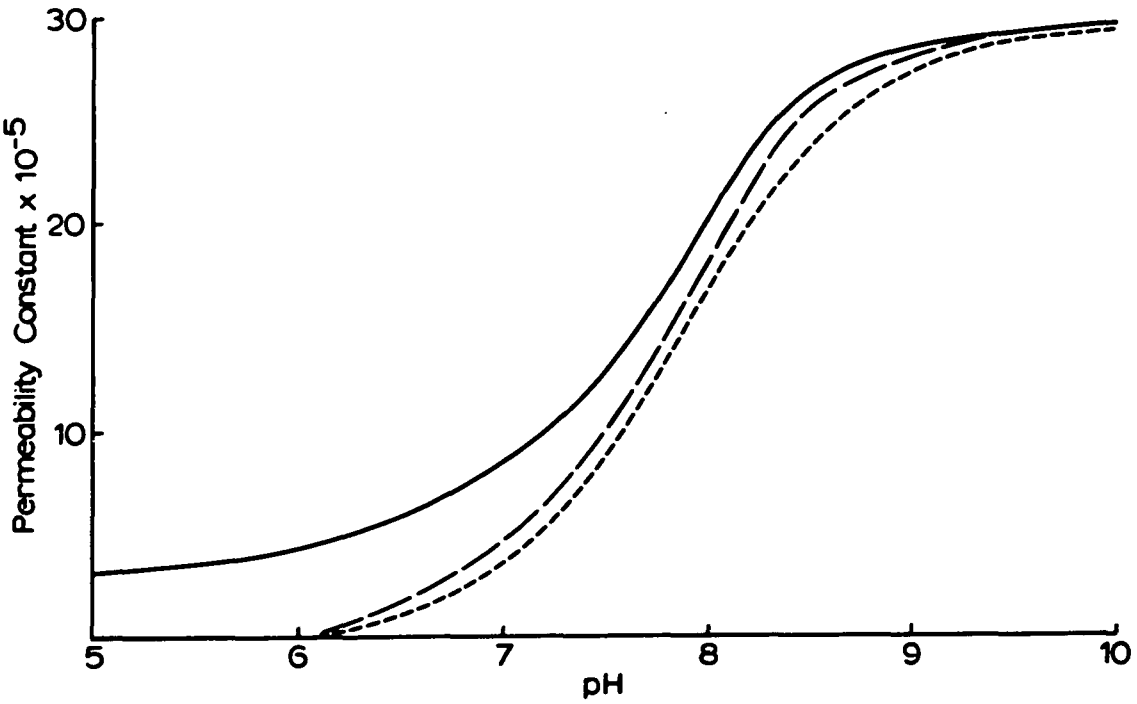


Fig. 20

Theoretical dissociation curve of lidocaine (pK_a 7·85) superimposed on line in Fig. 18.

Solid line, experimental curve (from Fig. 18); small dashed line, dissociation curve; large dashed line, experimental curve corrected for permeability of sheath at pH 5·5 e.g. at pH 7·85 (lidocaine 50% dissociated) half permeability constant for lidocaine at pH 5·5 was subtracted from solid line.

Ordinate: for solid and large dashed line, permeability constant in cm/sec: for small dashed line, ordinate is fraction of lidocaine as base, with 100% base equal to 30 units, and 0% base equal to zero units on permeability scale.

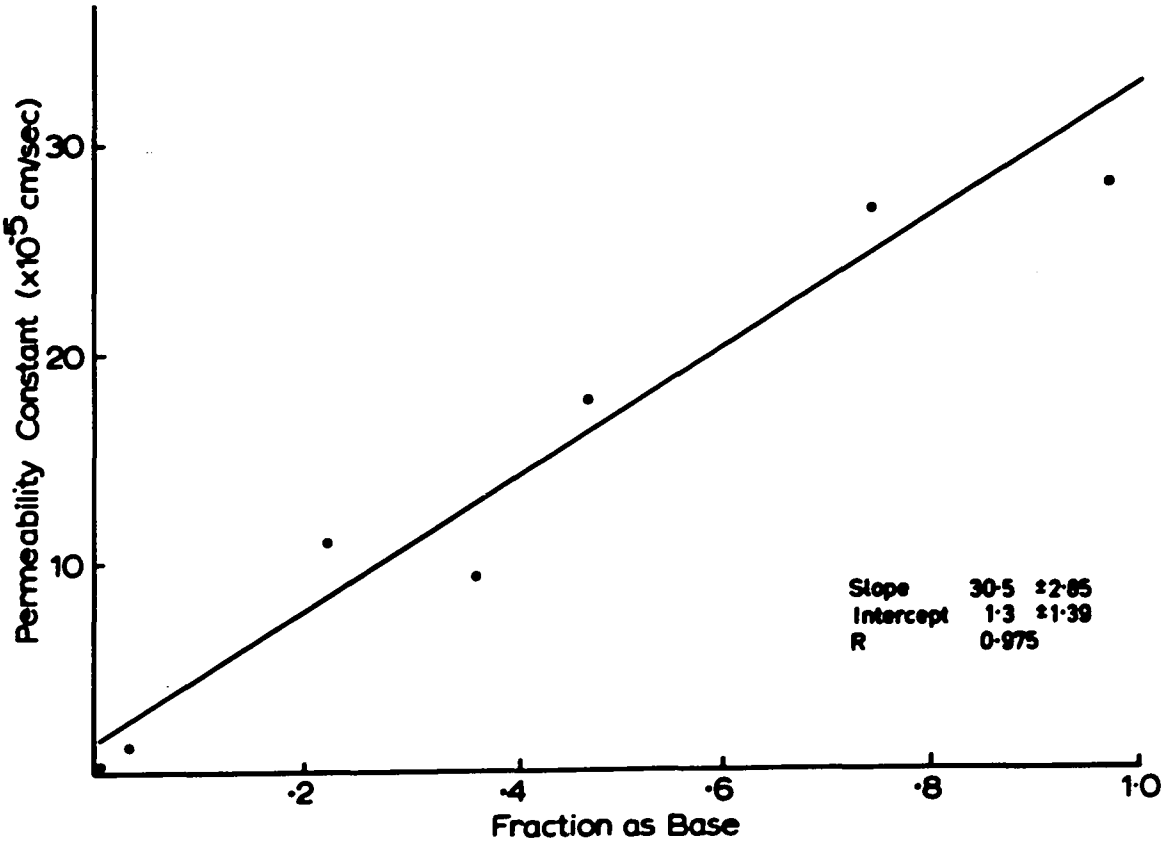


Fig. 21

Regression of permeability constant for lidocaine on fraction of lidocaine ionized. Points are from Table 4. Each point corrected for P of lidocaine at pH 5·5. Line fitted by method of least squares. Abscissa, fraction of lidocaine as base.

