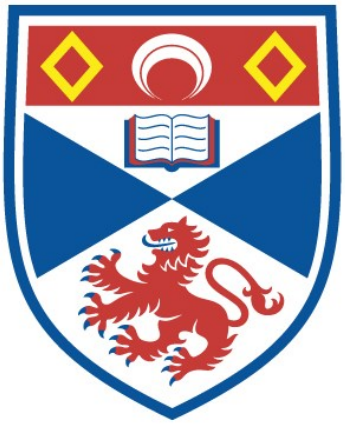
THE PERMEABILITY OF THE SHEATH AND THE SELECTIVE UPTAKE OF NICOTINIC ACID IN CRAB AXONS

John Moss Armson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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THE PERMEABILITY OF THE SHEATH AND THE SELECTIVE UPTAKE OF NICOTINIC ACID IN CRAB AXONS

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DECLARATION

I hereby declare that the work recorded in this Thesis has been carried out by myself, that it is of my own composition and that it has not been submitted in any previous application for a higher degree

SUPERVISOR'S CERTIFICATE

I certify that John Moss Armson has fulfilled the conditions laid down in the regulations for a Degree of Doctor of Philosophy, under the Ordinance No. 16 of the University Court of the University of St. Andrews and that he has accordingly qualified to submit this Thesis for the degree of Doctor of Philosophy.

G.A. Horridge,
Supervisor.

P R E A M B L E

From the work described in the first part of this thesis the conclusion can be drawn that nicotinic acid enters alike the sensory and motor fibres in the nerve trunk of a crab leg, and that the gradient of radioactivity seen in the nerve after labelled doses of NA can be explained by a consideration of the anatomy of the nervous system in the leg. When the work was commenced there was in hand Van der Kloot's report (1960) that NA had a connection with the motor transmitter substance in Crustacea to put the work into a larger context. But as it turned out, this context was destroyed by the conclusion that the link between NA and the transmitter does not exist, which, although a negative result, is none the less valuable for that.

The second part, which was triggered off by ideas rooted in the NA work, has borne more fruit in the form of positive results. The low permeability of the sheath around the Carcinus motor axons to a small concentration range is most curious: that it is a genuine result is certain from the consistency with which it appeared. What it means is less clear, but it has been suggested that it represents a safety mechanism of advantage to this particular species. Since this work was completed, a beautiful report has appeared by Nicholls and Kuffler (1964)

of their work on the permeability of the sheath around the neurons of the leech central nervous system. They suggest that the sheath does not act in this preparation in an active fashion since neither cooling the preparation to 1-4°C nor removal of most of the glia had any effect on the rate or size of the effect caused by changing the external potassium or sodium. Unfortunately in all their experiments one layer around the neurons - the endothelial layer outside the connective tissue capsule - was damaged, and they do not exclude the possibility that ions are leaking to and from the fibres through the damaged areas. With reference to the crustacean preparation again: what would be of interest now would be knowledge of the structure and function of nerve sheaths in other types related to Carcinus. For example, do those animals which regulate their blood composition more carefully have such an anatomically and physiologically complex sheath?

C O N T E N T S

Contents (continued)

PART I

INTRODUCTION

Acetylcholine as a crustacean transmitter

Of recent years there has been an increasing avalanche on the problems of chemical transmission at the junction between nerves and the end-organ, be it muscle, endocrine tissue or nerve. But most of the work, not surprisingly, ever since the idea of chemical transmission was first adumbrated in 1923 by Loewi, has been on vertebrates, and, more especially, on mammals, as a result of which there is now good evidence for the identification of at least two of the neurotransmitters in vertebrates.

In this field, the investigation of invertebrates has lagged far behind, if only because it has not the immediate medical value that accompanies work on those animals more nearly related to man. Not a single compound has yet been identified as a transmitter substance in any of the invertebrate phyla. Acetylcholine (ACh), almost certainly the link between the somatic motor nerves and the skeletal muscle in vertebrates, is almost certainly present in Crustacea, and this has led many to infer for it transmitter function in these species. Its presence has never actually been shown by the standard chemical and chromatographic tests, but rather deduced from the presence of cholinesterase, in which the crustacean nerve is unusually rich: the level in the cerebral ganglia is about the same as that in the brain of Rana esculenta, but is about five times greater than this in the

abdominal ganglion (Walop and Boot, 1950). Crustacean nerve is known to be able to synthesize ACh (Easton, 1950); Walop has confirmed this for Carcinus maenas (1950). Florey and Biederman (1960) claim that ACh is present in the peripheral nervous system of Cancer, but only in the sensory fibres. They only use bioassay methods to establish this. Of course, it is still possible to argue that the nerves really contain a closely related choline ester, but it seems unlikely.

There is no evidence to suggest that in the Crustacea, unlike the vertebrates, ACh is a transmitter substance. Attempts have been made to put ACh on the motor nerve endings, but, even when allowance is made for the difficulties of getting the substance actually to the site of the ending, no unequivocably positive results have been obtained. Florey (1954) reported success in causing a contraction with an ACh injection into crustacean muscle, but Wiersma (1961) criticises this on the grounds that evidence was lacking that the nerve itself was not stimulated by the concentration of ACh used. Florey and Biederman (1960) find no ACh in motor fibres and categorically deny that it is the neuromuscular junction transmitter.

ACh has often been seen to have a stimulant effect on the crustacean heart, however, although just where it acts is not clear. There is some evidence to suggest that ACh has some role in controlling the activity of the abdominal ganglion in the crayfish and cockroach (Twarog and Roeder, 1957) and also as a possible transmitter between the longitudinal giant fibres and motor neurons to the abdominal flexor muscles in

the crayfish (Welsh, 1961), but in neither case is the evidence very convincing. Furshpan and Potter (1959) think that these synapses may not use chemical transmission at all.

Thus the field is open for ideas about the nature of the motor transmitter in Crustacea. The vertebrate transmitter ACh is ruled out, and adrenaline and noradrenaline, which have not yet been shown to be present naturally in Crustacea, have only a slight effect on the neuromuscular junction, if any (Wiersma, 1961). Likewise 5-hydroxytrypamine has little or no effect on these junctions. The latest idea, which is well founded, is that L-glutamate, or a substance closely related to it, may be the motor transmitter. Robbins (1959) and Van Harreveld and Mendelson (1959) found that glutamate applied to crustacean muscle so closely mimicked nervous stimulation of the muscle that they suggested identity or close relation with the transmitter. Florey (1964) points out, however, that the glutamate concentration in the blood of these animals is 100 times above threshold for the action at neuromuscular junction, but that blood is not able to stimulate these junctions, and suggests that either there must be some 'neutralising' agent in the blood as well, or that the glutamate must be present in an inert form. It may be that there is some equivalent of a 'blood-brain' barrier between the blood and the neuromuscular junction, and, of course, in large concentrations, like ACh at the vertebrate end-plate, L-glutamate has an inhibitory effect on the crustacean receptor (Takeuchi and Takeuchi, 1964). In the same place, these authors also show that the

areas on the muscle which are sensitive to applied glutamate are exceedingly localised, and correspond to the end-plate regions.

But the Takeuchis make only the cautious conclusion that the receptors which respond to the L-glutamate are normal neuroreceptors, for it is notoriously difficult to be certain about the identity of a transmitter substance: to the critical it is indeed impossible. The way γ-aminobutyric acid (GABA) climbed to fame as the inhibitory transmitter in Crustacea was matched only by the way it sank again into disfavour, if not oblivion. The classical tests of Dale, even as expanded by Florey (1960), for the testing of hypotheses relating to transmitters are not sufficient to be certain. The application of GABA to crustacean organs mimics the action of the inhibitory transmitter to such a degree that no difference may be detected between them. 'Substance I [the supposed inhibitory transmitter], as far as we could ascertain,' said Florey in 1960, 'acts in a manner identical with that of GABA'; and in the same place he says, 'It was surprising when Chapman and I discovered that there are only traces of GABA present in the peripheral nervous system of crustacean species from which we could extract potent Factor I.' A year later he published a paper on 'The Non-identity of the Transmitter Substance of Crustacea Inhibitory Neurones and GABA' (1961).

Pharmacology of the crab heart

Another example of the way in which a compound was almost certainly identified, and then later discredited as a transmitter, is ACh in the crustacean heart. ACh was known to have an effect on the entire heart but it was not certain just where this action was taking place. Preparations of the heart included ganglion cells in the dorsal wall and it could be that the ACh was acting on these, on the junction between them and the nerves which supplied them; on the muscle directly or on the junction between the muscle and the ganglion cells. Garray (1942) in Limulus, and Florey (1963) in Homarus dissected away the nervous parts and showed that the cardiac muscle was quite unaffected by ACh.

In 1939, Welsh said that since

(i) ACh mimicked closely the acceleratory transmitter,

(ii) this effect was blocked by atropine,

(iii) the effect was potentiated by eserine, and

(iv) ACh occurred naturally in the system,

'the active principle in question is, with little doubt, acetylcholine'. The case for identifying it with the transmitter may have looked well founded then, but it was clearly based on ideas inherited from vertebrate physiology, and even when Smith in 1947 found that eserine and atropine, whatever they might do to the effect of applied ACh, did nothing to affect the normal beating of the heart, he did not doubt the proposal of Welsh. Not until 1954 did the Floreys follow this up

and conclude that ACh was not the transmitter. It was then widely thought that an adrenaline-like compound might take its place. The displacement of ACh was completed later when Florey showed that there was none to be found in the cardio-acceleratory fibres, nor in the cardiac ganglion; nor was there more than a trace of cholinesterase in the heart muscle or ganglion. The response of the heart to stimulation through the normal nervous channels was not in any way affected by the previous application of ACh, nor was any ACh found in perfusates of the eserinised heart.

The crab heart responds to a number of other well-known pharmacological agents. Alexandrowicz and Carlisle (1953) saw that adrenaline and noradrenaline, known to be transmitters in the vertebrates, increased the size and rate of the beat of Cancer heart, and Baylor (1942) notes the same for whole Daphnia. In Maia the former authors noted that tyramine slowed the heart and diminished the size of the beat in low concentrations, and that this was followed by the reverse effect if the concentration was greater. Davenport (1941) said that nicotine in concentrations of 1:20,000 increased the beat rate and size in Cancer, and that this effect was not quickly reversed when the heart was washed. The effect of different ionic environments on the heart was investigated by Hogben in 1925 when he described how low calcium around the heart, especially with low potassium as well, sent the heart into tetany, whilst excess calcium had the reverse effect. Increased potassium alone first excited and then depressed the heart.

The more recent ideas of the nature of the transmitter substance in the cardiac ganglion start with that of the Floreys in 1954, who suggested that it might be 5-hydroxytryptamine. Carlisle (1956), however, was unable to find any evidence that 5-hydroxytryptamine is present. He did find an ortho-dihydroxyindole alkylamine, though, which is tentatively identified with 5,6-dihydroxytryptamine.

Pharmacology of crab somatic muscle

Few of the compounds which act on the crustacean heart act visibly on the somatic musculature: ACh, curare-like compounds and anti-cholinesterases do not affect the muscle (Ellis et al., 1942) and neither do nicotine nor GABA have any effect on muscle when added alone (Davenport, 1941; Van Harreveld and Mendelson, 1959).

The effect of glutamate on crustacean muscle has been widely studied. Van Harreveld and Mendelson in 1959 reported that L-glutamate when put on to muscle caused it to contract with a slow tetany. This effect seems to be very specific, for glutamine was only 1/70th as powerful in causing a contraction, and D-glutamate and aspartate were even less effective. After the addition of glutamate, the normal response to nervous stimulation is much reduced, though direct faradic stimulation of the muscle still has its usual effect. The response to applied glutamate can be reduced by stimulation of the inhibitory fibres to the muscle, and the concentration of glutamate needed to elicit

a standard contraction is much higher if it is first mixed with GABA. Robbins found that while concentrations of 10^{-4} of glutamate excite, higher levels block contraction. Lower levels (10^{-5}) facilitated nervous stimulation of the muscle.

Factor S

At a time when interest in GABA as the inhibitory transmitter was at its height, another compound was brought to the fore by Van der Kloot (1960) with his proposal that there might be some link between the motor transmitter and a metabolite of nicotinic acid (NA). His conclusions were based on several observations which formed the starting-point for this present work:

(i) A substance, which he proposed to call Factor S, in extracts of crayfish and lobster nerve stimulated contraction of the closer muscle in 'low' concentrations. Moreover the resulting contractions were quick and transient as they would be if the extract truly contained the transmitter.

(ii) The same substance was also found in perfusates from stimulated, but not from unstimulated, claws.

(iii) Chemical tests and chromatography suggested that the compound and nicotinic acid were related.

He was not able to identify the compound fully and therefore not able to synthesise it, which means that one important test for its being the transmitter is missing. So too there is no information about blocking the effect. But it is clear that the compound in question is present in nervous tissue in large amounts - a matter which proved embarrassing for supporters of the transmitter functions of both ACh in the heart, and GABA in the limbs.

Repeating some of the work of Van der Kloot, I perfused stimulated crab legs and looked for physiological activity in the effluent solution. I also injected crabs with labelled NA in an attempt to follow it in the crab metabolism. Hints as to where it might go are few indeed, for here, as in the case of transmitters, most of the work on NA has been done using vertebrates. Factor S could be separated from crude extracts of crayfish and lobster which were themselves physiologically active, by a method described by Von Euler in 1948 for the isolation of catecholamines. Nevertheless, after paper chromatography of the separated active fraction it appeared from the spot tests performed that the compound was not a catecholamine but an l-substituted derivative of nicotinamide, and Van der Kloot, having searched the literature, thinks that it might be identical with 'catechol-4' discovered by Östlund in 1954. The distribution studies of the two compounds do not overlap as they have so far been worked out, and whereas catechol-4 is said to be present in several insects, and other phyla, Factor S is so far only listed as being present in 'crayfish, lobsters, the pupa of

Hyalophora cecropia and in the brains of the rat and cow'.

It is clear from the story of ACh in crustacean heart and GABA in crustacean limbs that finding an effect when a compound is applied to an organ is no guarantee that this is the compound which normally effects that operation, and it may well be thought that Van der Kloot's conclusion, though tentative, is a little premature. There are also certain queries one would like to raise in connection with the paper: for example, he goes out of his way to say that although his extraction technique has always been regarded as specific for catecholamines, there is no reason to suppose that Factor S is such a type. But surely this is begging the question? If the extraction technique is specific, that is good reason to suppose that Factor S is a catecholamine. If he has other reasons for thinking the compound is something else, or that the extraction is not specific, they are not presented in his paper. Östlund, in the initial description of the extraction of catechol-4, clearly believes it to be a catecholamine, for he says that the method of Von Euler is 'highly specific'. The same words were used by Von Euler himself in 1948.

In some respects it has been difficult to repeat Van der Kloot's work. For example, he does not indicate what organs he used for extracting Factor S, or whether he used whole animals. In all the work reported in this thesis only limb nerves were extracted for analysis. Another example is his description of the spot tests he performed: 'To study the chemical nature of Factor S,' he says, 'the spots on the

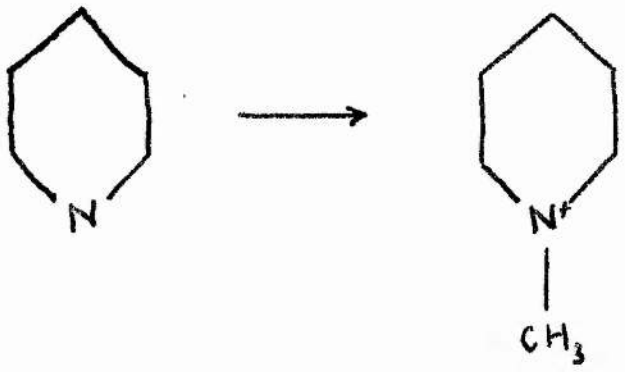
chromatograms were tested with 33 different spray and dip tests.' Only the results of five are given. There is also a misprint in the list of chromatogram solvents he used: the last solvent in Table 1 of the paper is listed as containing l-butyric acid, but it should probably read iso-butyric acid.

A preliminary report of an investigation into 'Factor S' has already appeared in print (Armson and Horridge, 1964).

The metabolism of nicotinic acid

In not all animals is NA strictly a vitamin, for most of those investigated - including man - are able to synthesise it from the aminoacid tryptophan (Krehl et al., 1945, and 1946; Hundley, 1954), cats being one exception (Da Silva et al., 1952) and hence it may well not be so among the Crustacea either. The link with tryptophan was of interest because of some of the results to be described later, but it is sufficient to note here that, as far as the literature goes, the link is one-way only: from tryptophan to NA. It would be mere guessing to propose that crabs can put this into reverse.

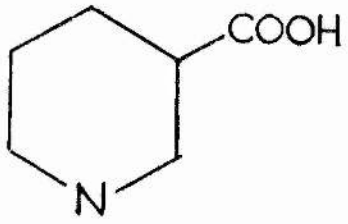
The normal detoxicating response of plants and animals to amino groups is methylation, and this occurs especially readily on a heterocyclic nitrogen atom. Thus pyridine and quinolic derivatives are converted into quaternary ionic compounds in many higher animals,



and probably in insects (Ellinger et al., 1947). N-methyl pyidinium hydroxide is known to occur naturally in crabs and other invertebrates (Smith, 1964). Williams (1959) says that because the capacity for this reaction is limited, another route of detoxication which may be important is by conjugation with glycine (and presumably ornithine in birds), and he cites the case of a large injected dose of NA.

N-methyl nicotinamide (II) is a characteristic excretory product of NA in animals. The enzyme for making this (nicotinamide methyl transferase) is found only in the liver (Ellinger, 1948) and is quite specific for nicotinamide: it will not methylate the original acid. This, says Goodwin (1963), is why trigonelline (N-methyl nicotinic acid) is not found in animal tissues. But, of course, trigonelline is found in animal tissues: in vertebrates (Liusa and Barbiroli, 1962, who found nicotinamide methyl transferase in the eye), in Crustacea (Ackermann and List, 1957), and in the Anthozoa (Ackermann, 1953), at least. Presumably then there is some difference here between the biochemistry of the vertebrates (on which Goodwin probably based his remark) and these invertebrates.

I



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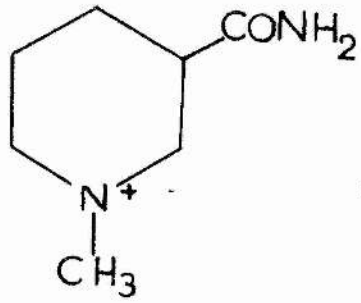

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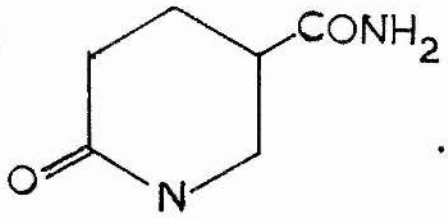

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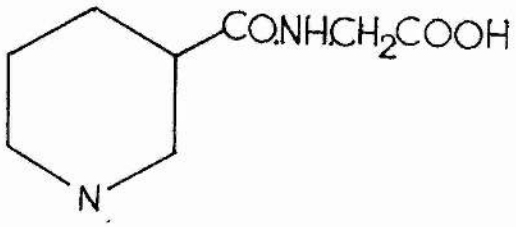

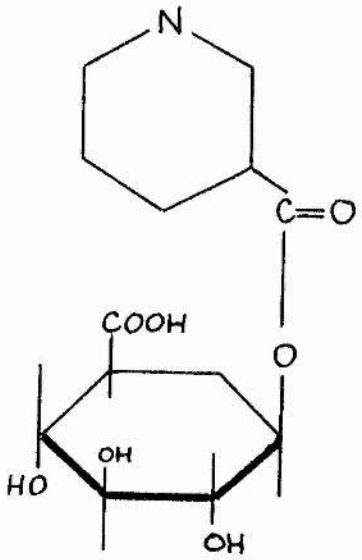

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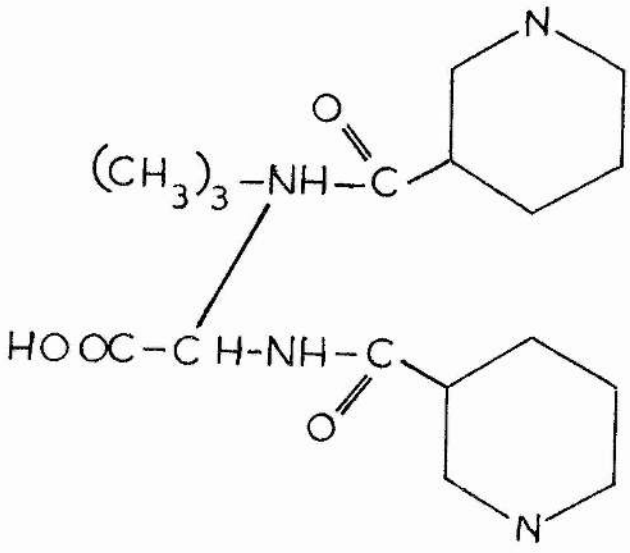
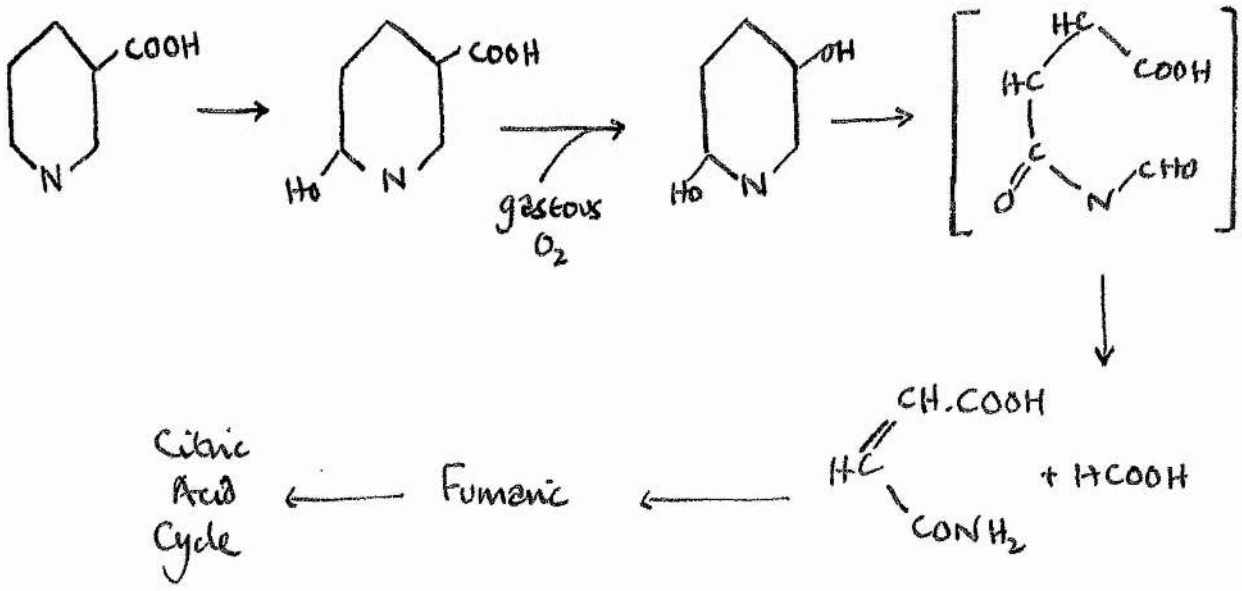


VI

In animals excreting N-methyl nicotinamide, oxidation of the waste product also occurs to give N-methyl-6-pyridone (III).

Nicotinic acid may also be detoxicated into three compounds especially: nicotinuric acid (IV), α, δ, and α-δ-di forms of nicotinoylornithine (V) in chicken and β-nicotinoyl-D-glucuronide (VI). According to Haraxy (1957) NA may also break down to give fumaric acid and thus link up with the citric acid cycle:



In this series it will be noted that the carboxyl group is lost early in the degradation, a point which will be raised later when the distribution of 14-C is discussed after the injection of carboxyl-labelled NA into crabs.

Before proceeding to the Argument it is necessary to describe briefly the anatomy of the crab leg nerve. Unlike vertebrates, in which the general somatic afferent fibres have their cell bodies centrally, the cell bodies of the sensory fibres in Crustacea are located peripherally, in or near the receptor organ. The motor fibres all have their cell bodies in the CNS, as in vertebrates. The different types of fibre travel along the length of the leg together, though they may be in different bundles within the nerve trunk. In all the preliminary extractions described here, therefore, when 'whole nerve' is used, the material contains sensory, inhibitory and motor fibres, of which sensory form by far the greatest number and volume.

If the hypothesis is accepted that most of the chemical intake into a neuron is through the soma rather than the axon membrane, and that metabolites travel along the length of the axon from the cell body to the nerve endings, then one of the consequences of the different siting of the motor and sensory cell bodies is that if either takes up a compound to a greater degree than the other, there will be a concentration gradient set up along the length of the nerve in the limb. If both take up the compound equally, one would expect to see at first high concentrations at each end of the nerve near the respective cell bodies, and a low concentration in the middle part of the nerve, and later equal concentrations throughout, or a reversal of the initial picture.

Argument

If the motor transmitter substance is a metabolite of nicotinic acid, then it is not unreasonable to suppose that there may be a special relationship between motor neurons and the compound. Such a relationship could mean that the motor fibres would contain a greater amount of NA, and this could be detected in two ways:

(i) by the assay of NA and its metabolites in the different types of axon, or

(ii) by the presence of a concentration gradient of NA along the length of the nerve as described above.

Moreover, a transmitter might well exhibit physiological activity on other preparations than that on which it normally acts - such as the heart of the same animal.

Accordingly, radioactive NA was injected into crabs and the different parts of the nervous system assayed after various intervals. The radioactive compound extracted from the nerves was tested for physiological activity on the crab heart and closer muscle.

METHODS

Animals

The shore crab, Carcinus maenas, and the swimming crab, Portunus puber, were obtained fresh from the sea and results from each have been pooled as no specific differences were detected. They were kept in aerated seawater until required when they were injected into the haemocoel just under the cuticle at the base of one of the rear legs with a measured amount of labelled nicotinic acid (*NA). The *NA was supplied by the Radiochemical Centre at Amersham in solid form in 100 μc lots, with the carboxyl group labelled with 14-C. If this was dissolved in 5 mls of seawater, a conveniently sized injection could be given. Crabs are not very tolerant of injections into the haemocoel of amounts greater than 1 ml. After the labelled injection, the crabs were given, after 24 and 48 hours, a dose of non-labelled acid, each of about 100 times the amount of NA as the labelled dose, the point being to wash out any of the labelled NA not firmly incorporated in either the original or a modified form in the animal.

