THE EFFECT OF STIMULATION ON THE OPACITY OF A CRUSTACEAN NERVE TRUNK AND ITS RELATION TO FIBRE DIAMETER

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It has been shown (Hill & Keynes, 1949) that the opacity to white light of a crustacean nerve trunk undergoes a reversible change when the nerve is stimulated. The cause of this phenomenon was at first obscure, though the inclination was to ascribe it to some change in the optical properties of the axoplasm or membrane of the fibres. The possibility that the effect was due to a change in fibre size was at first not considered, but this would now seem to be a sufficient explanation of what is observed, and the evidence pointing in this direction is set out here. Further support for this view has been provided by showing that the single large fibre of the cuttlefish does actually undergo a change in diameter when it is stimulated (Hill, 1950).

METHOD

Apparatus. The apparatus for holding the nerve was designed to allow recording of the opacity change brought about by an alteration in the composition of the electrolyte solution surrounding the nerve (Figs. 1, 2). The nerve could also be stimulated and the action potentials recorded. In this way the opacity change caused by stimulation could be compared with the change due to a known swelling or shrinking of the fibres, brought about by altering the osmotic pressure of the surrounding medium.

The photocell arrangement is a differential one, the balancing being done by controlling the light entering one cell. The recording circuit is shown in Fig. 3. The nerve chamber was constructed by milling and drilling out a block of Perspex, and polishing the optical surfaces. The solutions to be passed through the nerve chamber were contained in glass reservoirs, held at such a height as to give a head of pressure of about 2 ft. The substitution of one fluid for another was made by turning a tap, the second fluid then replacing the first in a matter of minutes. The flow of experimental solution round the nerve was controlled at about 0·3 c.c./min. by a capillary resistance in the outflow tube, and was sufficiently rapid to allow the medium to be completely changed in a few minutes, but slow enough to avoid disturbing the nerve by turbulence: in this way the opacity could be recorded while the change of medium was in progress. The solutions were previously filtered through a Berkfeld filter, to remove minute particles which might lead to fluctuations in the transmitted light. The solutions were oxygenated. Recording was done by hand from the galvanometer, using a stop-watch for timing. The nerve was stimulated for 10 or 15 sec. at 50 or 100 impulses per sec. with a multi-vibrator unit: the action potentials were observed on a Cossor oscilloscope (model 339 A).

It was found necessary to pay particular attention to two features in the design of the apparatus: in the first place, the light beams must not traverse long air paths, for variations in the refraction of the air due to the heat of the lamp may lead to considerable fluctuations in the reading. In the present apparatus the rapid mixing of the air in the lamp housing, due to the constant stream of cooling air, prevents any such fluctuations which might arise from the movement of hot air close to the lamp; secondly, the different components of the system must have short, very rigid connexions
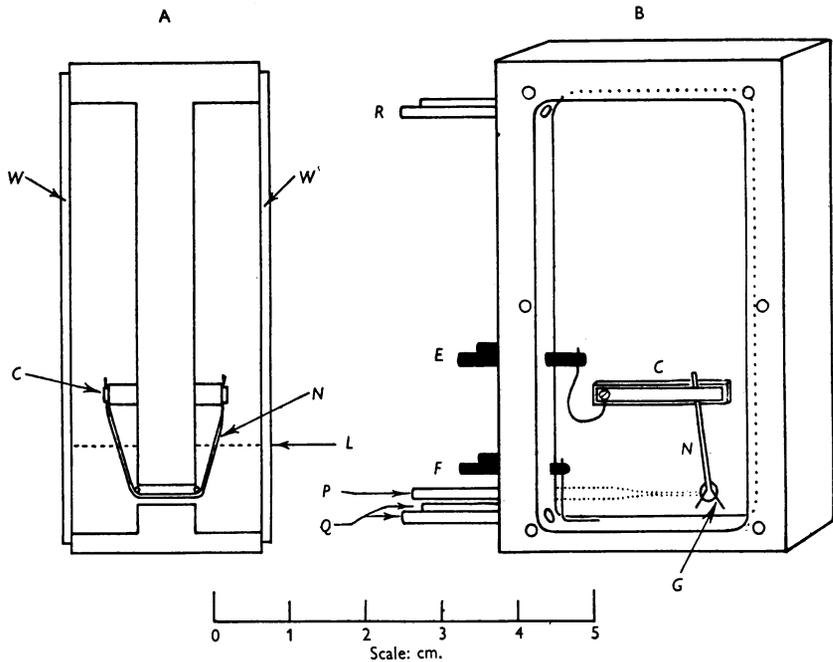

Fig. 1. The nerve chamber. A, vertical section through 'nerve tube'. *N*, nerve; *L*, level of sea water; *W*, window screwed on to side of nerve chamber after nerve is fixed in position (a rubber washer gives a watertight seal); *C*, clamp for holding nerve (also acts as electrode). B, side-view of nerve chamber. *N*, nerve; *C*, clamp for holding nerve (this is made of brass and allows electrical connexion with terminal *E*, for stimulation or recording of action potentials); *F*, terminals for 'earth' connexion to the sea water in the lower part of the chamber, through a chlorided silver wire; *P*, inflow tube: the continuation of this tube to the 'nerve-tube' through the centre of the block of Perspex is indicated by the dotted lines. The nerve is held in the centre of the 'nerve-tube' by glass loops, *G*, which are fixed to the Perspex with cement. The bathing solution leaves the chamber through the out-flow tubes, *Q*. The tubes, *R*, at the top of the nerve chamber, fitted with rubber tubes and clips (not shown), are used for controlling the level of the solution in the chamber. In the side view the Perspex windows are removed, but the six screw holes for fastening are shown.

with one another, to minimize disturbances due to external mechanical movements, such as might be caused by a person walking across the room. The previous apparatus (Hill & Keynes, 1949) had its components mounted on different stands, and although these stands were massive and the table a very stout one, the slightest pressure of a finger on the table would give a very large movement of the galvanometer spot when working at high amplification. The present system was almost entirely free from this failing, being constructed in a very robust way, but to ensure even more complete freedom from disturbance it was mounted on a 3 ft. square slab of 1 in. slate, which itself rested on sorbo-rubber pads on the bench.

The shutter controlling the light entering the balancing photocell had to be capable of very fine adjustment. The system shown diagrammatically in Fig. 2 was built up from a reduction-gear unit, taken from an ex-R.A.F. bomb-sight. One turn of the controlling wheel moves the shutter $17\,\mu$., and the minimum adjustment is about $1\,\mu$. When working at the highest amplification this balancing adjustment has to be used to its finest limit when centring the galvanometer spot.
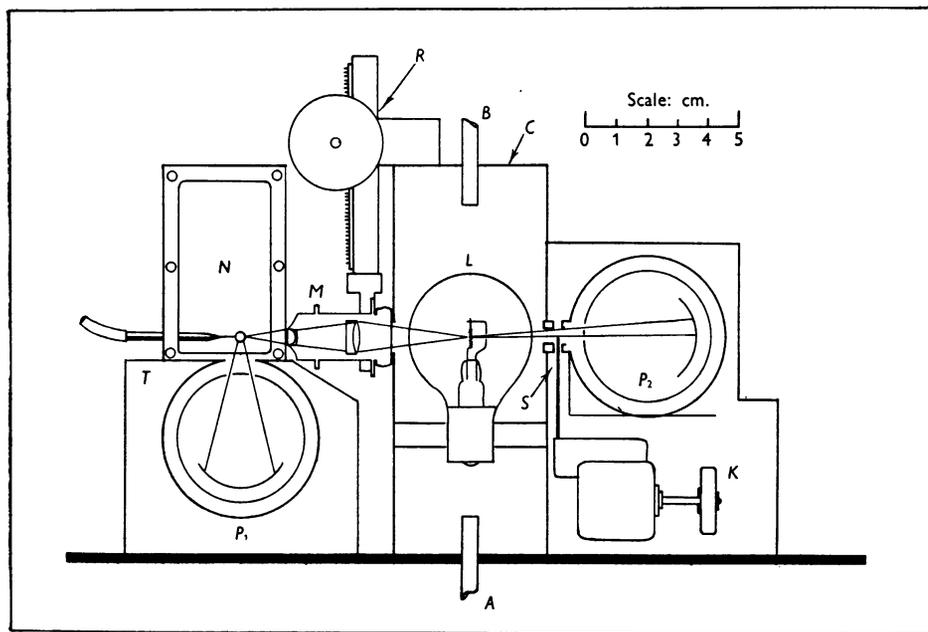