drawn through these points with a slope of $30 \cdot 5 \pm 2 \cdot 85 \times 10^{-5}$ cm/sec and a correlation coefficient, r^2 of $0 \cdot 975$. Although these calculations show that P and pH are related, there is still a difference from the relationship predicted. The possibility that the pK_a of 1 mM lidocaine in Ringer's solution might be lower than $7 \cdot 85$ was disproved by the results of four titrations of lidocaine in buffer-free Ringer, which showed that the pK_a did not differ significantly from $7 \cdot 85$.

Hydrogen ions may directly increase the permeability of the sheath to cation, but in fact a fall in pH should decrease the ionization of acidic groups in the membrane and therefore tend to lower the permeability of the sheath to cations. Furthermore, Fig. 22 shows that the permeability of the sheath to sucrose is not significantly changed, either by an increase or decrease in pH. The small random fluctuations in efflux in this figure are well within the error of the method, and are probably not significant.

Several other estimations of D for lidocaine are available for comparison. Assuming a sheath thickness of

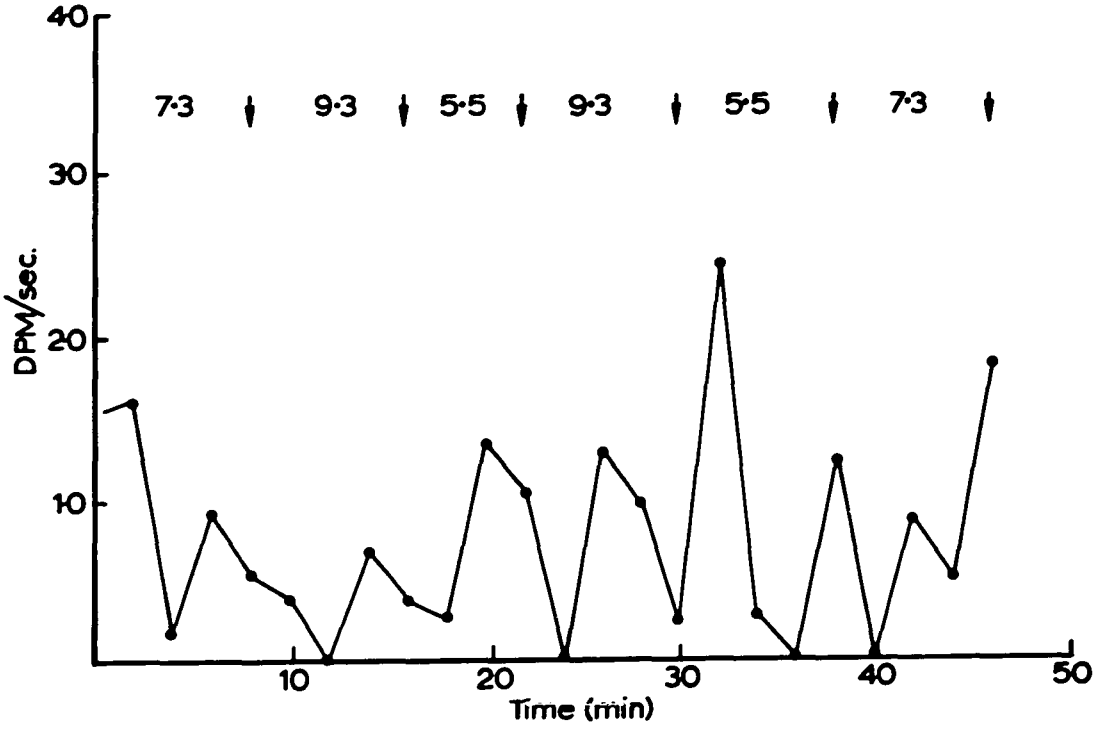


Fig. 22

The effect of pH on flux of ^{14}C sucrose through a nerve sheath.

Vertical arrows at top of figure indicate time at which solution was changed. Numbers between arrows are pHs of perfusing solutions. Ordinate is efflux from sheath corrected to hypothetical perfusate concentration of 500×10^3 DPM/ml.

30u as before, the calculated D_{app} of lidocaine at pH 9·3 was 8·4 x 10^{-7} cm^2/sec. By extrapolating the regression line of Fig. 21, the D_{app} for the base alone was estimated as 10 x 10^{-7} cm^2/sec, compared to the experimental finding of 1·4 x 10^{-7} cm^2/sec for the cation. The free diffusion coefficient for lidocaine has apparently not been measured. Weidling (1961) gives the values of Rud and Ehrenberg as "the diffusion coefficient for lidocaine", but he does not make it clear that the values were for whole frog nerves. The manufacturers of lidocaine were not able to supply the datum.

The D for lidocaine in sheathed nerves has been measured by Ehrenberg (1948), Rud (1961) and Strobel and Bianchi (1970 b) as between 1·67 and 3·2 x 10^{-7} cm^2/sec. The pH of the measurements was about 7·3 (7·2 - 7·4). In the present studies, the D_{app} at pH 7·3 calculated from Fig. 18 was 3·5 x 10^{-7} cm^2/sec, which agrees reasonably well with these other authors. But it must be emphasised that the result from the present studies is at best an estimate, and is for the sheath only, whereas the other authors' results were for the whole sheathed nerve. The

agreement must be further qualified because the method used by Ehrenberg and by Rud is open to some criticism. They adapted Hill's method (1928) for calculating the diffusion of O_2 into a homogeneous cylinder. This required measurement of nerve radius, knowledge of the effective blocking concentration of local anaesthetic inside the nerve trunk, and of the time taken to completely block conduction in the frog sciatic nerve-muscle preparation.

