J. Physiol. (1951) 114, 151–182

# THE SODIUM AND POTASSIUM CONTENT OF CEPHALOPOD NERVE FIBRES

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(*Received* 4 *October* 1950)

In investigating the part played by sodium and potassium in processes such as nervous conduction it is important to have fairly reliable values for the intracellular ionic concentrations. Neither measurements of the electric potentials across the nerve membrane, nor the results of experiments with radioactive tracers, can be interpreted without knowing the sizes of the ionic concentration gradients. In the preceding paper (Keynes, 1951*b*) it has been seen that although the absolute values of the inward ionic fluxes can be measured directly with $Na^{24}$ and $K^{42}$, the outward fluxes can only be found by observing the rate constants for loss of radioactive ions in an inactive solution, and their absolute values can only be calculated when the true internal concentrations are known. There was originally such uncertainty as to the exact sodium content of the *Sepia* axons used in the tracer experiments that it was impossible to decide whether there was a net loss or a net gain of sodium during activity (Keynes, 1949*a*, *b*). It was therefore difficult to make any further progress until analyses had been made of the sodium and potassium contents of these axons.

Steinbach & Spiegelman (1943), in their very valuable study of the sodium and potassium in squid axoplasm, were working at the limit of sensitivity of conventional analytical methods, and their procedures could not have been applied to axons any smaller than those which they used. More sensitive microanalytical methods have been described (Glick, 1949), but they are difficult to perform and would be very cumbersome for any work involving large numbers of samples. Some time was accordingly spent in developing a new analytical procedure for simultaneous determination of sodium and potassium—and, as it turned out, of chloride as well—using the technique of activation analysis.

Activation analysis, to the possibilities of which our attention was first drawn by the papers of Tobias & Dunn (1949) and Brown & Goldberg (1949), consists in principle of irradiating samples with neutrons and then determining the activity of the various radioisotopes which have been formed, as a measure of the amounts of the parent elements in the samples. It can be used to

estimate any element which gives reasonable yields of a suitable radioisotope, but has hitherto been employed chiefly for microanalysis of trace quantities of elements having a particularly large neutron capture cross section (Boyd, 1949). In such applications it is generally necessary to separate the different isotopes in the irradiated samples by the usual routines of radiochemistry before their radioactivity can be measured. On testing the method for sodium and potassium it was found that when they were major constituents of the sample, as they are in most biological material, the $Na^{24}$ and $K^{42}$ formed during irradiation could be estimated accurately without any chemical separation, by virtue of their characteristic radiation and half-lives. The only other radioisotopes found in any quantity in the samples were $P^{32}$ and $S^{35}$. These could be prevented from interfering seriously with the counts of $Na^{24}$ and $K^{42}$ by using suitable filters, and exact allowance could be made for the small residual error due to $P^{32}$ by taking further counts after the short-lived $Na^{24}$ and $K^{42}$ had disappeared. Both sodium and potassium give large yields of their radioisotopes, so that amounts down to $0{\cdot}3\,\mu g.$ could be estimated precisely and without difficulty. It also happened that the $S^{35}$ originated almost entirely from $Cl^{35}$, making it possible to obtain a fairly reliable estimate of the chloride in some of the samples.

As soon as it had been established that the method of analysis was practicable, a visit was made to Plymouth in order to collect material to be analysed. The main objective was to compare the sodium and potassium contents of resting *Sepia* axons with those of axons which had been stimulated for some time, in order to determine the net losses and gains of ions during nervous activity. The design of the experiments suffered slightly from the fact that all the axons had to be collected and stored before any of the analyses had been done, and the period of stimulation was made rather longer than in fact it need have been, in order to make sure of obtaining significant effects. Fortunately, the results were more reproducible than had been anticipated, and the figures obtained showed clearly the extent of the resting leakage of potassium in exchange for sodium, as well as the changes on stimulation. Some samples of extruded squid axoplasm were also collected, and the net losses and gains of ions during activity determined.

The use of this technique for sodium and potassium analysis has been advocated by the authors already quoted, but it has apparently not been applied before to animal tissues, so that part of this paper will necessarily be concerned with the development and reliability of the analytical method. Although it was realized from the start that the $Na^{24}$ could be estimated without any chemical manipulation by taking $\gamma$-counts, the $K^{42}$ in about one-third of the samples was separated chemically and counted in a liquid counter. In the section on 'methods' the procedure which was finally adopted is described first, and then the chemical procedure, together with an account of some of the errors in the two methods. In the following sections the experiments on which

the method is based are discussed, and the results for *Sepia* and squid axons are then given.

A preliminary account of these experiments has appeared in *Nature* (Keynes & Lewis, 1950).

## METHODS

### *Dissection of* Sepia *axons*

Giant axons, with diameters ranging from 150 to 300 $\mu$., were dissected from the mantle of *Sepia officinalis*. Isolated axons tend to degenerate at their cut ends, and the method of dissection described in the preceding paper (Keynes, 1951*b*) was therefore modified so that several axons could be taken from each half of the mantle, without severing them at their central ends until after they had been completely cleaned. The nerve trunks were cut through distally, as far from the stellate ganglion as possible, and dissected out of the mantle muscle back towards the ganglion, instead of the other way round. When several nerve trunks had thus been freed, the remainder were cut, and ganglion and nerves were moved to another dish, where the giant axons were cleaned under a binocular microscope in the usual way. Up to six axons were obtained from each half of the mantle. This method of dissection also yielded rather longer axons than the earlier procedure.

While the dissection of axons from the first half of the mantle was in progress, the other half was stored in fresh sea water in a refrigerator. Dissection and cleaning took about 1 hr. for each axon, so that the second half of the mantle generally remained in the refrigerator for about 5 hr. All the dissections were done in sea water previously cooled in the refrigerator, and heat filters were used to prevent the water in the dissecting dishes from warming up too much. The average temperature during dissection was about 10° C.; room temperature was 22° C.

### *Stimulation and storage of* Sepia *axons*

The condition of each of the cleaned axons was tested by grasping the ganglion and the distal end in forceps, raising them just out of the sea water, applying square wave shocks to one pair of forceps, and observing the action potentials on a Cossor oscilloscope connected to the other. Any axon which did not give a large action potential at a low threshold over the whole of its length was rejected; only about one in ten was not fully excitable. The diameter of each axon was measured at about six points, with a high power micrometer eyepiece in the dissecting microscope (over-all magnification 115 ×).

Axons which were to be analysed as unstimulated controls were cut off the ganglion as soon as their diameters had been measured. The distal end of the axon had by then been in sea water for some time, and probably contained abnormal amounts of ions; 10 mm. of axon was therefore cut off at this end. The central part of the axon, usually about 30 mm. long, was put in a small dish containing a choline chloride solution isotonic with sea water, in order to wash off all the extracellular sodium. This solution was made up according to the formula for artificial sea water used earlier (Keynes, 1951*b*), an equimolar quantity of choline chloride being substituted for the sodium chloride. After being washed for 5–10 min. the axon was quickly blotted, put on the waxed bottom of a specially constructed micro-weighing bottle, and weighed on a micro-balance. It was then hung over a very fine quartz thread and dried in an oven at 120° C. The dried axons were finally transferred, together with about 1 mm. of the quartz thread, into transparent quartz tubes about 3 cm. long with an internal diameter of about 2 mm., which were sealed off at both ends in a gas-oxygen flame. The tubes were labelled, and kept until required for analysis.

Other axons were stimulated before being stored. Shocks were applied to one pair of forceps, usually that in which the ganglion was held, and the exposed ends of the axon were lowered momentarily beneath the surface once every ½ min. in order to prevent them from drying up. The pulse generator was synchronized with the a.c. mains, so that the pulse frequency was an exact multiple of 50 imp./sec. Stimulation was generally maintained for 20 min. at a frequency of 100 imp./sec. During this period the threshold did not alter if the axon was in perfect condition;

in the few cases in which it rose appreciably the axon was preserved for analysis, but the results were not finally used. After stimulation the axon was severed from the ganglion, its distal end was cut off, and it was washed in choline and stored as described above.

*Collection of squid axoplasm samples*

Giant axons in the first stellar nerve of medium-sized specimens of *Loligo forbesi* were used. The axoplasm was extruded according to the method described by Steinbach (1941), with some modifications to make doubly sure that there was no possibility of contamination by extracellular material. The ganglion and nerve trunk were dissected out of the mantle as far as the first large branch of the giant axon, where the trunk was tied and cut. Two threads were tied round the trunk about 15 mm. from the distal end, and it was cut between them. The short segment of the giant axon was used as the unstimulated control. It was quickly cleaned for a distance of about 5 mm. from one end, put in a dish of choline solution, and taken to the balance room for extrusion. Here it was laid on filter paper, the last 3 mm. were cut off, and the axoplasm was extruded on to a waxed cover slip by gentle pressure with a small rod. The axoplasm was immediately picked up with a fine quartz rod, transferred to the weighing bottle, and weighed on a micro-balance. It was then hung over a quartz thread, dried, and bottled as before, using rather wider quartz tubes. Great care was taken to see that none of the sample was lost in the process.