Fig. 2. General view of apparatus. N, nerve chamber; it is clamped down rigidly on the platform, T, by a device which is not shown in the diagram. The source of light, L, is a 36 W. 6 V. headlamp bulb in a metal housing, C, and is cooled by a stream of air entering by tube A, leaving by tube B. (The lamp filament is actually horizontal, and not vertical as indicated in the diagram.) A 2/3 in. microscope objective, M, focuses an image of the filament (about 0·5 mm. wide) on the nerve. The exact positioning of the filament image on the centre of the nerve is done by raising and lowering the objective by the rack and pinion, R; the rack is then firmly clamped by a screw. The light scattered by the nerve enters the photocell, P_1. The light entering the balancing photocell, P_2, is controlled by a shutter, S, mounted on a reduction gear unit which is controlled by the knob, K. One turn of this knob moves the shutter through a distance of $17\,\mu$., and the minimum adjustment is about $1\,\mu$. The recording instrument measures the *difference* in the photocurrents from P_1 and P_2, and initially this difference is made zero by the balancing control. The photocells are 'Cintel' V.A. 17 (vacuum).

It was found that the stability was not so good with the perfusion fluid running as it was with the latter turned off. It is not certain whether this was due to particles being swept through the beam of light, or to movement of the nerve caused by the stream.

Preparation of nerve. The limb nerve of the common spider-crab, *Maia squinado*, was used. It was shown that the effects with this nerve are essentially similar to those obtained with the nerve of *Carcinus maenas*, used in the earlier experiments by Hill & Keynes (1949). The nerve was prepared from the proximal link of one of the walking legs by the 'pulling out' method of Furusawa (1929). Cowan (1934) disapproved of this method, on the grounds that the resting potential of a nerve

isolated in this way was considerably lower than the potential recorded from a nerve prepared by cutting open the leg after the manner of Levin (1927). Although this would seem to indicate that the 'pulled out' nerve suffers some damage in the process, it was not found in the present experiments to differ from the nerve prepared by dissection, and survival for many hours testified to its good condition.

A compact bundle of fibres, generally about one-fifth part of the whole nerve, was freed from the rest, then tied at both ends, and any loose strands of fibres carefully removed. This bundle of fibres was then set up in the chamber, and oxygenated filtered solution was run through the chamber
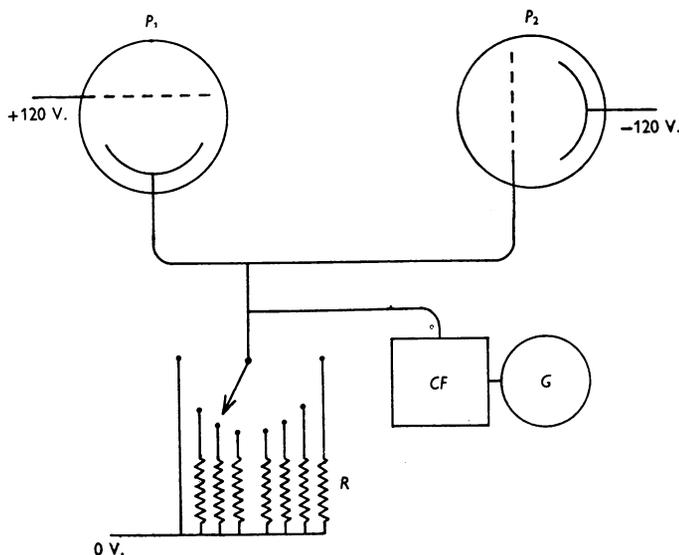

Fig. 3. The photocurrent amplifier circuit. The *difference* in the photocurrents from the recording cell, P_1, and the balancing cell, P_2, passes through one of the resistances, R, selected by a switch. The voltage developed across the resistance is measured by a cathode-follower unit, CF, which is similar to that described by Hill (1948), the final recording instrument being a Unicam 'Spot' galvanometer, G, resistance 1070 Ω., sensitivity 2 μA. for full-scale deflexion, and period 2·5 sec. The resistances, R, are 0·1, 1·0, 3·3, 10, 30, 60, 200 MΩ. (approximate values), and the ratios of amplification at each resistance to the amplification at 0·1 MΩ. are 1·0, 9·4, 33, 132, 409, 939, 2820 (measured values). The photocells are 'Cintel' V.A. 17 (vacuum).

continuously for 3–4 hr. to allow time for the nerve to attain equilibrium with its surroundings. The solutions used for bathing the nerve consisted either of sea water or of an artificially prepared *Carcinus* Ringer solution. In the experiments designed to investigate the effect of a change in external medium, the alternative solution was one of the above 'basic' solutions modified, either by dilution or by the addition of certain salts, as described later.

The composition of the sea water (calculated from Harvey's (1945) data) as regards major constituents, is as follows:

Na^+	471 mM./l.	Cl^-	549 mM./l.
K^+	9·9	SO_4^-	28·2
Ca^{++}	10·2	HCO_3^-	2·3
Mg^{++}	53·6		

Total ionic molarity, 1124·2 mM./l. Small variations in total molarity (or 'salinity') are to be expected from sample to sample.

The *Carcinus* Ringer solution was made according to Pantin's (1946) formula, and is based on Webb's (1940) analysis of *Carcinus* blood. Its ionic composition is as follows:

Na$^+$	493·6 mM./l.	Cl$^-$	535·2 mM./l.
K$^+$	11·3	SO$_4^-$	15·4
Ca^{++}	12·3	HCO$_3^-$	0·2
Mg^{++}	18·3		

Total ionic molarity, 1086·3 mM./l.

Hill & Keynes (1949), in the earlier experiments on the opacity of *Carcinus* nerve, used exclusively *Carcinus* Ringer solution. In the present series of experiments with *Maia*, sea water appeared to be equally effective in maintaining the excitability of the nerve, but it was found that there was a very conspicuous difference in the form of the opacity change following stimulation, as between nerves bathed in sea water and nerves bathed in *Carcinus* Ringer solution. This difference is described below. Under all conditions so far tested *Carcinus* and *Maia* nerves behave in the same way.

The level of the solution in the chamber was maintained above the electrodes throughout the experiment if stimulation was not required; otherwise it was lowered about an hour before the first run to such a level as permitted stimulation and recording of the response. The lamp and cooling air were also turned on an hour before the first run, so that the 'drift' of the galvanometer spot was generally quite slow by the time the photocells were finally balanced.