Their assumptions were that the nerve, including the sheath, was a uniform diffusion barrier, that the base was the only diffusible form, and that all fibres required the same concentration of base to block conduction. Consequently, their values must include a substantial degree of variation.

Strobel and Bianchi determined D in frog nerves by the washout of ^{14}C lidocaine from the nerves. For desheathed and sheathed nerves at pH 7·2, D was $8{\cdot}5 \times 10^{-7}$ and $2{\cdot}9 \times 10^{-7}$ cm^2/sec, respectively; the presence of the sheath decreased the coefficient substantially. Allowing for the relative diffusion distances, approximately 400u

from the centre of the nerve and 30u through the sheath, the apparent coefficient for diffusion through the sheath must be small, perhaps of the order of 5 x 10^{-8} cm^2/sec. Correcting for the proportion of base at this pH, the estimate is 2·7 x 10^{-7} cm^2/sec. This is in reasonable agreement with the present estimates at pH 7·2 of 2·1 x 10^{-7} cm^2/sec.

These authors also found that the efflux was less when the external pH was 8·2, contrary to the present study. Their observation is explained by the larger concentration of base at the higher pH outside, causing a smaller gradient for the base from inside to out.

The results from the present studies are further evidence that the nerve sheath is a barrier to diffusion, particularly to ionized and polar molecules (the local anaesthetic cation, and sucrose respectively), and less so to lipoid soluble molecules such as the local anaesthetic base.

The Site of Action of Local Anaesthetics

It is now well established that the most active form of the local anaesthetics is the cation, at least in the frog (Dettbarn, 1962; Strobel and Bianchi, 1970), rabbit (Ritchie et al, 1965), and in the squid giant axon (Narahashi et al, 1970, 1969; Frazier et al, 1970). Where this cation acts is not so clear.

In the present experiments, desheathed nerves were more rapidly blocked by lidocaine with 50% CO_2 at a low pH when compared to the effect when the pH of the solution was high (Fig. 9). At the higher external pH, more of the local anaesthetic exists as the base, so that diffusion into the axon could be expected to be greater at the higher pH, particularly in the presence of CO_2. The result of these actions would be a higher concentration of cation within the axon when the external pH was high compared to the concentration when the external pH was low. Despite a higher concentration of cation within the axon, the lower pH blocked more effectively. The conclusion is that the local anaesthetic probably acted on the outside of the membrane.

The results illustrated in Figure 13 offer additional evidence for this conclusion. In these experiments the desheathed nerve was only partially blocked by local anaesthetic at pH 7·3; when pH was changed to 5·5 there was a rapid increase in the rate of block for several minutes, which on returning to the pH 7·3 solution was rapidly reversed to above the initial level. Presumably only the external pH was changed, causing an increased cation concentration on the outside of the axon at the lower pH.

Another possibility is that at the higher pH local anaesthetic is lost into the axons by a concentrating effect (see below), through the large surface area of the axons. This may have lowered the concentration of local anaesthetic in the immediate vicinity of the site of action and allowed conduction of the action potential. This argument supports the idea that the site of action is on the outer surface of the axons, rather than the inside.

These results are in conflict with the very strong evidence presented by Narahashi *et al* (1970) and Frazier *et al* (1970), that lidocaine acts only on the internal surface

of the squid axon. It is worth noting that TTX acts only on the external surface of the squid axon. Thus, there appears to be a species difference in the site of action of local anaesthetics.

Mechanisms by which CO_2 might Potentiate Local Anaesthetics

1. By increasing the permeability of the sheath to local anaesthetics:-
   - through a direct action of CO_2
   - indirectly by its effect on pH

2. By increasing the fraction of the cationic form of local anaesthetic at the site of action;

3. By accelerating diffusion of local anaesthetic into the nerve;

4. By an action on the axon membrane:-
   - a direct action of CO_2
   - by its effect on intra- or extra-axonal pH
   - interfering with cellular metabolism causing an alteration in Na^+ or H^+ pumping in the axon.

1. The effect of CO_2 on Sheath Permeability.

The sheath perfusion experiments with lidocaine and sucrose, in which CO_2 was equilibrated with the bath and

perfusate solutions, clearly indicate that CO_2 did not increase the permeability. Similarly H^+ did not change the permeability to sucrose (Fig. 21). Waddell and Butler (1957) tentatively suggested that CO_2 may directly increase the permeability of the blood-brain barrier, but they did not develop the idea.

2. CO_2 increases the proportion of local anaesthetic present as the cation.

This mechanism is quite possible. The best evidence for this view from the present studies is given in Fig. 13, in which desheathed nerves were blocked faster at the lower pH, which gives a higher concentration of cation. Against this explanation is the finding that while 1 mM lidocaine at pH 5·5 and 7·3 blocked desheathed nerves only a little, equilibrating these solutions with CO_2 caused much greater block. The concentration of cation was unchanged, but the number of fibres blocked was increased. In this case the effect of CO_2 was unlikely to have been mediated by change in cation concentration.

3. Accelerated Diffusion.

This mechanism was suggested by Bromage et al (1967). By maintaining a lower internal pH, the CO_2 creates and tends to maintain a large difference between the concentration of basic local anaesthetic outside and inside the nerve sheath. At a pH higher than the pK_a (7·85 for lidocaine), the proportion of base is greater than 50%. Base which diffuses to a region of lower pH is converted to the less diffusible cation. This has the effect of trapping as cation the local anaesthetic at the region of the lower pH, and at the same time maintains a relatively large gradient of concentration of base across the sheath, thus facilitating further influx.