In the meantime the ganglion and remainder of the nerve trunk, about 25 mm. long, were mounted on forceps and stimulated in the same way as the *Sepia* axons. The raised ends were only about 2 mm. above the surface, and were lowered once every 2 min. to prevent drying. Shocks were applied for 30 min. at a frequency of 200 imp./sec. All the axons conducted perfectly for this period, with no perceptible rise in threshold. The end of the giant axon was then cleaned, its diameter was measured, and the axoplasm was extruded, weighed, and stored as before.

*Analytical procedure for sodium and potassium*

The nerve samples were irradiated in the large (B.E.P.O.) neutron pile at Harwell for 1 week. Once the analytical technique had been fully worked out they were irradiated in batches of about twenty, but earlier not more than six were sent each week. The tubes were held in a bundle by elastic bands, and packed in cotton wool in standard aluminium cans, 1 in. in diameter and 3 in. long. Three tubes containing about 20 mg. each of 'Specpure' $K_2CO_3$, three of $Na_2CO_3$, and one of $KH_2PO_4$, were spaced evenly round the bundle.

When the can was returned to Cambridge, each tube was broken open, and the nerve was transferred with fine forceps to a drop of inactive $K_2CO_3$ solution in the centre of a numbered nickel dish, or, in the earlier analyses, of a small piece of platinum foil. The inside of the tube was washed several times with distilled water, using a very fine pipette, and the washings were added to the drop on the dish, making a total volume of about 0·15 ml. The dish was then dried under an electric fire, the $K_2CO_3$ ensuring that the salts were spread in an even layer.

Counts were taken with a conventional single-ended Geiger counter (G.E.C. Type GM4), in a lead castle in which the tray carrying the samples was 15 mm. below the end-window, brass filters being inserted in a slot 7 mm. below the window. The routine for each sample consisted in taking first a 10-min. count with a thick brass filter (4·6 g./cm.$^2$), then two 5-min. counts with a thinner brass filter (0·46 g./cm.$^2$), and finally another 10-min. count with the thick filter. For samples giving exceptionally low counting rates the counting times were increased so as to give a total of the order of 20,000 counts with each of the filters, making the standard error generally less than $\pm 1\%$. These counts were almost all taken between 4.0 p.m. and midnight on the day the samples were removed from the pile (at 6.30 a.m.); a few were not done until the following morning. After being counted the samples were either preserved for subsequent determination of the $P^{32}$ and $S^{35}$, or treated chemically, as described below, in order to estimate the amount of $K^{42}$ separately.

*Standardization*

The tubes containing the standard samples were opened and their contents were tipped or washed into 25 ml. volumetric flasks. The carbonates were then titrated against 0·1 N-HCl, using

brom-phenol blue as indicator, to determine the total alkali present, and the flasks were made up to the mark. Duplicate samples of about 30 mg. of solution were weighed out from each flask on to nickel dishes, one drop of the $K_2CO_3$ solution was added, and the dishes were dried. The Na standards were counted with the thick $\gamma$-filter, and the K standards with the thinner $\beta$-filter. On several occasions the standards were counted with both filters, in order to obtain an accurate value for the relative counting rates for $\beta$- and $\gamma$-radiation.

*Calculation of results*

For $Na^{24}$ the relative counting rate with $\beta$- and $\gamma$-filters was 1·130:1. For $K^{42}$ it was 126:1. If the sample gave average counts of $C_\beta$ and $C_\gamma$ with the $\beta$- and $\gamma$-filters respectively, and if the $P^{32}$ in the sample gave a count $C_P$ with the $\beta$-filter, then the count due to $\gamma$-radiation from $Na^{24}$ was

$$C_{Na} = 1{\cdot}009[C_\gamma - 0{\cdot}008(C_\beta - C_P)],$$

and the count due to $\beta$-radiation from $K^{42}$ was

$$C_K = 1{\cdot}009(C_\beta - C_P - 1{\cdot}130\,C_\gamma).$$

After calculating the moles of Na or K corresponding to 1 count/min. with the appropriate filter at the mean time at which each of the samples was counted, their Na and K contents could be worked out.

*Procedure for chemical separation of $K^{42}$*

After the $\gamma$-counts had been taken to determine the amount of $Na^{24}$ present, the samples were incinerated in an electric furnace for 2–3 hr. at a temperature rising from 200° to 500° C. In the earlier analyses the samples were on platinum foil, and were incinerated on the foil in covered silica crucibles. After incineration they were washed off the foil into platinum crucibles with about 1 ml. of hot distilled water, and then evaporated dry in an oven at 110° C. In the later analyses they were washed off the nickel dishes into platinum crucibles before incineration.

The ash was dissolved in 0·2 ml. of water, and 0·5 ml. of lithium dipicrylaminate reagent (Keynes & Lewis, 1951) was added drop by drop. After gently warming to facilitate crystallization, the crucibles were left at room temperature for an hour, and then put in an ice bath for at least 3 hr. The precipitate of potassium dipicrylaminate was filtered at 0° C. in the ice bath with a B.T.L. Grade 4 sintered glass filter stick, fitted as described by Lewis (1951*a*). It was then washed with four 0·1 ml. portions of a mixture of N/30 lithium and sodium dipicrylaminates cooled to 0° C. The precipitate was next dissolved in a few ml. of aqueous acetone and siphoned into a 25 or 50 ml. volumetric flask. The filter stick was washed with several portions of hot water, which were added to the contents of the flask. The flask was then made up to the mark with water.

The $K^{42}$ was counted in an automatic-filling liquid counter of the type shown in Fig. 1. Counts were also taken with solutions of known strength made up from the $K^{42}$ standards.

*Phosphorus determinations*

The $P^{32}$ in all the samples which had not been treated chemically was counted about a fortnight after irradiation, using a mica filter of thickness 25 mg./cm.$^2$ to cut out soft $\beta$-radiation from $S^{35}$. The counts were standardized against the $P^{32}$ in samples of $KH_2PO_4$ which were irradiated with each batch of nerves.

*Chloride determinations*

Some samples were counted again about 3 months after irradiation to determine the amount of $S^{35}$, and hence of chloride, in them. A mica window counter was used (G.E.C. Type EHM2), and the counting rates were of the order of 100–400 counts/min./mg. nerve. Parallel counts were taken with a mica filter (25 mg./cm.$^2$) so that correction could be made for the small amount of $P^{32}$ (about 20 counts/min.) which was still present. The counts were standardized against the $S^{35}$ in a sample of about 5 mg. of NaCl which had been irradiated with the nerves. Portions of the NaCl were weighed accurately on to nickel dishes, the same quantity of $K_2CO_3$ added as for the nerve samples, and the dishes were dried under an electric fire.

There were two sources of error in counting the $S^{35}$ which were not appreciable in counting $Na^{24}$, $K^{42}$, or $P^{32}$. These were radiation losses from self-absorption in the samples, and losses of $S^{35}$ as $H_2S$ in drying the samples. The standards and the samples were treated in exactly the same fashion so as to reduce these errors to a minimum. They might have introduced a further S.E. of the order of $\pm 6\%$ in the results, though it will be seen from the figures quoted later that they probably had less effect than this.

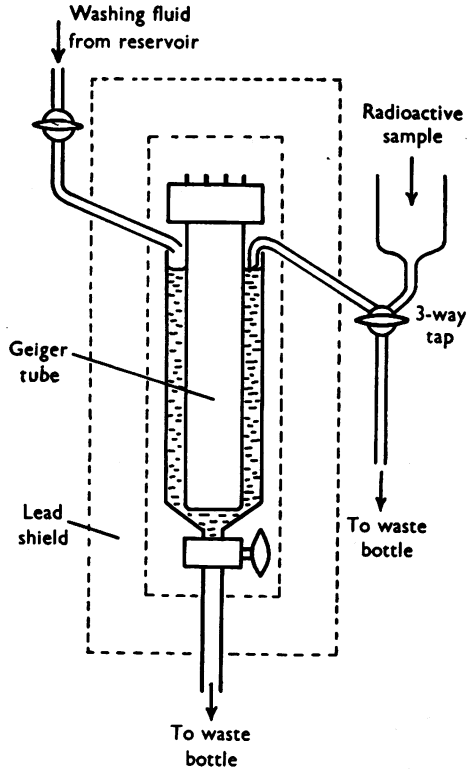


Fig. 1. Diagram (not to scale) of liquid counter used in measuring radioactivity of $K^{42}$ samples. The siphon arrangement ensured that it was always filled to exactly the same level. About 15 ml. of fluid was required for one filling.