Alternative apparatus for rapid automatic recording. A few records were made using an entirely different apparatus, which was primarily designed for measuring the change in diameter of a single *Sepia* fibre following stimulation (Hill, 1950). A small strand of *Maia* nerve, about 200 μ. across, was set up in this apparatus. The slit in the focal plane of the eyepiece was enlarged to take in view the greater part of the strand, and the change in the intensity of the scattered light due to stimulation could be recorded. The high amplification obtained with the 'multiplier' phototube enabled records to be made on paper with an ink-writing milliammeter, run from a single-stage amplifier. Automatic recording of this sort was useful for following the more rapid early phases of the opacity response.

RESULTS

The effect of a change in the osmotic pressure of the external medium. The nerve is set up in the way described above, and one of the two normal solutions (i.e. sea water, or *Carcinus* Ringer solution) is passed through the chamber for at least an hour, until equilibration is complete. The second reservoir is filled with 'normal' solution to which has been added a known quantity of sodium chloride. The opacity of the nerve changes when this new solution is run in (Fig. 4). It is seen that an increase in 'strength' of the medium gives rise to an increase in the opacity. One result of the addition of solute to the external medium is to increase its refractive index, and the difference between the refractive indices of the medium and the nerve membrane is reduced. This, by itself, must result in a *decrease* in the scattering. In actual fact, the scattering *increases*. Another substance, magnesium sulphate, which is presumably also to be regarded as inert in respect of the resting nerve membrane, gives a similar result. It therefore seems reasonable to conclude that the increase in scattering is associated with, or accompanies, a decrease in fibre size, brought about by osmotic shrinkage. (The words: 'associated with, or accompanies' are intentionally used in this non-committal way, in order to avoid stating definitely that the effect is *due* to a change in fibre size, for there is no rigid proof that this is so.)

The refractive index is not, however, a negligible factor. If the added solute has a high molecular weight, the change in refractive index becomes relatively more important. In the case of sucrose the two opposing tendencies become nearly equal. The effect of an addition of sucrose has on one occasion been found to be as great as 40% of that due to an equi-osmotic increment of sodium chloride, but generally it is less, and on two occasions has been found to be almost zero. With magnesium sulphate there is less variability, but it is always found that the 'refractive index factor' reduces the 'osmotic effect' appreciably.
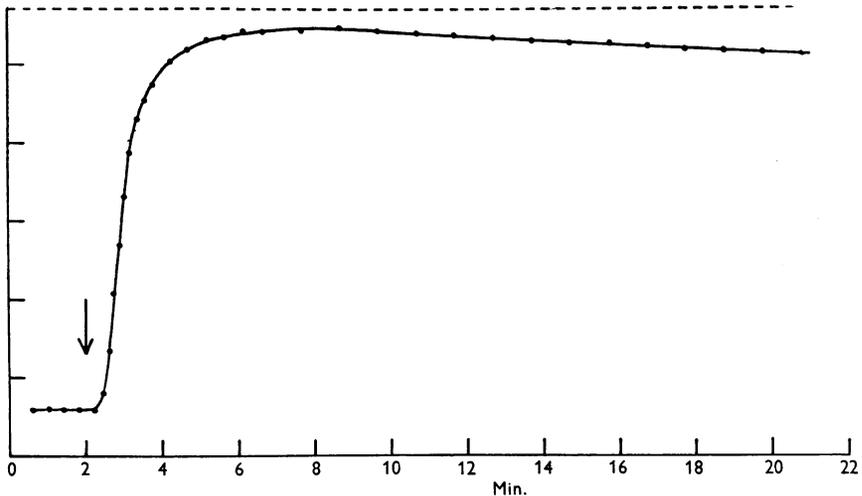

Fig. 4. The change in the intensity of light scattered by the nerve as the result of the addition of 26·8 mм. NaCl/l. to the medium. Normal medium, sea water. The switch from one reservoir to the other was made at a time indicated by the arrow.

As it is proposed to use the 'sodium chloride' standard for relating changes of scattering with changes in fibre diameter, it is necessary to estimate the extent to which refractive index is an important factor in this case. The molecular weight of sodium chloride is 58, of sucrose, 342. Taking the relative osmotic coefficients as 0·8:1·0, it follows that 58 g. of NaCl is equivalent, osmotically, to 615 g. of sucrose. The change of refractive index of an aqueous solution is nearly proportional to the weight of solute added, and the effects of sodium chloride and sucrose are practically the same, weight for weight. The 'refractive index factor' and the 'osmotic factor' are about equal for sucrose, so it follows that the effect with sodium chloride is reduced about 10% by the 'refractive index factor'. It will be seen later that the calculation of the apparent change in fibre diameter resulting from stimulation has necessarily to be rather inaccurate, and the above rather rough estimate of the importance of the refractive index is sufficiently precise for the purpose.

The kinetics of the penetration of water across the nerve membrane cannot be deduced from the form of the curve (such as that shown in Fig. 4), because the actual time course of the penetration of water is largely determined by the rate at which the new medium replaces the old between the fibres: the second fluid does not immediately replace the first in the nerve tube of the chamber (it is seen in Fig. 4 that there is a delay of about 30 sec. before the response even commences), and there is further delay due to the slowness of diffusion between the fibres. The rate of flow through the nerve chamber is purposely kept low to avoid movement of the nerve, and if the rate of flow is increased the attainment of the final equilibrium is hastened, for then the more rapid turbulent flow speeds up the complete exchange of fluid in the nerve tube.

The relation between scattering and osmotic pressure is approximately linear. This was shown by taking two solutions, one of which had an osmotic pressure 20% above, and the other 20% below, normal. The readings with these solutions, with normal solution as reference, were as follows:

Readings with normal solution as reference

Osmotic pressure 20% low		Osmotic pressure 20% high	
(2)	−238	(1)	+400
(4)	−338	(3)	+355
(6)	−354	(5)	+329
(8)	−349	(7)	+316
(10)	−311	(9)	+283

The runs were made in the order indicated by the numbers in brackets. At the start of the series, which took 3 hr. to complete, the effect of shrinkage exceeded the effect of swelling, but towards the end the position was reversed.