The advantage of using crabs for this work is that they are easily obtainable in large numbers locally, and that they have certain advantages which can be exploited for neurochemical work: the leg provides several centimetres of a nerve bundle which can be easily dissected, and from which identified single axons may be obtained, and the number of legs gives opportunity for repetition of results and for taking samples at different times.

The dissection of axons

The isolation of single axons was carried out essentially as described by Hodgkin in 1938. After cutting the tendons of the adductor and abductor muscles at their insertions, the meropodite-carpopodite joint of the isolated leg was broken by bending it gently but firmly sideways, and the cuticle of the meropodite would then be pulled off carefully to reveal the nerve trunk of the meropodite. The nerve, which lies between the muscles, was rarely removed, although sometimes damage to it occurred whilst the joint was being broken or the cuticle pulled off. This manifested itself as a tetanic contraction of the leg muscles for a second or two, and such legs were generally of no use, for after this the nerve often ceased to conduct along its whole length, and although the exposed portion might well still be viable, in the early stages of the dissection it is a great help to be able to identify the motor axons by stimulating the cut end and watching the contractions of the leg muscles.

After the leg had been clamped in the dish (fig. 1a) any remaining muscle was cut away and the nerve trunk fully exposed. Usually there is at this stage one large and either one or two smaller bundles, one of which may be in close apposition to the blood vessel. The large one invariably contains the fast and slow closer of the dactylopodite, and if there are two other bundles, then one contains the opener and the other the two benders of the carpopodite-propodite joint, usually. Otherwise they lie together in the same small bundle.

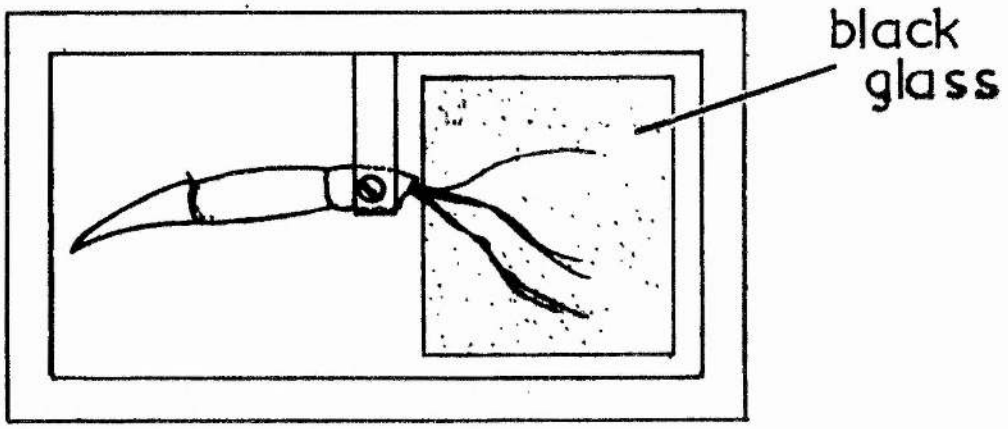


Fig. 1a Crab leg clamped in dissecting dish. The electrode system shown in fig. 1B fitted exactly into the area occupied by the black glass.

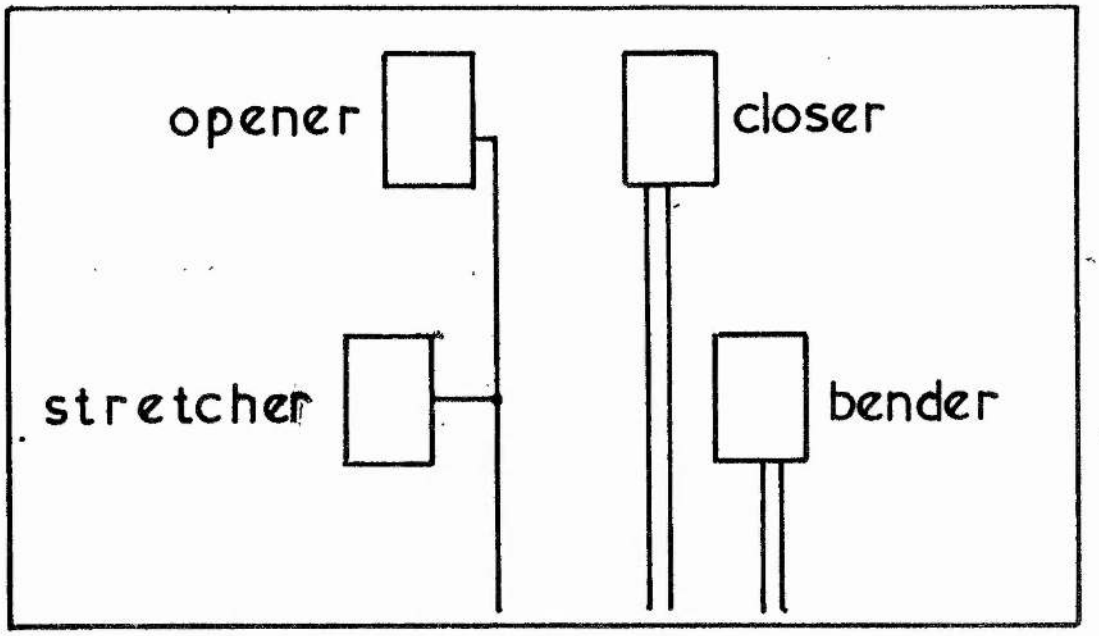


Fig. 1b The motor innervation pattern of the distal segments in the walking legs of the Brachyura (after Wiersma and Ripley, 1952).

Single motor axons could be obtained by gently dividing the bundle into two parts at the free (central) end, and then pulling the two parts away from each other with fine forceps which had been ground down. The nerve was divided into two roughly equal halves, usually, but sometimes after the motor axon had been spotted it was sufficiently clear to be able to pull it free of quite a large bundle. The appearance of a single motor axon is quite distinctive under reflected light: there are transverse markings on it, not exactly striations, but rather more diffuse areas resembling an agglutinous precipitate, alternating with clear regions (fig. 1). This contrasts clearly with sensory bundles which have more distinct markings on them, more closely resembling striations. The reason for either of these markings is not known, so far as I am aware: I do not think the suggestion of Chapman (1963) that the striations in small bundles are due to small fibres passing to and fro in the bundle is correct.

Solutions

Axons could be dissected out in a seawater bath, where they would stay alive for several hours at least. But sometimes, a whole run of experiments would be done when no response at all could be elicited from even the unsplit nerve. It seemed as if in these cases the bath contained some toxic substance, such as copper from the pipes bringing seawater from the storage tanks, and artifical seawater was then used as being more

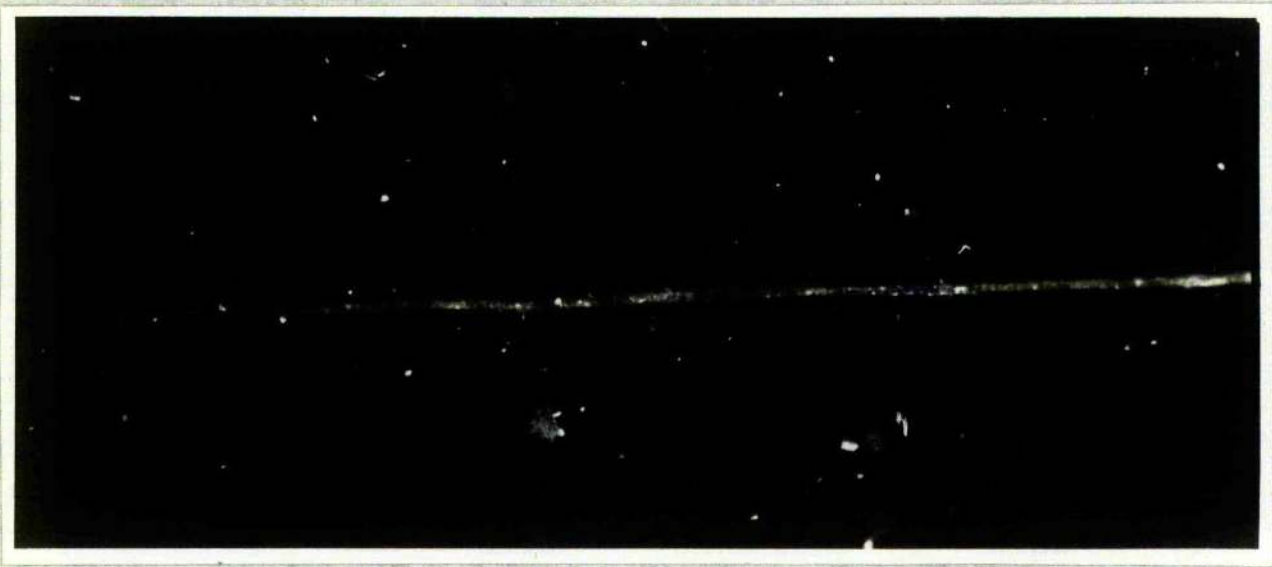


Fig. 1 Photograph of a single motor axon seen under the dissecting micrscope. The patchy appearance described in the text was more pronounced than this photograph would suggest.

reliable. The compound action potential of a large bundle was monitored, and it was seen that the saline below was best for keeping the nerve alive. The constituents were made up in this order, to help get the calcium into solution, and because the calcium and magnesium chlorides are deliquescent, they were first dissolved in water and then titrated with a chloride meter, and the appropriate amounts of the solutions added.

NaCl	23.486 gms/litre
KCl	0.739 "
Na_2SO_4	0.953 "
$MgCl_2$	5.103 "
$CaCl_2$	1.124 "
$NaHCO_3$	0.210 "

Assay of C^{14}

The estimation of small amounts of C^{14} (a weak β emittor) was most easily carried out by means of a liquid scintillation counter, but the use of this apparatus carries with it certain limitations. The extract to be counted, for example, may be dissolved only in a solvent which is fully miscible with the phosphor, and which does not cause an undue amount of quenching, that is reduce the efficiency of the light emission by the phosphor.

The leg nerve to be assayed was dissected out, blotted to remove the superficial extracellular water, and weighed on an aluminium planchette. It was then transferred to a glass tissue grinder with a volume of not more than 2 mls of acid alcohol (99 mls ethanol : 1 ml concentrated hydrochloric acid), and the empty planchette weighed. This solvent was found to be the best for the requirements by previous measurement, as is shown in the following table in which the results were obtained by extracting all the legs of a single crab on the same day, each in a different solvent. The counts differ of course because (i) there is always a difference between each of the legs of any one crab when they are extracted at the same time, even when the same solvent is used in each case (table 2); (ii) they affect the quenching of the phosphor to different degrees; and (iii) some of the solvents used extract the labelled compound better than others.

Table 1

Solvent	Counts per mg of nerve
Acetone	0
Acid alcohol	64
Alcohol	5
Ether	0
Seawater	29
Sulphuric acid, 0.1N	29
Trichloracetic acid, 10%	36

No correction has been made for the effect of pH on the phosphor, although this is said to make a slight difference. This table only

indicates which is the best solvent considering all the above factors, and does not enable their several effects to be determined. Thus the reason why there is no detected radioactivity when acetone is used as a solvent could be either because it is a marked quencher (which in fact it is, according to Kerr et al., 1957) and/or because it is not extracting the radioactive fraction from the nerve. Later evidence points to a combination of both these factors. The aqueous solvents were immiscible with the phosphor, and had to be used in small amounts so as to minimise the cloudiness which resulted from their mixing. This undoubtedly affected the efficiency of the counting. Likewise the miscible solvents could be used in greater quantities relative to the phosphor, but the increased accuracy gained by this had to be off-set against the increased quenching they would thereby cause. Ether does not quench toluene phosphors, at least at a concentration of 20 gms in a litre of phosphor (Kerr et al., 1957) but presumably it does not dissolve the radioactive compound either. This fits with the idea presented later that the compound is a peptide.

The quenching which different solvents cause with the phosphor used in this work (toluene-PPO-NPO) has been measured by Kerr et al. for low concentrations of solvents in the phosphor, and in this work for higher concentrations. Although alcohol is not a very strong quencher according to them (20 gms per litre give a fall to 94% of the true count) measurements here suggest that the quenching of the alcohol used in these extractions, where there is a concentration of 200 gms per litre, was as

great as 75% (fig. 2), but as all measurements were relative to a standard made up in the same amount of alcohol, it was only necessary to keep the proportions of alcohol to phosphor constant and not measure exactly the amount of quenching, nor correct for it.

The phosphor used in this work was based on the toluene-PPO phosphor described by Francis et al. (1959). It was made up with 50 mgs of 2-(1-naphthyl)-5-phenyloxazole (NPO) and 3 gms of 2:5-diphenyloxazole (PPO) in a litre of toluene. The NPO has the effect of shifting the emission spectrum of the PPO back towards the wavelength of the maximum efficiency for light collection of the photomultiplier tube, since the use of toluene as a solvent moves the spectrum away from this peak towards a higher frequency.

When samples were estimated, 10 mls of phosphor were thoroughly mixed with 2 mls of the alcoholic extract and counted in a vanadium bottle specially designed to fit the Ekco scintillation counter used, and whitened on the outside to increase efficiency. On occasion, only 5 mls of the phosphor were used, but this does reduce the count by a considerable amount, and in cases where the activity was low anyway, as high a count above background as possible was greatly needed.

The photomultiplier tube was calibrated so as to ensure the best distinction between background and source, and the voltages chosen for this, as seen from fig. 2, were 1100 volts for the EHT and 5 volts for the discriminator bias. The source during these calibrations and the standard in the assays was made by dissolving 0·1 mls of the original *NA solution (100 μc in 5 mls of water) in 1·9 mls of ethanol.

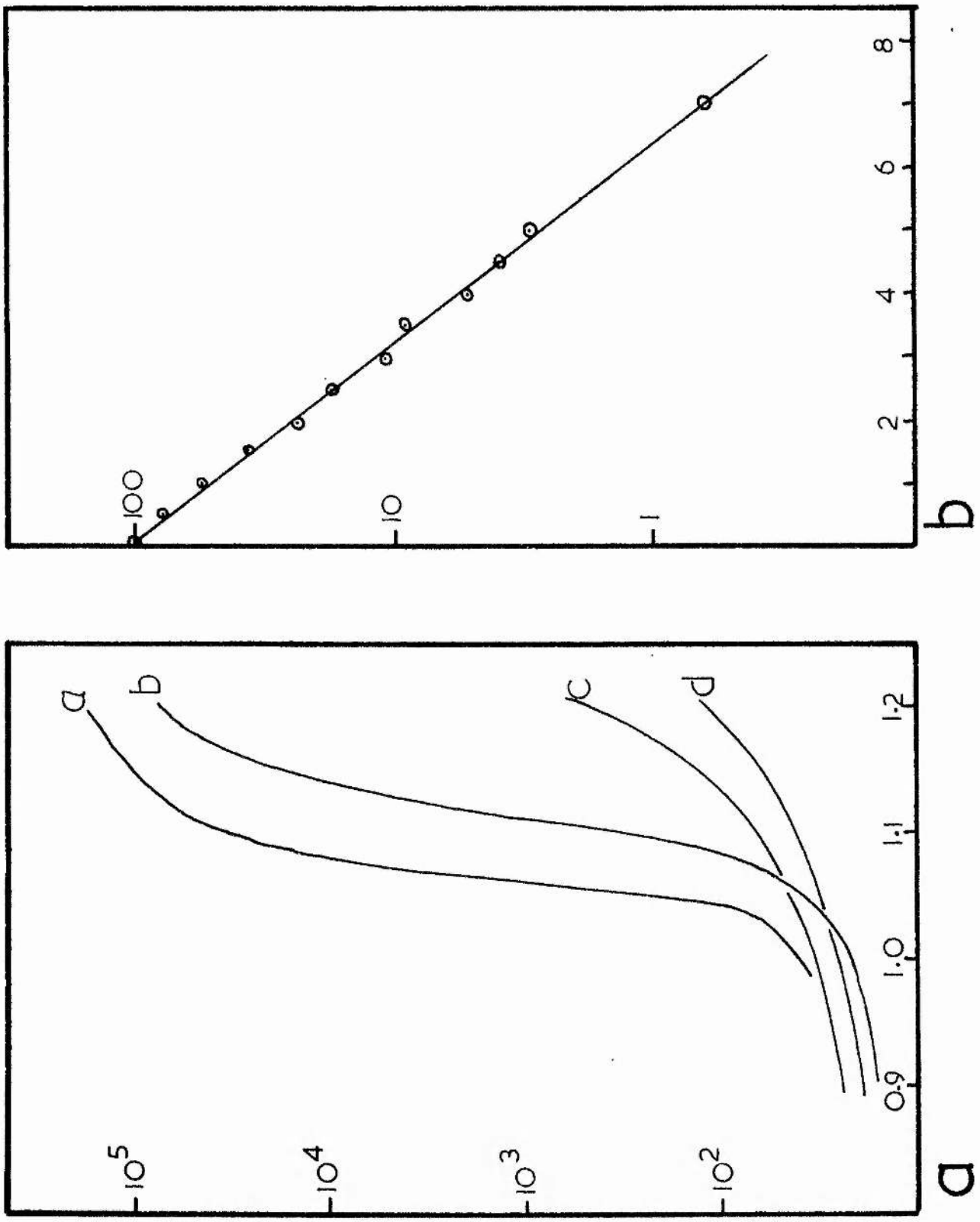


Fig. 2 A. Calibration of photomultiplier tube with source (curves a and b) and background (curves c and d). The discriminator bias was 5v in curves a and c; 15v in b and d. Ordinate: counts per 100 secs; abscissa: EHT (kv).

B. Quenching effect of alcohol in toluene phosphor. Ordinate: % 'true' count; abscissa: mls alcohol in 10 mls phosphor.

Assay of single axons

The method of counting using the scintillation counter was of no use for the assay of the activity in single, isolated axons as the amount of material was far too small. Indeed, with the general efficiency of the method (a scintillation counter such as ours cannot be expected to do much better than 30% due to geometric factors, etc.) and the quenching of the alcohol, in some cases the activity in a sample of whole nerve, weighing 0·3 mgs, could only just be measured, and the amount of material in a single fibre is much less than this. For this reason, another method was used which depended on the fact that radioactive material will expose a photographic plate. The axons were dissected out as described, and gently pulled up a moist glass plate which partly dipped beneath the surface of the dissecting dish. Once laid out on this plate side by side, the plate was dried and as much as possible of the salt removed without disturbing the axons. The plate was then pressed against an Ilford HP3 photographic plate for 28 days by means of bulldog clips. At the end of that time, the photographic plate, developed in ID 13, showed conspicuous darkening where it had been in contact with an axon, though even after exposure of a month, it was never completely exposed by any one fibre, and thus there was a method of comparing the activity in different fibres. Because of the great difference in size between motor and sensory fibres, the activity in motor fibres was compared with that in small bundles of only sensory fibres of the same diameter as the motor axons. In earlier experiments, bundles of 100 μ or more, known

to contain motor fibres, were compared with bundles of the same size known to be free of motor fibres.

Separation of the radioactive fraction by chromatography

Extracts of the nerve made up by grinding the leg nerve with alcohol as described above were spotted on to chromatography paper to separate the radioactive fraction. The following solvents were used for chromatography:-

t.BuM	tert.butanol:methanol:water	40:50:10
EtAm	ethanol:ammonia:water	180:10:10
BuA	n.butanol:acetic acid:water (upper phase)	120:30:50
BuP	n.butanol:pyridine:water	60:60:60
FORM t.Bu	tert.butanol:methylethylketone:formic acid:water	40:30:15:15
Ph	phenol:water	80gms:20
	iso.butyric acid:ammonia:water	661:329:10

All composition figures, except where otherwise stated, are in volumes. The one which gave the best separation of the other material from the radioactive spot was t.BuM at room temperature. The radioactive spot was located by scanning the paper after development in the various solvents with a thin (2·2 mg cm^{-2}) end-window geiger counter. The counter was enclosed in an aluminium cylinder, and the window covered except for a small slit (0·1" x 1·0"), through which radiation could enter, aligned with its long axis parallel to the solvent front on the paper beneath it. To start with a device was used to scan the paper consisting of a kymograph around the drum of which the paper passed, the

end-window counter, and a ratemeter which was used to drive a pen recorder, but later the paper was counted by holding the end-winkow counter over discrete areas of the paper for periods of five minutes or longer, and merely taking the number of counts in that period. A plot of the counts thus obtained against the distance along the paper from the origin clearly showed where the activity lay (fig. 9).

Autoradiograms of the papers were made using Kodak 'Royal Blue' and Ilford 'Industrial G' film. The latter was not fast enough, and the former, kindly supplied and developed by the local hospital, still needed an exposure of up to six months for satisfactory results.

On developed chromatogram papers which were stained with ninhydrin, one of the series of blue and purple spots which appeared corresponded with the area of peak radioactivity. This correspondence was equally precise in four different solvent systems: t.BuM, EtAm, BuA and BuP and was a great help when eluting the papers.

Once the region of radioactivity had been found, the paper could be eluted by cutting the active region out and letting it hang from a piece of filter paper which dipped into a trough containing the solvent. The capillary action of the solvent between the two papers served to keep it in place. The choice of the eluting solvent depended on what the eluate was to be used for: if it were to be put on to the heart or leg muscle preparations from the crab, the solvent was seawater or saline respectively, but for subsequent chemical work, it was more convenient to use distilled water or alcohol. That elution had been

successful and efficient could be checked by counting and staining the piece removed afterwards, and by staining too the rest of the chromatogram - especially around the area cut away. It was important to choose a solvent when developing the chromatogram which could be completely removed from the paper. Phenolic solvents, for example, are difficult to remove and would certainly have an effect if included in an eluate to be assayed on crab heart.

Perfusion of crab leg

To obtain any active fraction that there might be in working crab legs, isolated legs were perfused with the saline described on p. 25 by pushing the cut end of the meropodite into a piece of rubber tubing which fitted tightly over it. This was connected to a saline reservoir over which was a constant pressure equivalent to four feet of water. The tip of the dactylopodite was cut off, and the saline collected as it dripped from this end. The leg was stimulated for 0·5 seconds every second at a frequency of 50 cps by means of a kymograph time-marker connected to the leg through two platinum wire electrodes, and a potentiometer adjusted to give just suprathreshold shocks. In order to obtain as high a concentration as possible of any interesting substance, a small quantity of saline was passed through the limb more than once until the leg was fatigued by the stimulus. When this occurred, sometimes the saline was then passed through another limb.

Assay with crab heart and leg muscle

To test the eluate from chromatograms, and the perfusate from legs for physiological activity, two biological preparations were used: the crab heart and a closer muscle preparation.

(a) The crab heart: A ligature was tied around each of the alary muscles of the isolated heart of a spider crab, Hyas araneus, one serving to anchor the heart to the bottom of a 10 ml aerated seawater bath, and the other being attached to a kymograph lever. The dissection and subsequent experiment were both done in a 10°C constant temperature room.

Crab hearts, at least from the smaller crabs, do not need to be perfused to stay alive in aerated seawater and this experiment could be run for several hours before the heart showed any signs of abnormality. Large crab hearts were tried, as were lobster hearts, in an attempt to get a better trace, but these died in a short time after being dissected out, even in an aerated bath.

(b) The closer muscle preparation: The nerve trunk in the meropodite segment of the leg of a Cancinus was dissected out and split to isolate the small bundle which contains the two closer axons. The leg clamped at the propodite segment moved a kymograph lever by closure of the propodite-dactylopodite joint. The nerve bundle was lifted up into paraffin and stimulated at 50 cps for half a second every five seconds with a neon stimulator adjusted to give just suprathreshold shocks. Injections of the test substance were made through a hole

drilled previously in the cuticle of the propodite, and thus had immediate access to the surface of the closer muscle, although it is debatable whether it reaches the inside of the muscle - and the neuromuscular junctions - for some time after this. For reasons given later, it was found that test substances for injection had to be dissolved in a saline, the composition of which is NaCl = 31·0; KCl = 0·99; $MgCl_2$ = 2·35; $CaCl_2$ = 1·87 (all figures in grams per litre). The saline of Pantin (1934), on which this is based, produced a marked inhibition of the response to electrical stimulation when injected alone, and had to be modified to the above, in which the Ca^{++} is raised from 1·37 gms/litre to avoid this background reaction.

RESULTS

Introduction

The suggestion by Van der Kloot that nicotinic acid is related to the motor transmitter may be followed up in the manner outlined on p. 18, and these results describe the uptake in whole nerve and single axons accordingly.

Legs from crabs injected with NA were removed at varying times after the injection, and the radioactivity in the nerve trunk of the meropodite, carpopodite and propodite segments was estimated. It soon became clear that there was considerable variety in the results due to the natural variation which is always shown by living creatures, and in an attempt to measure the extent of this, all the legs from one crab were removed on the same day and the difference in levels of activity was recorded, after they had all been extracted in the same manner. The results of this assay are shown in table 2. The figure for muscle is included for comparison: it was obtained from muscle taken from all the legs and segments, and pooled.

Table 2

Limb	Tissue	Activity in meropodite (μμc/mg)	Activity in carpo-propodite (μμc/mg)
1	Nerve	6·28	8·1
2	"	9·21	11·3
3	"	10·2	10·2
4	"	10·6	11·2
5	"	17·7	16·2
6	"	15·1	15·5
7	"	13·6	10·3
8	"	11·9	-
	Muscle	1·15	-

Using/....

Using $\sigma^2 = \frac{\Sigma x^2}{n} - m^2$ where x = reading; n = number of readings; m = mean; the standard deviation, σ, when n = 15, is 3·07. The mean reading is 11·82 μμc/mg for nerve.

The limbs numbered 7 and 8 were the swimming legs and furnished very little material, so that in one case it was hot possible to make an assay of the radioactivity in it. All the legs in this experiment were counted on the eighth day after the labelled dose had been given - that is six days after the second dose of non-labelled acid. In another case, the crab looked like dying after 24 hrs., and instead of giving it the non-labelled injections it was sacrificed immediately and the nerves in the meropodite segments counted:

Table 3

Limb	Tissue	Activity in meropodite (μμc/mg)
1	Nerve	10·3
2	"	9·34
3	"	9·64
4	"	8·37
5	"	10·4
6	"	12·1
7	"	30·4
8	"	37·8

when n = 6, m = 10 and σ = 1·1.

The last two readings in this table are so far away from the norm that they can reasonably be ascribed to some artefact: the most likely

explanation is that they were the two limbs next to the site of injection, and that there is still a deal of free *M in the vicinity (no washing dose had been given in this crab). The formula used to determine the standard deviation is not really applicable here as there should be a minimum of ten readings before it may be used properly.

Variations of this order, which are not excessive for biological assays, mean that there is great need for duplication of results if any confidence is to be placed in them.