CO_2 diffuses readily through biological membranes (Halpern and Binaghi, 1959; Caldwell, 1958), whereas H^+ does so only very slowly (Spyropoulos, 1960; Waddell and Bates, 1969). Consequently, CO_2 can lower the pH inside the nerve sheath and axon while the external pH is high. Presumably, the fall in pH will be less inside the axon because the buffering capacity of the axoplasm is likely to be greater than that of the nerve extracellular fluid.

The evidence from the present experiments that this may happen is as follows: the results in Table 3 show that the presence of CO_2 in the external radio-active solution significantly increased the net flux of lidocaine through the nerve sheath. The observed small drop in internal pH suggests that the acceleration of local anaesthetic influx could have been due to the mechanism proposed by Bromage et al (1967). In the presence of CO_2, the influx at pH 7·3 was about equal to that from a solution at pH 8·3 without CO_2. The fact that there is 3·4 times more base at pH 8·3 than at 7·3 indicates the magnitude of the CO_2 effect.

The perfusing solution was deliberately buffer-free to accentuate the pH change. With the same buffering capacity as normal extracellular fluid, there would have been less fall in pH. However the rate of flow of fluid in a nerve is normally negligible, which would allow full equilibration of the fluid with CO_2. In the perfusion experiments, the level of pH indicates that full equilibration was not reached, and to some extent this would compensate for the absence of buffer in the perfusate.

If acceleration of diffusion does occur, the block of sheathed nerves should be proportional to the effect of CO_2 on the pH of the nerve extracellular fluid. When the T_3 of the curves in Fig. 11 are plotted against the concentration of CO_2 in the local anaesthetic solution applied to the nerves, one obtains a sigmoid relationship reaching a maximum at about 20% CO_2 (Fig. 23). For comparison, the change of pH caused by equilibrating CO_2 with Ringer containing 2 mM bicarbonate was plotted against the concentration of CO_2 (Fig. 24).

The solubility (moles/l) of CO_2 in Ringer's solution is (Umbreit et al, 1964):-

$$CO_2 = \frac{P \times \alpha \times Z \times 1000}{760 \times 22{,}400 \times 100}$$

where P = atmospheric pressure (mm Hg)

Z = concentration of CO_2 in air (ml/100 ml)

α = solubility coefficient of CO_2 in Ringer at 20° C (ml/100ml)

22,400 is Avagadro's Number.

The pH of the Ringer's solution was calculated from the Henderson-Hasselbach equation:-

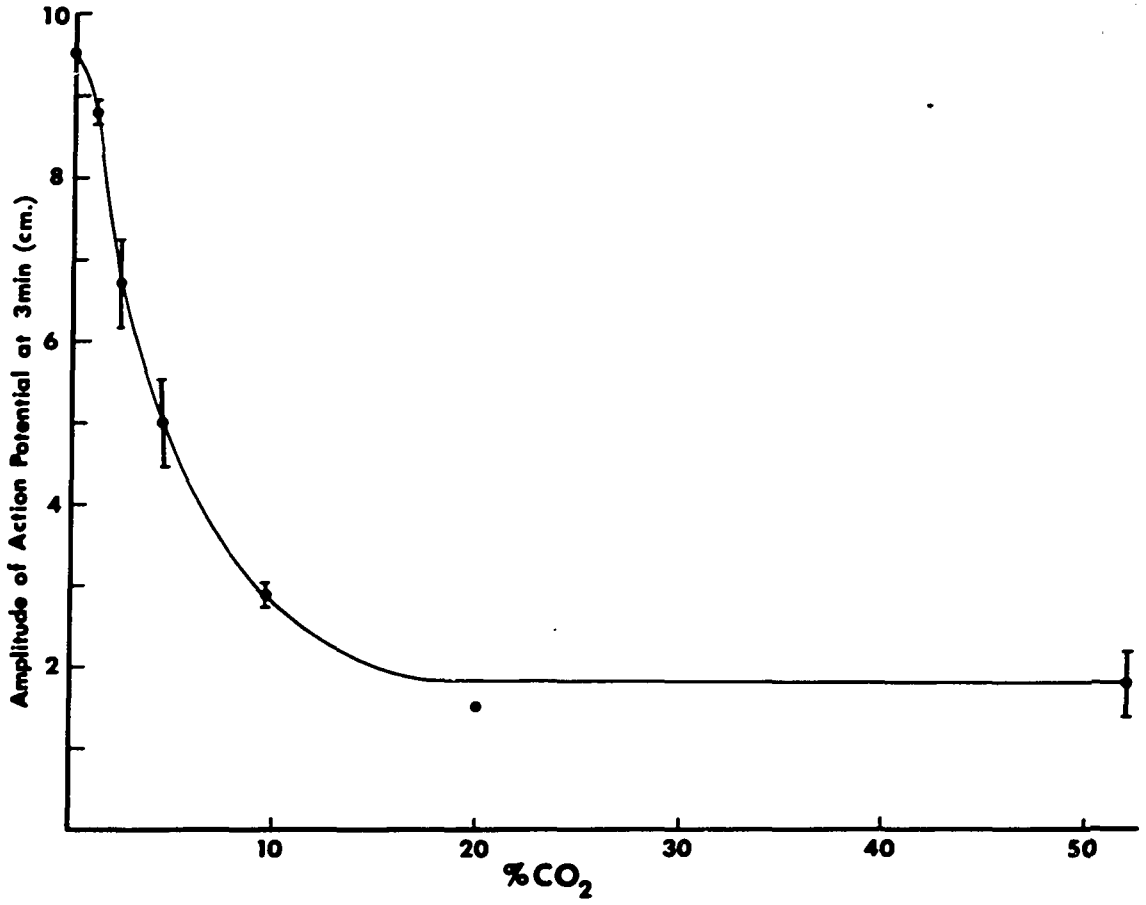


Fig. 23

Height of the compound action potential after three minutes application of lidocaine solutions (1 mM, pH 7·3) containing different concentrations of CO_2, plotted against the concentration of CO_2. (From Fig. 11).

Points are mean $\pm$ 1 S.E. Point at 20% CO_2 is one reading only.