*Sources of error in the analytical procedure*

Direct measurements were made wherever possible to check the magnitudes of the errors. They were as follows:

(*a*) *Variation in the neutron flux.* It was tacitly assumed in the calculations that the neutron flux during irradiation was the same for all the samples and standards. It is unlikely that the neutron flux varied by more than 1% within an empty can, but there might have been point-to-point variations greater than this if the can had contained specimens with too large a neutron absorption factor. The use of chlorides as standards for sodium and potassium was therefore avoided—the neutron absorption factor of KCl is given by A.E.R.E. as 0·30 cm.²/g., whereas for $K_2CO_3$ it is only 0·034 cm.²/g.—and the carbonates were irradiated in tubes whose internal diameter was always under 2 mm. The nerve samples weighed less than a milligram, so that there can have been no appreciable self-shielding effects in them. The absorption factor for silicon is only 0·0054 cm.²/g., so that absorption of neutrons by the quartz tubes cannot possibly have reduced

the neutron flux at the centre of the can by more than 0·4%. An upper limit can be set to this source of error by examining the counts taken for the standard samples of sodium and potassium carbonate, of which there were in all six tubes spaced regularly through each can. The average standard deviation of the individual figures for the specific activity was ±1·4%. The average S.E. arising from statistical fluctuation in the counting rate was ±0·8%, leaving ±1·1% to be accounted for by all other sources. Most of this must have been due to errors in titration and weighing, and to variation in the position of the samples beneath the Geiger tube, so that the variation in neutron flux during irradiation must have been rather small.

(*b*) *Errors due to the method of storing the axons.* The dried axons were rather hygroscopic, and some of their salt content was always deposited on the walls of the quartz tubes in which they were stored. After removal of the axons the tubes were thoroughly washed out with water, and the washings were added to the samples on their nickel dishes. In a series of tubes which were washed out twice, the second washing contained only 0·5% of the total radioactivity, so that losses from this source were probably less than 1%. Counts taken of the washings of four empty tubes which were sealed off and irradiated in the usual way showed that there was only about 0·001 μg. of sodium on the walls of a single tube. This would have caused a maximum error of +0·3%. The small piece of quartz thread on which each sample was suspended while it dried did not contain more than 0·00001 μg. of sodium, so that counting it with the sample caused no appreciable error.

Further experience has shown that these errors can be reduced to negligible proportions by using wider quartz tubing (an internal diameter of 3 mm. is convenient), and by drying the samples at room temperature, when they do not become hygroscopic. With these modifications the method of storing the samples in quartz tubes has the great advantage that they can be handled freely before and after irradiation without fear of contamination.

(*c*) *Weighing errors.* In analysing the contents of single *Sepia* axons, which varied in weight between 0·3 and 1·5 mg., weighing errors were one of the most serious sources of error. This is further discussed in considering the actual results obtained.

(*d*) *Counting errors.* The counting rates were never less than 100 counts/min., and the S.E.'s of the counts were never greater than ±2%. When the counting rate was more than 1000 counts/min. correction was made for the paralysis time of the scaler. Errors due to positional variation were reduced by adding a drop of $K_2CO_3$ solution to each dish, which ensured that the samples dried evenly, and cannot have been large for the reason discussed in (*a*) above.

(*e*) *Errors in the chemical procedure.* These were determined, where possible, by using highly active samples containing known amounts of radioactivity, and measuring the losses or contamination at each stage directly. The separate sources of error were estimated as:

| | |
|---|---|
| Volatilization of $K^{42}$ during incineration | −1% |
| Incomplete removal of $K^{42}$ from nickel dish | −0·3% |
| $Na^{24}$ included with the $K^{42}$ | +0·2% |
| $P^{32}$ included with the $K^{42}$ | +0·6% |
| $K^{42}$ lost in precipitation | −0·5% |
| $K^{42}$ lost in washing precipitate | −0·5% |
| $K^{42}$ lost by exchange with inactive K in vessels and filter stick | −2% |

The over-all error of the $K^{42}$ estimate was determined by incinerating small pieces of dried, unirradiated crab nerve with known amounts of $K^{42}$, and then separating and estimating the $K^{42}$ by the normal routine. The average result was low by 3·7±0·6%. This agrees well with the figure obtained by adding together the separate errors.

The best estimate of the actual over-all error in chemical determinations of the $K^{42}$ in *Sepia* axon samples was obtained by making dual measurements, the $K^{42}$ being determined first by the radiation discrimination method and then separated chemically. For twenty samples treated in this way the chemical method gave a result 4·4±0·6% low. Two further samples gave results respectively 12 and 23% low, this being so far outside the normal range that it must have been caused by some accident in the procedure.

## RESULTS

### *The radioactive isotopes in irradiated nerve samples*

It can be calculated from published figures for the yields of isotopes during neutron irradiation (all isotope characteristics quoted in this paper are taken from Siri, 1949, or from Friedlander & Kennedy, 1949) that only four of the elements present as major constituents in invertebrate nerve are likely to make

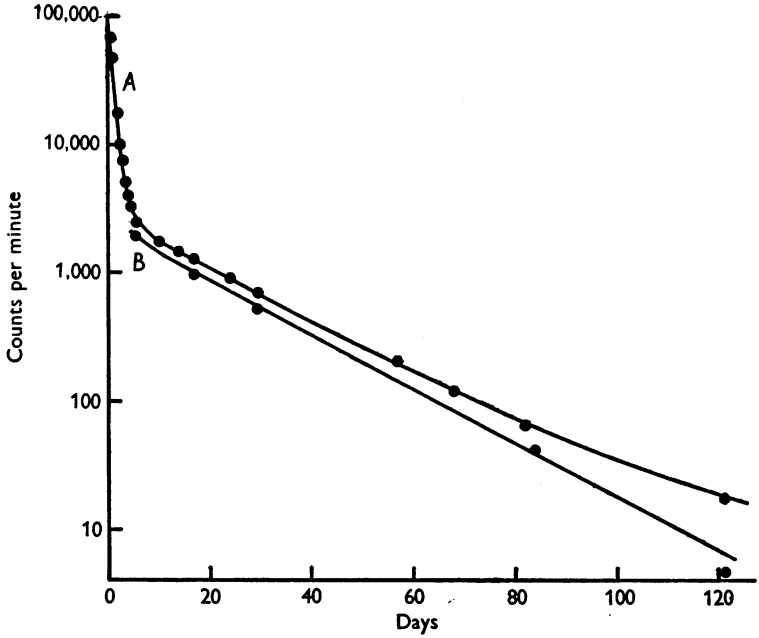


Fig. 2. Decay of $\beta$-radiation from an irradiated sample of *Sepia* nerve. *A*, with no filter. *B*, counts taken with a mica filter of thickness 25 mg./cm.$^2$.

appreciable contributions to the radioactivity of irradiated nerve samples. These are sodium, potassium, and phosphorus, which give $Na^{24}$, $K^{42}$, and $P^{32}$ respectively by $n,\gamma$ reactions, and chlorine, which gives $S^{35}$ by an $n,p$ reaction. The other elements found in biological material in relatively high concentrations—H, C, O, N, Mg, Ca, S, and Fe—give little radioactivity, since their isotopes either have such short half-lives that they have disappeared before any counts are taken, or such long half-lives that the counting rates obtained are very small. This also applies to the two chlorine isotopes given by $n,\gamma$ reactions with stable chlorine.

The correctness of these calculations is borne out by the decay curves given in Figs. 2 and 3. Fig. 2 shows the decay of $\beta$-radiation from 11·3 mg. of *Sepia* nerve which had previously been irradiated in the neutron pile for a week. The initial half-period of decay was about 14 hr., this being intermediate between

the values for $Na^{24}$ (14·8 hr.) and $K^{42}$ (12·4 hr.). After 6 days decay slowed to a half-period of 14 days, characteristic of $P^{32}$ (14·3 days), and continued at this rate for about 3 months until only $S^{35}$ (87·1 days) was left, with traces of other long-lived isotopes. Counts taken with a mica filter of thickness 25 mg./cm.$^2$, which would have cut out all the rather soft radiation from $S^{35}$, decayed to zero with a half-life (calculated by the method of least squares) of $14·4 \pm 0·3$ days, so that after the $Na^{24}$ and $K^{42}$ had gone $P^{32}$ was almost certainly the only isotope giving strong radiation which still remained. In this sample the $S^{35}$ was somewhat under-represented, since the nerve trunk was not incinerated before

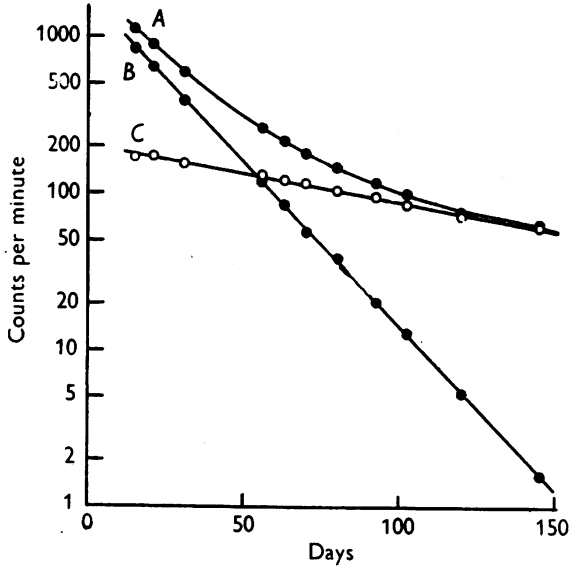


Fig. 3. Decay of $\beta$-radiation from an irradiated sample of squid axoplasm. *A*, with no filter. *B*, counts taken with a mica filter of thickness 25 mg./cm.$^2$. *C*, the component giving very soft $\beta$-radiation, calculated from the difference between *A* and *B*.

it was counted, and was so thick that much of the soft radiation must have been lost by self-absorption. The decay curve shown in Fig. 3 was obtained with a sample of squid axoplasm whose contents were spread thinly and evenly over the nickel dish. It gave considerably more $S^{35}$ relative to the amount of $P^{32}$ than the *Sepia* nerve trunk. Again the counts taken with a mica filter decayed to zero at exactly the right rate for $P^{32}$ ($14·4 \pm 0·2$ days), and in this case it was also possible to calculate the rate of decay of the soft-radiating component, from the difference between the filtered and unfiltered counts, which had a half-life of $83 \pm 2$ days.