Solutes capable of penetrating the nerve membrane. If the solute added to the normal medium is capable of penetrating the nerve membrane the initial increase in scattering is followed by a regression as the penetration proceeds. This method has been used for studying the penetration of potassium chloride (Fig. 5) and urea (Fig. 6). The response to potassium chloride is dependent on the excess concentration. With a small addition, e.g. 9 mm./l., which about doubles the normal concentration, the regression to the base-line is more or less rapid, and considerable variability is found in the rate at which penetration occurs (Fig. 5). If a large excess of potassium is added, e.g. 50 mm./l., the initial 'osmotic' effect is very soon followed by a *decrease* in scattering, which is too large to be accounted for by the 'refractive index factor', and is probably to be attributed to the 'stimulating' action of potassium chloride in such concentration, akin to that produced by normal excitation (discussed below). The situation is complicated, because even in the absence of any 'stimulation', and basing a theoretical calculation simply on the assumption that a Donnan equilibrium governs the distribution of ions, the net volume change following the addition of potassium chloride cannot be estimated with any accuracy. This point is discussed later.
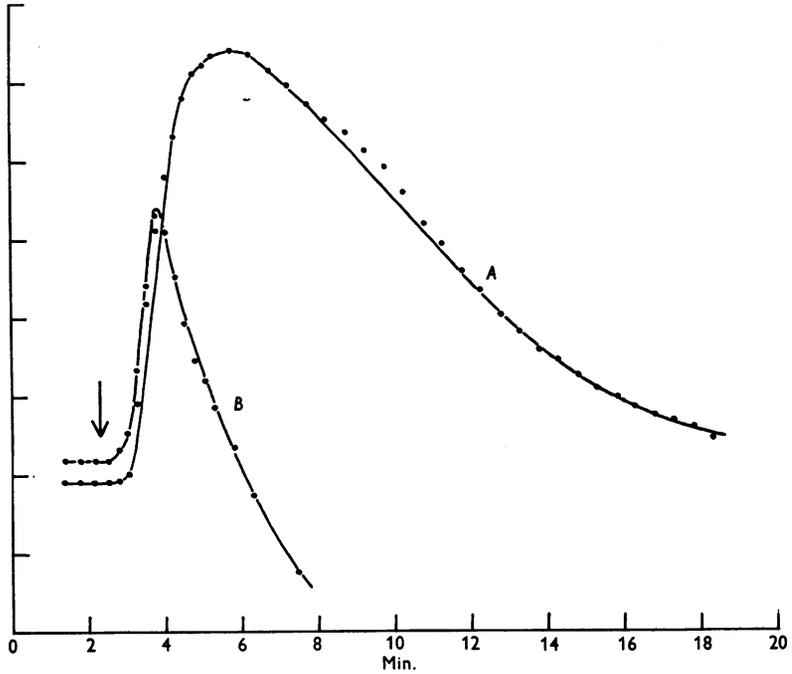
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Fig. 5. The change in scattering following the addition of potassium chloride to the medium.
Normal medium, sea water. *A*, addition of 9·1 mM. KCl/l.; *B*, addition of 53·6 mM. KCl/l.
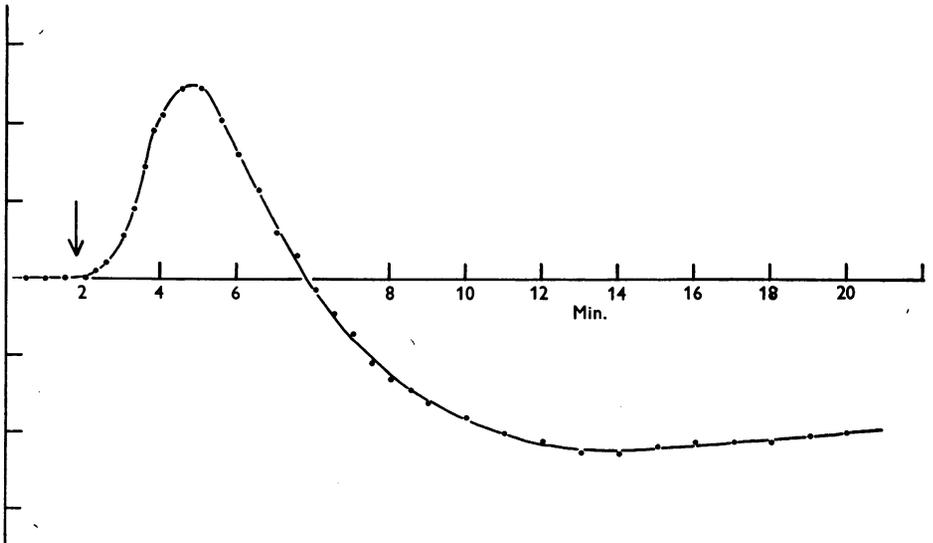The switch from one reservoir to the other was made at a time indicated by the arrow.

Fig. 6. The change in scattering following the addition of 66·6 mM. urea/l. to the medium (norma
medium, sea water). The switch from one reservoir to the other was made at a time indicated
by the arrow.

In the case of urea (Fig. 6), the return to a final level on the reverse side of the base-line can be attributed entirely to the 'refractive index factor', and does not indicate a concentration of urea by the nerve fibre.

The kinetics of penetration are to be deduced from the curves only when the substance in question penetrates very slowly. When the penetration is rapid, e.g. with urea, the time course is largely determined by the rate at which the new medium penetrates into the inter-fibre spaces.
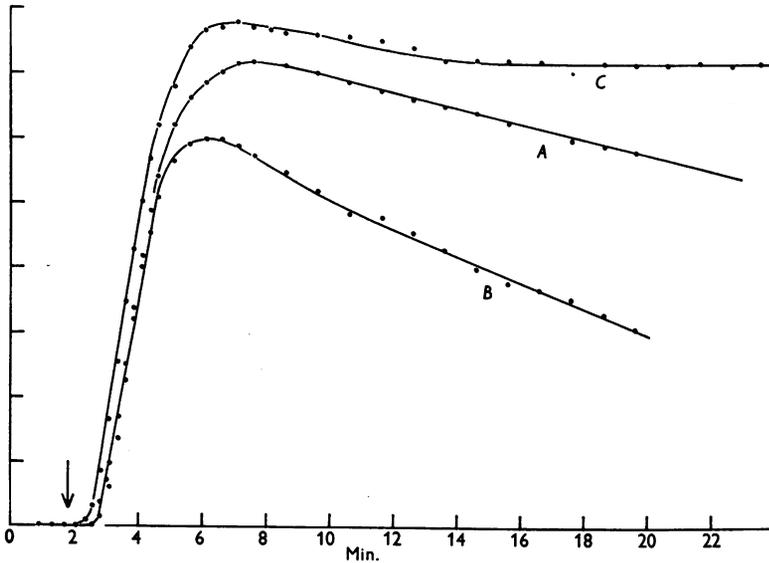

Fig. 7. The effect on the permeability to sodium chloride of altering the calcium concentration of the bathing solution. In each case 27·4 mM. NaCl/l. was added to the reference solution. *A*, reference solution was *Carcinus* Ringer solution with slightly subnormal calcium concentration (9·9 mM. Ca; normal is 12·3 mM. Ca). *B*, reference solution was Ca-free *Carcinus* Ringer solution. *C*, reference solution was *Carcinus* Ringer solution with additional calcium (total 52·7 mM. Ca). Runs made in the order *A, B, C*. Run *A* was taken 1 hr. after setting up the nerve in the chamber; *B*, after 3 hr. perfusion with Ca-free solution; *C*, after 2 hr. perfusion with the Ca-rich solution. The switch from one reservoir to the other was made at a time indicated by the arrow.

The effect of calcium on the permeability to sodium chloride. One instance of slow penetration, that of sodium chloride into the resting nerve, has been studied by this method. It will be seen from Fig. 4 that the osmotic shrinkage, following the addition of sodium chloride to the medium, shows a slow decline; this indicates, presumably, that sodium chloride is entering the fibre. This slow regression was always found to be present, but its rate was rather variable.