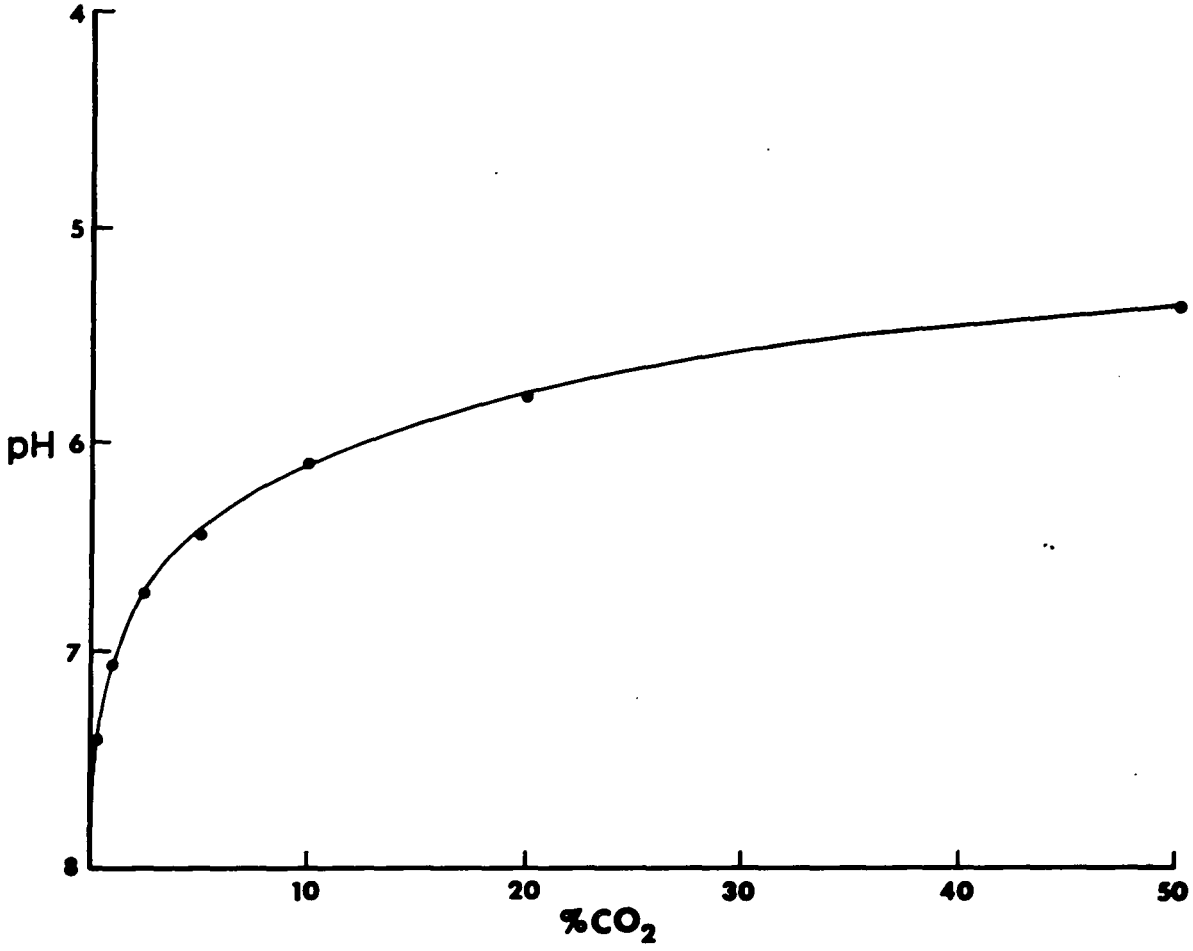


Fig. 24

The effect of CO_2 on pH of Ringer's solution containing 2 mM HCO_3^-. Points were calculated from the Henderson-Hasselbach Equation, $pH = pk + \log \frac{[HCO_3^-]}{[CO_2]}$. pK_a for CO_2 in Ringer at 20° C is 6·39. (Umbreit et al, 1964).

$$pH = pK_a + \log \frac{[HCO_3^-]}{[CO_2]}$$

$$= pK_a + \log [HCO_3^-] - \log [CO_2]$$

The resulting curve is similar in slope to that in Figure 23, with the most rapid change between 10 - 20%, and a much slower change from there to 50%. In other words, the effect of CO_2 on pH within the sheath is similar to its effect on the block of the action potential. This is consistent with the proposed accelerating mechanism.

Similar conclusions can be drawn from Figures 7, 8, 9. Sheathed nerves were only slightly blocked by lidocaine at pH 7·3 (Fig. 8) or CO_2 in Ringer at pH 7·3 (Fig.7), yet when lidocaine with 50% CO_2 at pH 7·3 was applied to these nerves, the rate and degree of block was greatly increased (Fig. 9). The result was more than simple summation, suggesting that the CO_2 caused more local anaesthetic to reach the site of action. It should be pointed out, however, that summation could just conceivably account for the result in Fig. 9, if both CO_2 and local anaesthetic separately reduced the "safety factor" to just short of blocking conduction. In this case, summation of each would cause a block.

Taken together, these results provide evidence for the operation of a mechanism for accelerating diffusion of local anaesthetics.

4. The direct effect of CO_2 on the Axon Membrane

CO_2 in Ringer's solution caused a partial block of both sheathed and desheathed nerves (Fig. 7), with somewhat greater block at the lower pH in the desheathed nerve. This may simply be an additional effect of the low pH (Fig. 6). Despite the fact that CO_2 diffused freely throughout the nerve, the effect on the desheathed nerve was significantly greater than on the sheathed nerve. One explanation for this may be that 50% CO_2 causes reversible swelling of the axons which stretches and even disrupts the membrane and thus blocks conduction. The swelling would be prevented by the nerve sheath.

While the effect on the sheathed nerve was not great, there may have been substantial effects which were not detectable from the compound action potential. No effect would be seen until the "safety margin" for conduction is

reduced from its normal of 4 - 5 (Katz, 1966, p. 92), to nearly 1, when only a small additional effect would be sufficient to cause a marked block. This fact may account for the rather dramatic results to be described in the following paragraph.