These curves do not by themselves provide adequate proof that the only radioactive isotopes in the samples were those which have been mentioned so

far. The question of interference by other isotopes will be considered more fully in the discussion which follows of the methods by which the contributions of the four most important radioisotopes were determined.

*The estimation of* $Na^{24}$ *and* $K^{42}$ *in irradiated samples*

Table 1 shows the counting rates for the different isotopes in a typical *Sepia* axon sample, 10 hr. after removal from the neutron pile. The problem is to separate $Na^{24}$ and $K^{42}$ from each other, and from the two longer-lived isotopes. It is a fairly simple matter to deal with $Na^{24}$, because it gives extremely strong $\gamma$-radiation. $K^{42}$ also gives some $\gamma$-radiation, but whereas $Na^{24}$ emits two $\gamma$-rays of energies 1·4 and 2·8 MeV. respectively for each disintegration, $K^{42}$ only gives

TABLE 1. The principal radioactive isotopes in a typical irradiated *Sepia* axon. The figures show the counting rates, in counts/min., given by a sample weighing 1 mg., 10 hr. after removal from the neutron pile

| | No filter | With $\beta$-filter (0·46 g./cm.$^2$) | With $\gamma$-filter (4·6 g./cm.$^2$) |
|---|---|---|---|
| $Na^{24}$ | 15,000 | 450 | 400 |
| $K^{42}$ | 12,000 | 3,000 | 20 |
| $P^{32}$ | 1,500 | 15 | 0 |
| $S^{35}$ | 150 | 0 | 0 |

one $\gamma$-ray of 1·5 MeV. in 25 % of its disintegrations and none in the others. The $\gamma$-radiation from $Na^{24}$ is hence counted over 10 times as efficiently as that from $K^{42}$. $P^{32}$ and $S^{35}$ do not give any $\gamma$-radiation. The yield of $Na^{24}$ during irradiation, expressed per mole of sodium, is also about 10 times the yield of $K^{42}$ (since the stable isotope $Na^{23}$ is relatively more abundant than $K^{41}$), with the result that, as Table 1 shows, about 95 % of the total recorded $\gamma$-radiation given by a *Sepia* axon sample originates from $Na^{24}$. The sodium content of the samples could therefore be determined by taking a $\gamma$-count, and the only correction necessary was a small one for $K^{42}$. The validity of this procedure is illustrated by Fig. 4, which shows the decay of $\gamma$-radiation from the sample of *Sepia* nerve which was used for Fig. 2. The count was reduced from 17,000 to 2 counts/min. with a half-period of 15·03 $\pm$ 0·06 hr., and there was no significant amount of any long-lived $\gamma$-active component. The discrepancy between the observed half-period and the correct half-period for $Na^{24}$ could have been due to the presence of enough $Br^{82}$, whose half-life is 34 hr., to contribute about 0·5 % of the initial count. The only disadvantage of determining $Na^{24}$ solely by its $\gamma$-count is that there may be slight errors from the presence of traces of heavy elements giving exceptionally large yields of $\gamma$-active isotopes whose half-lives are comparable with 15 hr., which will of course be counted as sodium. This possibility will be discussed in detail in the next section, and it need only be noted here that it was found not to cause an error of more than 1 % in the sodium analyses.

The method used at first to estimate the amount of $K^{42}$ in the samples was to add inactive $K_2CO_3$ as carrier, and to separate the $K^{42}$ chemically from the

other isotopes, so that it could be counted in a liquid counter. The conventional reagents for precipitating potassium were unsuitable for this purpose—cobaltinitrite because it is a double salt of Na and K, and would bring down $Na^{24}$ as well as $K^{42}$, and perchlorate and platinichloride because their relatively high solubility would entail the use of inconveniently large amounts of carrier. The reagent chosen was the dipicrylaminate, which had already been tested fairly extensively for colorimetric estimation of small amounts of potassium in crab nerve and frog muscle (Keynes & Lewis, 1951; Lewis, 1951*a*), and which

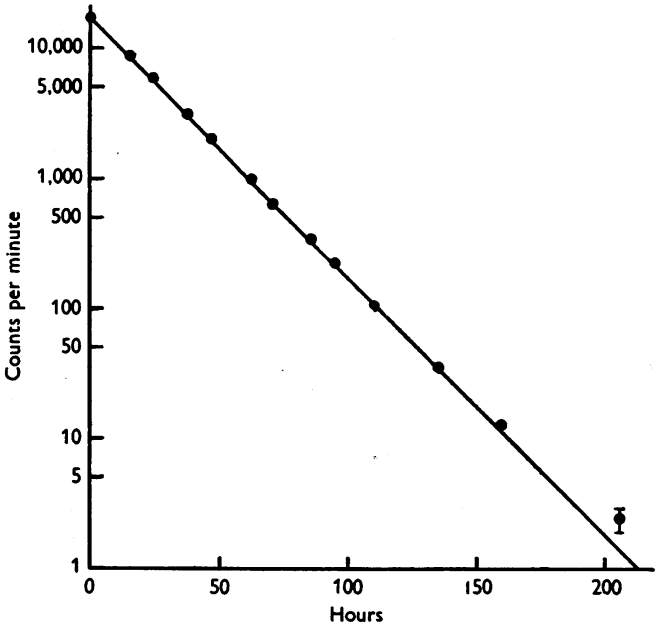


Fig. 4. Decay of $\gamma$-radiation from an irradiated sample of *Sepia* nerve (counts taken with a brass filter of thickness 4·6 g./cm.$^2$). Counting was begun 14 hr. after removal of the sample from the neutron pile. The bar drawn through the last point indicates $\pm$ the S.E. of the count. The errors for the other counts were too small to be shown in this way.

was known to be suitable for precipitating potassium with no danger of co-precipitation of sodium. The exact procedure adopted has been outlined in the section on 'methods'. It proved difficult, even under the best conditions, to recover more than 96% of the $K^{42}$, and occasionally the losses were even greater for no obvious reason.

It was realized later that $K^{42}$ could be estimated without separating it chemically, by taking advantage of the exceptional strength of its $\beta$-radiation. The principle involved should be clear from Fig. 5, which shows the variation in counting rates for $K^{42}$, $Na^{24}$, and $P^{32}$ when filters of different thicknesses are inserted between the tray carrying the radioactive sample and the Geiger counter. A single-ended Geiger counter of the type used (G.E.C. Type GM4)

is rather inefficient for detecting $\gamma$-radiation, but fairly efficient for $\beta$-particles. For small filter thicknesses the counting rate is attenuated roughly in inverse proportion to the $\beta$-particle energy, so that the counts for $Na^{24}$ (1·4 MeV.) and $P^{32}$ (1·7 MeV.) are reduced much more rapidly than that for $K^{42}$ (3·6 and 2·1 MeV.). With very thick filters a plateau is reached where most of the count is due to secondary $\beta$-particles produced by the $\gamma$-rays; for $K^{42}$ this plateau is not shown on the figure, since it only occurs at about 0·2% of the initial count.

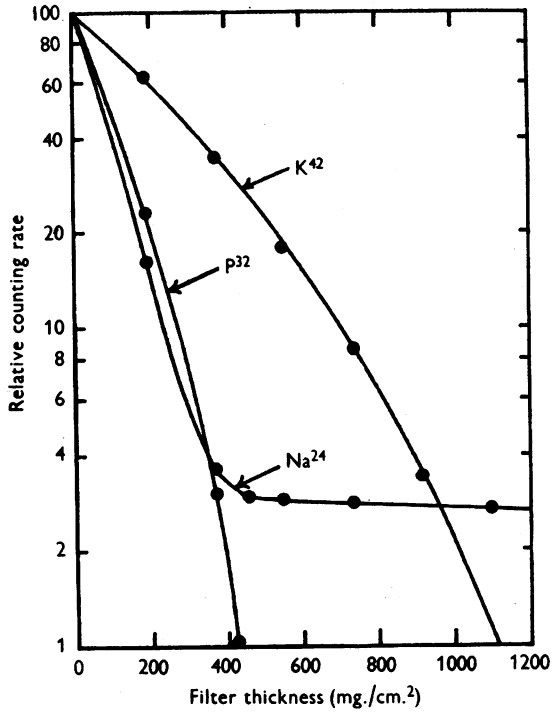


Fig. 5. Variation in counting rate with filter thickness for $K^{42}$, $P^{32}$, and $Na^{24}$. Filters were thin sheets of nickel or brass. Radioactive samples were 15 mm. below window of GM4 Geiger tube, and filters were inserted about 7 mm. below window.