Although there is this variation between the amount of radioactivity in one nerve and the next, there is a clear difference between the content of nerve and of other tissues in the body. As is shown in tables 2 and 4, the activity in the muscle taken from the same region of the animal as the nerve assayed with it, is only about one tenth that of the nerve. Every time muscle was assayed - in all about half a dozen times - it was found to be an order less active than the limb nerve, but of the same order of activity as the other tissues in the body. Thus:

Table 4

Tissue	Crab 1: Activity, μμc/mg. Day 9.
Nerve, mean for whole leg	20·44
Muscle, " " " "	3·35
Ventral ganglion	3·11
Brain	undetectable
Eye	0·17
Liver	2·05
Testis	0·64
Heart	0·09
Blood	4·00

The figures for heart and brain in this table are probably too low as the tissues were fixed in Bouin's fluid before being counted, and the yellow colour this imparts to the tissue extract was later seen to have a marked quenching effect in the scintillation counter. In one crab (no. 20) the mean activity in the nerve, extracted 48 hours after the labelled injection, and after only one non-labelled dose of NA, was 16·8 μμc/mg whereas in the muscle it was 0·80 μμc/mg. In this crab, too, the activity in the blood was 0·78 μμc/mg - that is about the same level as in the muscle, which suggests that the activity in the muscle is only due to passive accumulation of the radioactive substance from the bloodstream. Clearly this cannot be the case with peripheral nerve.

Uptake by nerves in vivo

Two relationships may be studied in the eight walking legs of the crab in connection with the uptake of *NA to help evaluate the idea that there is a connection between NA and the motor transmitter. Both stem from the argument presented in the introduction on p. 18: the amount of radioactivity in each part of the leg nerve relative to the other parts, and the amount of activity in leg nerve relative to the time after the initial injection. It is more or less impossible to combine directly the results from different crabs when plotting the time course because of the wide variation between them - even when the initial injection was kept constant, as it usually was, at 10 μc. In a few cases it was reduced to 5 μc, but the general level of activity was much nearer to background in

the small samples, and this was not very satisfactory.

When the nerve is exposed, it may be divided into three longitudinal sections corresponding to the segment of the leg which contained it. As is shown in fig. 3 in nine times out of ten, the distal segments contain more activity than the proximal. The full data for this figure are given in table 5 where it can be seen that each point on the graph is the mean for one, two or three crabs. Only one crab (no. 17) was ever counted for longer than three weeks, so that the points at that end of the figure are naturally from that one crab alone. There is often a considerable difference between the levels of activity in different crabs but by taking the ratios of activity in the meropodite and propodite it is possible to combine the results from more than one crab. The activity in the carpopodite, though not expressed in fig. 4 - merely for the sake of clarity - was usually mid-way between the levels in the other two segments. In the one experiment where the activity in it was measured, the dactylopodite nerve had the highest level of radioactivity of the segments.

The other relationship which may be readily examined in these experiments is the time course of the activity in the leg nerves. A plot of the uptake of *NA (or rather, the nett uptake and loss of the radioactive NA metabolite) against time reveals a shape of curve which is similar in all cases tried, and a shape which would be expected from first principles (fig. 4). There is an initial hump in the curve shortly after the injection, corresponding perhaps to the arrival in

Table 5

Day	Crab number							Mean
	1	3	4	12	14	17	20	
1								
2		0·76					0·45	0·61
3	1·58							1·58
4		0·59			0·64			0·62
5	0·30				0·86			0·58
6			0·89		0·63			0·76
7	4·65				0·73	0·78		2·08
8	0·48	0·68	1·36					0·80
9	0·19			0·36				0·28
10		0·88						0·88
11					0·71			0·71
12					0·56			0·56
13		1·47	1·15	0·79				1·14
14								
15								
16								
17		0·62	1·14					0·88
18								
19								
20		0·65						0·65
21						0·35		0·35
22								
23								
24								
25								
26								
27						0·58		0·58
28								
29								
30								
31								
32								
33								
34								
35								
36						2·74		2·74

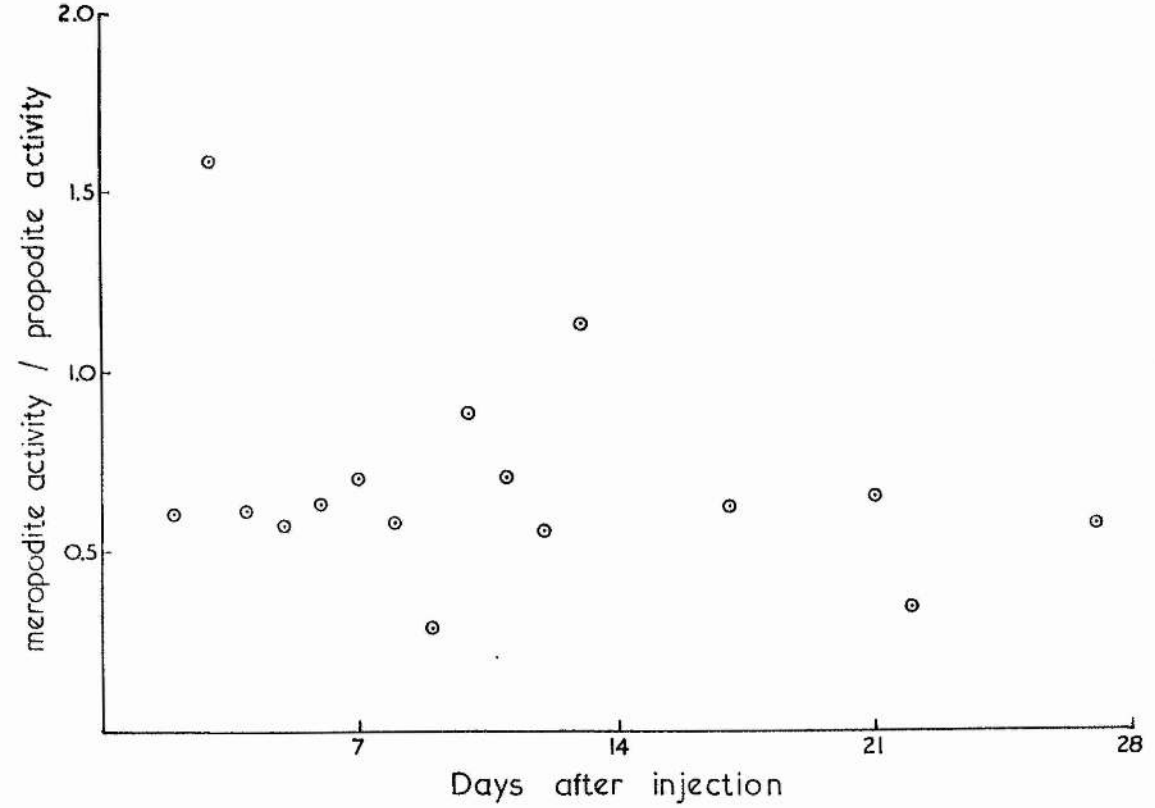


Fig. 3 Ratio of radioactivity in the nerve of the meropodite and propodite segments plotted against time after the injection of a standard dose of *NA. There is consistently more activity in the propodite segments over a period of four weeks. Each point is the mean from two or three crabs.

Fig. 4 Uptake of *NA in the propodite (distal) and meropodite (proximal) segments of the leg nerve plotted against time. Figures shown are from one crab but are typical of twelve, the results from which are not easily combined as readings were taken at different intervals, and the basal level of activity varied between crabs. The standard deviation of the readings (see text) is 15.8% of the mean, and is shown by the vertical lines on the left-hand side.

the tissues of the *NA. This hump is not removed however by the two non-labelled injections, and is due therefore not to the presence in the tissues of unchanged *NA but to some bound form, or metabolite which is rapidly formed there, for the whole point in giving the non-labelled dose is to wash out any free *NA by the principle of exchange. Of course, the same principle will no doubt mean that some unlabelled NA will exchange with acid already incorporated into the body, but presumably this will be to a much lesser extent than the former. The figure above shows the results from one crab which, as explained above, is typical qualitatively, if not quantitatively, of the others. The shape of the curve is still humped after account is taken of the scatter between different legs of the same animal.

The general impression, and it can scarcely be called anything more grand than that, with the scatter between the legs of any one crab and between one crab and the next, is that after the initial hump there is a slower rise in the level of activity. The amount of labelled substance in the blood remains more or less constant throughout this time, and the results suggest that there is a continuous uptake of this compound, whether it be unchanged *NA or some metabolite already formed, into the peripheral nervous system. In those crabs where the activity in the muscles was measured for a comparable period to the nerve, the level of activity also shows a continuous rise during this time, and unless one supposes that muscle is taking it up from the blood (albeit at a much lower rate than nerve, or, at least, to an equilibrium much further to

the left hand side) this is difficult to understand. Moreover, presumably the *NA is being changed into a stored form which keeps the pumping-in mechanism going. There is also the problem of where the store is which supplies the blood; as all the tissues in the body which were assayed had a much lower count than the nerve, it can only be the body as a whole which is storing the acid. This point is considered further in the Discussion.

One general result in the time courses from different crabs is that the activity per mg of nerve in the three segments estimated, and in the muscle from the same segments, follow the same shaped curve. The difference then looks like being differences between legs as a whole This too will be discussed in more detail later.

Uptake by nerves in vitro

With the idea that there might be some difference in the permeability of the sheath in the peripheral part of the nerve which allowed more *NA to enter the fibres there, and thus produce the gradient just described, it was decided to measure the uptake of *NA by whole nerves in vitro. Nerves dissected out from the whole length of meropodite, carpopodite and propodite were immersed in a bath of seawater containing *NA in various concentrations. Each end of the nerve was hooked up on to a pair of silver/silver chloride electrodes; through one pair a stimulating pulse was applied and through the other the propagated compound action potential could be monitored. Thus it was ensured that

the nerve remained alive throughout the experiment. The monitoring used a pulse once a minute just suprathreshold for most of the fibres. On the other hand, in other nerves the stimulation was repeated at a frequency of 10 cps for half a second every second which meant that it was possible to find out if there was any difference between the uptake in active and comparatively resting nerves.

In passing, the action potential recorded showed well the differing propagation speeds in the one nerve. A quick measurement gave a conduction velocity of 7.5 m/sec for the fastest and 2.1 m/sec for the slowest fibres. The so-called 'slow' fibres, that is those which respond only to a high stimulation rate, have a conduction velocity slightly less than the 'fast' fibres - 5.54 m/sec.

The activities of resting and stimulated nerves were compared, with the results obtained from nerves which had previously been killed by immersion in water at 70°C for a minute. It appeared that there was no difference in the activity (and therefore the nett uptake and loss of *NA) between the three conditions and that living and dead tissues behave in the same manner in this respect. Moreover the membranes from this appeared to be just as permeable at the central end as at the peripheral, and in no part to be especially active with regard to the uptake of the acid. However it should be borne in mind with respect to active transport that the experiment only ran for three or four hours, and that in this time, the internal concentration of the nerves was never more than that in the bath, and does not indicate either way whether the entry is active or not.

Uptake by axons in vivo

One of the most obvious ways of clearing a lot of the ambiguity attaching to the results in the previous section is to measure the uptake in single axons. A distinction between the two types would then be immediately apparent, and instead of having to deduce the conclusion of the experiment from the results, as in the previous measurements, the result and the conclusion would be one.

The dissection of small bundles and single axons was as described on p. 21 and the amount of radioactivity in them determined by autoradiography, for reasons already given. The technique of 'stripping film' which is used in autoradiography of histological sections was also used here, but it was also quite adequate to apply the film to the axons as described above, and the practice was discontinued in favour of the simpler method. Because it was difficult to dry thoroughly the larger bundles (that is, those of more than 100 μ) they would often interfere with the exposure of the plate and produce areas where the emulsion was completely wrecked (figs. 5 and 6). This was one of the reasons why later only single axons and small bundles of comparable size were used.

The resultant blackening of the radioactive material in the nerves was estimated crudely on an arbitrary scale of five units of darkening, 1 being the darkest. If the plates were examined under the microscope the silver grains per unit area could be counted, and this afforded a more accurate way of expressing the results. In either case, however, the results were the same: there was no difference in the density of

Fig. 5 Photograph of axons and bundles (a) laid out on a glass sheet compared with a print of the autoradiogram (b) obtained by pressing a photographic plate against the glass sheet. From the top down, the fibres or bundles (all of which are taken from the meropodite unless stated) are: i, ii, iii sensory; iv, v sensory from propodite; vi bender; vii opener; viii sensory; ix closer; x closer; xi sensory; xii sensory from propodite. The central end of each fibre is to the l.h.s. The two circles towards the top r.h. corner were caused by dried seawater. Scale = 1 cm.

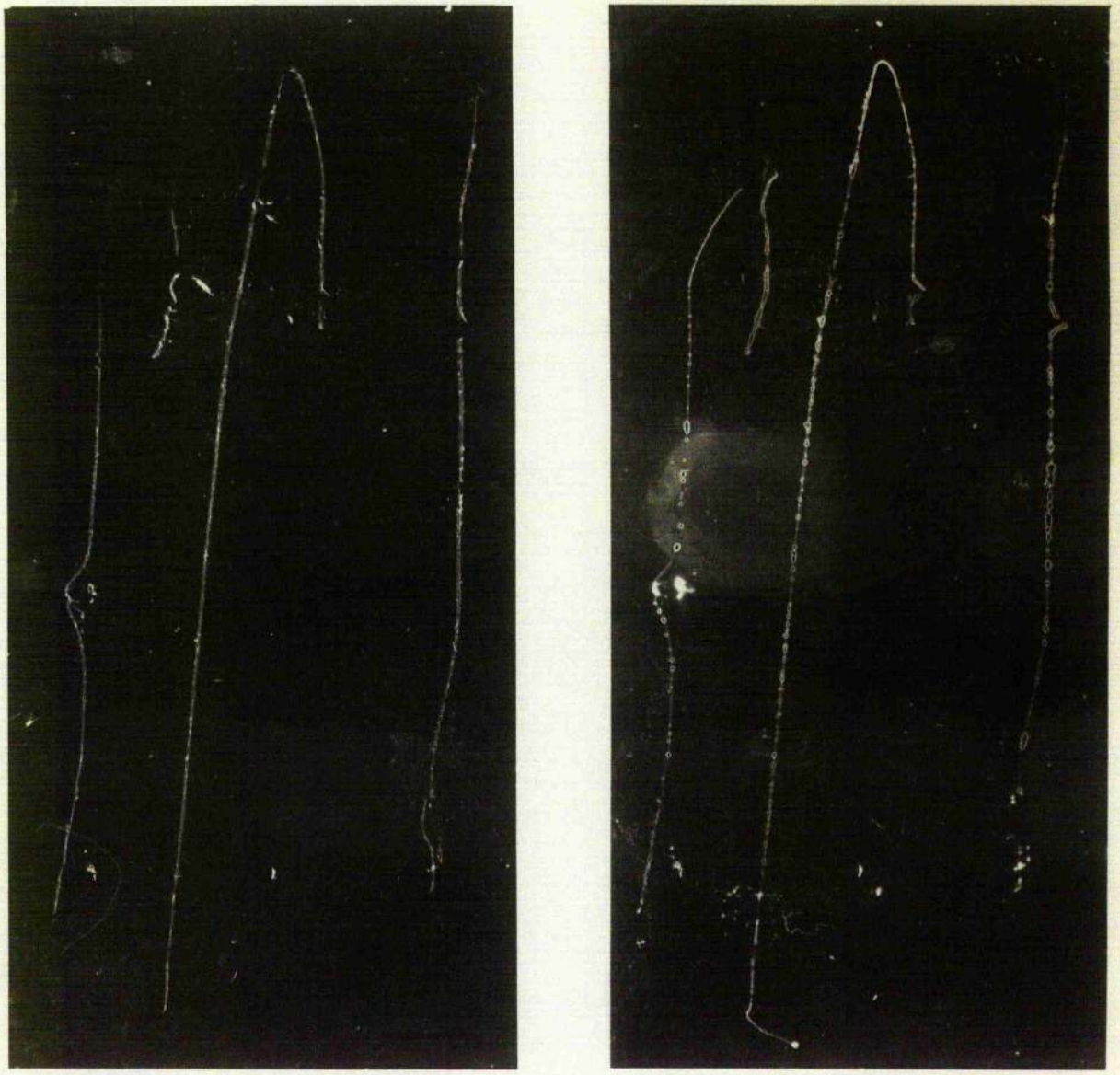


Fig. 6 A photograph (a) and an autoradiogram (b) of three long sensory bundles arranged with their peripheral ends at the top. This was the only case where a slight indication of a gradient was seen. The small circles on the autoradiogram were caused by seawater within the bundle. Scale = 1 cm.

blackening caused by the two types of fibre.

A plot of the number of fibres producing the different effects shows that they are divided into sensory and motor in almost exactly equal parts (fig. 7). Moreover, no sign of a gradient was seen in any of the exposures, except in the case of three large sensory bundles taken from the whole length of the limb - about 8 cms in all. In these, the peripheral end of the bundle shows a greater development of the plate than the central end, but as the number of such examples found was so small, even though the gradient is in the direction expected, little can be built on them with certainty. In all about 60 pairs were compared, though mostly they were of much shorter length than the three with the graded activity, being about 2 cms long.

Chromatography

When chromatogrammed extract of the peripheral nerve of a crab injected with *NA was examined, a radioactive spot was found at Rf 0·23 using the tert.butanol:methanol:water (t.BuM) solvent described on p. 31. The finding of this spot was first attempted by the scanning device described earlier, but as can be seen from the trace below (fig. 8), the method was not sufficiently discriminating to reveal clearly where the active spot was. The resolution of the system depended upon the relative speeds of the kymograph, carrying the chromatogram under the window of the counter, and the pen recorder; and upon the width of the window, the integrating time of the ratemeter (1 sec.), and other factors, and while

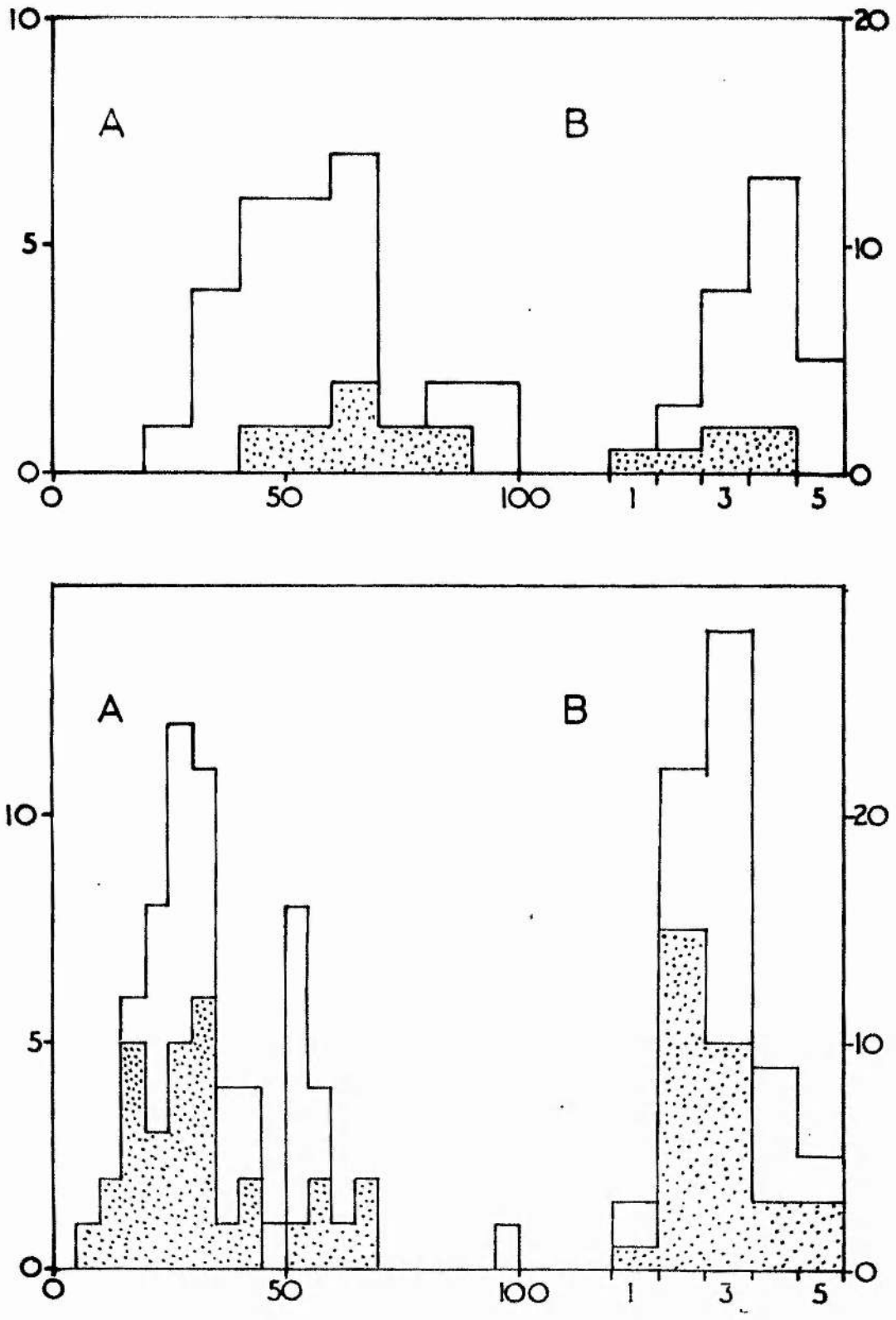


Fig. 7 The uptake into motor fibres (stippled area) is as common as that into sensory (clear area). The upper histogram was obtained from bundles over 100u in diameter; the lower from fibres and bundles less than that. Ordinate: number of fibres; abscissa: (a) blackening on autoradiogram (expressed in 5 arbitary units) as ratio of diameter, and (b) blackening irrespective of diameter.

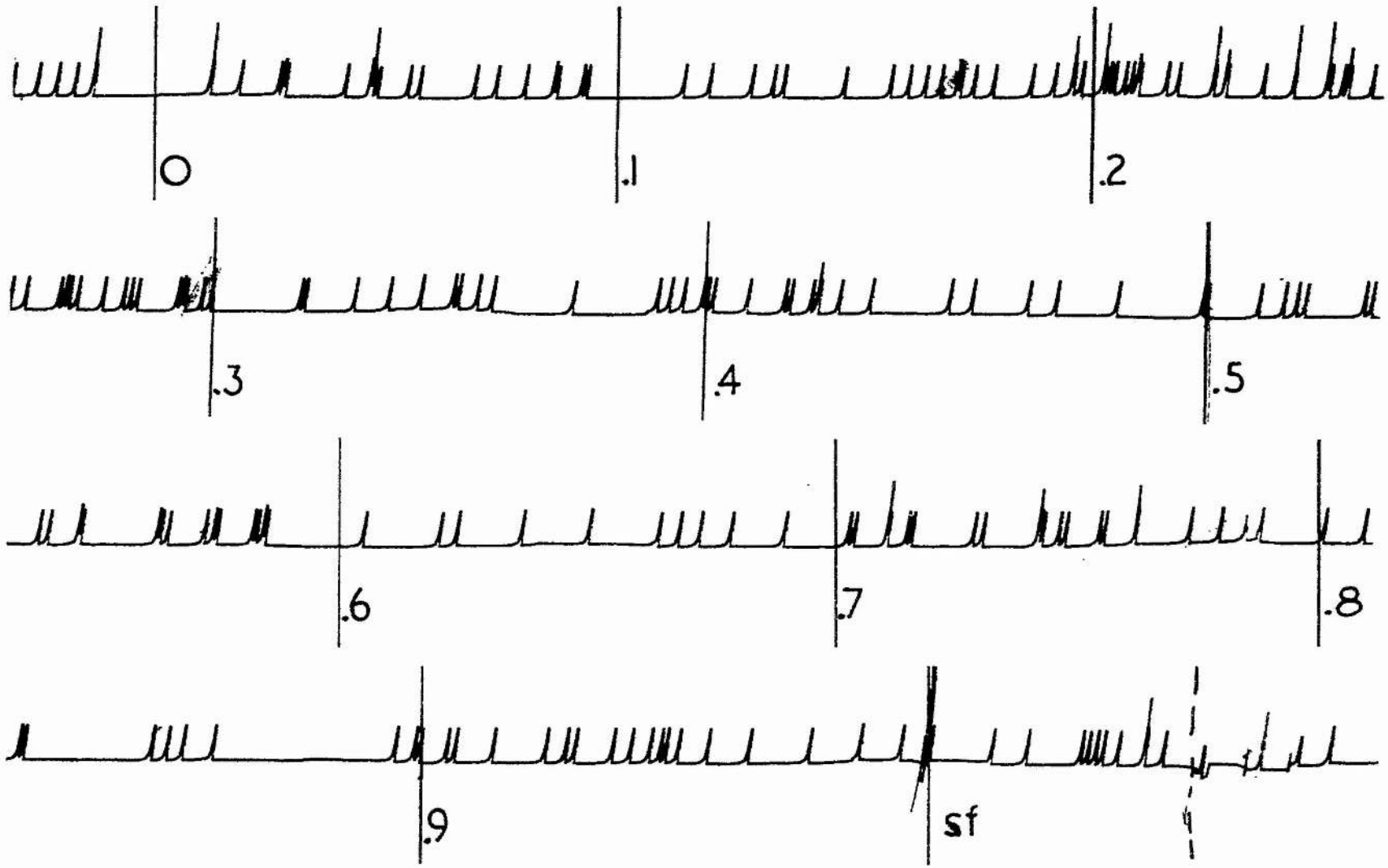


Fig. 8 Saan of the chromatogram analysed in fig. 9 (upper) using the pen-recorder device described under 'methods'. It does not reveal clearly the peak shown in fig. 9 which is at R_f 0.23. Figures = R_f values; sf = solvent front.

most of these were probably sufficient for the purpose, the pen recorder speed could not be reduced below 0·5 inches per minute, and therefore any advantage gained by slowing the kymograph was annulled by the great length of paper records obtained.

The method of counting discrete areas of the paper for given times, on the other hand, showed immediately where the activity was. The upper trace in fig. 9 shows that nearly all the activity was at Rf 0·23, but on another chromatogram (fig. 9b) there was a trace at Rf 0·70 also - the Rf of unchanged NA in this solvent.

Another way of finding the radioactivity on the paper, which defined the position more accurately, but which took much longer to do, was to expose the paper to an X-ray film. The results of this technique are shown in fig. 10, and although it was quite clear from this just where and how large the radioactive spot was, the exposure time to get satisfactory results was six months.