The effect of changing the calcium content of the medium is illustrated in Fig. 7. In a stream of calcium-free solution, three to four hours' soaking greatly increases the permeability. In this particular case (Fig. 7), the regression

with normal calcium concentration (10 mM./l.) was rather faster than it generally is. An increase in calcium well above the normal value brings about an almost complete stoppage of the regression. The slight initial decline from the peak in the high-calcium curve has been found in every experiment.
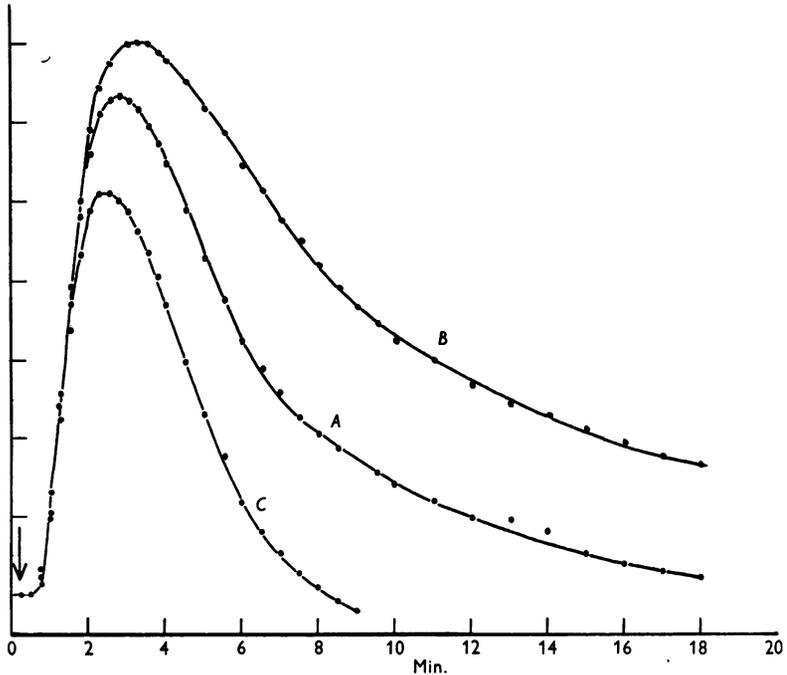

Fig. 8. The effect on the permeability to potassium chloride of altering the calcium concentration of the bathing solution. In each case 20 mM. KCl/l. was added to the reference solution. *A*, reference solution was normal sea water (10·2 mM. Ca); *B*, reference solution was sea water with additional calcium (total 65·2 mM.); *C*, reference solution was normal sea water. Runs made in the order *A*, *B*, *C*. Run *A* was made 1 hr. after setting up the nerve in the chamber; *B*, after 2½ hr. perfusion with Ca-rich solution; *C*, after further 2½ hr. perfusion with normal sea water. The switch from one reservoir to the other was made at a time indicated by the arrow.

The effect of calcium on the permeability to potassium chloride. The situation with potassium chloride (Fig. 8) is complicated by what has been referred to above as its 'stimulating' action. This gives rise to a swelling which, particularly at high concentrations, soon masks the osmotic effect (Fig. 5). A sign of this tendency is seen in curve *C* of Fig. 8, even though the potassium concentration was only three times its normal value. In calcium-free solution the initial shrinkage may be absent altogether. This 'de-sensitization' of the nerve by calcium makes the interpretation of the curves in Fig. 8 somewhat doubtful, although the conclusion that calcium decreases the passive permeability to potassium chloride would seem to be a reasonable one.

The effect of stimulation

The time course of the change in sea water

The early phase. When the nerve is soaked in sea water the opacity change due to stimulation at 50/sec. for 15 sec. is of the form shown in Fig. 9. The main response is a *decrease* in opacity. The maximum response does not coincide with the end of the period of stimulation, but it is delayed. With automatic recording on moving paper this initial stage is more clearly displayed (Fig. 10). Besides this 'overshoot', another feature which may be connected with it is also seen in Fig. 10: this is a lag in starting up at the beginning. The lag is partly due to the slowness of the recording instrument, but this can be allowed for (Fig. 10).
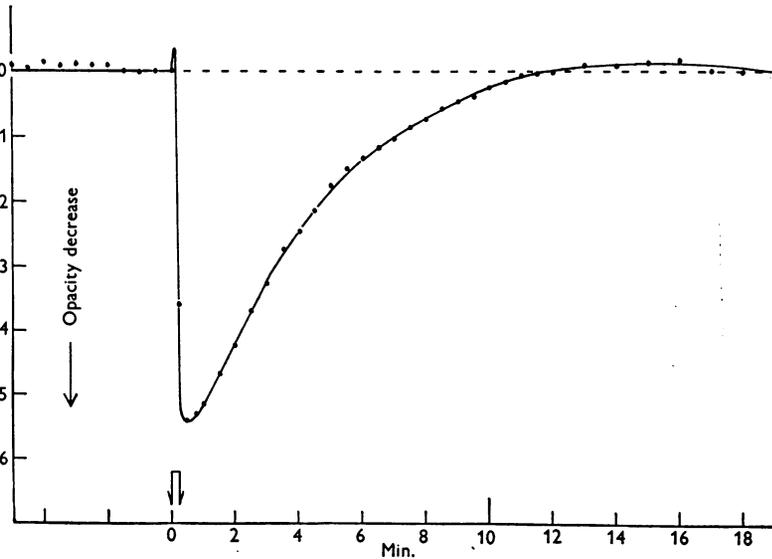

Fig. 9. The response to stimulation at 50/sec. for 15 sec. The stimulation period is indicated by arrows. *Maia* nerve in normal oxygenated sea water. Room temperature 18·5° C. Series resistance 60 MΩ. The ordinate scale is in arbitrary units. Base-line corrected for 'drift'. Recording by galvanometer; readings at 0·25, 0·5 or 1·0 min. intervals. An early positive opacity change is shown, but it could not be recorded accurately.

The recovery phase. Fig. 9 shows a prolonged recovery following the initial peak. In the earlier paper, Hill & Keynes (1949) were unable to follow this phase with any regularity, but it now appears that it follows about the same time course as the recovery heat production of *Maia* nerve. A. V. Hill (1929, p. 165) gives the heat production in 30 sec. intervals following a 6 sec. stimulus of *Maia* nerve at 16·8° C. Ignoring the heat production in the first 30 sec. interval (to avoid confusion with the 'initial heat'), half of the remaining heat is produced by 5·5 min. after the beginning of the stimulus. By comparison, the curve of Fig. 9 has returned from the peak half-way to the base-line in

3·7 min. (18·5° C.). The run selected for Fig. 9 is not entirely representative of the sixteen which have been recorded under similar conditions on other nerves, because in most instances the curve either did not completely return to the base-line, or it overshot, and there was a tendency for the drift rate of the base-line to undergo a change. However, the curves in their general form were not greatly different from the one in Fig. 9, but the 'half times' of recovery are not sufficiently well defined to make it worth pursuing the comparison with heat production. The recovery phase has also been recorded with solution flowing
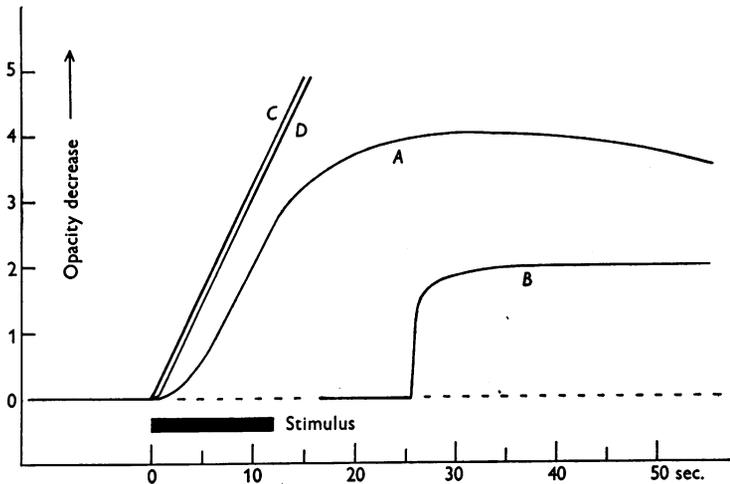

Fig. 10. Curve *A*, record of the early stages of the response to 12 sec. stimulation at 50/sec. of a nerve soaked in sea water. Room temperature 15·0° C. The curve is a tracing of the record made on moving paper with the ink-writing milliammeter. The positive opacity change was entirely absent. The 'instrumental' lag is calculated with the aid of the control curve, *B*, which is the response, with reduced gain on the amplifier, when the light is suddenly switched off. The line, *C*, represents an imaginary opacity change building up at a constant rate from time zero, and recorded without lag. The instrumental lag would cause a delay in recording, and the actual response would be as indicated by *D*. It is seen that there is a further, non-instrumental, lag in the response of the nerve to stimulation.

continuously through the chamber. This was done because it was thought possible that the temperature in the nerve tube might be considerably elevated owing to the intense light, and that the recovery might consequently be speeded up. However, the curve was not perceptibly affected.