The potentiation of the action of local anaesthetics on sheathed nerves by the direct effect of CO_2 is well seen in Figure 14, where 9·6% CO_2 at pH 5·5 increased the degree of block caused by 0·25 mM lidocaine at pH 8·3. Because the external pH was reduced, the increased degree of block in this case could not be a manifestation of the concentrating effect - that is, there would be no increased diffusion of local anaesthetic through the sheath. The increased block may have been caused either by formation of cation (owing to the pH changing from about 7·3 in the extracellular fluid to about 6·5) or by an interaction of CO_2 and local anaesthetic. In view of the effect of CO_2 on nerve function, and the formation of cation, the direct action seems the more likely mechanism.

The potentiation of block by the CO_2 lasted about two minutes and then started to reverse. The max-

imum effect of CO_2 alone in the present experiments (Fig. 7) occurred after about 2 minutes. Together these observations also suggest that the potentiation was due to CO_2, but at the same time some other process must have been occurring to account for the recovery. The other process may be the diffusion of local anaesthetic out of the nerve into the region of low pH. After being in the test solution for 1/2 to 1 hour, the total concentrations of local anaesthetic inside and outside are likely to be approximately equal. The pH inside the sheath was about 6·4 (9·6% CO_2), so that there was a concentration gradient for base from inside to out. When the outside solution was changed to a high pH without CO_2, the reversal of block continued over the following 2 minutes. The interpretation of this effect is that just as it took several minutes for CO_2 to have its effect initially, so it took some time for the effect to abate, despite the reversed concentration gradient for the base. In time, the concentration of local anaesthetic at the site of action increased and the block was intensified.

The effects when procaine was used in the experiments above were similar, but somewhat greater. The reason for this may have been that procaine was more diffusible than lidocaine, so that the rate of change at the site of action was greater, causing faster changes in block. The D_{app} for lidocaine and procaine at pH 7·39 into a sheathed nerve are 1·67 and 1·68 x 10^{-7} cm^2/sec respectively, but since at this pH procaine is 97% ionized and lidocaine only 77%, procaine is probably more diffusible (Löfgren, 1948).

Further support for a direct action of CO^2 comes from the effect of local anaesthetic, at pH 5·5 and 7·3, with and without CO_2, on the desheathed nerve. The rate and degree of block with CO_2 was substantially greater at both pHs when CO_2 was added, yet because the pH was constant, the concentration of cation was unchanged. These results also indicate that CO_2 has a direct effect on the axon. On the same basis, it can be argued that the action of CO_2 is probably not mediated by changes in external pH. These results do not exclude the possibility that intracellular pH changes are an important factor, but this has not been examined in the present studies.

Mechanism of Action of CO_2 on the Nerve Membrane.

In the present studies, there is little evidence on the mode of action of CO_2 except for the observation that it has effects similar to those of local anaesthetics, such as causing a decrease in the height of the compound action potential and an increase in its latency. Following the demonstration by Walker and Brown (1969) that CO^2 caused an increase in g_{Cl} in Aplysia neurones, tests were made with 50% CO_2 in which methylsulphate was substituted for Cl^-, but no significant change was detected (Fig. 25).

The action of CO_2 on Aplysia neurones can be depolarizing (Chalazonitis, 1963), hyperpolarizing or both (Brown and Berman, 1970; Walker and Brown, 1969). There were similarly varied actions on lumbar neurones (Speckman et al, 1970; Papajewski et al, 1969) and also on other neurones. CO_2 causes excitation of carotid chemoreceptors without affecting adjacent baroreceptors (Eyzaguirre and Zapata, 1968; Heymans and Neil, 1958), depolarization and excitation of mammalian respiratory centre neurones (Von Euler and Söderberg, 1952), but on the other hand, it also causes hyperpolarization of phrenic motor neurones (Gill