After they had been counted, the chromatograms were stained with ninhydrin. Among the many spots which appeared (fig. 11) was one which exactly corresponded to the radioactive area on four one-dimensional chromatograms, each in a different solvent (as listed on p. 31), and on a two-dimensional chromatogram using the n.BuA solvent followed by EtAm (fig. 12). Immediately ahead of the corresponding purple spot was another, even after the two-way run, which stained yellow with ninhydrin.

Much the same pattern of spots was obtained when extracts of crab legs from untreated crabs were run with the same solvents. In particular,

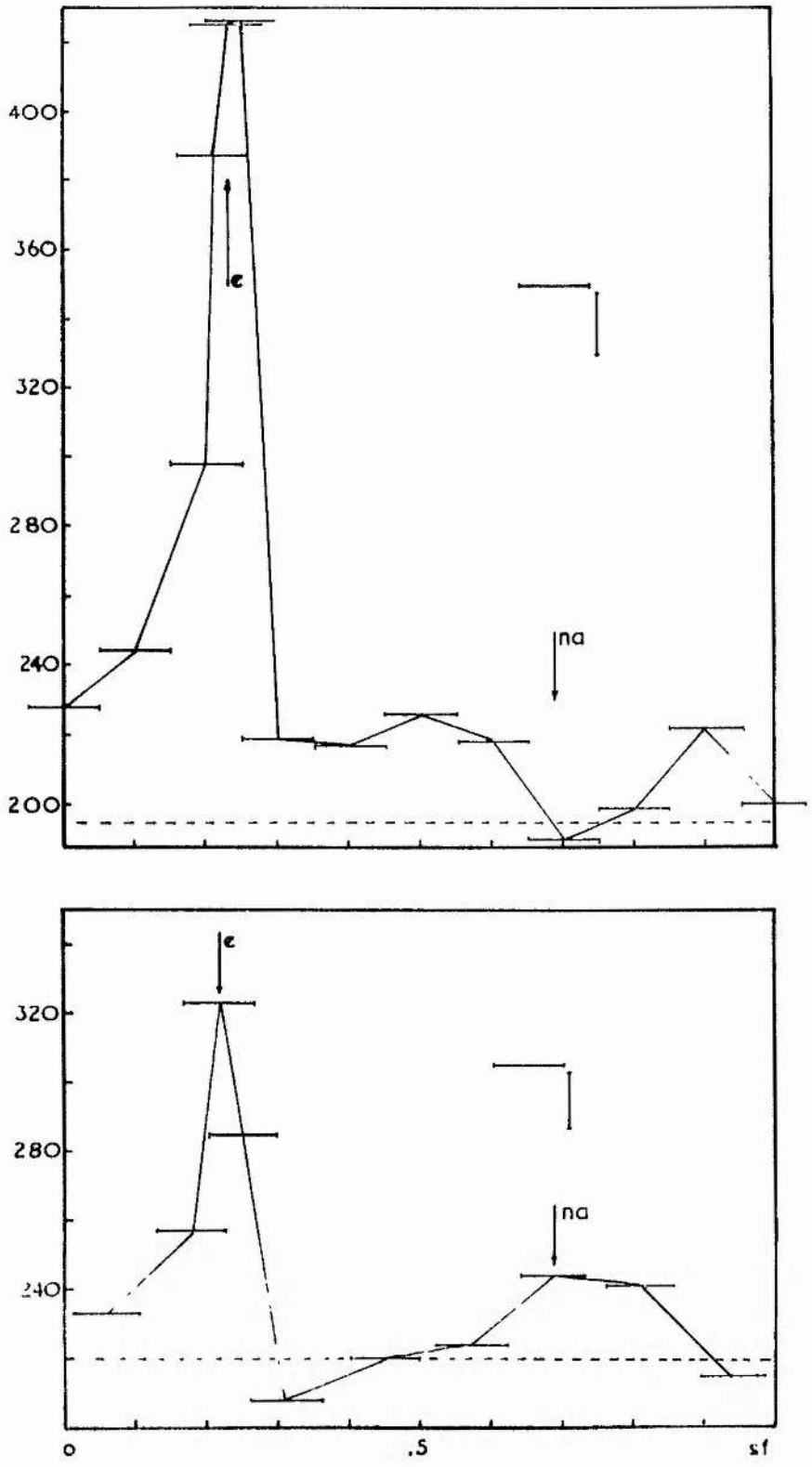


Fig. 9 Two examples of counting areas on chromatograms spotted with extract of leg nerve from crabs previously injected with *NA. In the lower there is a slight rise of activity at R_f 0.7 which is probably due to unchanged *NA. The R_f of the purple spot F is shown. Horizontal ambiguity is due to the width of the counter window, and is shown. The vertical ambiguity (= $\sqrt{}$ the number of counts) is also shown. The dotted line gives the background activity.

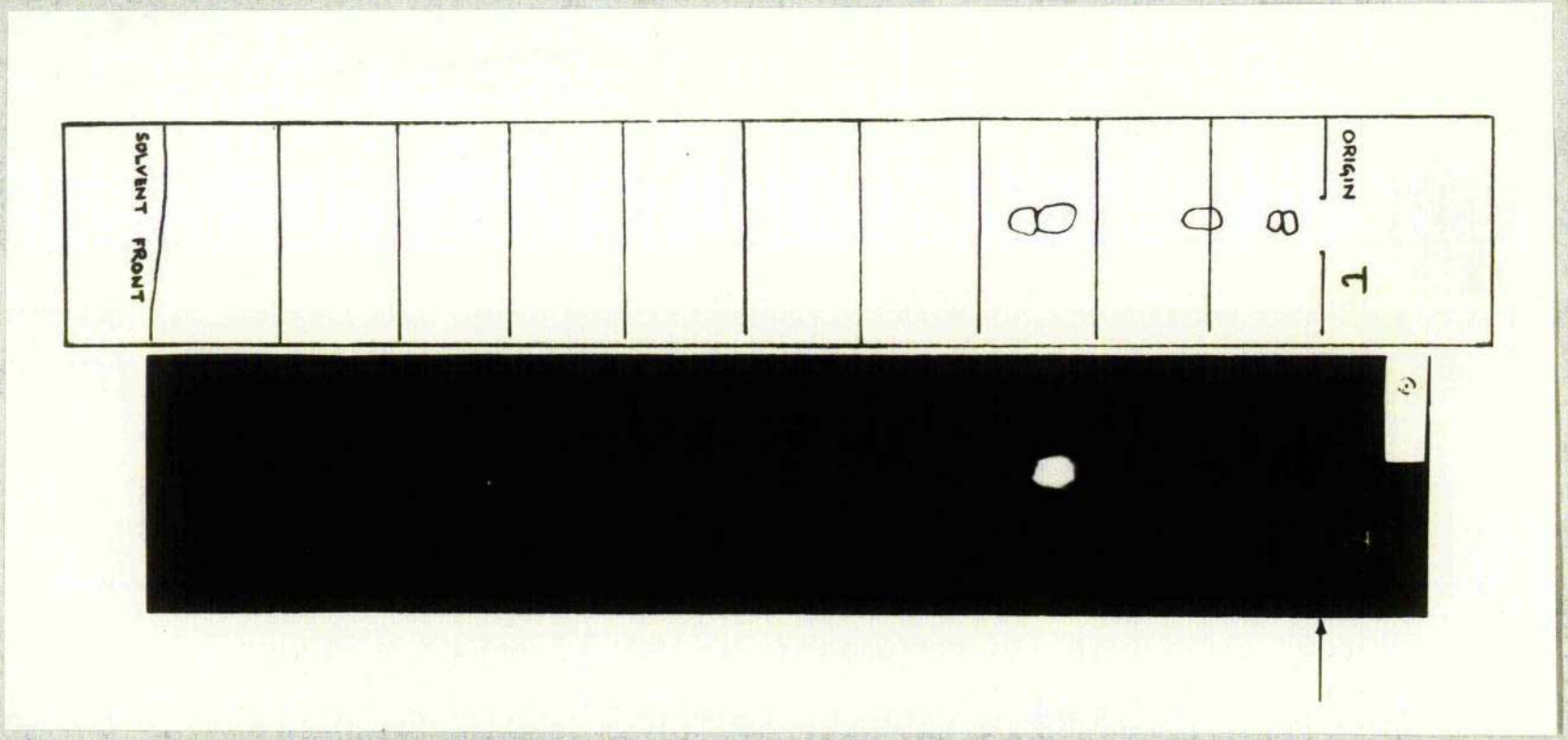


Fig. 10 Photograph of a chromatogram showing the ninhydrin-positive spots, and the autoradiogram obtained from it. The position and number of the radioactive spots is quite clear.

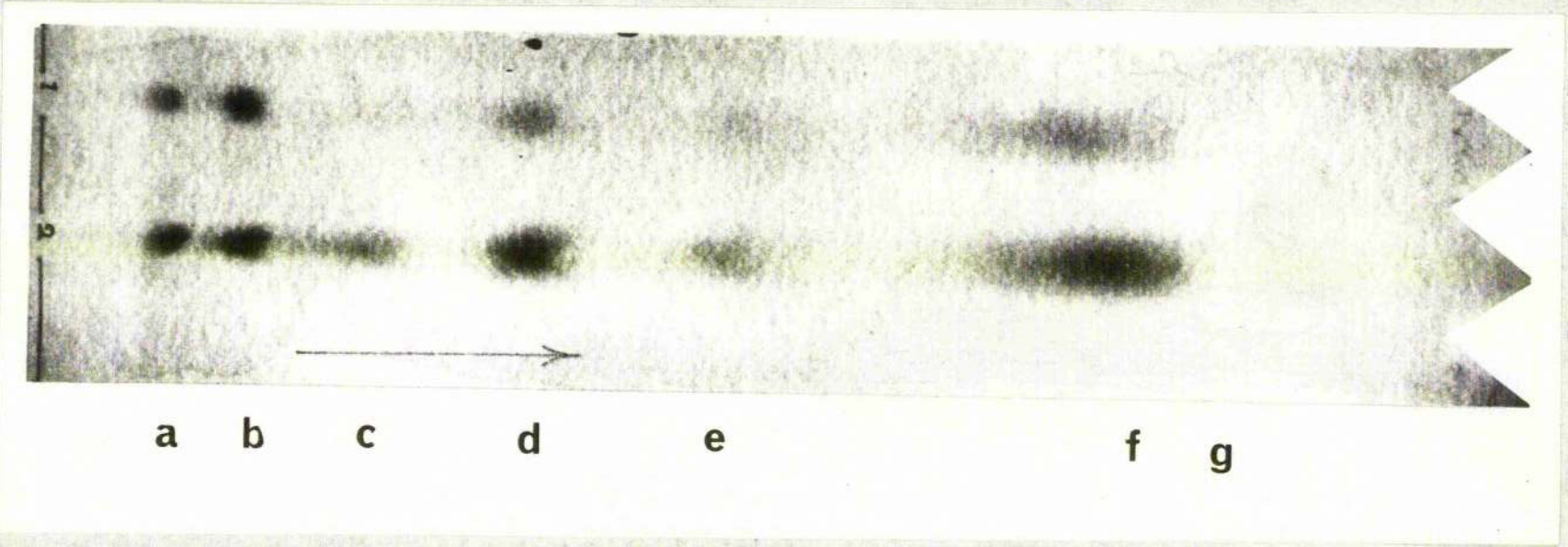


Fig. 11 Photograph of a chromatogram showing the position of the main ninhydrin-positive spots. On this chromatogram, which was run in t.BuM (descending) the solvent front had passed off the paper for some time and the front edge represents about Rf 0.3. The two bands are of alcoholic extract of leg nerve from Carcinus and Hyas respectively.

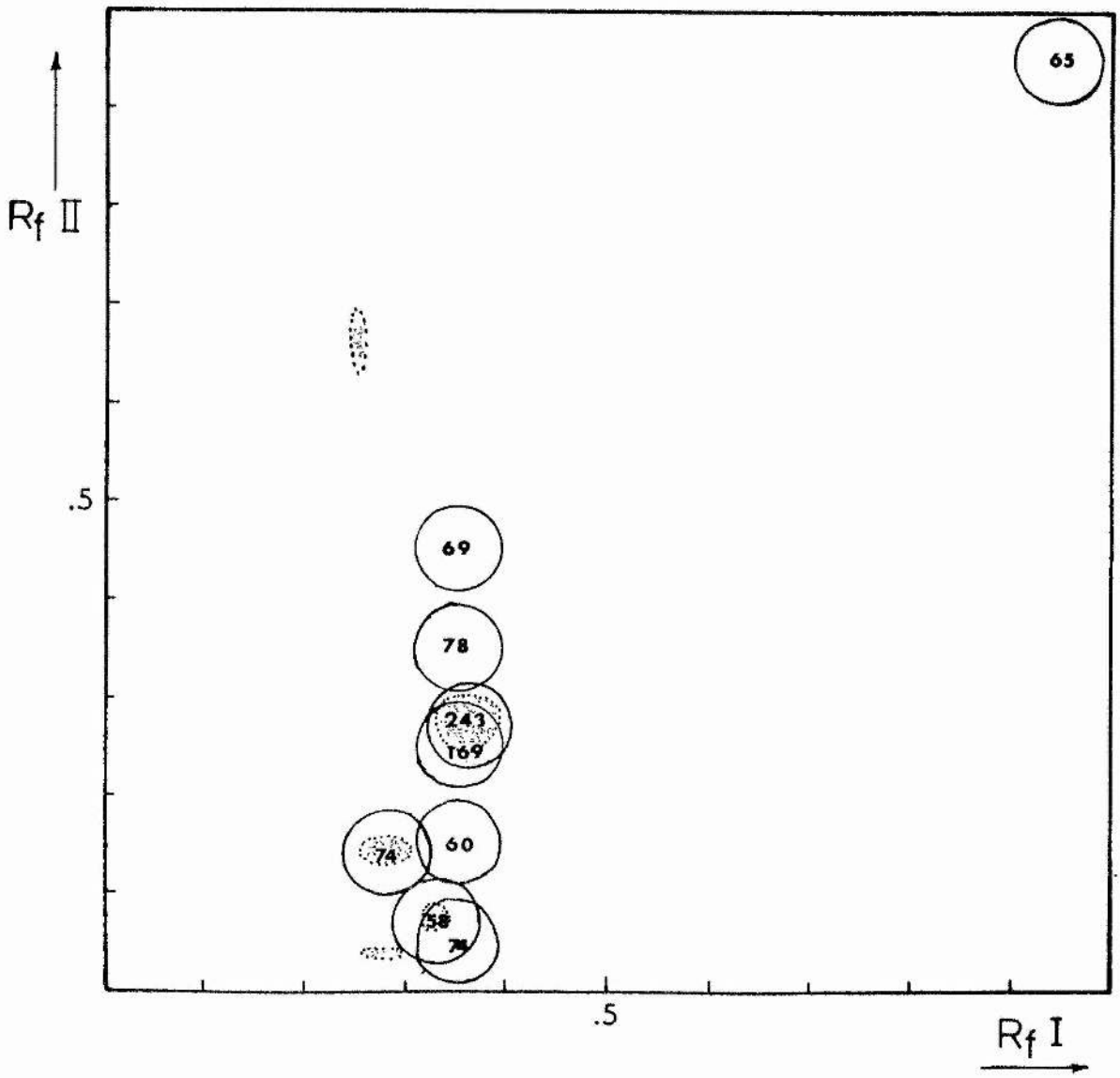


Fig. 12 Two-way chromatogram of crab nerve extract. The ninhydrin-positive spots are shown in purple and the counts per 300 secs are shown in the areas covered by the window of the counter. As an initial scan of the paper after the first run (in BuA) showed all the activity to be at Rf 0.37, it was only necessary after the second run (in EtAm) to count from this point. There is good correlation between one of the spots and the area of highest radioactivity. The background count of 65 c/300 secs was measured at the solvent front of each of the solvents.

the purple spot at Rf 0·23 and the yellow one just ahead of it were both present in extracts of Carcinus, Portunus and Hyas nerve. Presumably, therefore, the compound at this point on the paper, into which the *NA is being incorporated, is a normal metabolite of nerve in these animals.

Spot tests

In an attempt to characterise the compound at Rf 0·23 (referred to hereafter for convenience as substance or spot F) more fully, various chemical spot tests were performed on it, and on the whole extract spotted on to filter paper. The tests were usually carried out as described in Dawson et al. (1959), and the references given below are to pages in that book: other references are at the end of the table.

In this table some of the tests shown are far more specific than others and it is only by considering them as a whole, together with the information presented by Rf values, that it is possible to draw any clear conclusions from it.

The choice of tests was partially governed by what chemicals were available in a non-chemical laboratory, but it was also possible to obtain samples of materials from outside. I am grateful to British Drug Houses for making a supply of o.acetoacetylphenol available for me, and to the University Chemistry Laboratories for much help in obtaining the less usual compounds.

Some of the tests, when carried out on controls, did not work satisfactorily in my hands. Thus I was unable to derive any positive

Table 6

No.	Test	Observation	Inference
1	N-methylation; Dragendorff's reagent	-ve	not a basic substance with quaternary ammonium atom. Not NA. (217)
2	o.acetoacetylphenol	-ve	not a primary aliphatic amine. (218)
3	diazotised p.nitraniline	-ve	not an aromatic amine. (219)
4	diazotised p.bromaniline	-ve	" " " " . "
5	sodium nitrite and alk. β naphthol	-ve	not an ammo-phenol. (220)
6	sodium nitrite and phenol	-ve	not an amino-phenolic acid. (220)
7	α.nitroso-β.naphthol with HNO_3	-ve	not a substituted phenol, e.g. tyrosine, tyramine. (220)
8	α.nitroso-β.naphthol	-ve	not a 5-hydroxyindole. (220)
9	Ehrlich's reagent (p.dimethyl-aminobenzaldehyde)	-ve	not various amines, e.g. aminobenzoic acid, sulphonamides. (220; 231)
10	Folin-Ciocalteau's reagent	-ve	not enteramine, octopamine, tyramine, etc. (221)
11	ammoniacal silver nitrate	-ve	not enteramine, 3-hydroxy sulphanilic acid or amide. Not aldose or ketose. (221)
12	alloxan	red-brown spot.	suggests aminoacid. (223)
13	Folin's reagent (β.naphtho-quinone sulphonate)	light rose spot.	" "

"/....

Table 6 (contd.)

No.	Test	Observation	Inference
13	Folin's reagent with sodium hydroxide	ochre spot.	suggests tryptophane, GSH, proline or hydroxproline.
14	ninhydrin with acetic acid	-ve	not 5-HT and other tryptamine compounds.
15	salicylaldehyde	-ve	not an aminoacid. (223)
16	sublimed iodine	brown spot.	suggests little definitely; could be aminoacid, peptide, etc. (224; 236, etc.)
17	formol-bromomthymol blue	yellow spots on blue ground.	suggests aminoacid. (224)
18	UV light after drying	whitish fluorescence.	suggests aminoacid, peptide. (224; 233)
19	orcinol	could-n't get to work.	(224)
20	isatin	-ve	not proline or hydroxyproline; not pipecolinic acid. (225)
21	nitroprusside-acetaldehyde	-ve	not proline. (225)
22	phenol-hypochloride	-ve	not an aminoacid. (226)
23	diacetyl reaction	couldn't get to work.	
24	nitroprusside	-ve	not an -SH or S-S compound. (228)
25	perchloric acid	-ve	not tryptophan. (231)
26	p.aminophenol	-ve	not glucose, fructose, sucrose, maltose, etc. (237)

27/....

Table 6 (contd.)

No.	Test	Observation	Inference
27	p.aminohippuric acid	-ve	not a hexose or pentose. (237)
28	ninhydrin with butanol	purple spot.	not hexosamine. (243)
29	Reinecke's salt	-ve	not a lipid. (247)
30	sudan black	-ve	not a lipid. (249)
31	UV light	no absorption.	not a protein (252); not a pyrimidine derivative (256) not purine derivative.
32	picryl chloride	-ve	not NA.
33	1-fluoro-2:4-dinitrobenzene	-ve	not NA.
34	potassium cyanide followed by UV light	-ve	not oxidised pridine nucleotide, NMN, nor riboside. nicotinamide. (258)

References:- No.1: Kariyone and Hashimoto, 1951.

No. 13: Giri and Nagabhushanam, 1952.

No. 14: Jepson and Stevens, 1953.

No. 23: Eggleston et al., 1943.

results from the orcinol test for aminoacids (no. 19) - which depends on the partial hydrolysis of the chromatography paper by sulphuric acid except where the amino acid is able to buffer the acid - even using known solutions of amino acids as controls. Another test, alkaline α-naphthol with diacetyl (23) (Eggleston et al., 1943), which is supposed to give yellow colours with homarine, coloured all the paper yellow for me, and to that extent was unsatisfactory. The reason for this may be to do with the solvents in which the chromatogram was run, and although, when a control test with known homarine was run, the spots were slightly darker than the background, the difference was so slight as to make it pointless trying the test on experimental chromatograms with their small concentrations of material.

As a reagent for staining chromatograms, a 0·1% solution in acetone of ninhydrin is useful as a general stain. As is shown in fig. 11, there were several ninhydrin-positive spots on the developed chromatogram of crab leg nerve extract, most of which are purple. The test is not at all specific, and reacts with any compound which has on it a free amino group. Spots A and B were bluer than the rest, and, considering the fact that they had low Rf's and that they were UV absorbing, were probably polypeptides. Nothing can really be concluded about the others below spot F (fig. 12) on the paper. The yellow spot immediately in front of F stained yellow with ninhydrin, but was not proline or hydroxyproline - at least, it did not stain with isatin (no. 20), nor had it the same Rf as those aminoacids. Its Rf was the same as that of 5 HT,

a compound which stained with ninhydrin, but it had none of the other properties of this compound, so far as it was tested. Ninhydrin can be made up in other ways than with acetone, and when, for example, it is dissolved in butanol, it may be used as a test for hexose amines (no. 28). Again, it was used by Jepson and Stevens (1953) with acetic acid (no. 14) as a test for 5 HT and other tryptamine compounds.

Ehrlich's reagent too may be applied in a number of ways to bring out the presence of different groups of compounds. In no. 9 it was dissolved in 3% HCl and colours yellow with primary aromatic amines, green-blue with indoles: exposure to hydrochloric acid vapour after staining turns the tryptophan yellow spot to purple. If the reagent is applied in acetone with 10% HCl, the spot given by tryptophan is purple to start with. The interest in tryptophan springs from the fact that (i) it is linked metabolically with NA (although, as discussed on p. 13, the only pathway described is probably irreversibly towards NA), and (ii) the Rf of tryptophan in the t.BuM solvent is about the same as that of the NA derivative. It was soon clear, though, that the radioactive spot on the paper was not tryptophan.

The possibility that the purple spot at F was due to an amine was ruled out by the several negative results obtained when this group was tested for. Dragendorff's reagent, which reacts with most basic quaternary-N compounds, also indicates that adrenaline and pyridoxine, as well as the loosely related NA, are not present here (Kariyone and Hashimoto, 1951). o.Acetoacetylphenol (no. 2) is specific for primary aliphatic

amines and a negative result with it does not eliminate secondary or aromatic amines (nor aminoacids), but the absence of aromatic amines is indicated by the negative results from both diazo tests (nos. 3 and 4.).

All the positive results point towards the compound at F being an aminoacid, or something bigger, such as a peptide or polypeptide, but none of them by itself is conclusive. Nor are they of much help in distinguishing between different aminoacids. The red colour given with alloxan (no. 12) is typical of 23 of the 46 aminoacids listed by Harris and Pollock (1953) in their table of aminoacid reactions. Folin's reagent (no. 13) gave a light rose-coloured spot at first, and then an ochre patch after development in the alcoholic alkali. In the table of Giri and Nagabhushanam (1952), on which this test is based, the colour ochre does not appear; proline and hydroxyproline are listed as giving orange; β aminobutyric acid brown. Peptides produce various shades of green according to them - and certainly the spots A and B changed to green after the second part of the test. Glutathione is listed as giving a brown colour, but this compound is not confirmed by use of nitroprusside (no. 24).

The formol reaction (17) is quite a good test, but by contrast the subliming of iodine (16) on to the paper does not really tell very much: too many compounds react with it for it to be of great help. Among other compounds which do react with it to give brown spots are two compounds related to NA, trigonelline (N-methylnicotinic acid betaine) and homarine (N-methylpicolinic acid betaine) (p. 15). Both these

compounds are known to occur in Crustacea: Ackermann and List (1957) presented evidence for their presence in the tissues of Crustacea generally, Leonard and MacDonald (1963) for the occurrence of homarine in marine Crustacea muscle and Kravitz et al. (1963) for the occurrence of the same compound in crustacean nerve. However, if samples of these compounds were run side by side with extract of nerve, it was clear that their Rf's were markedly different from that of the radioactive spot (homarine = 0·40; trigonelline = 0·26 in the t.BuM solvent). They are both strong absorbers of UV light, and their position on the chromatogram may be determined under the UV lamp. This is quite different from spot F; and neither stains with ninhydrin. However, two spots in extract of nerve were noted which did absorb UV light, at the same Rf's as synthetic homarine and trigonelline; they were presumably both present in Carcinus and Portunus nerve therefore.