It is evident that for optimum discrimination between $K^{42}$ and $N^{24}$ the filter should have a thickness of about 400 mg./cm.$^2$; furthermore it should be of a light material such as aluminium. For various practical reasons the filter actually used was brass, and had a thickness 460 mg./cm.$^2$. This gave a discrimination reasonably close to the maximum, and at the same time almost completely cut out the radiation from $P^{32}$. For an unstimulated *Sepia* axon (see Table 1) the recorded $\beta$-count taken with this filter was due mostly to $K^{42}$, the proportion contributed by $Na^{24}$ being about 13%, and by $P^{32}$ less than 1%. Knowing the exact relative counting rates for the $\gamma$- and $\beta$-filters for $Na^{24}$ and $K^{42}$ it was hence only necessary to count each sample with the two filters in

turn, and the separate contributions of the two isotopes could then be calculated (see 'Methods'). The correction for $P^{32}$ could be made by allowing the $Na^{24}$ and $K^{42}$ to decay and taking a count with a mica filter, from which its contribution to the $\beta$-count at the time of the original measurements could be worked out.

This method of determining $K^{42}$ suffers from the same disadvantage of possible interference by trace elements as does the determination of $Na^{24}$ from its $\gamma$-count alone, though this is actually a smaller source of error than the inevitable losses which occur at various stages in the chemical procedure. The

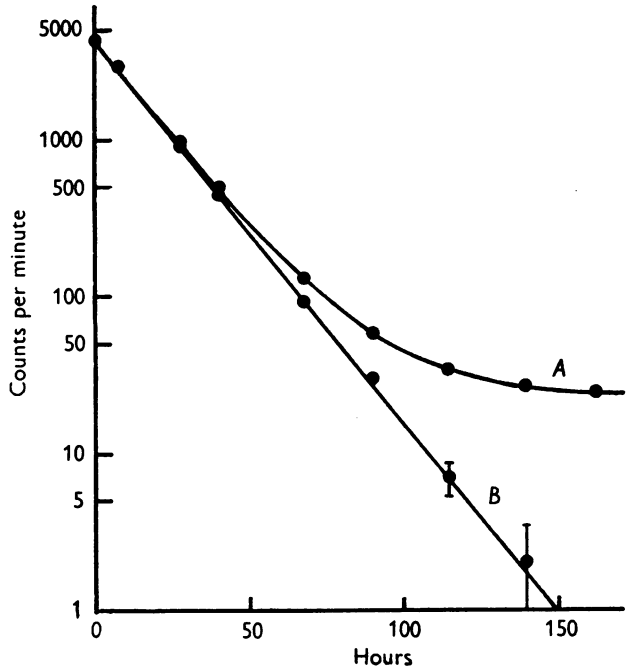


Fig. 6. Decay of filtered $\beta$-radiation from an irradiated sample of *Carcinus* nerve. Counts were taken with a brass filter of thickness 0·46 g./cm.$^2$. *A*, uncorrected $\beta$-count. *B*, $\beta$-count with contributions from $Na^{24}$ and $P^{32}$ subtracted. Vertical bars through two of the points indicate $\pm$ the S.E. of the count.

curves in Fig. 6 show the decay of filtered $\beta$-radiation from an irradiated sample of *Carcinus* nerve. The contribution of $Na^{24}$ was found by taking a $\gamma$-count at the beginning of the series of measurements; it was initially 93 counts/min., the total sodium content of the nerve having been reduced by soaking it in a choline solution to remove extracellular sodium before storing it. The contribution of $P^{32}$ was determined by taking a mica filter count after 10 days, and corresponded to an initial count of 33 counts/min. By subtracting these from the $\beta$-count the $K^{42}$ count was obtained. This decayed with a half-period of $12{\cdot}3 \pm 0{\cdot}1$ hr., which confirms that there were no appreciable quantities of any other strongly $\beta$-active isotopes in the sample.

*Interference caused by trace elements*

Most elements give rise to radioactive isotopes when they are irradiated with slow neutrons, and some of the heavier ones give enormous yields. It was important to see whether any of the elements known to be present in trace quantities in biological material might cause serious interference. The error due

TABLE 2. Interference by trace elements in estimating sodium and potassium by activation analysis

| Isotope | Half-life | Energy of radiation (MeV.) | Activity relative to $Na^{24}$ or $K^{42}$ (%) | Probable conc. in inv. nerve (mg./kg.) | Error in $Na^{24}$ or $K^{42}$ (%) |
|---|---|---|---|---|---|
| | | (*a*) $\beta$-emitters; activity and errors relative to $K^{42}$ | | | |
| $Si^{31}$ | 170m. | 1·8 | 0·2 | 100[1] | 0·002 |
| $P^{32}$ | 14·3d. | 1·7 | 6·3 | 1000[2] | 0·6 |
| $Cl^{38}$ | 38m. | 1·2, 2·8, 5·0 | 0·09 | 4600[2] | 0·04 |
| $Ca^{49}$ | 2·5h. | 2·3 | 0·015 | 300[2] | 0·0005 |
| $Sc^{46}$ | 85d. | 0·4, 1·5 | 2·0 | 1[3] | 0·0002 |
| $Mn^{56}$ | 2·6h. | 0·8, 1·0, 2·8 | 610 | 0·4[4] | 0·024 |
| $Ni^{65}$ | 2·6h. | 1·9 | 0·2 | 20[1] | 0·0004 |
| $As^{76}$ | 26·8h. | 1·3, 2·5, 3·0 | 3500 | 0·2[2] | 0·07 |
| $Br^{80}$ | 4·4h. | 2·0 | 155 | 10[2] | 0·15 |
| $Rb^{86}$ | 19·5d. | 0·7, 1·8 | 5·5 | 0·5[4] | 0·0003 |
| $Ba^{139}$ | 85m. | 2·3 | 0·3 | 10[1] | 0·0003 |
| | | (*b*) $\gamma$-emitters; activity and errors relative to $Na^{24}$ | | | |
| $Cl^{38}$ | 38m. | 1·6, 2·1 | 0·0025 | 4600[2] | 0·012 |
| $Ca^{49}$ | 2·5h. | 0·8 | 0·0007 | 300[2] | 0·0002 |
| $Sc^{46}$ | 85d. | 0·9, 1·1 | 52 | 1[3] | 0·05 |
| $Cr^{51}$ | 26·5d. | 0·32 | 0·56 | 10[1] | 0·006 |
| $Mn^{56}$ | 2·6h. | 0·8, 1·8, 2·1 | 37·5 | 0·4[2] | 0·015 |
| $Fe^{59}$ | 46d. | 1·1, 1·3 | 0·002 | 125[1] | 0·0003 |
| $Co^{60}$ | 5·3y. | 0·3 | 2·3 | 3[1] | 0·007 |
| $Ni^{65}$ | 2·6h. | 1·1 | 0·03 | 20[1] | 0·0006 |
| $Cu^{64}$ | 12·8h. | 1·3 | 5·0 | 1·8[2] | 0·009 |
| $Zn^{69}$ | 13·8h. | 0·44 | 0·3 | 200[1] | 0·06 |
| $As^{76}$ | 26·8h. | 0·5, 1·2, 1·7 | 29 | 0·2[2] | 0·006 |
| $Br^{82}$ | 34h. | 0·5, 0·8, 1·4 | 38 | 10[2] | 0·4 |
| $Rb^{86}$ | 19·5d. | 1·1 | 0·3 | 0·5[4] | 0·0002 |
| $Cs^{134}$ | 2y. | 0·6, 0·8, 1·4 | 2·1 | 0·05[4] | 0·0001 |
| $Ba^{139}$ | 85m. | 0·2, 1·0 | 0·02 | 10[1] | 0·0002 |

All activities are calculated for counts taken 9·5 hr. after the end of 1 week's irradiation in a neutron pile. The $\beta$-activity for each element is expressed as a percentage of that for an equal weight of potassium, counts being taken with a filter of thickness 0·46 g./cm.$^2$. Similarly $\gamma$-activities are given relative to sodium, with a filter of thickness 4·6 g./cm.$^2$. Capture cross-sections and decay schemes were taken from Siri (1949). Potassium concentration taken as 10,000 mg./kg., sodium as 1000 mg./kg. Figures for the probable concentrations of trace elements in invertebrate nerve were derived as follows:

[1] Maximum amount in invertebrate tissues (Webb, 1937).
[2] Figure discussed elsewhere in this paper.
[3] Maximum figure (Harvey, 1945).
[4] Assuming same ratio to potassium as in sea water (Harvey, 1945).

to a given element depends on four factors—its concentration in the tissue, the yield of its radioactive isotope, its half-life, and the efficiency with which its characteristic radiation is detected. As the samples were never counted until

at least 9·5 hr. after they had been removed from the pile, no isotope with a half-life of less than 40 min. could still have been present in any quantity when the counts were taken. This eliminates a number of elements, notably chlorine, whose short-lived isotope $Cl^{38}$ has a half-life of only 38 min. Many other isotopes can be ignored because of the thickness of the filters used in taking the counts; there are fortunately very few which give radiation as strong as that given by $Na^{24}$ and $K^{42}$. The list of possible interfering isotopes given in Table 2 includes all those elements remaining which have been reported in animal tissues, and gives some indication as to which isotopes are most likely to cause trouble. The list does not include various elements which certainly would interfere, but which have never been found occurring naturally in tissues of marine animals. In this category those which would have the largest effect are Y, In, Sb, La, Pr, Eu, Dy, Ho, W, Re, Ir, and Au. The sources from which the figures were derived are given at the foot of the table; many of the figures are unavoidably very approximate. Only isotopes formed by $n,\gamma$ reactions have been included, since the only $n,p$ reactions which are important in this connexion are those in which $C^{14}$, $S^{35}$, and $P^{32}$ are formed, and corrections can in any case be made for these isotopes.