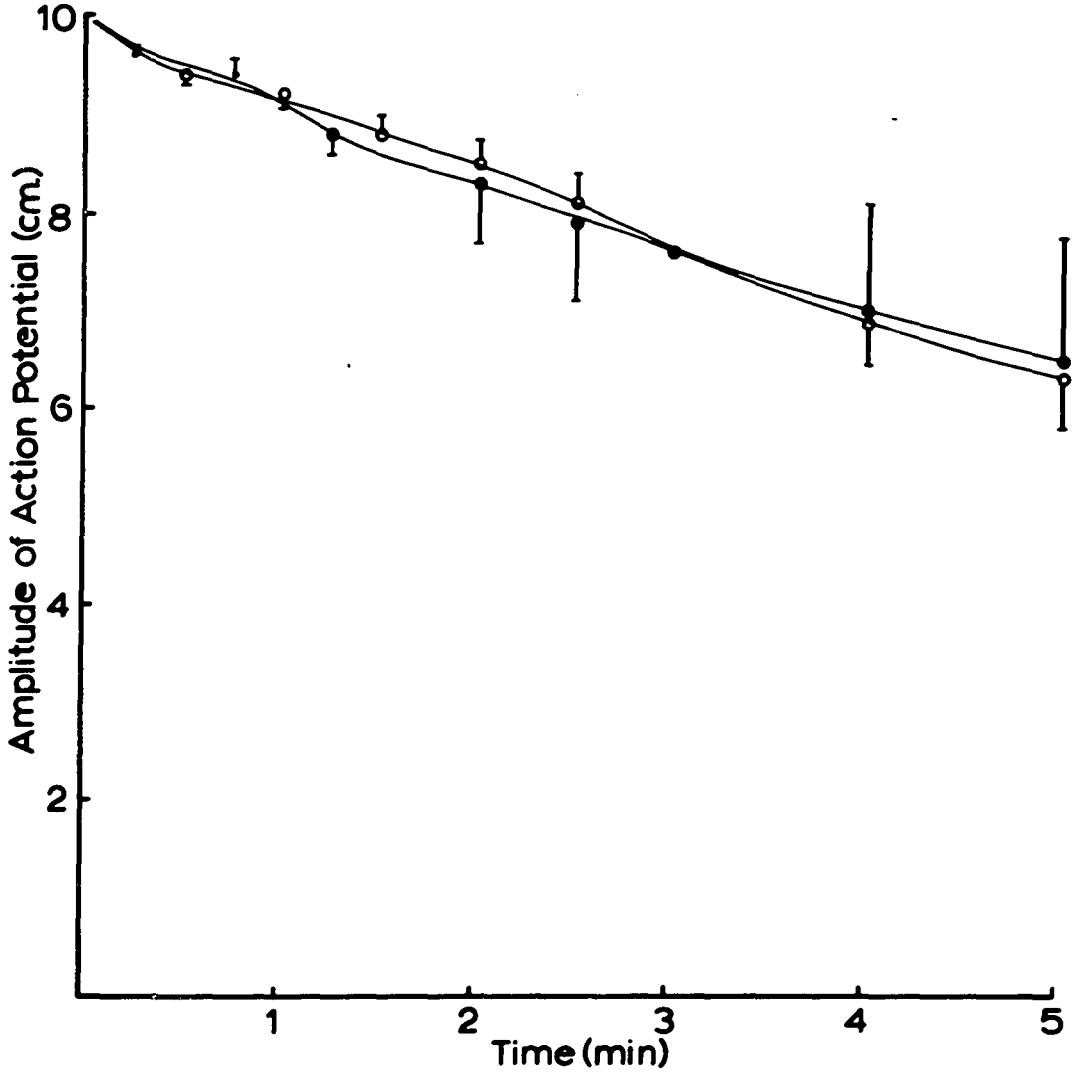


Fig. 25

The effect of substituting sodium methylsulphate for NaCl on the action of 50% CO_2 and 1 mM lidocaine at pH 7·3 on compound action potential of desheathed frog sciatic nerves. Points are mean ± 1 S.E. (n = 7)

Open circles, normal Ringer; closed circles, substituted Ringer.

and Kuno, 1963), depression of monosynaptic reflexes in lumbar motor neurones (Esplin and Rosenstein, 1963), and hyperpolarization of cortical neurones (Krnjević et al, 1965).

As mentioned previously, Brown et al (1970), and Brown and Berman (1970) have shown that CO_2 increases both the chloride and potassium conductance of Aplysia neurones through its effect on pH; they have suggested that the wide spectrum of action of CO_2 depends on the E_{Cl} of the neurone involved. They imply that this is the mechanism in all excitable cells affected by CO_2, although they do not state this directly. A large increase in g_{Cl} when the E_{Cl} was more negative than the membrane potential (E_m) would cause hyperpolarization, and when $E_{Cl} > E_m$ depolarization would result. So far, this phenomenon has not been examined in the nervous tissue of other species.

Further support for the concept that changes in pH mediate the action of CO_2 comes from Hille (1968 b), who found in the frog node of Ranvier preparation that as fixed acid was added, the g_{Na} fell. The effect on g_{Na} is in contrast to that seen in Aplysia, but is further sup-

ported by Straub (1956) who tested CO_2 on frog nerves in low Na^+ and Na^+-free solutions, and concluded that CO_2 affected the Na^+ channels. Papajewski et al (1969) always found an increase in membrane resistance and decreased post synaptic potential associated with the variable hyperpolarization or depolarization of the cells they studied. However, they do not have an explanation for these effects.

Local anaesthetics and CO_2 have been applied independently to the isolated rabbit atrium (Laborit et al, 1969), where both agents reduced the rate and amplitude of contraction; these parameters were only slowly corrected by the addition of Ca^{++}, but if Ca^{++} was added first, there was a small increase in rate after CO_2 and procaine, but no change in amplitude. These authors believe that CO_2 and local anaesthetics act in a similar manner to prevent movements of Ca^{++} across the cell membrane.

Sears and Eisenberg (1961) proposed that CO_2 acted by increasing the packing of the lipoid molecules in the nerve membrane, causing an increase in membrane resistance and so making the cell less excitable. Feinstein (1964) showed actions of Ca^{++} and local anaesthetics on lecithin

and cholesterol membranes which were very similar, and suggested that local anaesthetics competed with Ca^{++} for sites on the membrane. Shanes et al (1959) using the voltage clamp technique, showed a relationship between Ca^{++} and local anaesthetics. Hille (1968 b) noted that the effects of Ca^{++} and H^{+} in decreasing g_{Na} were interrelated.

Thus, while there is good evidence that in Aplysia CO_2 acts mainly by lowering the pH, which leads to an increase in the membrane g_{Cl}, and to a lesser extent, g_K, the evidence from other species suggests that the effect of CO_2 may be similar to that of local anaesthetics, and may involve Ca^{++} movements, causing a block of the transient increase in g_{Na} which occurs during excitation.

From the evidence presented above, it is possible to offer an account of the way in which CO_2 potentiates the action of local anaesthetics.

It is most likely that CO_2 reaches the axon before the local anaesthetic, with the earliest phase of the block

due to CO_2 acting through its effect on local pH. Direct effect of the CO_2 on the membrane seems less likely.

The acceleration of diffusion of the local anaesthetic by CO_2 should occur concurrently because the CO_2 need only change the pH just at the inner surface of the sheath to be effective. However, time dependent diffusion from this point to the centre of the axon is required for complete block, and it is likely to take longer for local anaesthetic to reach a sufficient concentration to potentiate the block by CO_2. The effects of local anaesthetics will follow closely behind those of CO_2.

The effect of CO_2 on pH and indirectly on the fraction of cation, will ensure that all local anaesthetic present is active and only the minimum amount of local anaesthetic need diffuse to the site of action.

Condouris and Shakalis (1963, 1964) tested the effect of local anaesthetic together with a range of concentrations of CO_2 on sheathed rat sciatic nerves. While recording the compound action potential height, they exposed the nerve first to Tyrode's solution equilibrated

with CO_2 and then applied the local anaesthetic (cocaine or procaine) equilibrated with the same CO_2. Generally, the findings of these authors are in accord with the results of the present experiments, except that they found that the maximum potentiation occurred at 5% CO_2. However, their paper gave no data for comparison with the present results.