The list by Harris and Pollock (1953) already referred to is useful in distinguishing between aminoacids, but is limited by the ambiguity of such terms as 'deep rose' and 'yellow blue'. A standard way of identifying individual aminoacids is by making a 'map' on which the Rf of known samples of different aminoacids is plotted alongside a sample of the unknown. This was done here, with particular reference to three aminoacids known to be strongly present in lobster nerve metabolism: glutamate, aspartate and malate (Waelsch and Cheng, 1963). As can be seen from fig. 13, the only aminoacids with Rf approaching that of the interesting unknown spot were tryptophan, tyramine and tyrosine. As

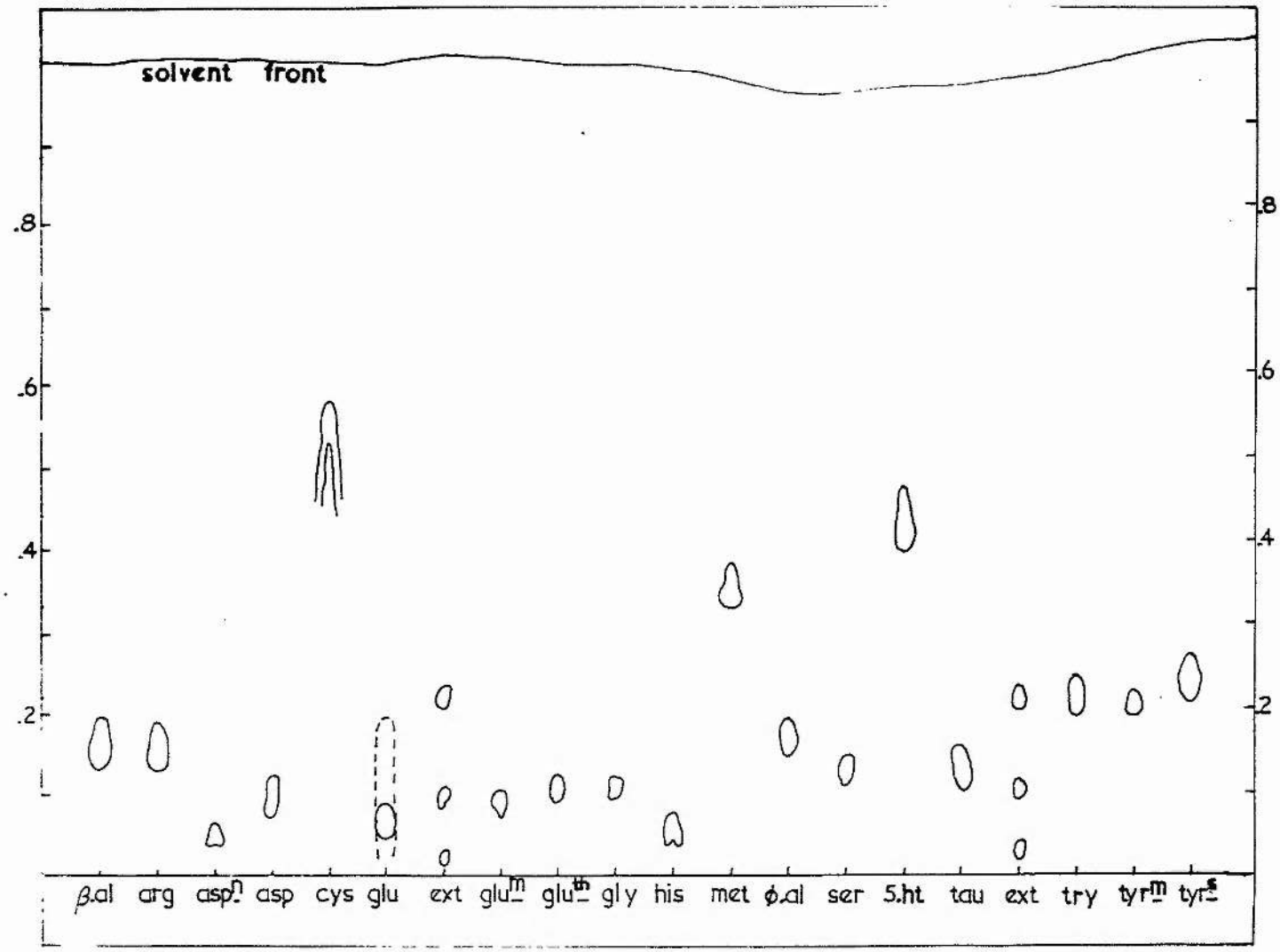


Fig. 13 An aminoacid 'map' run in t.BuM. The abbreviations are those customary for aminoacids except for asp.^{n} = asparagine; ext. = extract of nerve; glu.^{m} = glutamine; glu.th = glutathione; tyr.^{m} = tyramine; tyr.^{s} = tyrosine.

discussed on p. 62, the material at F is almost certainly not tryptophan, and the other two stained a different colour with ninhydrin.

The use of Rf values in identifying compounds is limited by the large number of possible compounds and the resolution possible on a chromatogram, say, 50 cms.long between the different values. The useful work of Fink et al. (1963) only serves to emphasise this, for they list over 400 naturally occurring compounds with their Rf's in ten different solvents. Unfortunately this work came out after most of the chromatography described here had been done, and although they do cater for so many solvent systems, the main one used in the work (t.BuM) is not listed. They do include, however, one which I had used - n.butanol:acetic acid:-water (n.BuA), and list 18 compounds with Rf's between 0·38 and 0·42, which was the Rf of spot F in this system. Some of them are too distant chemically from NA to warrant serious consideration - sedoheptulose, for example. And also, of course, the compound of interest here stains with ninhydrin, which rules out such as fructose or mannitol which bear no $-NH_2$ group. On one chromatogram, the extract was developed in tert.butanol:ethylmethylketone:formic acid:water (FORM t.Bu), a solvent listed in Fink's tables, and the Rf of the compound of interest compared with the values in their tables. As on this occasion the initial extract was not radioactive, it was not possible to be absolutely certain which of the ninhydrin-positive spots corresponded to spot F in the t.BuM system, but one there was, ahead of which was a yellow spot after ninhydrin. In every other system used the compound at F has been preceded by a single

yellow spot, including the two-way run, and it is not unreasonable to suppose that this is the case here too. If this is assumed, the Rf of the spot of interest is 0·67. Nowhere is there any correlation between a compound of this Rf in FORM t.Bu and one of Rf 0·38-0·42 in BuA.

All the indications from the initial series of tests described on pp. 58-60 carried out on spot F suggest that it is either an aminoacid or a peptide or polypeptide. Lewis (1952) found that 25% of crustacean peripheral nerve is made up by free aminoacids, but comparison with Fink's tables and with my own aminoacid map make the latter possibility far more probable than the former, and to test further for this, the compound was hydrolysed. The spot was eluted from the paper in distilled water and boiled with 6N hydrochloric acid for half an hour, when after re-chromatogramming, it was seen that the one spot had become three. Any less vigorous treatment of the substance (e.g. 6N hydrochloric acid at 60°C for half an hour) did not break it up thus, but this is by no means excessive for hydrolysis of peptides (cf. Consden et al., 1947, who use 6N hydrochloric acid at 105°C overnight). The three spots which appeared after staining the chromatogram with ninhydrin had values 0·21-0·19 with a tail, 0·32-0·29 with a tail, and 0·51. Of these, the spot at Rf 0·51 was by far the least concentrated on the paper, the other two giving about the same colour density with the stain.

This splitting of the original ninhydrin-positive compound into three such substances strongly suggests that the original was a small peptide, but is not conclusive. Clearly the original might be any

substance which breaks down with acid to give three components with $-NH_2$ groups, and any number of others without this group. The matter is discussed further later.

The assay of substance F on heart and muscle

If the substance at F has any physiological significance as a transmitter for example, it may well be that it has an action on crab heart, which is known to respond to such compounds (pp. 6 ff.) and maybe too (if indeed it is the motor transmitter) on the muscle or neuromuscular junctions of the crab leg. The crab heart and closer muscle were used to test this possibility.

(a) The crab heart preparation, consisting of the heart muscle, and the ganglion on the dorsal surface, is sensitive to a wide range of compounds and is a frequently used system for physiological assay. In this case, when testing substances were added to the seawater bath containing the heart, the trace made by the cardiac contraction was examined on a smoked kymograph drum.

The addition of such compounds as adrenaline, 5 HT, had the effects already described and was useful in discovering how long the latent period of the system was: that is, how long it took for the extract to reach the active site in the preparation, assuming that the extract from crab leg nerves acts at the same place in the heart as adrenaline, etc. In the case of GABA at a concentration of 10^{-6} gms/ml it was seen to be of the order of 60 seconds.

As is shown in fig. 15b, the application of the eluate of spot F produces an immediate and quickly reversible speeding of the heart-beat. On the other hand, extract of whole peripheral nerve (fig. 15a) speeds the heart-beat and increases the size of the contraction, but the effect is slow to appear, and to be reversed by washing.

On one occasion when an extract was used which had been eluted in distilled water, an increase was also observed. In case this was due to the distilled water, a control was made using only distilled water, and the beat was seen to slow down from 42 to 12 beats/min. The effect of adding 0·1 mls of distilled water to the bath cannot be very great (it equals a 1% change in the molarity of the bath), but the heart responds to it dramatically.

(b) The closer muscle preparation, set up as described on p. 34, when tested with the eluate of the spot F did not respond in a detectable manner. At first it seemed that the muscle was being inhibited by the extract for the response to the stimulus dropped in size considerably when the extract was injected. But when a control solution, of ordinary seawater, in which the extract had been dissolved, was injected into the leg, the same response was obtained (fig. 16). The Carcinus saline of Pantin (1934) was then used, but the result was the same as with seawater - an inhibition of the contraction. Now it is known that the Ca^{++}/Mg^{++} ratio is an important factor in the response of a muscle to nervous stimulation, and it seemed possible that here the inhibition caused by the seawater and saline might be due to a low $Ca^{++}:Mg^{++}$ ratio. Absence

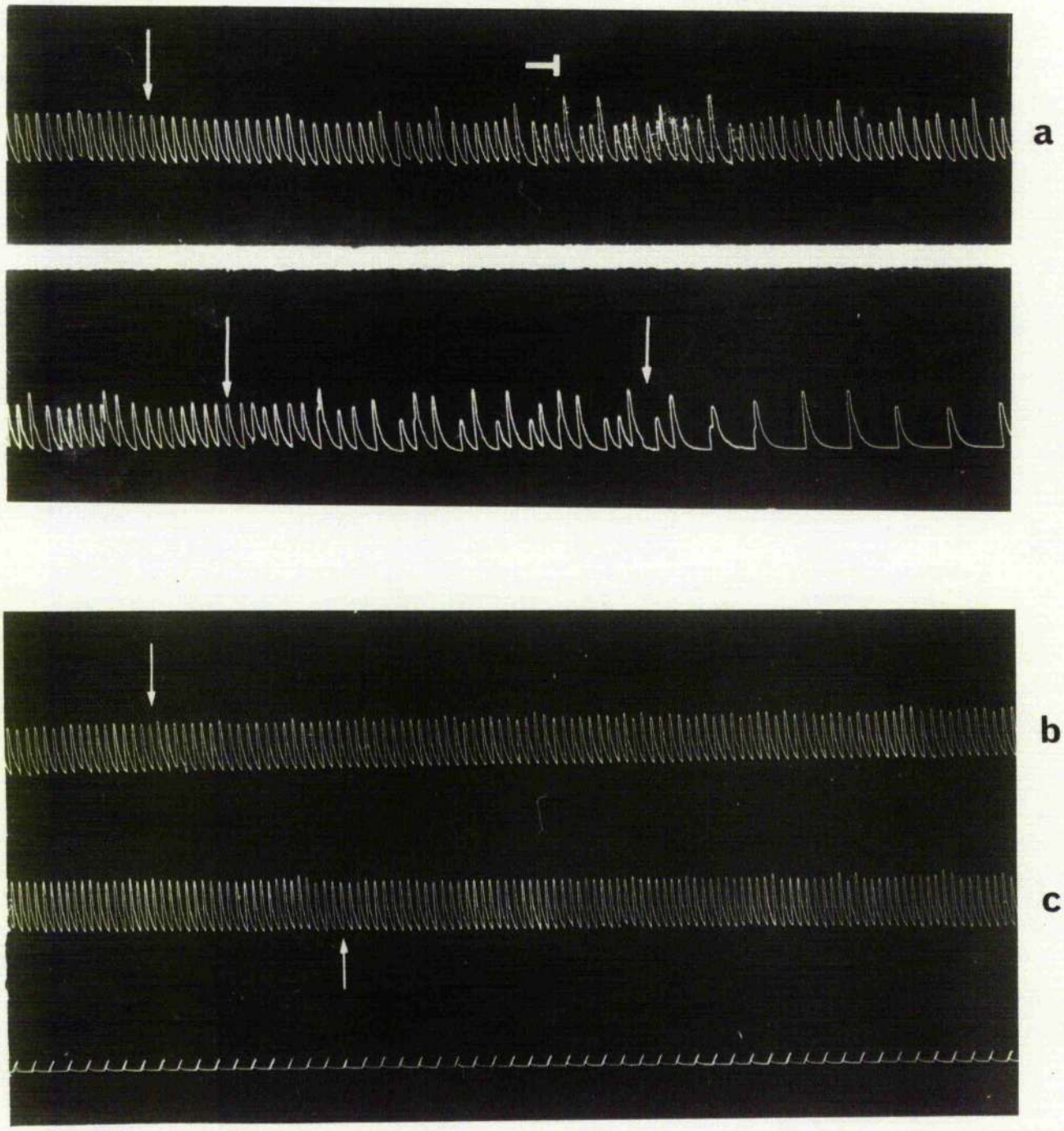


Fig. 14 Effect of various substances on the crab heart. (a) GABA at concentrations of 10^{-6}, 10^{-5}, 10^{-4} gms per ml respectively. The initial dose was washed off at —|, and the two parts of the trace are continuous. (b) homarine and (c) trigonelline at 10^{-6} gms/ml have no effect. Time marker = 10 secs.

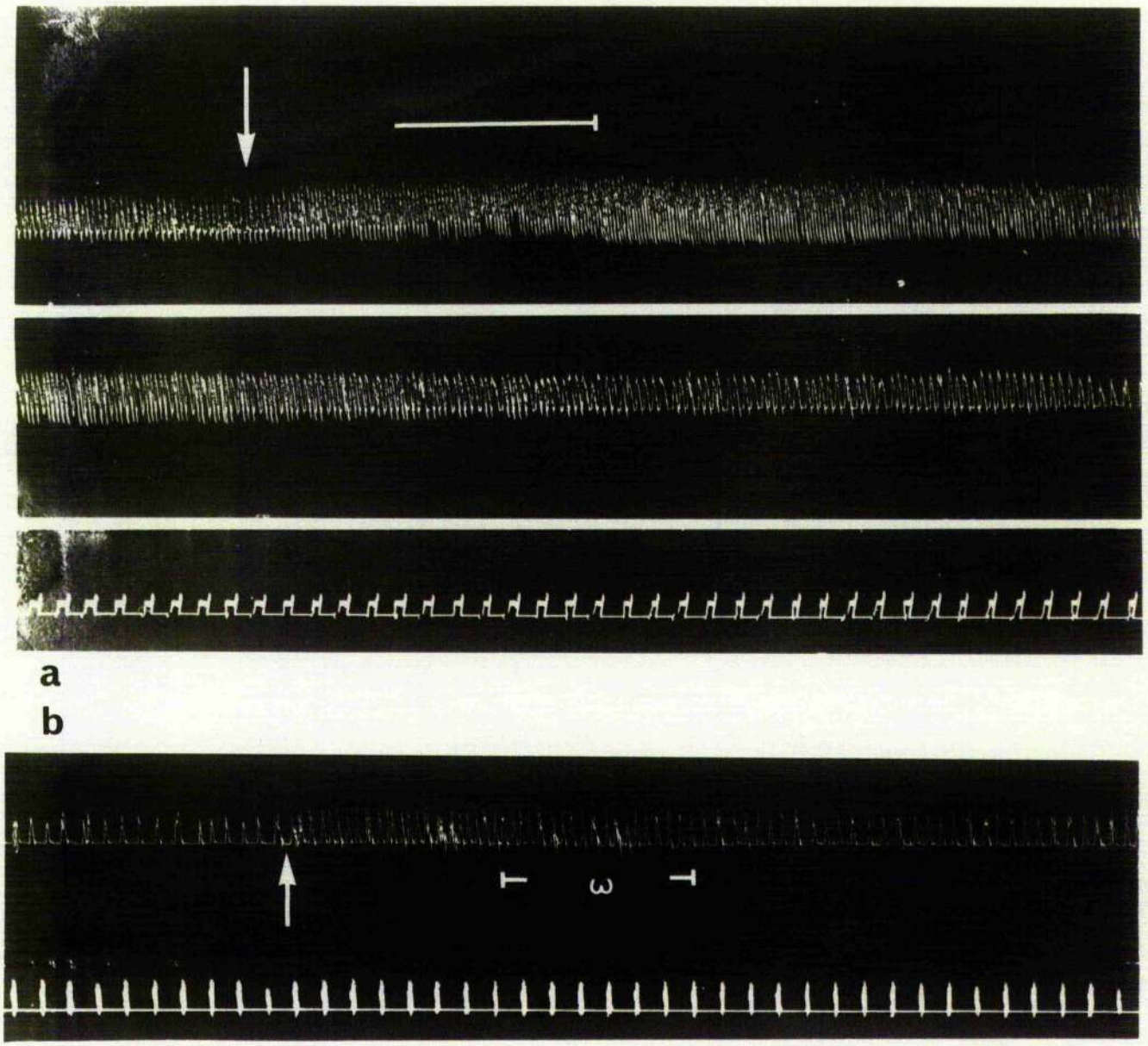


Fig. 15 Two examples of the excitatory effect of crab leg nerve on crab heart. The two, continuous parts of the upper trace (a) are of cardiac contractions whilst the crude alcoholic-extract of leg nerve was applied for the period shown. (b) shows the effect of the addition of an eluate of spot F. Time marker = (a) 10; (b) 5 secs.

of Ca^{++} in crustacean muscle prevents the formation of muscle action potentials (Fatt and Ginsborg, 1958) and in vertebrates low Ca^{++} interferes with the release of ACh vesicles by the nerve endings (Hubbard, 1961). If this latter effect is a property of nerve membranes in general, the low Ca^{++} effect here should be countered by increasing the number of nerve impulses, and when the stimulus frequency or duration was increased, a contraction was obtained more nearly normal than that caused by the usual stimulation of the nerve. Increasing the strength of the (already suprathreshold) stimulus naturally had no effect on contraction (fig. 17a). Accordingly the Ca^{++} concentration was raised by increasing the amount of calcium chloride in the saline until a value was reached which just did not cause a detectable inhibition: 18-20 mM (fig. 16). It is important not to go beyond this point for increasing the level further results in a tetanic contraction of the muscle.

If now the chromatograms were eluted with this saline, and the injections into the closer muscle also made using it, any effect caused by the extract should be manifest. No change could be seen, however, in the response, and it was concluded that the extract had no detectable effect either on the muscle directly or on the neuromuscular junction. The significance of this is discussed later.

On the other hand, injection of the perfusate from another active leg contained in the raised-calcium saline into the closer muscle produced an inhibition of the contraction of that muscle when its nerve

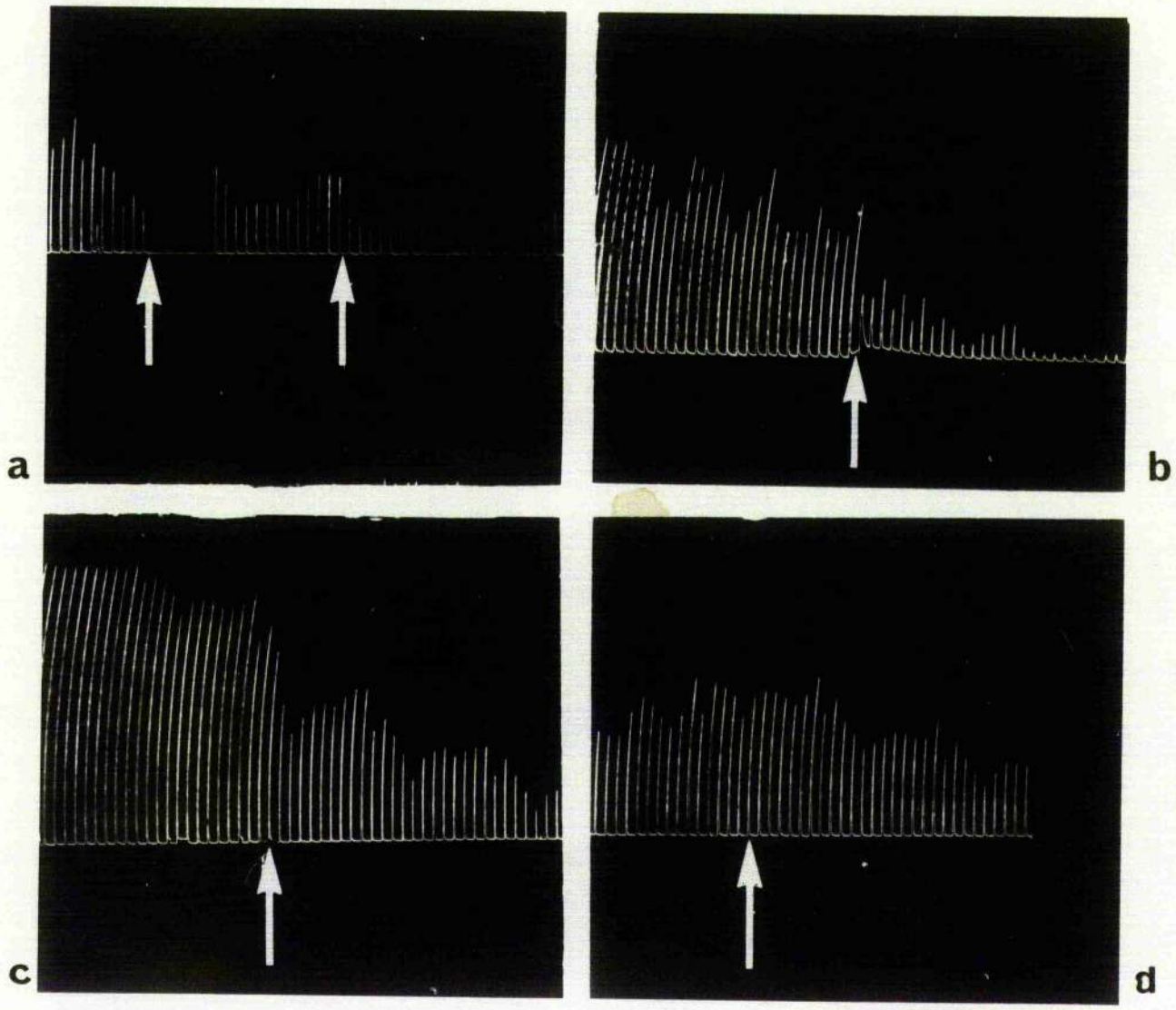
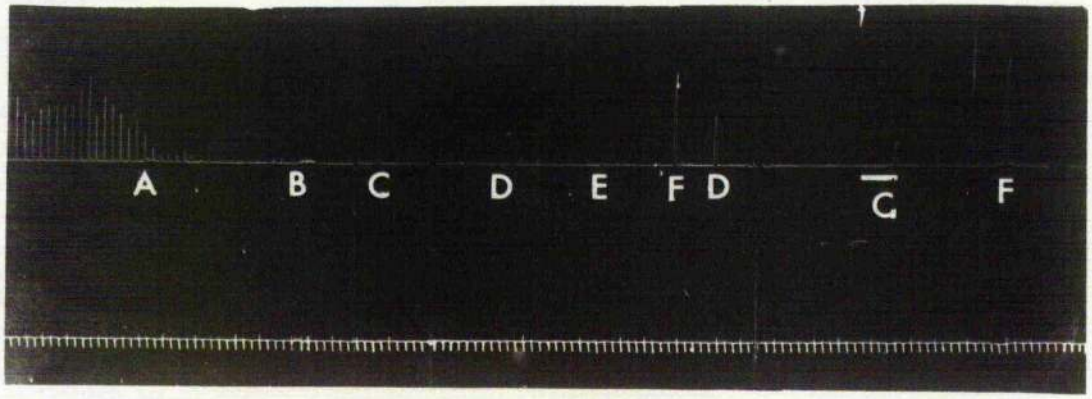
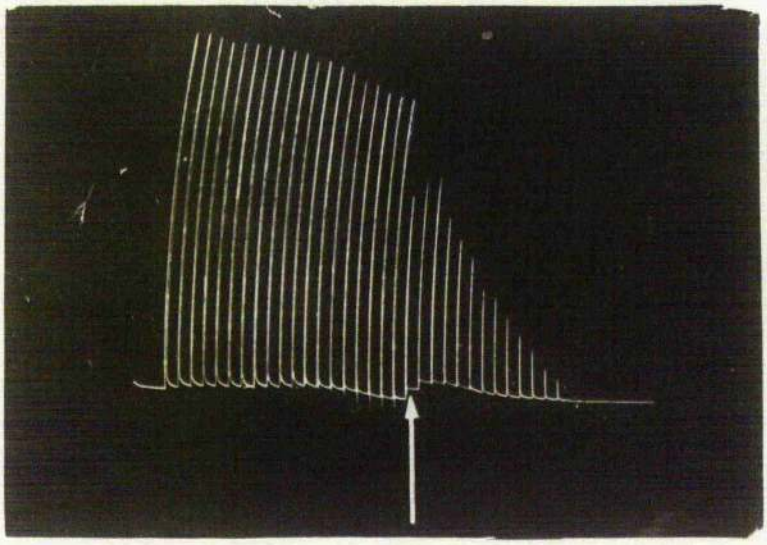


Fig. 16 The effect of an injection into the closer muscle of a crab leg of (a) artificial seawater, with Ca^{++} = 12.34 mM, and (b) - (d) saline with Ca^{++} = 14.59, 16.84, and 18.20 mM respectively. Even the last causes a slight inhibition of contraction.



a



b

Fig. 17a The effect of seawater injected into the closer muscle. Where f = frequency (cps) of stimulation and d = duration (secs) of pulse, at

A 0.1 mls seawater totally inhibit contraction caused by stimulation of the nerve at f = 50, d = 0.5 every 5 secs.
B f = 50, d = 0.5 repeated every sec.
C 50 1.0
D 100 0.5
E 25 2.0
F 250 0.5
G 25 5.0

Fig. 17b Injection of perfusate af leg contained in saline (Ca^{++} = 18.20mM) into the closer muscle caused marked inhibition of contraction caused by stimulation of the nerve as at A above.

supply was stimulated. In view of the inhibitory effect of saline, such a result had to be examined carefully, but controls performed using blank saline injections showed the effect to be genuine. It was also seen, however, when the leg supplying the perfusate had not been active.

DISCUSSION

The idea that NA bears a special relationship to the motor transmitter in Crustacea, as previously noted, could be linked with the idea that it also has a special relationship with the motor axons of these animals, and it was to investigate this situation that the uptake of NA was measured in the experiments described above.