Comparison of the effect of stimulation with a change in external osmotic pressure. In the argument to correlate the effect of stimulation with changes in fibre size, there seems to be no possibility of deriving a theoretical relation, on the basis of geometrical optics, between the intensity of scattered light and the fibre size. In any case it may not necessarily be true that the factor primarily determining the opacity is the size *per se*, for another possible factor is the degree of dilution of the internal colloidal particles. If this were the operative factor, there is even

less hope of calculating the relation between size and opacity, because the required relation between opacity and dilution is not known. However, without prejudging the real nature of the effect, one may experimentally compare the change due to activity with the effect of an approximately known change in fibre size brought about by an alteration in external osmotic pressure. As a measure of the 'activity' effect one takes the peak value of the response (Fig. 9), and compares this effect with a known dilution of the sea water surrounding the nerve. This was done for eight different nerves, each stimulated at 50 impulses/sec. for 10 sec. (500 impulses). Allowance must be made for the 'refractive index factor', taken as for sodium chloride. The results were as follows:

Nerve	Equivalent dilution (%)	$\Delta I/I$
1	0·62	—
2	0·58	—
3	0·28	—
4	0·37	1:164
5	0·63	1:92
6	0·52	1:90
7	0·45	1:109
8	0·73	1:79
Mean	0·52	
Mean*	0·47	

* With allowance for 'refractive index factor'.

The 'activity effect' was taken in each case as the mean of three, and the 'dilution effect' as the mean of four observations. The dilute sea water 'standard' was 90 % sea water + 10 % distilled water. (It was convenient to use a 'dilution effect' which was greater than the 'activity effect', and the gain of the amplifier was altered between the two sets of readings, but owing to there being a nearly linear relation between intensity of scattered light and dilution, this did not affect the result.) In the last five runs the relative change in the light scattered by the nerve was also measured. The symbol I represents the intensity of light scattered by the unstimulated nerve, and ΔI the peak change due to 500 impulses, and the ratios $\Delta I/I$ for these five runs are included in the above table. The measurement of the intensity I involved the removal of the nerve from the chamber, in order to make allowance for the light scattered into the photocell by the chamber itself.

The ratio $\Delta I/I$ can be slightly increased by using blue light, obtained by placing a colour filter in the light beam. The advantage gained by increasing the ratio was, however, more than offset by having to work with a greatly reduced light intensity.

The time course of the change in Carcinus *Ringer solution or in diluted sea water*

The records made by Hill & Keynes (1949) showed a large initial *increase* in opacity following stimulation. Subsequently, when further experiments on similar lines were done with the modified apparatus described here, it was

found that this early phase of *increase* was either relatively small (as in Fig. 9) or was often completely missing (as in Fig. 10), the response being simply a decrease in opacity. *Carcinus* and *Maia* nerves behaved similarly in this respect. This difference was found to be due to the fact that in the earlier experiments the nerve was soaked in *Carcinus* Ringer solution, while in the later ones sea water was used (the nerve survived equally well in either solution). The transition from one type of response to the other could be made simply by changing
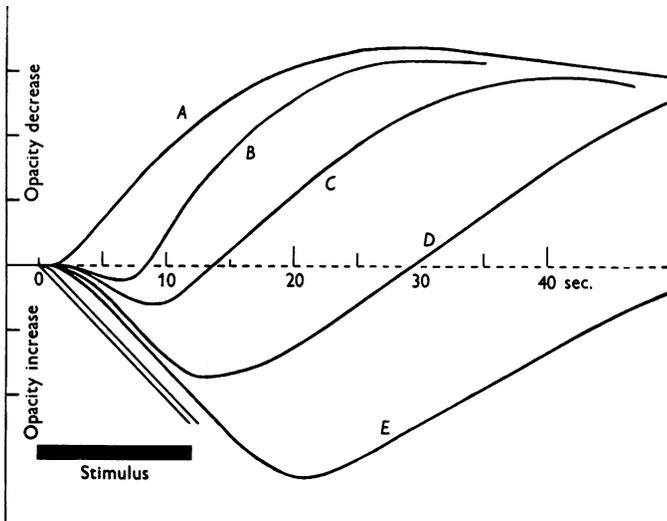

Fig. 11. The response to stimulation in dilute sea water, or in *Carcinus* Ringer solution. The curves are not tracings of actual records, because a series as complete as this has not been recorded on any one nerve. Curve *E* approximately represents the response to 12 sec. stimulation at 50/sec. of a nerve transferred to 80 % sea water 10 min. earlier. The two lines to the left of curve *E* have the same significance as those shown in Fig. 10, and illustrate the lag in commencement of the response. Curve *D* is typical of records made with 90–95 % sea water or in *Carcinus* Ringer solution. Curves *B* and *C* are types which may be observed with the nerve in normal sea water; or if the normal response does not commence with a positive phase (curve *A*), some dilution is required to bring out the forms *B* or *C*. At any given dilution there is considerable variability, as between different nerves, in the relative proportions of the positive and negative phases, and in the time at which the curve crosses the base-line.

the solution around the nerve: after about 5 min. pause the nerve could be stimulated and the transition found to be complete. At first it was thought that this must depend on the difference in the relative proportions of the various ions in the two solutions, but by making suitable tests this was found not to be the explanation. It turned out that a difference in total concentration (or osmotic pressure) was alone responsible. An initial positive phase could be made to appear simply by diluting the sea water surrounding the nerve, and the relative size of this phase, and the time at which it reversed were dependent on the degree of dilution (Fig. 11). To confirm that the effects with sea water

and *Carcinus* Ringer solution differ for this same reason, the osmotic pressures were compared by measuring the change in opacity of the nerve when one solution was replaced by the other, and by standardizing this difference against a known dilution of sea water. It was found that the osmotic pressure of *Carcinus* Ringer solution is equal to that of 95·1 % sea water + 4·9 % distilled water. A 5 % dilution of the sea water bathing the nerve caused the appearance of the positive phase, and it was then relatively about as large as it is with the same nerve in *Carcinus* Ringer solution.

The effect of *increasing* the osmotic pressure of the solution by 10 % above the normal value for sea water was a reduction in the size of the negative opacity phase. This is the reverse of what might be expected. The test was made by comparing the peak values of the negative opacity changes following 50/sec. stimulation for 10 sec. in normal sea water and in sea water enriched with 3·0 g./l. sodium chloride, giving a difference in osmotic pressure of about 10 %. Observations were taken in the following order:

> 110 % sea water, three runs, peak values 19, 20, 21 units;
> 100 % sea water, two runs, peak values   46, 37 units;
> 110 % sea water, three runs, peak values 20, 25, 25 units;
> 100 % sea water, three runs, peak values 35, 37, 35 units.