When this table was first compiled it appeared that the isotopes most likely to cause interference were $Mn^{56}$, $Cu^{64}$, $As^{76}$, and $Br^{82}$. Though there are figures in the literature for the amounts of these elements in whole marine animals and in various organs, no analyses of invertebrate nerve have been reported. The quantities present were therefore determined by activation analysis. Samples of about 30 mg. (wet weight) of *Carcinus* leg nerve were irradiated in the usual way. 1 mg. each of inactive Cu, As, and Mn were added to the irradiated samples as carriers, and, after incinerating, the metals were separated as sulphides by conventional methods. Each element was reprecipitated several times in the presence of more inactive carriers, so as to reduce mutual contamination to a minimum. Radiation characteristics and decay curves were determined for each sample isolated, and compared with those of standard samples of $MnSO_4$, $As_2O_3$, and $CuSO_4$ which had been irradiated at the same time. The average figures obtained from duplicate determinations for the concentrations of each element in *Carcinus* nerve were: Cu, 1·8 mg./kg.; Mn, 0·4 mg./kg.; and As, 0·2 mg./kg. The figures for Cu and Mn are rather lower than might have been expected from the spectrographic analyses of Webb (1937), but most of his results refer to whole animals or soft parts, and the impression gained from the analyses of Phillips (1917) is that these two elements occur mostly in the blood and digestive organs. No figures could be found with which to compare the arsenic analyses. Each of these elements would have to occur in concentrations over 10 times as great as was actually found before they caused serious errors.

Determinations of Br by activation analysis did not give satisfactory results, owing to the difficulty of precipitating AgBr without contamination by other

isotopes. It seems likely, however, that the ratio of bromine to chlorine in the axons will be roughly the same as in sea water, since there is no evidence of any marked discrimination between the two elements in marine animals (Krogh, 1939), and tracer studies have shown the distribution of bromide and chloride between tissue and serum to be very similar (Hevesy, 1948). On this basis $Br^{82}$ might cause a maximum error of 1% in the apparent sodium content of *Sepia* axons or whole nerve trunks—a figure not inconsistent with the estimate of 0·5% from the decay curve given in Fig. 4. In squid axoplasm samples, containing less chloride, the error would be smaller. A sample of pure choline chloride was irradiated and found to have only a slightly lower bromine-chlorine ratio than sea water, though it apparently contained no other undesirable trace elements. The washing of the axons in choline solution would therefore not have affected the error due to $Br^{82}$.

A useful check on the errors caused by trace elements was to take duplicate counts of the samples about a day after the original counts had been taken. The error due to $Br^{82}$ would then have been about three times greater for the second count than for the first, owing to the difference in half-lives. For four *Sepia* axons counted twice in this way the average alterations in the apparent sodium and potassium contents for the recount were $+0{\cdot}0 \pm 0{\cdot}2\%$ and $+1{\cdot}6 \pm 0{\cdot}6\%$ respectively, while for four *Carcinus* nerves they were $-0{\cdot}9 \pm 1{\cdot}4\%$ and $-1{\cdot}6 \pm 0{\cdot}4\%$. There was thus no appreciable change in the results. Yet another check was to determine the potassium content of four *Carcinus* nerves first by activation analysis and then by the dipicrylaminate method (Keynes & Lewis, 1951). The second determination gave an average potassium content lower by $1{\cdot}3 \pm 1{\cdot}3\%$, so that again there was no significant discrepancy between the two sets of values.

### *The sodium and potassium contents of resting* Sepia *axons*

The results of all the reliable analyses made on unstimulated *Sepia* axons are plotted in Figs. 7 and 8. All the sodium analyses were done by the same method—a $\gamma$-count of the unincinerated sample. The earlier potassium analyses were done by separating the $K^{42}$ chemically, and the results were corrected by $+4{\cdot}4\%$, which was the average discrepancy between this method and the more reliable $\beta$-count method. Values corrected in this way are plotted as +'s in Fig. 8 to distinguish them from those obtained later. The only results rejected were those for axons which were recorded as having been damaged in any way or whose thresholds for excitation were higher than normal, and those for two axons whose volumes calculated from their weights were over 20% lower than their measured volumes.

It soon became clear that there was a correlation between the sodium and potassium contents and the time which had elapsed between decapitating each animal and storing its axons for analysis. The results showed a steady gain of sodium and loss of potassium. This was not unexpected, since tracer studies had already shown a resting potassium leakage in *Sepia* axons (Keynes, 1951*b*), and Steinbach & Spiegelman (1943) found that there was a considerable exchange of sodium and potassium in dissected squid axons soaked in sea water. It also became clear that the shortest axons contained appreciably

more sodium and less potassium than the average. This again agreed with the tracer experiments, which had shown an abnormally high potassium leakage

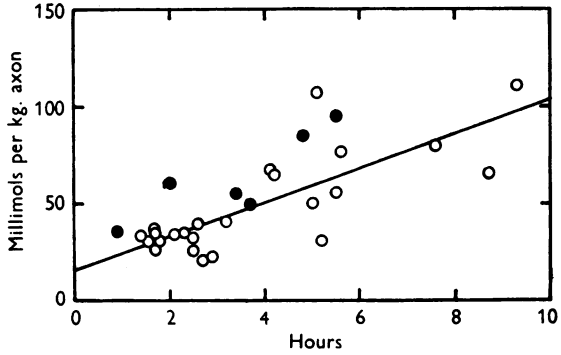


Fig. 7. The sodium content of resting *Sepia* axons. Ordinate: m.mol. Na/kg. wet weight of whole axon. Abscissa: time in hours from decapitation of the animal. The filled-in circles are values for axons which were 21 mm. and less in length. The line was calculated for best fit by the method of least squares, omitting the values for the short axons. Average axon diameter 213 $\mu$. Average axon length 29 mm. Temperature 10° C.

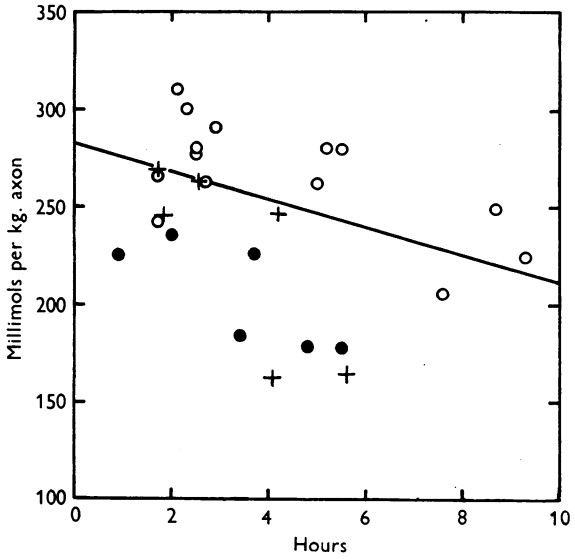


Fig. 8. The potassium content of resting *Sepia* axons. Ordinate: m.mol. K/kg. wet weight of whole axon. Abscissa: time in hours from decapitation of the animal. ○, $K^{42}$ determined by $\beta$-count. +, $K^{42}$ determined in liquid counter after chemical separation. ●, axons 21 mm. and less in length. The line was calculated for best fit by the method of least squares, using all values except those for the short axons. Average axon diameter 220 $\mu$. Average axon length 29 mm. Temperature 10° C.

from short axons. In calculating the resting changes in sodium and potassium content, the values obtained for axons 21 mm. and less in length were therefore

omitted. This limit was chosen rather arbitrarily, because it happened to coincide with an accidental grouping of the axon lengths. As the end 10 mm. of each axon had been cut off just before it was blotted and weighed, the axons rejected were actually those whose lengths during the period of cleaning were less than about 30 mm. The average length of the remaining axons after their ends had been cut off was 29 mm. Since the dissection of axons from each specimen often occupied as much as 10 hr., changes in ionic content with time made it impossible to tell whether there was any systematic variation between individual animals, and no attempt was made to group the results according to the particular animal used.