The uptake in whole nerve showed that there was a greater concentration of *NA, or its radioactive metabolites, in the peripheral part of the leg nerve than in the central part. That it is not, in fact, *NA was shown by the chemical spot tests performed on it. The meaning of such a gradient is not immediately clear for there are several factors which complicate the picture. It is true that in the central two sections of the leg studied (the meropodite and the carpopodite) there are motor fibres whereas in the propodite and dactylopodite there are not. For this reason, a concentration gradient with the maximum centrally could be explained in terms of uptake by the motor fibres. But the gradient actually seen was in the opposite sense to this, and in any case there are other factors which make such an interpretation of a gradient in either sense difficult. For one thing, the ratio of motor to sensory fibres is very small - of the order of 5 to 5,000 in the meropodite, although the mass ratio would not be quite so small owing to the large size of the few motor fibres. Again, there are the inhibitory fibres (3 of them in the meropodite) about which nothing has been said; also there is the fact already mentioned that the cell bodies of

the motor and sensory fibres are at opposite ends of the nerve - all the sensory cells being situated peripherally, and the motor being in the CNS. Now the position of the cell bodies is important for they contain most of the DNA and RNA of the neurons, and therefore represent the site of most of the chemical building which the cells carry out. The cine-films of Willmar (private communication) and more recently Weiss (1962) show pretty conclusively that axonal transport of material occurs, and it is not unreasonable to think of the metabolic precursors being absorbed mainly through the cell body surface, where the enzymes are situated, rather than through the axonal surface, and then passed along the axon. If this be assumed, then, that the *NA is absorbed primarily through the somatic membrane, and passed down the axons in its changed form, or even still as *NA to be metabolised in the axon, an explanation of the concentration gradient can be given. Equal uptake in the two types of axon would mean initially a high concentration of radioactivity at each end of the nerve, where the cell bodies are, and a lower concentration in the length of the nerve which only contains fibres. This has to be qualified slightly in the particular case studied here because the part of the nerve examined in fact contains neither type of cell body: the peripheral parts of the nerve, however, are nearer to the sensory bodies, which are situated in or by sense organs distributed over the whole surface of the leg, than the central are to the motor, and hence the high concentration levels might be expected to reach the peripheral parts of the nerve examined sooner than the central parts. Moreover, it may be argued that in such a case, as time passes, the gradient will disappear

only to re-appear in the opposite sense, as the radioactive substance absorbed by the axon for the period between the initial labelled, and subsequent non-labelled doses of NA passes along the fibres. One can scarcely hope for such a clear picture as this with the different speeds of transport in different axons, etc., but some indication should be visible in the time course of radioactivity graphs (fig. 4).

The results do show a gradient with the higher level peripherally, but do not show any reliable signs of gradient reversal. In one case (crab 17) it was seen after 36 days that the ratio of activity in the meropodite and propodite changed from figures consistently less than unity to 2·74, but, as explained earlier, such is the variation between one leg and the next that no confidence can be placed in single cases like this.

Hence the tentative conclusion (subject to the assumptions about absorption and transport made above) may be drawn that either both types of fibre are taking up the *NA in equal amounts, or, less likely, that the sensory fibres are taking it up preferentially. It is almost certain, though, that the motor fibres are not especially involved in the matter.

The only way to clear up the indecision of the above results was to measure the uptake in single, identified axons and compare the two types. As the sensory fibres are very small - they range in size from a couple of μ down to 1/10th μ - a comparison was made between a single motor fibre and a bundle, known to contain only sensory fibres, of the same diameter. The results from this work show quite definitely that there

is no difference detectable between the two types of fibre as regards their uptaking *NA. The degree of blackening on the photographic plate used for the assays did vary from one fibre or bundle to another, but this was due to the same reasons which led to differences in the results of the counting of extracts of whole nerve. When the fibres are classed according to the arbitrary degrees of darkening devised, equal numbers of sensory and motor fibres appear in each class. It should be possible, of course, to detect a gradient in single fibres by this technique, if one exists. In fact, the exposure caused by the radioactivity in any one fibre over a length of a few centimeters was always the same along the whole length, except for three examples in which it appeared that there was less activity centrally than peripherally. It would seem, then, that the hypothesis that there is a relationship between the motor axons and NA particularly is one which is best discarded, although it is also clear from the results that it is true to talk of a special relationship between the peripheral nerve as a whole and NA, when its content is related to other tissue. The nerves do take up NA from the blood and raise the concentration of radioactivity in themselves to ten times that of the blood and other tissues. It is not necessary, of course, to envisage an 'active transport' mechanism, in the sense of transport up a concentration gradient, for this, for it may be that the NA is converted into some other compound as soon as it enters the fibres, and thereby is effectively removed. The experiment with the in vitro fibres was not designed to show, nor did it give any indications about the possibility of active transport.

The biochemistry of NA has been discussed in the introduction, and there appears to be no known reason why nervous tissue should take up so much more NA than other tissues - about ten times as much, in fact. It has, of course, no connection with nicotine, with its autonomic nervous system associations, and the one or two special facets of NA metabolism which are associated with nervous tissue do not seem to explain this satisfactorily - for example, NA derivatives are known to inhibit the hexokinase reaction in brain (Kuriyama, 1962), and there is described too an excitatory activity in man for small doses, and an inhibitory effect for larger (Nikolov, 1962). In all cells, NA is used for the production of NAD and NADP, the coenzymes, but far from being a process which might be especially associated with nerve cells, the maximum concentration of these nucleotides would be expected to occur in those cells respiring at the highest level - i.e. the liver cells. But far from containing more, the liver only contained one tenth as much activity as the leg nerve - that is, about the mean for the body. The brain and ventral ganglion cells took up very little, and in any case the chemical tests done on the extracted radioactive compound make it unlikely that it is either of these nucleotides.

The hydrolysis of the compound succeeded in splitting it into three ninhydrin-positive substances. This, though not conclusive in itself (for, as already mentioned, any compound which contains three amino groups and any number of other groups might give this reaction when boiled with acid), taken with the evidence of the Rf, the spot tests,

and such things as the response to UV light after heating the paper strongly, suggests peptides. As there were only three amino residues detected after hydrolysis, it is probably a small peptide. There may, of course, have been more in concentrations too small to be detected, sensitive though the ninhydrin reaction be. This raises the question why there is so much of the substance in the nerve. Generally speaking, peptides only occur in relatively small amounts - like insulin, for example - and yet the spot at F, when stained with ninhydrin, is among the three most dense on the paper. The other two, however, which compare with it also give reactions for peptides, and they too have small Rf's (A = 0·03 and B = 0·05, fig. 11).

The -COOH group on the NA molecule is certainly the easiest, and probably the first, to be lopped off. The excretion of NA in nearly all cases leaves the pyridine ring intact, but the ^{14}C in the acid group may well leave the molecule before this. Generally, such an action would result in the formation of carbon dioxide and thus body carbonate, which would mean that the concentration of ^{14}C in any tissue would be small - and equal throughout the body. The fact that I found detectable amounts of it in one tissue particularly makes this unlikely, therefore.

The same arguments apply to the idea that the ^{14}C may be being split off to become formate, and thence enter the active-carbon pool. Such an action would result in there being a low, generally-equal level of activity in the body.

There is one report I have found of a bound form of NA. Sarkar

et al. (1962) describe the purification and some properties of 'niacinogen', a peptide bound with NA, and found in rice bran. They find that it contains 30% peptide, 6.4% glucose and 2% NA. Although it is ninhydrin-positive, it also stains strongly with Folin-Ciocalteau's reagent (no. 10, p. 58) which the spot F on the chromatograms did not. Moreover my tests on F for glucose were negative in contrast to their results.

There remains to explain the rise of activity level in the nerves and muscles from the legs with time (fig. 4), if this is a true result, and not an artefact depending on the scatter of results. It will be recalled that there appears to be this slow rise in the level of activity in these two tissues, although the muscle level remains at about 1/10th that of the nerve. Clearly the body level generally cannot be rising, for there was only one dose of *NA given, and that was followed by two much bigger doses of ordinary NA. These washing-out doses mean too that it is unlikely that there is store of *NA in the body anywhere in particular, or even everywhere in general, for such a store would exchange rapidly with the non-labelled acid, and after the second doses, the nerves would be accumulating virtually only non-labelled NA. The only explanation which will fit is one which depends on the nerves having quickly snatched the first, labelled NA and incorporated it into themselves - maybe in a form akin to 'niacinogen' - but mainly in some part of them which is not included in the assay method used. From this region, the radioactive compound then travels to the parts assayed as time passes. This of course fits in with the idea of absorption being mainly through

the somatic membrane, and with that of axonal transport of metabolites.

The absence of a connection between NA and the motor transmitter is borne out by the results of the bio-assay methods used. The substance F had an excitatory effect on crab heart, it is true, but so do many other compounds. What is more interesting here is the fact that it had no effect when injected into the closer muscle, in direct contrast to Factor S of Van der Kloot which was "highly active in exciting the crayfish closer muscle". Also he found that perfusates from active, but not inactive, legs were able to stimulate contraction in other legs. In the case described earlier here, perfusates from active and inactive legs alike inhibited contraction in the assay preparation. But crude perfusates were used here - collected and then immediately injected, whereas in Van der Kloot's experiments the perfusates were subjected to considerable chemical processing before injection into the assay preparation, and this may have not only separated out a small fraction, but introduced some foreign material.

PART II

INTRODUCTION

Following on from the work described in the previous section, where some aspects of the chemistry and physiology of substance F, a metabolite of nicotinic acid extracted from crab nerves, were discussed, it was decided to measure the effects, if any, of substance F when applied to single axons of the crab, Carcinus maenas. Once the apparatus for this work had been set up, further studies on single axon physiology were conducted, such as, for example, the effects of different external potassium concentrations. The motor axons furnish a sensitive physiological assay method, for they exhibit a characteristic response to direct current stimulation.

It has been known since the work of Biederman (1896) that crustacean motor fibres are capable of firing repetitively when stimulated by a maintained, constant depolarising current. Hodgkin (1948) classified the types of response into three groups:

> "1. Axons which were capable of responding over a wide range of frequencies (5-150/sec.).
>
> "2. Axons with a pronounced supernormal phase. This class gave a train of impulses of frequency 75-150/sec. which was relatively insensitive to the strength of the applied current.
>
> "3. Axons with high threshold and low safety factor which either failed to repeat or succeeded only if the current strength was much greater than rheaobase."

Chapman (1963) has elaborated this classification by subdividing

each of the groups, and he also found that any one fibre might change from one group to another in the course of an experiment. Hodgkin notes that the third group are found when the preparation is damaged slightly, or after some time in the seawater bath - even though the axon may have been responding according to one of the other ways before. In contrast to Wright and Coleman (1954) who found that an axon of one particular function always exhibited the properties of one particular class, and who correlated response with function in the animal, Chapman saw that any fibre in the crab leg might manifest any of the responses, and that it was not true to talk of the fast closer being in class 1, for example. Wright and Adelman (1956) described how an increase in the external potassium concentration caused fibres which had previously exhibited a repetitive response to become non-repetitive; they became in fact like the giant fibres of the ventral nerve cord in this respect, and also in that the local response was shortened. Lowering the external sodium had the same effects.

The nature of the sheath

One of the possible, though improbable, connections with the three responses obtained with a DC stimulus to a single axon, is the type of sheath around the fibre. The nature of the sheath in these tissues, as in all others, also introduces difficulties about the accessibility of the fibre to applied chemicals. Many a doubtful result, for example, has been encouraged by the argument that access to the end-plate regions

is hard to achieve and ensure. For this reason the sheaths of named axons have been examined here, and compared with the type of response obtained from them.

Crab nerve fibres have a variety of types of sheath around them, as described by Horridge and Chapman (1964). The smaller fibres were seen by them to be quite unsheathed, and in direct contact with other axons - and only other axons - whereas the larger motor fibres, of 20 μ or more diameter, have a thick complex sheath around each fibre.

According to Young (1936) a 15 μ fibre may be surrounded by a sheath such as to make the total diameter 80 μ, but none of the fibres which either Horridge and Chapman or I looked at had anything quite as big as that: more usually a 20 μ fibre would carry a 5 μ thick sheath.

The sheath of the large axon described by Horridge and Chapman consists of an outer region of alternating bands of sheath cells and extracellular material and a much smaller 'inner pavement sheath' of flattened cells, usually two deep, which completely encircle the axon. The meeting of two such cells on the surface of the axon shows a highly interdigitating junction which might be up to 20 μ in length. Moreover the width of this junction region is so constant that Horridge and Chapman conclude that they are not 'channels' in the normal sense, but in fact intercellular regions which are filled with a rigid substance holding the two cells apart, or, as they point out, together. They argue that as channels, the intercellular space is badly adapted: it is long and convoluted, and also runs into the layers of the outer sheath.

Structure and permeability in other sheaths

As work has been done on the permeability of sheaths in other animals, it is of interest to note how they compare with those in Carcinus. The work of Hess (1958, 1960) on the sheaths of the cockroach peripheral and central nerves shows that these fibres are surrounded by a sheath much smaller than the comparable fibres of Crustacea, and that there is a considerable difference between the structure of the sheaths in the peripheral and central nervous systems (as described by Twarog and Roeder, 1956) and in other insects. Hoyle (1954), for example, describes the sheath of the motor axons in the locust leg. These large fibres share a common sheath consisting of big sheath cells surrounded by a thin neural lamella and a fatty layer outside that. Although the cockroach sheath is thinner than that in Carcinus, it is still considerable in size, and it is not surprising that desheathing a nerve lowers its threshold to applied chemicals. Both Roys (1958) and Twarog and Roeder found this to be the case using desheathed nerve cords in the cockroach, and Roys found that the sensitivity of the exposed nerve fibre was greater even than that of the 'specialised' end organ to applied sodium chloride, and other substances. The effect of applying chemicals to a normal nerve was qualitatively the same as a desheathed nerve, but a higher concentration was needed to elicit the response.

In contrast to Roys, Hoyle (1953) found that in the locust the nerve sheath acted as an effective barrier to applied potassium. In the whole animal, the blood potassium level may change appreciably, and it is not

surprising that the nerve sheath has some part to play in protecting the axons from potassium concentrations which would depolarise them to an extent which would render them non-conducting. The tracheolated membranes round locust muscle only acted to delay the entry of potassium.

Twarog and Roeder (1956) measured the time taken to block conduction in the cockroach nerve cord by raised potassium. They found that if the cord were desheathed the time fell from twenty minutes to twenty seconds. Likewise the recovery from this treatment when the bathing solution was returned to normal was greatly reduced by desheathing.

It should be noted in passing that the term 'desheathing', used by both Roys, and Twarog and Roeder in the work quoted above, is really of rather vague meaning. Exactly how exposed the nerve fibres are once the outer sheath has been removed is not clear, but presumably they still have their individual sheaths. It may be noted here that all attempts to 'desheath' the single motor axons of Carcinus have failed: the sheath is so closely attached to the fibre that any attempt I have made has killed the axon.

Treherne's recent work (1961, 1962) on the permeability of cockroach nerve cord to ions is also of interest in this connection. His conclusions are somewhat in contrast to those of Hoyle and Twarog and Roeder, for he finds that the sheath around the cockroach nerve cord is highly permeable to sodium ions. The loss of sodium from a nerve cord previously labelled with Na^{24} was seen to be in two parts: a fast component (half time, $t_{0.5}$, 33 seconds and unaffected by metabolic inhibition), and a

slower component which was dependent upon metabolism, He showed that the faster part is due to the exchange of ions between the external solution and the extracellular space inside the central nervous system. The slower phase was due to the movement of ions out of the cells of the central nervous system. In a private communication, he expanded this idea by putting forward the hypothesis that the sheath itself contained ions 'bound' to the protein molecules there, but which could exchange easily and rapidly with ions on either side of the sheath. Thus concentrations could be quickly shifted from one side to the other by means of a 'chain' movement, although the ions themselves might not move more than a little way.

Hess (1958) points out that in many ways the cockroach and vertebrate sheaths are comparable. Work on the permeability of the vertebrate sheath by Shanes and Berman (1955) shows that sodium passes out of a toad axon labelled with Na^{24} as from a cockroach nerve cord at an initially fast ($t_{0.5}$ = approx. 2 minutes) and a subsequently slower ($t_{0.5}$ = approx. 8 minutes) rate. Much the same pattern was seen by Krnjević (1955), and Huxley and Stämpfli (1951) believe that changed external potassium concentrations are transmitted to the inside of the myelinated frog axons in less than one second. Kato (1936) showed that cocaine blocked conduction in a time of this order.

Work on the permeability of the squid giant axon by Tasaki (1963; Tasaki et al., 1961) showed that in the resting fibre both potassium and sodium may pass in or out of an axon, but there is no distinction in

this work between the axon membrane and the sheath. Villegas and Villegas (1960) have described the sheath of the giant axon which in some respects resembles that of Carcinus motor axons. Immediately around the axon is a layer, about 1 μ thick, of flat Schwann cells which are penetrated by slits passing from the extracellular space to the connective tissue layer outside. The slits may be up to 4.3 μ long for they meander between the two boundaries and although their usual width is 60Å, they appear to close down when the external medium is made hypotonic, suggesting that they are actively responsible for the passage of ions and water to and from the axon. The same authors have also calculated (Villegas et al., 1963) the theoretical equations to explain their observed diffusion through the barrier and conclude that apart from pores in the axolemma 4-4.5Å radius, there are channels through the Schwann cells which are of the same dimensions as those described above for the slits.

Permeability of Carcinus sheath

The complexity of the sheaths of the motor axons of Carcinus is greater than those of the nerve cord in Periplanata, but it might well be, none the less, that the passage of ions across them has to be understood in much the same manner as Treherne suggests for the cockroach. That they are permeable to potassium, and easily so, is shown by two observations: Hodgkin and Huxley (1947) saw that surrounding a single axon with paraffin oil made a great difference to the electrical properties of the axon - due to the fact that such treatment greatly reduced

the volume of the seawater layer around the fibre, thereby greatly increasing the effect of a given quantity of potassium which would leak from the fibre during activity. Also a decrease in the size of spike during a repetitive train is often seen, and is due presumably to the accumulation of potassium around the axon. But Chapman reported that a 5% change in the external potassium led to great changes in the axon properties, and Horridge and Chapman (1964) calculated that after one impulse, unless the leaked potassium could escape to the external medium, the concentration around the fibre - inside the sheath, that is - would more than double. Large concentration changes of this order are often big enough to render the fibre inexcitable (see below; Wright and Adelman, 1956).

Schmitt (1957) proposed four models for understanding the relationship between the axon and its environment. The first of these considers the case where the axon is in direct contact with the true extracellular medium, and is clearly not applicable in the case of the crab motor fibres with their complete and complex sheaths. The other three suggest roles for the Schwann cell in varying degrees of importance:

(a) where the axon is in communication with the extracellular space through channels between the Schwann cells;

(b) the same as (a), but with the Schwann cells acting like porters and maintaining the passage composition of the channel; and

(c) in which there is no direct link between the axon and the extracellular space, but in which all the ionic flow to and from the axon takes place through and across the Schwann cells.

If the conclusion of Horridge and Chapman about the 'channels' in the inner pavement sheath, that they are in fact filled with a cement substance, is correct, the only version of Schmitt's models which is appropriate is the last. The idea that cells might work in a sort of 'push-pull' symbiotic manner is one which has been developed very attractively by Willmer (1960), and it could be that there is such a mechanism at work here. Willmer has shown that cells may be broadly divided into two classes depending on whether they primarily take up or pump out ions through their 'vegetal' or 'animal' poles, and also that a nerve cell derived from one of these types is accompanied by a Schwann cell of the other, and vice versa.

If the last of Schmitt's models, where the changes in the extra-axonal space are closely controlled by the Schwann cells, be accepted, then, as being applicable to the crab motor fibres, it is clear that the idea that the constitution of that space is the same as the constitution of the extracellular space proper (wherever that be) has no foundation. Indeed there are no figures available which indicate the concentration of substances in the extra-axonal space, and we are left in the dark completely about what may be there. Nor is there very much information about how easily compounds may pass across this complex structure: whether it forms a completely impermeable barrier to some chemicals, whether there is active transport of the chemicals that do get across, or whether there is, after all, easy access to the axon membrane. It is not far-fetched to associate the complex sheath of these

animals with the fact that their blood concentration is liable to change: this will be discussed further later.

It is interesting to note, finally, that Tobias (1958), working on isolated lobster peripheral fibres found that if these were left in seawater for three hours, the structure of the sheath changed considerably, although no corresponding electrical changes were noted. Moreover, if the fibres were left in a solution of trypsin or α-chymotrypsin, they were still electrically excitable long after the sheath had been quite destroyed. A solution of phospholipase A or C, on the other hand, quickly rendered the axon unexcitable, but had no visible effects on the structure of the sheath. It is a pity that no measurements were made of the effect of changing the external ionic concentrations in axons known to have little or no sheath left about them.

In this part of the thesis work is described in which the sheaths of named motor axons from Carcinus were examined. The work of Horridge and Chapman (1964) suffers from this lack: that they only show pictures of, and describe one pair of axons, the identity of which is not clear.

Also described here are the results of experiments designed to show the permeability of the sheath to various compounds, and the effect they have on the repetitive response, and to attempt to throw some light on the question of whether the sheath controls the composition of the extra-axonal space, or allows it to follow the larger extracellular space.

METHODS

Histology

The first histology of single axons was attempted using wax sections, but this was soon abandoned in favour of araldite as an embedding medium. After axons had been fixed and embedded in wax, even if they were previously stained strongly, they were rarely seen again.

Single axons were dissected out most carefully, identified and sucked up in a small drop of seawater in a pipette. They were then extruded into a 5% solution of gluteraldehyde in filtered seawater buffered with phosphate to pH 7·4. This way, undue strain on the axon during transport from one dish to another was avoided. After fixing for half an hour, the axons were transferred to a 2% solution of osmic acid in seawater to colour them black.

After partial dehydration through ascending concentrations of alcohol, the specimens were left in acetone, which had been previously stored over metallic calcium, and a piece of metallic calcium placed alongside the axon. For the short time the calcium and the acetone are in contact, reaction between them is negligible, though after longer periods by-products are formed. The dried axons were then placed in a 25% solution of araldite in acetone for a couple of hours, and thus through ascending concentrations of araldite until they reached the 100% mixture where they remained for 24 hours or longer, so that the araldite might fully penetrate. The blocks were cured in an oven at

65°C on a piece of polythene in a watch glass for a further 24 hours. When set, the blocks were transparent and about the size of a farthing, and they could be conveniently trimmed down, as the specimen was easily seen. They were mounted on wooden dowel with sealing wax ready for cutting in a Porter-Blum microtome.

Sections of up to 0·5 μ thick were cut and transferred in a drop of 5% acetone in distilled water to a clean slide. These were then quickly dried on a hot-plate at about 70°C and the sections stained at this temperature with a solution of toluidene blue in saturated borax. Photographs were taken with a box camera using Ilford G 30 plates and yellow light. Some phase contrast pictures were also taken using a Zeiss camera and microscope.

Electrode systems

An excellent method of applying substances to an artificially produced node on an isolated crab axon is the pipe electrode system. The set-up was essentially that described by Hodgkin in 1947 with some of the modifications of Chapman (1963) and one form is shown in fig. 18.

Two holes were made in a polythene pipe, P, diametrically opposite each other, and of diameter about 60 μ. This size was chosen after careful trials with other sizes between 40 and 200 μ for a balance has to be reached between two factors: if the hole is large, seawater from within the pipe leaks out along the axon and reduces considerably the shunting resistance around the fibre - R_e. This can be reduced by

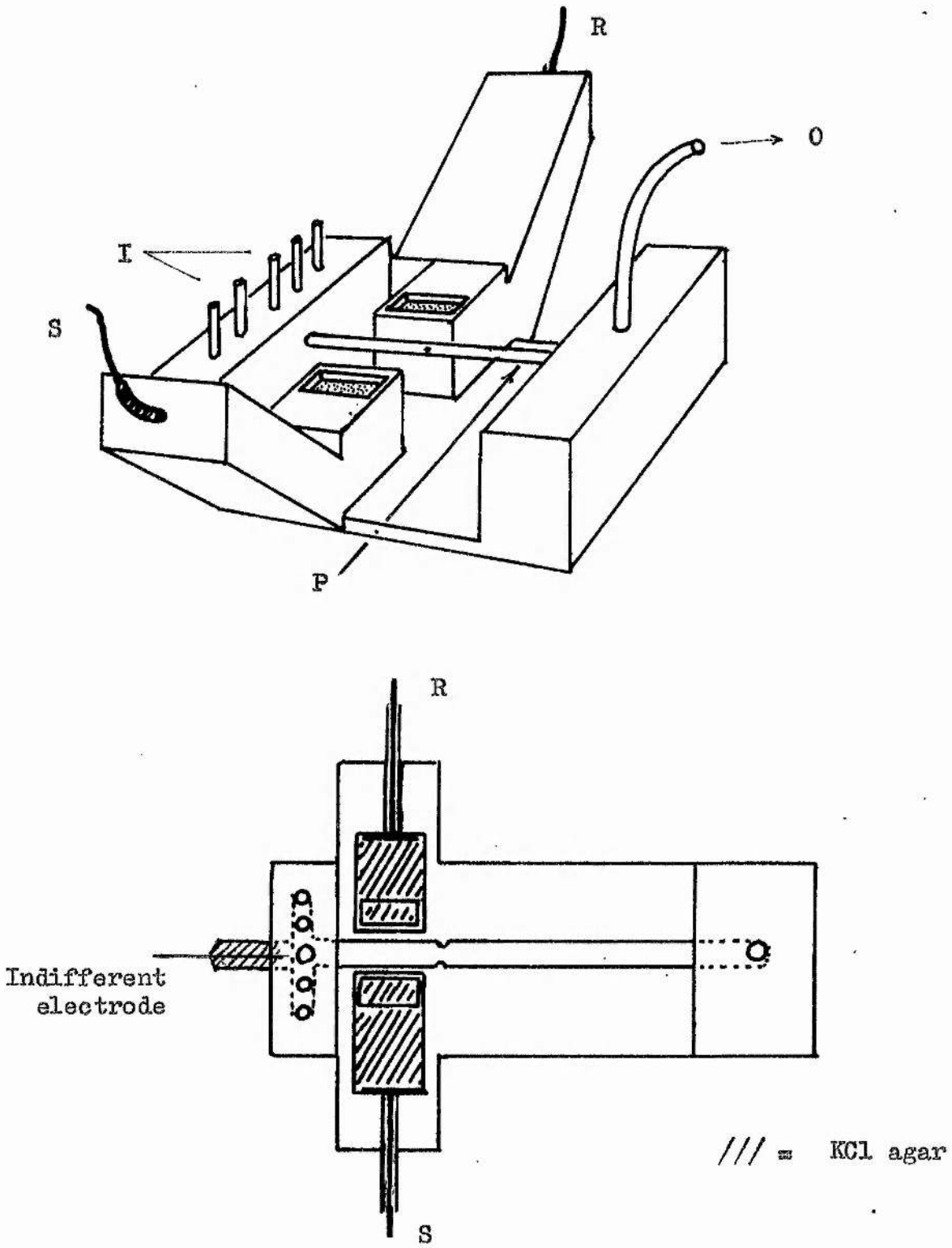


Fig. 18 Drawing and plan of the pipe electrode system. For details of use see text.

increasing the suction applied to the pipe (see below) when the paraffin around the fibre is drawn into the holes, but such a technique is liable to exert damaging tensions on the axon. It may even suck the axon away altogether if it is not securely anchored in forceps. If on the other hand the holes are much smaller than 60 μ the difficulty of threading the axon through is increased, and often the membrane is rendered non-repetitive if not inexcitable.