It is clear from these figures that the response is reduced by increasing the external osmotic pressure.

The question which now arises is this: which type of response must be considered as representing the 'normal' function? The change from the one form to the other is brought about by a remarkably small change in the conditions, for example, a 3 or 4 % dilution may cause the appearance of a large positive phase where none existed before. Although *Carcinus* Ringer solution is more like crustacean blood than is sea water as regards the relative proportions of the different ions, there is no good evidence that, to within 3 or 4 %, the osmotic pressure of *Carcinus* Ringer solution is more nearly equal to that of blood. The method which has been used in the attempt to decide this point involves the possibly questionable assumption that if the nerve is equilibrated for long enough in any one of these media the response should tend to become 'normal'. Making use of this criterion, it appears that a large early positive phase is the abnormal response. This was tested by soaking nerves for long periods in *Carcinus* Ringer solution or in diluted sea water. In either case the early positive phase, which was large at the start, diminished steadily, and at the end of 3–4 hr. soaking the response was practically the same as that of a 'control' nerve in normal sea water: the latter hardly altered with time. On the other hand, it is not so certain whether a *small* early positive phase should be considered as abnormal. In some instances it has persisted up to 4–5 hr. in sea water—as in the case shown in Fig. 9—but often it was neither seen at the start, nor did it appear later.

The time course of the positive phase, recorded when it is most pronounced shortly after dilution or the application of *Carcinus* Ringer solution, shows that there is a lag in starting off (Fig. 11). This lag is about the same as was found for the commencement of the negative phase for a nerve in normal sea water (Fig. 10).

<div align="center">DISCUSSION</div>

Stimulation of a nerve trunk has been seen to produce a decrease in opacity, and this may, in certain circumstances, be preceded by a phase of increased opacity. Concentrating attention in the first place on the *decrease*, which is invariably shown, evidence has been produced which suggests that this is due to an increase in diameter of the fibres, for when the fibres are made to swell by diluting the external medium, the opacity decreases. Hill (1950) has shown that a single giant fibre from the cuttlefish swells when it is stimulated, and there are good reasons why it should do so. The effect of stimulation is to increase the internal osmotic pressure of a nerve fibre, and this results in swelling due to the inward passage of water. The osmotic pressure increases for the following reasons: (*a*) stimulation causes an exchange of sodium and potassium across the nerve membrane, sodium entering and potassium leaving the fibre. Potassium is osmotically less active than sodium, and the internal osmotic pressure therefore increases; (*b*) in addition to the exchange of sodium and potassium, some sodium enters the fibre accompanied by chloride ions. In the first part of the discussion an attempt is made to correlate the opacity change with the movement of ions.

The second part of the discussion turns on the nature of the initial *increase* of opacity which may appear in some circumstances. This increase suggests a decrease in fibre size, but the possible reasons for a shrinkage are not so clear (Hill, 1950), and the discussion becomes rather more speculative.

Lastly, the question of the permeability of the resting fibre to sodium ions and to other substances is touched on.

It must be stressed that no proof can be advanced of the tacit assumption that the changes in opacity as observed are solely due to alterations in fibre size and not partly due to changes, independent of size, in opacity of the axoplasm or membrane of the individual fibre. Even if the effects *are* due to a swelling or shrinkage of the fibres, it is by no means plain why the opacity changes as it does. It was pointed out earlier that at present there is no criterion which enables one to decide whether the size *per se* is responsible, or whether the associated change in the degree of dilution of the internal colloidal particles is the operative factor.

The decrease in opacity following stimulation

The effect of 500 impulses has been found to produce a decrease in opacity equal to that brought about by diluting the external medium by 0·47 %. To

translate this figure into the corresponding actual change of diameter it is necessary to select an 'equivalent diameter' to represent the fibres as a whole. Young (1936) states that the axons of the leg nerves of *Maia* vary in diameter from 'less than $1\,\mu$. to $20\,\mu$.'. The majority of the fibres are at the small end of this range, and as it seems probable that the opacity of a nerve depends to a greater degree on the small fibres than on the large ones, the upper and lower limits of the 'equivalent diameter' are taken as 0.5 and $5\,\mu$. In calculating the corresponding increase of diameter for 0.47% dilution, allowance has to be made for a fraction of the volume of the fibre being osmotically inactive. Taking this fraction as one-quarter (estimated on the basis of evidence presented by Lucké, 1940) the increase in fibre diameter produced by 500 impulses is 0.18%, and therefore lies between 0.0009 and $0.009\,\mu$. Is this the order of magnitude one might expect? The extra potassium lost during stimulation from whole *Maia* nerve has been measured by Cowan (1934); he gives the figure as 2.92×10^{-10} mole K/g./impulse. Young's (1936) histological data suggest that there are roughly 2000 sq.cm. of membrane in 1 g. of nerve. This gives a figure of 0.15×10^{-12} mole K/cm²./impulse. Keynes (1949) finds about twice this value for the leakage of potassium from a stimulated *Carcinus* nerve. Cowan's nerves were 'stimulated to fatigue' and many axons may have become inexcitable before the end; it is also possible that considerable quantities of potassium may have been absorbed from the interstitial spaces of the nerve trunk during the 5 min. period of stimulation employed. Keynes, in referring to his results, remarks that the 'action potential always declined, often to half or one-third of its original size during the course of the experiment'. A figure rather greater than 0.3×10^{-12} mole K/cm.²/impulse should therefore be taken. In the single fibre of *Sepia* the outflow of potassium is 4.5×10^{-12} mole K/cm.²/impulse (Keynes, 1950), and in the smaller single fibres of *Carcinus* it is 1.7×10^{-12} mole K/cm.²/impulse (Hodgkin, 1947). A value of 1.0×10^{-12} mole K/cm.²/impulse cannot be far from the true value for the leakage of potassium in whole *Maia* nerve. What change of osmotic pressure and increase of fibre diameter results from an exchange of this quantity of potassium with an equivalent quantity of sodium? It is shown (Hill, 1950) that the exchange of one ion of sodium with one of potassium results in the entry into the fibre of 3.2 molecules of water. The exchange of 1.0×10^{-12} mole K/cm.²/impulse will, owing to this entry of water, result in an increase in fibre diameter of $1.1 \times 10^{-6}\,\mu$./impulse. For 500 impulses this is $0.0006\,\mu$., which is less than the minimum $0.0009\,\mu$. estimated to occur. The difference, $0.0003\,\mu$., may be assumed to be made up by the entry of sodium chloride (Hill, 1950). It can be shown that an entry of 3.3×10^{-14} mole NaCl/cm.²/impulse is sufficient to give an increase of $0.0003\,\mu$./ 500 impulses. This ionic *transfer* amounts to about 3.3% of the ionic *exchange*, and the amount of sodium entering the fibre during activity is 3.3% greater than the amount of potassium leaving, the excess of sodium being accompanied

by chloride. If one takes the upper limit of $0.009\,\mu$. for the actual change, the difference is $0.0084\,\mu$., and an entry of 9.2×10^{-13} mole NaCl/cm.^{2}/impulse is required. In this case the transfer amounts to 92% of the exchange.

Hill (1950) estimated that the transfer of sodium in a stimulated *Sepia* fibre was about 38% of the exchange.