While they suggested several possible mechanisms whereby CO_2 might potentiate local anaesthetics, they do not have conclusive evidence for a particular one. They thought that a change in pH was not the potentiating mechanism, because they maintained the pH at 7·4. It is clear that they believed that the perineurium of the rat sciatic nerve offered no barrier to diffusion of H^+. They say that "it is conceivable that the joint actions of the drugs and carbon dioxide on the membrane would augment each other." But to them this was unlikely in view of what they termed the "relief of local anaesthetic block by anodal polarization." Finally, they offered no satisfactory explanation for the potentiation.

Carbonated Local Anaesthetic in Clinical Anaesthesia

The use of carbonated local anaesthetic has been reported so far for two procedures, viz., epidural analgesia (Bromage, 1964, 1967; Bromage *et al*, 1967) and axillary brachial plexus block (Schulte-Steinberg *et al*, 1970). The lidocaine was in the base form (not the hydrochloride salt) dissolved in isotonic solution equilibrated with 100% CO_2, to form lidocaine $\cdot H_2CO_3$. The solution was heavily buffered with bicarbonate (approximately 50 mM) to pH about 6·5. The solution tends to bubble when opened, but PCO_2 subsequently remains constant for a long time if it is not agitated.

Even with the local anaesthetic buffered to 6·5 there would be only about 4% as the base and, as seen from the experiments reported here, diffusion to the site of action would be slow, and perhaps even slower than if no CO_2 has been added. Therefore, to explain the increased efficiency of the local anaesthetic *in vivo* on the present hypothesis,

the pH of the injected solution must rise. This would occur if CO_2 left the solution and allowed the pH to rise because of the bicarbonate buffer. At a PCO_2 of 100 mm Hg the pH would be 7·38, and at 40 mm Hg it would be 7·78, which makes available sufficient base for diffusion. There is no direct evidence about the rate at which CO_2 leaves the injected bolus, but the experiments on nerves at these pHs suggest that it does so rapidly.

The experiment to test this would be to monitor the pH or PCO_2 of a bolus injected into the epidural space with a pH or PCO_2 electrode or by taking serial samples of the injected solution. However the injected local anaesthetic spreads in all directions, leaving a very small volume which would be insufficient to sample. Unfortunately a sufficiently small pH electrode is not readily available, so this experiment has so far not been possible. The assumption then remains that CO_2 rapidly leaves the injected local anaesthetic and diffuses across the dura and coverings (dura and perineurium) of the nerve roots to the site or sites of local anaesthetic action (Bromage, 1967). On the other side of this diffusion barrier it lowers the pH, increasing the con-

centration of cation and decreasing that of base, and also acts directly on the axons to depress nerve activity, and potentiate the local anaesthetic. It does this either by increasing g_{Cl}, or more likely by reducing g_{Na}, perhaps by a direct action on the nerve membrane or through formation of H^+.

So far CO_2 has only been used together with lidocaine clinically, and with procaine, cocaine and lidocaine *in vitro*. From a clinical viewpoint the advantages of carbonating local anaesthetics are the possibility of speeding up the onset and increasing the intensity of block with a local anaesthetic which has the desirable characteristic of long action with an acceptable low level of toxicity, but whose onset is excessively slow (such as bupivacaine, the butyl analogue of mepivacaine).

SUMMARY

1. The ways in which CO_2 potentiates the action of local anaesthetics was investigated in the frog sciatic nerve preparation. While using conventional techniques to stimulate and record the compound action potential of the nerves, local anaesthetics with various combinations of CO_2 and pH were applied to a segment of the nerve.

2. It was found that lidocaine blocked the compound action potential of sheathed rat and frog sciatic nerves more effectively when the local anaesthetic was equilibrated with CO_2 (1 - 50%). The block was most effective when the pH of the solution also was high (> 8).

3. It was confirmed that 50% CO_2 has a direct depressant effect on conduction in frog nerves. This was greater when the nerve was desheathed.

The permeability of the frog sciatic nerve sheath was investigated by forming a tube of isolated sheath through which radioactive sucroce or lidocaine flowed. The efflux of radioactivity into a bath surrounding the tube was measured.

4. The permeability of the sheath to lidocaine at pH 9·3 was about 7 times greater than to lidocaine at pH 5·5. The apparent permeability of the sheath was linearly related to the concentration of the non-ionized lidocaine.

5. The sheath presented a greater barrier to diffusion of a polar compound and a positively charged ion (sucrose and lidocaine cation, respectively) than to diffusion of a lipoid soluble compound (lidocaine base).

6. The permeability of the sheath to sucrose and local anaesthetic was unchanged by 10% CO_2. Variations in pH did not alter the permeability of the sheath to sucrose.

7. Ten percent CO_2 was able to increase the flux of radioactive lidocaine through the nerve sheath, probably by lowering the pH inside the sheath. This was described as accelerated diffusion of the local anaesthetic by CO_2.

8. The evidence for the site of action of local anaesthetics was reviewed, and it was concluded that the most

likely site was the external surface of the axon membrane. This was in contrast to the squid axon where local anaesthetics act on the external surface.

9. CO_2 appears to act like local anaesthetics, by interfering with sodium channels in the membrane.

10. It was concluded that CO_2 potentiates local anaesthetics by three mechanisms. These are: a rapid direct blocking effect on the nerve fibre; an acceleration of the rate of entry of local anaesthetic into the nerve bundle; and by promoting the formation of cation through its effect on pH. The direct effect summates with the action of the local anaesthetic on the membrane.

11. Finally the ways in which local anaesthetics used clinically are enhanced by CO_2 was briefly discussed. It was apparent that an essential feature of the action of CO_2 when "carbonated" local anaesthetic solutions are injected may be that the CO_2 rapidly leaves the injected solution thus allowing its pH to rise.

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