To thread the axon, a 20 μ stainless steel wire was first passed through the holes and then doubled back on itself to form a loop through which the cut end of the axon could be placed. On withdrawing the wire the axon is thus drawn through the pipe, and although that part of it in immediate contact with the wire is usually damaged, gentle pulling on the end of the axon enables a fresh part to be drawn into the pipe. Hodgkin's method of threading a hair through the pipe suffers from two drawbacks: the difficulty of finding hairs fine enough, and then getting such through the pipe - their suppleness militated against that.

Solutions were introduced into the pipe from burettes attached via polythene tubes to the inlets, I. Flow through the pipe was controlled both by the burette taps and by the suction applied to the outlet, O, but was usually such that the time taken for solution to pass from the openings of the separate tubes to the site of the axon was 80 ms when the large (1 mm diameter) pipe was used, or 50 or 20 ms when the smaller pipes (0·8 and 0·5 mm) were used.

The outlet communicated with a large reservoir which could be

evacuated: usually it was sufficient to partially evacuate this at the beginning of an experiment and then isolate the system from the pump as a large suction was neither necessary nor desirable for the reasons given above.

Each end of the threaded axon was clamped in a pair of forceps mounted on manipulators and two wick electrodes introduced to the axon, one each side of the pipe, which were also mounted on a manipulator. These electrodes were made by threading an 8 cm length of pyrex glass tubing (for pulling microelectrodes) with a length of cotton which protruded at one end for about 0·5 cms, and with a long length of silver wire which protruded from the other end and was soldered to the amplifier leads. The length and thickness of the wire was made as great as conveniently possible to increase the surface area and thus minimise polarisation effects. The tube was then filled with KCl-agar which held the cotton and wire in place, and stiffened the cotton where it stuck out of the tube. The tube was surrounded by screening wire, and covered then with a polythene sheath.

Once the electrodes were in place and connected, the seawater in the bath was replaced with liquid paraffin, and a 0·1% solution of cocaine applied to the axon outside the pipe to render it non-conducting. Some paraffin could be sucked into the pipe to reduce the shunting resistance of the seawater film around the fibre. The distance between pipe and wicks was made fairly large (5 mm) to avoid complications from the 2 mm axon space-constant.

Prior to the use of wick electrodes, the system of chambers shown in fig. 18 was used, but this proved to be unsatisfactory because of the larger amount of manipulation required to mount the axon, and because of electrical leakages between the pipe (indifferent electrode) and the other two electrodes (R and S) mounted on the same perspex block. Both these factors were reduced by the use of wicks.

Solutions

The seawaterused for dissection and experiments in this section was made up in the same way, and for the same reasons as described on pp. 23-25. Changes in the potassium level were made by exchanging potassium chloride with sodium chloride to the required amount, so as to keep the total molarity constant. Shanes (1946) reported that such a method resulted in osmotic effects which produced swelling in the nerve, and a delayed recovery from potassium treatment, but his experiments were conducted over periods of up to half an hour using whole nerve, whereas those described here exposed single axons to potassium solution for never more than one minute; and recovery was always of the same order of time as was the appearance of the potassium effects.

In the initial experiments, axons were washed in isotonic sucrose to remove the seawater film outside the axon, and thus prevent conduction, but following a report from Hodgkin that such led to hyperpolarisation which affected the repetitive response of the axon, the practice

was discontinued in favour of cocaine. Likewise application of KCl to the axon in an attempt to measure the demarcation potential was stopped in case the depolarisation affected the membrane inside the pipe.

During the experiment the axon was surrounded outside the pipe by liquid paraffin obtained locally but not stored for more than a month following a report from Chapman (1963) that a change in the oil occurred after some time, to render it toxic to the axons.

Circuits

Recording of the membrane and action potentials was made through a two-channel differential pre-amplifier with negative capacity (Bak, 1958) to correct for distortion caused by the capacity of the leads between preparation and amplifier. The outputs from this were viewed on one (differential) channel of a Nagard oscilloscope, whilst on the other channel current flowing through the preparations could be monitored (fig. 19). A battery arrangement was used to get a suitable DC level for viewing on the former.

Square pulses were fed into the preparation from a two-channel Tektronix pulse generator triggered either from the time-base of the oscilloscope (which conveniently put the pulse at the same time on each sweep) or, when moving film recording meant that the time-base was switched off, from a Tektronix wave-form generator whose pulse interval could be varied continuously from 0·1 ms to 10 seconds. One channel of the pulse generator was used to produce negative (hyperpolarising)

pulses for measurement of membrane resistance, and the other positive (depolarising) pulses to produce action potentials. The length of the pulse was usually 40 msecs. All the results described later were taken from healthy (i.e. propagating with good action potentials) axons, but it was also of interest to look at less healthy axons and a battery circuit, shown in fig. 19, enabled low-resistance preparations to be hyperpolarised and thus produce spikes. An integrating circuit near the preparation was used to square-up the corners of the square pulses.

Prior to the advent of the Tektronix pulse generator, a home-made flip-flop circuit was used which produced single pulses of variable duration and height. It fed to the preparation through a radiofrequency isolation unit (Schmitt and Dubbert, 1949) and was powered by a home-made power pack. Likewise before the Nagard was available, the extra gain required with the use of a Cossor oscilloscope was achieved by a home-made pre-amplifier.

Pulses were recorded from a slave cathode-ray tube on Ilford R 55 oscilloscope film - either single frames, or moving film. Spikes cannot be filmed very satisfactorily with moving film, but an attempt at Z-modulation of the trace (feeding the output from the oscilloscope plates, amplified, back on to the cathode to brighten the pulse at each spike) failed because of reciprocal attenuation of the brightness on the screen after each spike.

To the burette used for supplying the potassium solution, the output from a low-frequency oscillator was fed. This way a 10-cycle

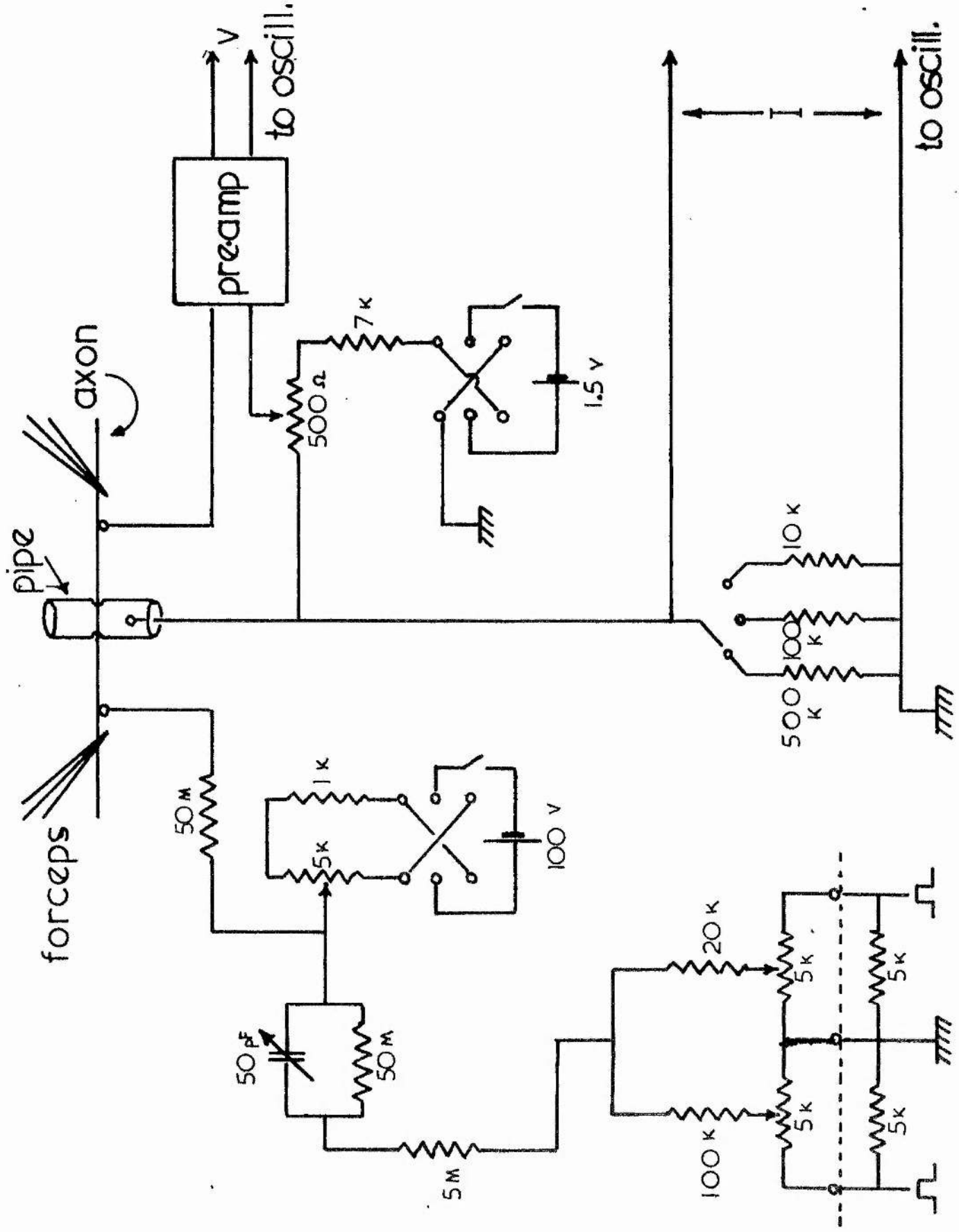


Fig. 19 Schematic drawing of the circuits used with the pipe electrode.

sine wave was seen on the axon current trace when the tap at the bottom of the burette was open, as the indifferent electrode was situated in the pipe (fig. 18). As explained above, the time taken for the potassium solution to arrive at the axon after the beginning of the sine wave was never more than 80 ms.

RESULTS

The structure of the sheath

Examination of named single axons from the crab leg nerve was carried out as described on p. 21. Using thin sections and high-power oil immersion, it is possible to make out the layers of the sheath easily but not, of course, to see the detailed structure described by Horridge and Chapman (1964) who used the electron microscope. Thus the inner cell layer seen by them to consist of flat glial cells, one or two deep, appeared under the light microscope as a thin dense layer immediately around the axon.

With this limitation in mind, however, it is possible to say from the light microscope pictures here, that the sheaths of each of the motor axons in the leg are all the same. Also that the sheath around the larger non-motor fibres looks very similar to that around the motor axons. Figs. 20 and 21 show transverse sections of the opener, closer and bender axons. They are all of comparable size, and the variety of shape may be attributed to changes occurring in the fixation. On account of the delicacy of the tissue, it was very difficult to prevent the fibre from flattening at some stage in the dehydration or embedding, and most of the first sections cut were of fibres which had become ribbon-like instead of the normal cylindrical shape. Horridge and Chapman draw attention to the fact that fibres are not necessarily round in cross-section, but it is probable that the large motor fibres

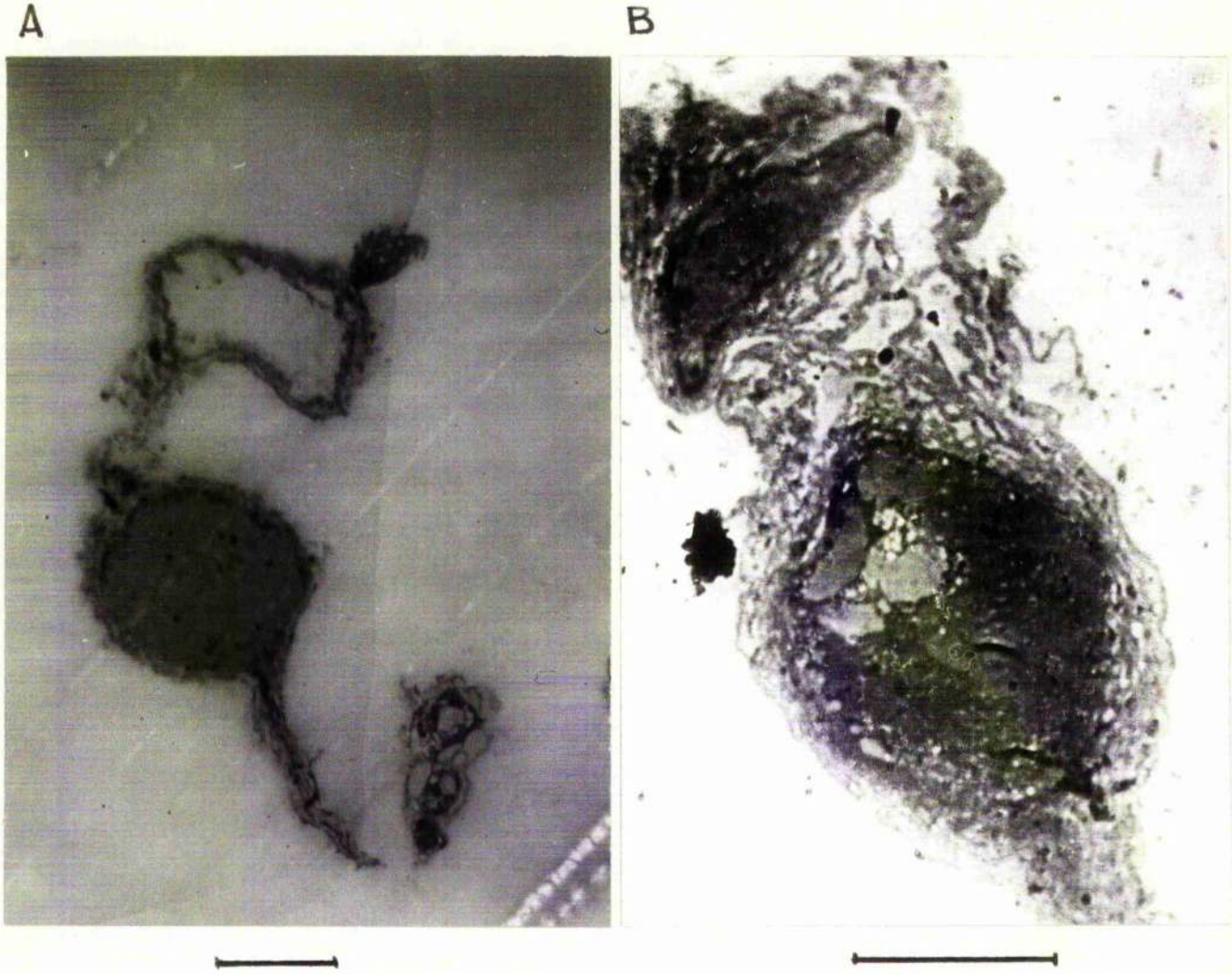


Fig. 20 Two transverse sections of the bender axons. (B) was taken with phase contrast; in both the scale is 10μ.

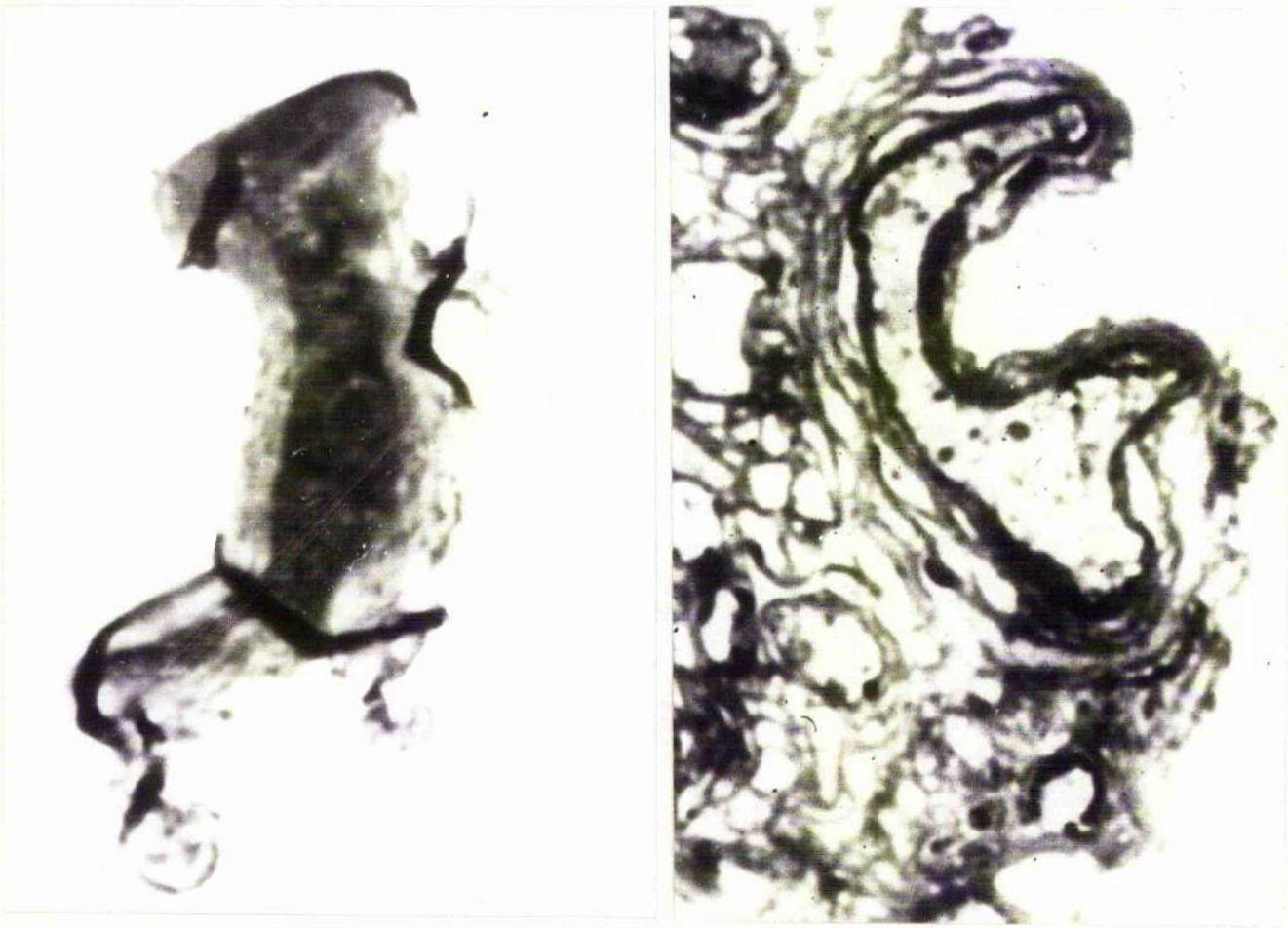


Fig. 21 T.S. Opener (a) and closer (b) axons. Scale equals 10μ.

are cylindrical *in vivo*, for when dissecting them under the binocular they never appear anything but that; that is, they always appear to be of constant width.

It was also difficult, using the x 100 oil immersion lens, to photograph sections which were accurately in focus, but the pictures in figs. 20 and 21 show clearly the inner sheath layer, consisting of the flattened layer of glial cells, and the outer fibrous layer of loose fibres between larger sheath cells. The average thickness of the sheath around a 20 μ fibre is 2 μ.

In the bundle shown in fig. 22, although slightly out of focus, four large axons can be seen. One of these was known to be the opener, by stimulating it before removal from the leg. When dissecting, there are always seen with the opener two other large axons which are immediately next to it. It is suggested that these others are the two inhibitory axons which supply the opener muscle - one of which is common to all the muscles of the last three segments (fig. 1a). At a section more proximal than the one shown in fig. 22, there were only three large axons in the bundle, but by this stage the common inhibitory has divided to supply the stretcher and/or bender. As far as one can tell from this photograph, all the fibres have the same type of sheath.

Changes/....

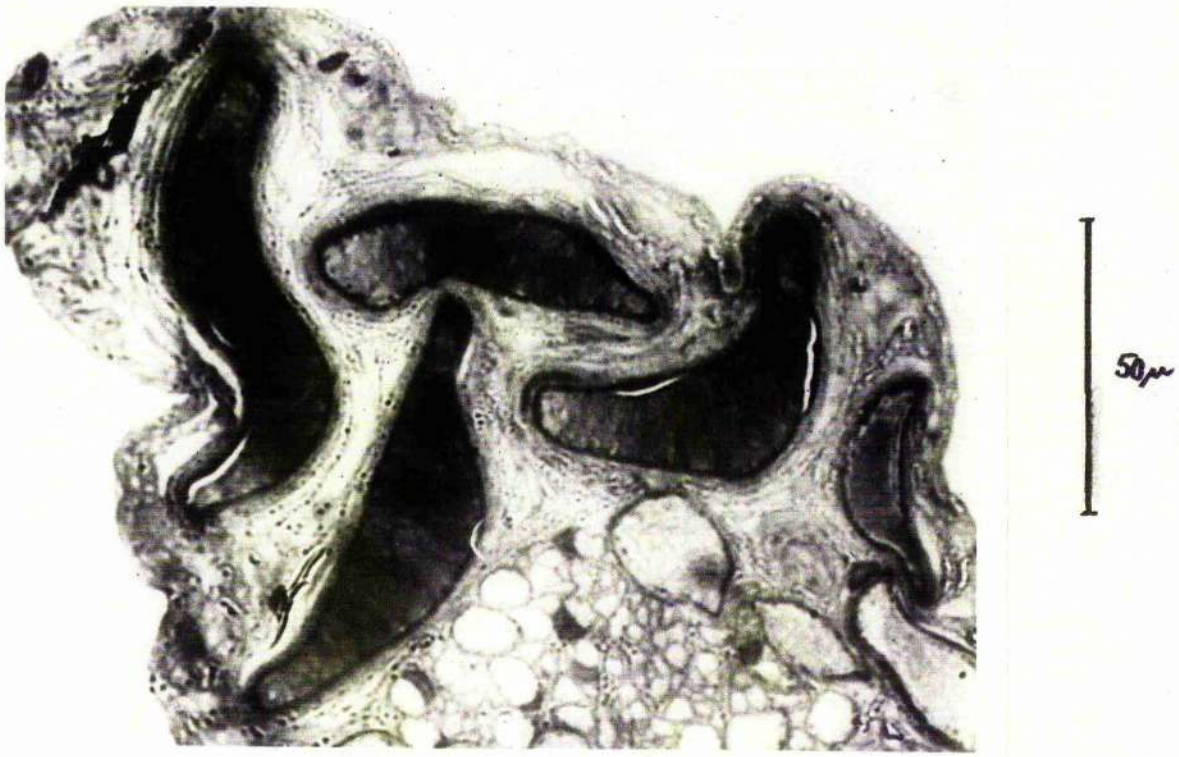


Fig. 22 T.S. bundle containing opener axon. The other three large axons are always seen with the opener when dissecting, and are presumably inhibitory.

Changes in axon environment

(a) Changes in external potassium. When the potassium ion concentration around a single motor axon (K_o) was changed over a small area of the fibre by means of the pipe electrode system described earlier, the well-known membrane effects were seen. The membrane conductance rises (resistance falls reciprocally) and the membrane potential, E_m, changes to a new resting level. The greater the increase in K_o, the greater were the effects, as is shown in fig. 24. It will be noted that this preparation follows the usual pattern for animal cells (e.g. Shanes, 1948, for crab nerve; Hodgkin, 1947, for isolated squid axons) where the relationship between log K_o and E_m is linear only over the range of high potassium concentrations - i.e. outside the physiological range. At low concentrations, those which are normally present in the extracellular space, the slope of the graph is smaller, and may reverse. The meaning of this is discussed further below.

Another interesting observation also came out of these experiments: when the potassium solution is applied, the change in E_m occurs over a definite and measurable time. This time, determined by recording the shift in the E_m trace of the oscilloscope on fast-moving film, was seen to be different for each potassium concentration applied, being shortest for highest values of K_o. A plot of the time taken for the effect to appear after application of the potassium against log concentration (fig. 23) shows however that the relationship is not simple, but has a peak value when the external potassium is raised to three times its

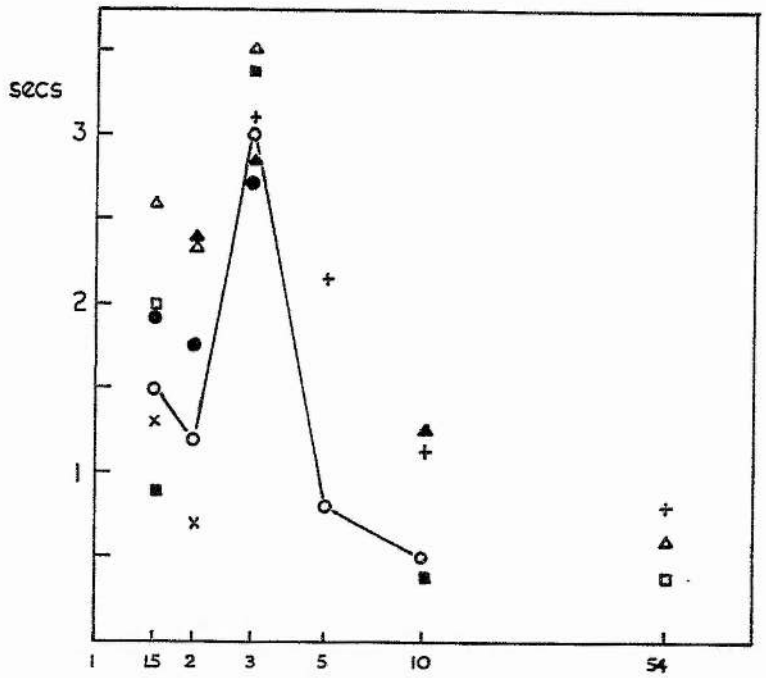


Fig. 23 Time taken (ordinate) for membrane potential to change by half the amount associated with each concentration of potassium outside. The abscissa shows relative external [K] — 1 being artificial seawater. Each series is mean from one axon.