The resting rate of gain of sodium, calculated by the method of least squares and omitting the figures for the short axons, was $8{\cdot}8 \pm 1{\cdot}5$ m.mol./kg. whole axon/hr. Inclusion of the short axons made no difference to the slope of the regression line, but increased the mean sodium content 2 hr. after decapitation from $32 \pm 4$ m.mol./kg. to $36 \pm 4$ m.mol./kg. The average sodium content of the short axons, $64 \pm 5$ m.mol./kg. at 3·4 hr. from decapitation, was certainly significantly greater than that of the remaining axons, which was $45 \pm 3$ m.mol./kg. at the same time ($P = 0{\cdot}001$).

The resting rate of loss of potassium, calculated in the same way, was $7{\cdot}1 \pm 3{\cdot}5$ m.mol./kg./hr. taking all the results except those for the short axons. Omitting the values obtained by the chemical separation method it was $6{\cdot}9 \pm 2{\cdot}3$ m.mol./kg./hr., and the potassium content 2 hr. after decapitation was increased from $268 \pm 11$ m.mol./kg. to $282 \pm 8$ m.mol./kg. The difference was largely due to two low values, one of which was for an axon only 23 mm. long. Both these axons gave a correspondingly high sodium content, so that the discrepancy was probably caused by their poor condition rather than by an analytical error; they were among the earliest axons dissected, and may have been less well treated than the others.

### *The extracellular space of* Sepia *axons*

All the axons were washed in choline solution for 5 or 10 min. before being stored for analysis. The values plotted in Figs. 7 and 8 are expressed in m.mol./kg. wet weight of whole axon—the quantity actually determined. In order to calculate the true intracellular ionic concentrations it is necessary to know the effective depth of the connective tissue layer. An estimate of this can be obtained by comparing the results for axons soaked in choline with those for axons taken straight from sea water, since if the figures are for the same time from decapitation the intracellular sodium concentrations should be the same, but the extracellular concentrations should be zero in one case and 455 mM.—the sodium concentration in sea water—in the other. After multiplying the observed figures by 1·04, the approximate density (in g./cm.$^3$) of an axon, to convert them from m.mol./kg. whole axon to m.mol./l. axon, then if

$[Na]_{CH}$ and $[Na]_{SW}$ are the sodium concentrations (in m.mol./l. whole axon) for axons taken at corresponding times from choline and from sea water respectively, and if $[Na]_I$ is the true internal sodium concentration (in m.mol./l. axoplasm), it is easy to see that

$$\pi(r+d)^2[Na]_{CH}=\pi r^2[Na]_I \tag{1}$$

and

$$\pi(r+d)^2[Na]_{SW}=\pi r^2[Na]_I+\pi\{(r+d)^2-r^2\}\,455, \tag{2}$$

where the effective depth of the extracellular layer is $d$, and the radius of the axon proper is $r$. After eliminating $[Na]_I$ this gives

$$\frac{(r+d)^2}{r^2}=\frac{455}{455+[Na]_{CH}-[Na]_{SW}}. \tag{3}$$

Table 3 shows the value of $d$ calculated from analyses of four axons taken direct from sea water and not washed in choline. The average result was 12·8 $\mu$. This estimate may not be truly representative since so few measurements were made, and the individual values ranged from 5·2 to 20·1 $\mu$. However, it is close to the average figure obtained by Weidmann (1951) in a study of the electrical characteristics of *Sepia* axons, and provides a reasonably reliable basis for calculating the intracellular concentrations in the other analyses. The value of the factor $(r+d)^2/r^2$ given by the results in Table 3 is 1·20, so that to convert the figures in

TABLE 3. Analyses of *Sepia* axons taken from sea water

| Axon diam. ($\mu$.) | Time from decap. (hr.) | K concentration (m.mol./l.) | Na concentration (m.mol./l.) | [Na] in choline soaked axon (m.mol./l.) | Calculated depth of extracellular layer ($d$) ($\mu$.) |
|---|---|---|---|---|---|
| 300 | 8·1 | 307 | 119 | 89 | 5·2 |
| 305 | 9·2 | 281 | 146 | 100 | 8·5 |
| 265 | 1·5 | 258 | 127 | 29 | 17·2 |
| 207 | 2·1 | 223 | 171 | 35 | 20·2 |
| Average: | | | | | |
| 269 | — | — | — | — | 12·8 |

The Na and K contents of the axons have here been multiplied by 1·04 to express them in m.mol./l. whole axon, the figures in the penultimate column being obtained from the regression line in Fig. 7. The value of $d$ was calculated by applying equation (3) as described in the text.

m.mol./kg. whole axon to m.mol./l. axoplasm they must (from equation (1)) be multiplied by $1{\cdot}04\times1{\cdot}20$, that is by 1·25. This assumes, of course, that the effective thickness of the extracellular layer is a constant fraction (about 4·8 %) of the axon diameter. Young (1936) has found that the sheath thickness does increase with axon diameter, though the sheath is relatively slightly thinner in the largest axons. It is simplest to assume a constant correcting factor for all analyses, and this assumption is unlikely to introduce any large errors.

### *Errors in analysing* Sepia *axons*

It has so far been assumed that the period of washing the axons in choline solution was long enough to remove all the extracellular sodium, but not so long that there was an appreciable loss of intracellular sodium. It can be seen from the figures given in the preceding paper (Keynes, 1951*b*) for the rate of removal of extracellular $Na^{24}$ from *Sepia* axons that even in 5 min. washing should have been virtually complete. This was confirmed directly by dipping a *Sepia* axon for a few minutes in $Na^{24}$ sea water and then putting it in three dishes of choline solution for successive periods of 10 min. All but 0·2% of the $Na^{24}$ was washed off in the first dish. It has also been found with $Na^{24}$ (Keynes, unpublished) that there is no immediate change in the outward sodium flux when axons are transferred from sea water to choline. Taking the resting sodium exchange constant as 0·21 hr.$^{-1}$ (Keynes, 1951*b*), only 4% of the intracellular sodium would have been lost in 10 min. For most of the analyses the loss was less than this, since the washing period was usually nearer 5 min. than 10. Experiments with $K^{42}$ have shown that there is no rapid change in the potassium content of axons on transfer from sea water to choline. Calculation shows that the loss of sodium and potassium by longitudinal diffusion from the cut ends was negligible. There is thus no reason to suppose that any appreciable errors were introduced into the results by washing the axons in choline solution.

The five main causes of variation in the results were: analytical errors, weighing errors, variation in the thickness of the extracellular layer, variation in the condition of the axons, and variation from one animal to another. Analytical errors were probably the smallest source of uncertainty, since the S.E. for the $\beta$- and $\gamma$-count determinations was only of the order of $\pm 2\%$. Weighing errors were somewhat larger, since some of the smaller axons weighed as little as 0·3 mg., and it was impossible to prevent them from drying slightly while they were being blotted and transferred to the weighing bottle. The S.E.'s of the weights were less than $\pm 7\%$, since the S.D. of the individual figures for the weight/volume ratio was $\pm 9{\cdot}3\%$ (52 axons), and the weights were almost certainly more accurate than the volume estimates. For each of the 14 axons whose potassium content was determined by the $\beta$-count method the S.D. of the sum of the sodium and potassium contents was $\pm 7\%$, the average value for [Na]+[K] being 311 m.mol./kg. axon. Including the chemical determinations of $K^{42}$ as well (26 axons in all) the S.D. of [Na]+[K] was $\pm 10\%$, the average sum then being 293 m.mol./kg. Weighing errors and variation in the amount of extracellular material would have affected the separate values for [Na] and [K] in each axon to the same extent, so that the two effects combined must have given rise to a S.E. of less than $\pm 10\%$. The S.E.'s for individual determinations of [Na] and [K] were rather larger, being $\pm 33$ and $\pm 14\%$ respectively. This would be consistent with a tendency for a low sodium content to be associated with high potassium and vice versa, the sum [Na]+[K] remaining relatively constant. Inspection of Figs. 7 and 8 shows that there was apparently a tendency of this sort, but taking the fourteen most reliable pairs of figures the partial correlation coefficient between sodium and potassium contents was found to be 0·41, so that the effect was not quite statistically significant ($P = 0{\cdot}1$). The evidence suggests, however, that there is not much variation in the total sodium plus potassium in individual axons, and it would be surprising if the net leakage rate were the same in all axons, hence the correlation seems more likely to be genuine than not.

### *The sodium and potassium contents of stimulated* Sepia *axons*

Fig. 9 shows the results of analyses of the sodium and potassium in sixteen *Sepia* axons which had been stimulated for some time before being stored. As before, the potassium figures obtained by the chemical separation method were corrected by $+4{\cdot}4\%$. In four cases no reliable figures were obtained for [K], owing to an accident during incineration. The only results rejected were those for one axon 20 mm. long, and for a few axons whose threshold rose appreciably during the period of stimulation. Most of the axons had conducted about

120,000 impulses, though one (marked with arrows) had conducted twice as many.