A direct analytical test of the relative quantities of sodium entering and potassium leaving the fibre during activity would help to narrow down the range of possibilities, but until this is available the potassium outflow must be employed as a guide to the whole ionic exchange. It appears, however, that the orders of magnitude are right, and the phase of decreased opacity is therefore attributable to swelling of the individual nerve fibres. The increase in diameter appears to lie between 0.0009 and $0.009\,\mu$. for 500 impulses.

The initial increase of opacity

It has been found that dilution of the solution around the nerve has a striking effect on the opacity change following stimulation, for a large initial *increase* in opacity is found then to precede the main decrease. A small early positive phase is often seen with a nerve in normal solution, but it is sometimes entirely absent. An increase in opacity indicates a decrease in fibre size.

It has been shown that a single *Sepia* fibre undergoes a very small shrinkage before the main swelling (Hill, 1950). The only explanation which could be offered was this: that in the rapid exchange of sodium and potassium, the potassium remains associated with its hydration water, which accompanies the ion through the membrane, and the sodium ion is forced to part with its hydration water, the latter following slowly afterwards under normal osmotic forces to replace that taken up by the sodium ion when it reaches the other side. Conway (1947) gives sodium as being hydrated with 8.0 molecules of water, and potassium 3.8 molecules. If sodium is entirely stripped of water and potassium not at all, the decrease in diameter of a nerve fibre would be $0.0007\,\mu$. This is therefore a possible explanation, because $0.0007\,\mu$. is comparable with the actual change and, although it may be more or less masked by the supervention of the phase of decreased opacity, a change of this order of magnitude might be observable. However, if this is the explanation it is not plain why the early positive phase should be so greatly exaggerated by dilution of the medium. For what may be called the 'dilution effect' another explanation must be sought. The only suggestion which seems at all plausible is that the hydrostatic pressure inside a swollen fibre is in some way responsible for the phenomenon. In the unstimulated fibre, the membrane of which is almost impermeable to sodium ions, dilution must result in a swelling which is permanent, or which regresses only slowly, for the conditions required for the Donnan equilibrium have to be fulfilled, and equality maintained in the activity products of the internal and external potassium and chloride ions. If, however, the perme-

ability to sodium is suddenly increased to a large value, as by stimulation, the Donnan equation no longer applies, sodium and chloride enter the fibre, potassium leaves, and the fibre swells: this has been discussed earlier. But if the interior of the fibre is initially under hydrostatic pressure due to a stretched membrane, there will be a tendency for the fibre to shrink, which could come about in the active fibre by a rapid extrusion of potassium and chloride ions. In this case water would pass out *with* the ions, whereas the water *follows* the ions if they move under concentration gradients. Thus the net ionic exchange may be inwards right from the start, and yet the form of the response be diphasic.

Perhaps the hydrostatic pressure in a fibre under normal conditions is appreciable, so the same explanation might be offered for the small initial positive phase seen with a nerve in a normal medium, and for the initial shrinkage of a *Sepia* fibre.

The permeability of the resting fibre to sodium ions and other substances

It has been shown that an increase of the osmotic pressure of the external medium has the immediate effect of causing a decrease in volume of the fibres. But this is not permanent: there tends to be a regression to the original fibre size. If the change in osmotic pressure is brought about by the addition of urea or potassium chloride this regression is easily accounted for: urea simply penetrates the fibre until its internal and external concentrations are the same, so the volume returns to its normal value; in the case of potassium chloride the controlling force is probably the unbalance in the products of the potassium and chloride activities, $[K] \times [Cl]$, inside and outside the fibre. This results in the entry of sodium chloride.

It has been shown by Boyle & Conway (1941) that there will be a return to exactly the original volume only if the potassium activity within the normal fibre is exactly one-half of the total internal ionic activity; although it appears that this condition obtains for the frog's sartorius muscle, the evidence (Shanes, 1946) that it does so for nerve is rather incomplete, and the volume of a nerve following the addition of potassium chloride to the medium should not necessarily be expected to return exactly to its original value.

There is some difficulty, however, in accounting for the slow reversal of the immediate change in volume due to the addition of sodium chloride. The immediate change, due to the passage of water, restores the osmotic balance across the membrane. The slight unbalance in the $[K] \times [Cl]$ ratio associated with this will contribute a further, but relatively small, decrease of volume; but it is not plain what forces produce the reversal, which in a calcium-free medium appears to restore the volume almost to its original value. One possible explanation is as follows: the permeability of the nerve membrane to sodium is not zero, but the inward leakage of sodium must normally be reversed by some active extrusion process. If, for some reason, the rate of entry of sodium is increased to such an extent that the extrusion process cannot cope with the

leakage, sodium chloride will enter the fibre. It is known that an isolated nerve in sea water, or other artificial medium, loses potassium and gains sodium, until it finally gets into a steady state: the rate of entry is then presumably just low enough to be reversed by the extrusion mechanism. A nerve in this condition should react to addition of sodium chloride in just the way that is seen; that is to say, the immediate response will be a shrinkage to restore osmotic balance: this will be followed by a regression, because the increased rate of entry of sodium brought about by the raised external concentration is greater than the extrusion process can deal with. The net intake of sodium ions is electrically balanced by an outflow of potassium ions, but also by an accompanying inward flow of chloride ions. It has been pointed out earlier that both of these processes result in swelling of the fibre. Lack of calcium increases the rate of regression, which seems to indicate that it raises the permeability to sodium, and an excess of calcium decreases it; or, alternatively, the rate of extrusion is affected. The complete stoppage of the regression in a solution containing five times the normal calcium concentration, suggests that the fibre is then in a condition such that the extrusion mechanism is capable of expelling sodium at a rate greater than the rate at which it enters in normal sea water; consequently, when the external concentration is raised it is still able to expel the total quantity which enters, and there is no net transfer. No explanation can be offered for the initial fall of the curve from the peak in high-calcium solution (Fig. 7).

The swelling due to dilution would be expected to regress slowly with time, owing to the *reduced* inflow of sodium, but this has not been tested.

SUMMARY

1. The change in the opacity to white light of a crustacean nerve trunk following repetitive stimulation has been further investigated. The effect is measured by making photoelectric recordings of the intensity of light scattered by the nerve at right angles to the incident beam.

2. The chamber for holding the nerve was designed so that, in addition to recording the effect of stimulation, the change in opacity brought about by alterations in the composition of the solution surrounding the nerve could also be investigated. It was found that the opacity of the nerve is very sensitive to changes in fibre diameter, brought about by altering the osmotic pressure of the solution. When the fibres swell the opacity decreases, when they shrink it increases.

3. The response to stimulation at 50–100/sec. in normal sea water consists of an initial small increase of opacity, which is rather variable and may be absent, followed by a larger decrease, which reaches its maximum shortly after the end of the 5–10 sec. period of stimulation, and then reverses slowly. At its peak the decrease in the intensity of the scattered light, due to stimulation at

50/sec. for 10 sec., is about 1 part in 100 of the resting intensity. Recovery appears to be complete in about 10–15 min. It is shown that this latter phase of decreased opacity can probably be attributed to an increase in diameter of the individual nerve fibres, and it is calculated that this increase lies between 0·0009 and 0·009 μ. for 500 impulses.

4. The initial increase of opacity due to stimulation can be greatly exaggerated by slight dilution of the solution surrounding the nerve. The reason for this is not clear.

5. The dependence of the opacity upon the fibre size has been made use of in studying the permeability of the fibre membrane to certain solutes. There is evidence which suggests that the permeability to sodium ions can be increased by removing calcium from the solution, and decreased by adding an excess.

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