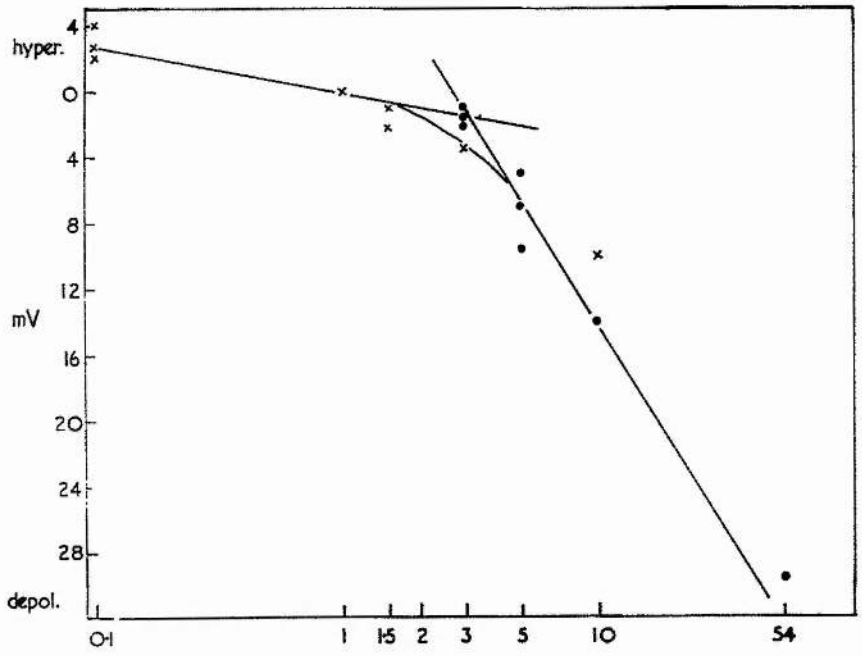
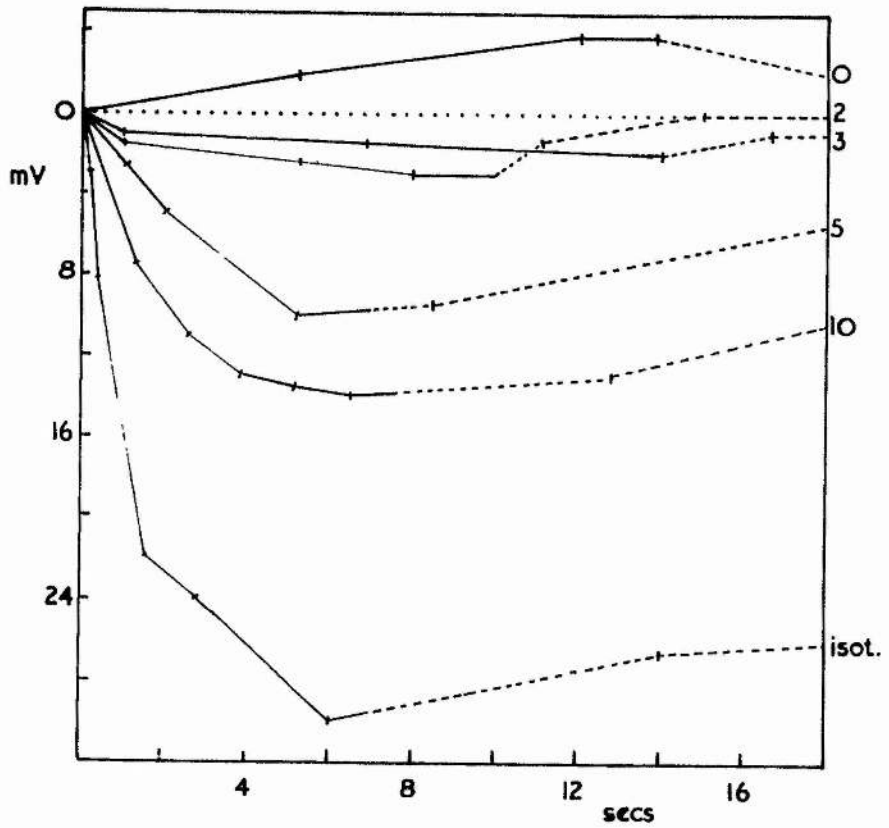


Fig. 24 Membrane potential (ordinate) from two axons plotted against external [K] The abscissa shows relative potassium concentrations, 1 being artificial seawater.

normal value. This time includes the very short period before any effect is seen at all, because of the time taken for the new solution to reach the axon after the taps are turned, although this is never more than 80 ms (see p. 100). Apart from the peak at 30 mM, the relationship is more or less exponential, as far as can be deduced from the points on fig. 23 and others taken but not shown in that figure, both for the total- and half-latency, where these terms are used to refer to the period over which E_m and R_m (membrane resistance) are changing. The term half-latency ($L_{0.5}$) was taken as the time in which the membrane potential changed half way towards its new resting level, and was much easier to measure than the total latency (L_t) s i m p l y because the effect was slow and (with small concentration changes) small, and because the film was moving fast, to obtain reasonable resolution between spikes, the trace might only shift by a millimetre in 30 cms of film. The change in E_m and R_m followed an exponential-like curve (figs. 25 and 26) which meant that $L_{0.5}$ was much less than half L_t.

In figs. 25 and 26, a plot is made from one axon only of the time course of changes in E_m and R_m for different concentrations of potassium around the fibre. Although this only shows the results from one experiment, every concentration was tested three times on the axon, and the shape of the curves are quite typical of all the ten axons tried. Each concentration was tried three times on each fibre, and although these three tests were usually done consecutively, the order in which the different concentrations were tried varied from one axon to the next,

Figs. 25 and 26 (following page): The time course of the action of different external potassium concentrations on membrane potential and resistance respectively. The figures to the right of the upper, and on the lower diagram give the factor by which K_o has been changed from normal saline. The solutions were applied for the solid part of the curve, and washed off during the broken part. The results shown here were obtained from one axon, but are quite typical of all tried. Ordinates: upper, membrane potential change (depolarisation downwards); lower, membrane resistance relative to value in normal saline.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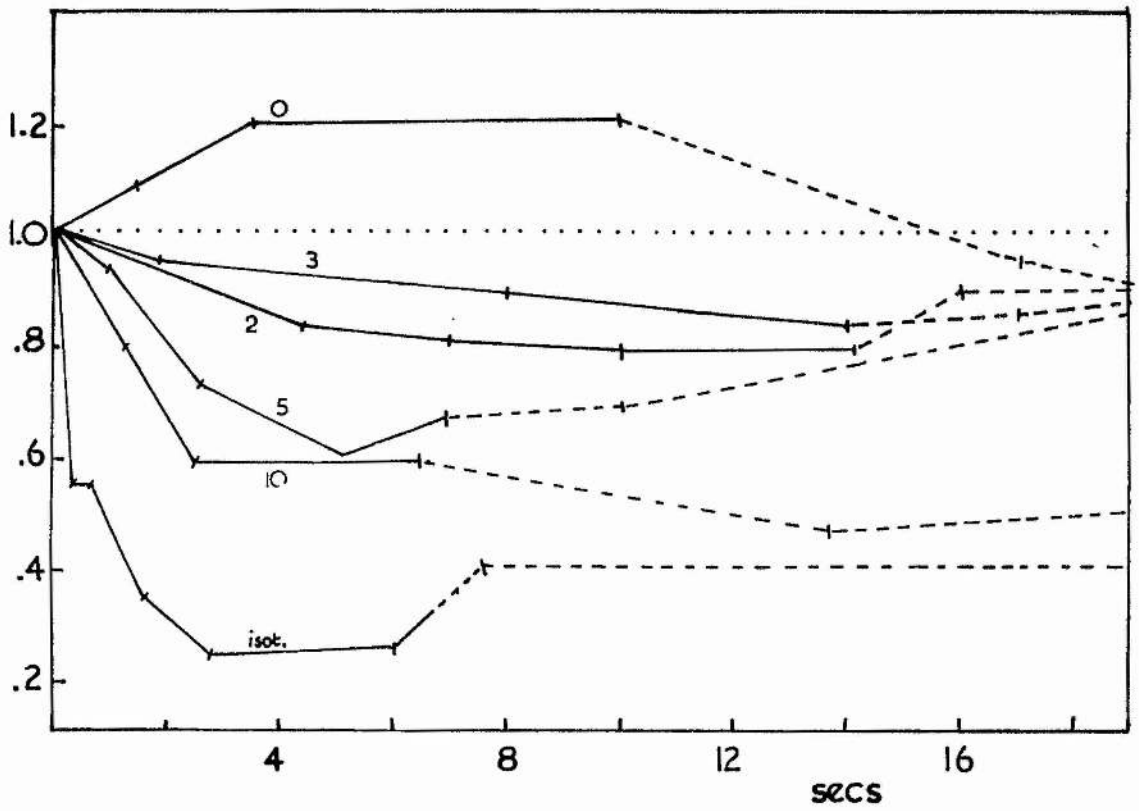
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except for isotonic KCl which was always tried last.

When the potassium solution was washed off, the fibre recovered with much the same time course as when the potassium was applied. That is to say, $L_{0.5}$ was usually the same at 'on' and 'off'. L_t was almost always slightly longer at 'off', especially with the higher concentrations of potassium. Recovery of E_m and R_m from isotonic KCl was always very prolonged, and usually washing had to be continued for more than five minutes. However, even when E_m and R_m had returned to their former value, the threshold for spike production, or, in the case of a repetitively firing axon, the repetitive response, usually remained away from its former level for some minutes.

It will be noticed in figs. 25 and 26 that the change in both E_m and R_m is less with three times K_o than with two times, and that the time taken for the effect to appear is longer. This was always seen with every axon tested.

Effect on spike height. Raising the potassium concentration around the axon to three or more times its normal level stopped any action potentials which were produced by the depolarising pulse applied to the axon: often, twice normal potassium had the same effect. The time taken for this disappearance varied with different concentrations, but was closely related to the latency for membrane resistance changes already described. The size of the spike was reduced by increased K_o, and increased in potassium-free solutions, as would be expected from the ionic theory of the action potential. In one or two cases, the effect on spike height

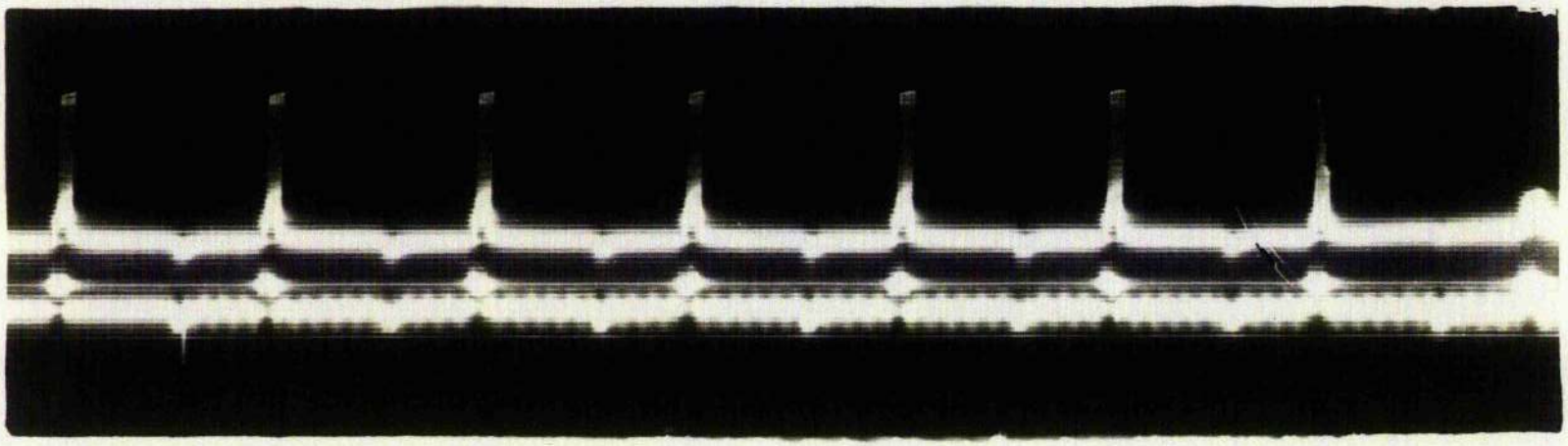

Fig. 27 An example of the effect of raising the external potassium concentration to twice its normal value. The solution was applied for the time indicated by the 10 cps sine wave on the lower trace. The spike size can be seen to diminish after 5.5 seconds.

was seen during the period of a repetitive response, and one such example is shown in fig. 27.

(b) Application of dinitrophenol. A study was made of the effects on the single fibre preparation of the metabolic inhibitor dinitrophenol (DNP). As is shown in fig. 28 the same results were obtained as had previously been described for single myelinated frog nerve fibres (Oomura and Hashimura, 1954a). The threshold stimulation for the production of one spike rises slowly when the DNP is applied in a concentration of 10^{-4}M. Solutions of lower concentration than this were not seen to have any effect in the time over which they were applied, but as they only affected the axon slowly, it may well be that had they been left on for longer periods, similar results would have been obtained.

(c) Acetylcholine, GABA and substance F. The effect of surrounding the axon with the above substances was looked at briefly. In accordance with the observations of others (e.g. Nachmansohn, 1959), acetylcholine (ACh) did not have any effect when applied to the fibre in concentrations of up to 10^{-2} gms/ml, probably for the same reasons as those given by Nachmansohn: that the ACh, being insoluble in lipids, does not enter the membrane. As he says, this observation is of no relevance to the arguments for and against the essential role of ACh in nervous transmission.

For the same reason possibly, substance F, the compound eluted from

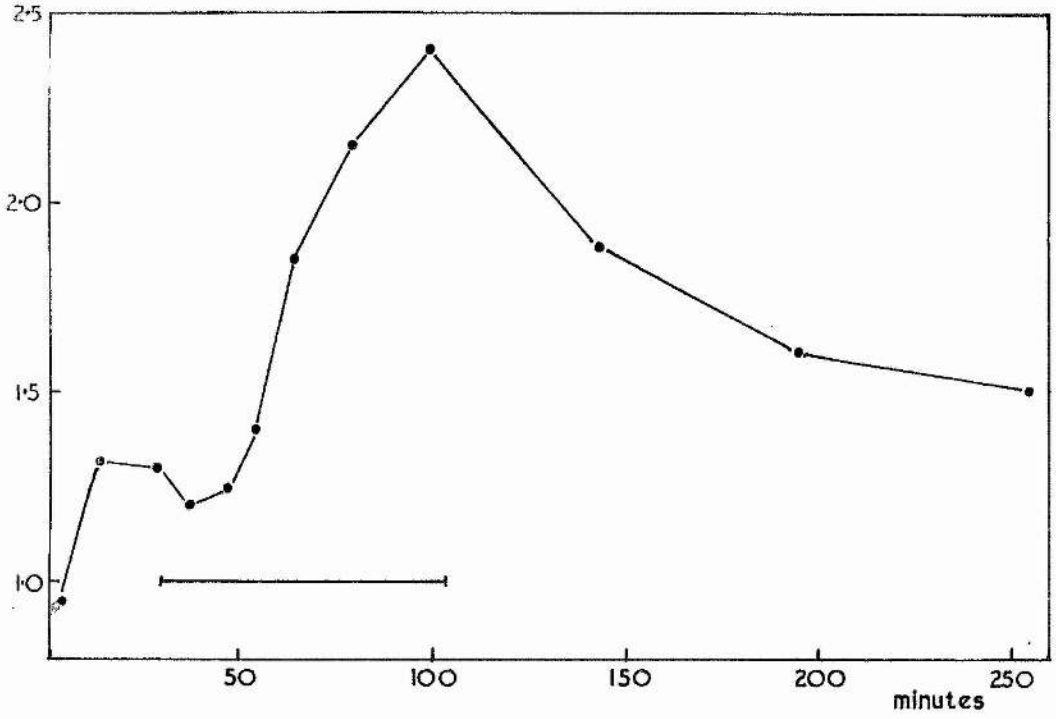

Fig 28 The effect on relative threshold for the production of a single spike in an isolated motor axon (ordinate) of dinitrophenol. The drug was applied for the time indicated by the horizontal line from 30 to 105 minutes.

chromatograms as described in the first part of this thesis, had no observed effects when applied to motor axons in concentrations which notably accelerated crab heart contractions.

Application of GABA too did not bring about any noticeable change in the fibre properties. The threshold for production of one spike remained constant, as did the number and frequency of the spikes in a repetitive response to a greater depolarisation.

D I S C U S S I O N

From the histological work described earlier it is probable that all the motor axons in the leg nerve of Carcinus are surrounded by the same type of sheath. Certainly when thin sections are examined by the light microscope, no differences can be detected which are not immediately attributable to an artefact such as fixation shrinkage, for example. The question was raised earlier as to whether the sheath had any connection with the type of repetitive response of the axon, which Wright and Coleman (1954) thought to be characteristic of a particular fibre, but from this result it would seem that such an idea is unlikely to be correct. Moreover, Chapman's work (1963) strongly suggests that it is not helpful to talk of one response being associated with one axon, for, as noted before, he saw one fibre exhibiting two or more types of response. Indeed the whole question of classification of responses which axons may give to a maintained depolarisation is one which, to my mind, is not very helpful. Chapman's sub-division of the three types of response was criticised by Hodgkin (private communication) on the grounds that the types Chapman saw were probably a product of his recording conditions - particularly the use of sucrose solution to render the axon outside the pipe non-conducting, which, by hyperpolarising the axon, probably caused its response to change. The fact that (i) Hodgkin admits that recording conditions may affect the response obtained;

(ii) Chapman saw fibres change from one type to another; and (iii) the class 3 fibres (those which did not repeat except with very large currents) appeared when dissection had been rough, or when the axon had been mounted under experimental conditions for some time, strongly suggests that all the classification may be capable of explanation in terms of factors which normally do not have any part to play in the fibre in vivo, but which, as yet, are unknown.

It is of interest to note here that it was extremely difficult to make an axon repeat when it had been mounted in a thin pipe. Three types of pipe were used: of diameters 1·0, 0·8 and 0·5 mm. With the largest of these, all axons except those which had been damaged in some way (manifested too by their short life) repeated according to the manner described by Hodgkin as Class 1: fibres which would respond over a large range of frequencies, depending on the size of the depolarisation. With the smaller sizes the appearance of anything except a single spike shortly after the beginning of the depolarising pulse was rare, and with the smallest sized pipe only seen once or twice altogether. A parallel may be drawn here with vertebrate physiology: frog myelinated fibres will fire repetitively to a steady depolarisation if three or four nodes of Ranvier are stimulated at the same time (Ōmura, private communication). Any number less than this will only give a single spike when depolarised, irrespective of the pulse size. It is as if in crabs too a large area of membrane is necessary before more than a single spike is obtained; a large area, that is, which may no longer be considered as uniform. The

more nearly uniform the membrane becomes, as when the stimulated area is reduced by use of a smaller pipe, or, in the case of the frog fibre, by reducing the number of nodes, the more difficult it is to make the fibre fire repetitively.

It is also by analogy with frog nerve fibres that the action of DNP on crab axons may be understood. It is known that the nerve fibre membrane is far from passive and that the term 'resting' potential is something of a misnomer: Oomura and Hashimura (1954a) showed that when DNP was applied to the nodes of these fibres the threshold rose. They also showed (1954b) that whereas pyruvate applied to the axon lowered threshold, this effect was abolished by DNP. The lowering of the threshold was accompanied by an increase in both oxygen consumption and membrane potential. ATP also serves to lower the threshold of a frog axon (Oomura and Hashimura, 1953; Caldwell and Keynes, 1960). The membrane is actively burning material (protein) to provide energy to maintain the electrochemical gradients across it, and the action of DNP, by uncoupling the oxidative phosphorylation mechanisms, quickly lets the membrane run down. As this happens, the membrane resistance also falls, and thus a given depolarising current pulse passing now through a smaller resistance will cause a smaller potential change. Hence that which previously had been sufficient to lower the membrane potential to the critical depolarisation level for spike production can no longer do so, and the observation is made that the threshold has risen. Strictly speaking, this may be considered to be incorrect, for the critical

depolarisation level may well remain unchanged even after the application of DNP. Certainly these experiments do not throw any light on that.

Nachmansohn's thesis of the generality of the action of ACh in nervous conduction prompted the application of that drug to the motor axons described above. As might have been expected, no effect was noted, although as Nachmansohn takes pains to point out (1959) this has no adverse effects on the theory. He has shown in fact by labelling ACh with N^{15} that when it is applied to squid axons, the ACh does not enter the axon to more than a trivial extent, whereas the uncharged tertiary amine eserine does enter the axon. By similar argument, any effect which either GABA or substance F might have on the axon could be prevented by permeability considerations, and a negative result is no indication that they are not having an effect. GABA was tried because of its connection with the inhibitory transmitter in Crustacea: it is known to have an effect on the nerve as well as the muscle membrane at the end-plate region. Substance F is also a constituent of peripheral nerve, and was tried partly because of its interest as a possible relative to the motor transmitter, though this by itself would scarcely provide the reason why it should have any effect on the motor axon membrane, and partly because a sensitive assay preparation was required for its estimation and this was tried as one possible.

The application of all these substances did not in any way help to elucidate the problem of the permeability of the sheath, for whereas a positive result - a change in the response of the axon to the stimulus -

might have told something, a negative result did not indicate either way whether the sheath had allowed the compounds in or not. Even the rise in threshold with DNP is not conclusive, though in view of the other work quoted it is highly suggestive that the inhibitor is entering the sheath. An alternative explanation, however, which is entirely acceptable in view of the ambiguity described above over the meaning of 'threshold', is that it is merely changing in some way the sheath cells. This could mean that the resistance of the sheath is lowered, and hence the term 'membrane resistance', which includes, of course, the sheath as well as the membrane proper, would he lowered. For this reason, by the arguments given above, the threshold would appear to rise.

The application of potassium, although a special case in many senses, is not so ambiguous, for although the changes in membrane resistance measured can be explained in terms of changes in the sheath cells, the effect on the action potential can only be understood as an effect of potassium on the axon membrane itself. As is described in the results section, there was a change in the type of response obtained with a given stimulus; both the frequency and size of the spikes of the repetitive response changed with applied potassium. It is clear, then, that the potassium is reaching the axon membrane and it is thus permissible to treat the changes in E_m and R_m as, at least in part, true effects of the potassium on the axon membrane. Needless to say, the manner in which measurements were made in these experiments does not distinguish the component parts of the changes: a change in the resting potential of the

sheath cells might give the same effect as a change in E_m proper. Villegas and Villegas (1963) have shown the sheath cells to possess resting potentials of their own in the squid; the same work also mentioned, however, that between cells in the sheath the potential was often seen, by their penetration technique, to drop to zero relative to the outside medium, which suggests that the region around the axon may be in continuity with that by a low-resistance pathway.

The effect which potassium has on the membrane is of interest in that it fits in with the pattern generally seen. In this, as in other preparations, the membrane potential, which over a large range of potassium concentrations behaves like a potassium electrode and follows the predictions of Nernst's equation, ceases to do so at low external concentrations - those values which normally surround the fibre. Curtis and Cole drew attention to this phenomenon in 1942 but had no explanation for it. They also pointed out that the action potential was extremely sensitive to changes in K_o which had no effect they could observe on the resting potential. The explanation given by Hodgkin (1951), that the permeability of the membrane to potassium ions decreases from the near-perfect condition at the higher concentrations, fits in with the observation made here as elsewhere that the membrane resistance changes by an amount smaller than that which would be expected if it obeyed a simple relationship with K_o.

When the time was measured for the potassium to have its effect the idea was to distinguish between the two possibilities: that the

potassium is reaching the axon through the active participation of the sheath cells which control the flow in and out of the extra-axonal space of water and ions, or that the passage to that site is dependent only upon simple diffusion considerations. If the former is the case, then access to the axon would not follow the simple diffusion curves which would apply if the latter were the truer picture. As the graph of the time for the effect to appear (latency) against concentration was constructed, it appeared at first as if there was a simple relationship between the logarithm of the external potassium concentration and the latency, for most of the points lay on a straight line. Closer examination revealed, however, that the latency when a saline containing three times the normal amount of potassium was applied was always more than if lower or higher values of K_o were considered. This is true whether the latency is considered as the time taken for the depolarisation of the membrane to change half-way towards its new value, $L_{0.5}$, as in fig. 23, or whether the time taken to reach the new value of E_m is considered (L_t).

The meaning of this is obscure. Certainly the present work is unable to clear the matter up, but it would appear that it is necessary to postulate two mechanisms which control the entry of potassium to the axon.

In the living creature, the blood concentration is liable to change by fairly large amounts as the crab is unable to regulate the ionic concentration of its blood when the surrounding medium varies, which

often does happen as it lives in shallow and brackish waters whose salinity may fall or rise considerably. It would be a great advantage to the animal, from the point of view of natural selection, if it possessed a mechanism by which changes in the blood ionic level were prevented from being reflected in the space around the axons - changes which otherwise might render them inexcitable and non-conducting. It would not be surprising therefore to find that access to the extra-axonal space depended on some biological factor and the sheath cells are admirably suited to perform this function, for they surround the fibre with an efficiency which earned the pia mater its name in vertebrates. The only known gap between the cells, Horridge and Chapman (1964) have filled with cement.

The peak value of $L_{0.5}$ and L_t when K_o equals 30 mM can only be explained by some active mechanism which depends on biological factors, and, as is clear from figs. 25 and 26, although the fibre takes longer to recover from 30 mM K_o than from 20 mM, the effect of this solution is less: it is as if the sheath is preventing the 30 mM solution from having its full effect on the fibre. It should be noted, though, that the particular case illustrated in those figures was more pronounced than in most tried, but in two cases the effect on E_m of 15 mM K_o was greater than a 20 mM solution.

This evidence, with the peak value of latency at three times external potassium, points to the sheath playing an active role in the regulation of the entry of potassium into the space around the axon. On the

other hand, the relationship between external potassium concentration and the depolarisation of the membrane at higher concentrations strongly suggests a purely passive entry of the ion. It could be that the sheath is able to play a part in the regulation of the extra-axonal concentrations when it is not presented with too great a task, but that when the normal condition is departed from by more than a little, the control mechanism breaks down. Such a state would be advantageous to the animal (to refer back to the argument on page 131) in which the external environment, though changeable, does not vary by anything like the extremes tried in these experiments. But such an idea is not particularly borne out by the present work; nor is it altogether satisfactory for, as has been noted before, with small changes in the external potassium level, the axon itself, by changes in its permeability to potassium, compensates for the change in concentration gradient, and maintains the resting potential level.

SUMMARY

1. Peripheral nerve from the crab walking leg was seen to take up nicotinic acid (NA) to an extent ten times greater than the surrounding tissues (p.37).
2. After a labelled dose of NA there was a gradient of radioactivity in the nerve trunk with the maximum peripherally (p.40).
3. Individual fibres from the trunk were seen to take the *NA up in equal amounts, irrespective of their function (p.47).
4. No such gradient was seen in a length of nerve immersed in a bath containing *NA (p.45).
5. A substance was extracted from the nerve after injection of the *NA which was radioactive (p.49), and which was seen to have reactions of a small peptide (p.57).
6. This substance was also seen to have an accelerating effect on the crab heart, but had no detected effect on the contractions of the closer muscle in the leg (p.68).

7. In the second part of the thesis, results show that there is no difference in the structure of the sheath around the different motor axons in the crab leg, as seen under the light microscope (p.108).
8. When the potassium concentration around an isolated axon was changed to three times its normal value, the resulting drop in the membrane potential and resistance was slower and smaller than for any other concentration tried (p.113).
9. The effect of applying to single fibres dinitrophenol and other compounds of pharmacological interest was studied; with the dinitrophenol the membrane resistance was seen to fall reversibly in the manner described for other preparations (p.120).

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