In every case the stimulated axons contained more sodium and less potassium than resting axons at the same time from decapitation, as can be seen from the regression lines for resting sodium and potassium drawn on Fig. 9, and from the numerical values given in Table 4. The net losses and gains were worked out by subtracting the appropriate resting values, and were then expressed as

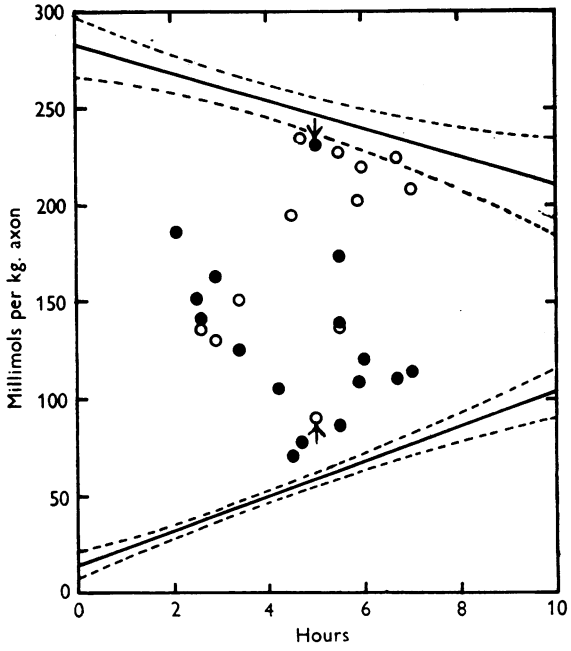


Fig. 9. The sodium (●) and potassium (○) contents of stimulated *Sepia* axons, expressed in m.mol./kg. of whole axon. Abscissa: time in hours from decapitation of the animal. The solid lines show the mean resting sodium and potassium values (from Figs. 7 and 8), and the dotted lines ± the S.E. of the mean. Most of the axons had conducted about 120,000 impulses. The values marked with arrows were for an axon which had been stimulated to exhaustion. For further information see Table 4.

the entry or exit per unit area of membrane per impulse. The over-all average gave a sodium entry of $3{\cdot}8 \times 10^{-12}$ mol./cm.$^2$/imp. and a potassium exit of $3{\cdot}6 \times 10^{-12}$ mol./cm.$^2$/imp. It is possible that these figures are not quite representative, since the axons stimulated at frequencies above 100 imp./sec. gave, averaged separately, a rather lower figure. The difference was statistically significant ($P = 0{\cdot}05$) in the case of sodium, but not ($P = 0{\cdot}2$) in the case of potassium. It would be reasonable to find a difference in this direction, since at the higher rate of stimulation each action potential would fall in the refractory period of the preceding one (the phase of positive after-potential lasts

about 10 msec.—Weidmann, 1951) and its size would be reduced. Part of the difference could, however, have arisen because the axons stimulated at the higher frequencies also happened to be rather larger than the average, or because they were left for somewhat longer periods from decapitation—the size of the action potential may well have been reduced as the condition of the axons deteriorated. The only axon stimulated at 50 imp./sec. gave a lower sodium entry and potassium exit than average, but in view of the appreciable errors in the analyses no great significance can be attached to a single observation. There was, in fact, insufficient data to show conclusively whether the ionic exchange varied much with frequency of stimulation or with the two other factors just mentioned.

TABLE 4. Analyses of stimulated *Sepia* axons

| Axon diam. ($\mu$.) | Time from decap. (hr.) | Na content: Stim. (m.mol./kg.) | Na content: Resting (m.mol./kg.) | Na entry ($\mu\mu$mol./cm.$^2$/imp.) | K content: Stim. (m.mol./kg.) | K content: Resting (m.mol./kg.) | K exit ($\mu\mu$mol./cm.$^2$/imp.) | Stimulation (min.) | Stimulation (imp./sec.) |
|---|---|---|---|---|---|---|---|---|---|
| 246 | 3·4 | 125 | 45 | 5·1 | *150 | 258 | 6·9 | 20 | 100 |
| 211 | 7·0 | 114 | 77 | 2·0 | *208 | 232 | 1·3 | 20 | 100 |
| 191 | 4·2 | 105 | 52 | 2·6 | — | — | — | 20 | 100 |
| 172 | 5·5 | 139 | 63 | 3·4 | *137 | 244 | 4·8 | 20 | 100 |
| 166 | 2·5 | 151 | 37 | 4·9 | — | — | — | 20 | 100 |
| 176 | 2·1 | 186 | 34 | 7·0 | — | — | — | 20 | 100 |
| 165 | 5·5 | 173 | 63 | 4·7 | — | — | — | 20 | 100 |
| 202 | 2·6 | 141 | 38 | 5·4 | 136 | 263 | 6·7 | 20 | 100 |
| 177 | 2·9 | 163 | 40 | 5·7 | 130 | 261 | 6·1 | 20 | 100 |
| 205 | 5·9 | 109 | 67 | 3·7 | 203 | 240 | 3·3 | 6 | 200 |
| 225 | 6·0 | 120 | 68 | 4·1 | 220 | 240 | 1·6 | 10 | 150 |
| 293 | 4·7 | 77 | 56 | 2·6 | 234 | 250 | 1·9 | 0·5 | 300 |
| | | | | | | | | +5·5 | 200 |
| 319 | 4·5 | 70 | 55 | 1·2 | 194 | 250 | 4·5 | 7 | 300 |
| 301 | 5·5 | 86 | 63 | 1·7 | 228 | 244 | 1·2 | 7 | 300 |
| 266 | 6·7 | 110 | 75 | 3·2 | 224 | 235 | 1·0 | 30 | 50 |
| 174 | 5·0 | †231 | 60 | 3·9 | †91 | 247 | 3·5 | c. 40 | 100 |
| Averages: | | | | | | | | | |
| 216 | — | — | — | 3·8±0·4 | | — | 3·6±0·7 | All results | |
| 192 | — | — | — | 4·4±0·4 | | — | 4·3±0·9 | Axons stim. at 50 or 100 imp./sec. | |
| 269 | — | — | — | 2·7±0·6 | | — | 2·5±0·6 | Axons stim. at 150–300 imp./sec. | |

The figures for resting axons were taken from the regression lines in Figs. 7 and 8. The three K analyses marked with an asterisk were done by the chemical separation method, the remainder by the $\beta$-count method. The net gains and losses were obtained from the differences between the pairs of figures, multiplied by 1·25 to convert them to m.mol./l. axoplasm (see text), and expressed in $\mu\mu$moles ($=10^{-12}$ moles) per cm.$^2$ membrane per impulse. The axon marked † was stimulated to exhaustion. The S.E. of the averages are shown.

Taking the S.D. for each analysis of the potassium in a stimulated axon as ±37 m.mol./kg. (from the results for resting axons), the S.E. of the mean of twelve determinations of the net K loss during activity would be about $\pm 37/\sqrt{12}$, or ±11 m.mol./kg. The twelve axons lost an average of 60 m.mol. K/kg., so

that on this basis the expected S.E. would be $\pm 18\%$ of the mean. The S.E. given in Table 4, which was worked out from the scatter of the results, was almost exactly the same, so that the individual variation in K exit could have been due wholly to sampling errors, rather than to real differences in K exit between the axons. The same is true for the sodium entry, where the expected value of the S.E. was $\pm 7\%$ of the mean net entry, as compared with an observed value of $\pm 10\%$.

Weidmann (1951) found a nearly equal net potassium leakage in *Sepia* axons, using the indirect method depending on changes in membrane resistance of axons stimulated in oil which was developed by Hodgkin & Huxley (1947). Tracer studies also gave a very similar result (Keynes, 1951*b*). The method used here was as direct as it could have been, so that the figure of $3{\cdot}6 \times 10^{-12}$ mol./cm.$^2$/imp. is probably the most reliable of the three estimates.

The total number of impulses conducted by each axon was about half the number they were capable of conducting before becoming exhausted. Two axons which were stimulated to exhaustion each conducted about 240,000 impulses at a rate of 100 imp./sec. before they finally became inexcitable. One of these was later analysed (see Table 4), and it was interesting to find that it did not fail until it had lost about two-thirds of the potassium it originally contained, and had gained an equivalent amount of sodium. This provides a striking example of the great margin of safety in the mechanism of nervous conduction.

*The sodium and potassium in squid axoplasm*

The values obtained for the concentrations of sodium and potassium in extruded squid axoplasm are given in Table 5. The average resting sodium content was $46 \pm 2$ m.mol./kg. axoplasm, which is close to Steinbach & Spiegelman's (1943) figure of 44 m.mol./kg. The potassium content was $323 \pm 10$ m.mol./kg., which is rather lower than their estimate of 369 m.mol./kg. The results were more consistent than those for *Sepia* axons, probably because weighing errors were smaller, and because there was no extracellular material with the axoplasm. No reliable indication of the net resting leakage rate was obtained, since the dissections were done fairly quickly, and there were no long intervals between the times when the samples were taken. Such changes as there were between the two resting samples taken from each animal were all in the direction of a loss of potassium and gain of sodium, but they were small, and were not statistically significant. Steinbach & Spiegelman found a net exchange of the order of 20 m.mol./kg./hr., but their experiments were apparently done at room temperature, whereas the dissections considered here were done at about 10° C.

Stimulation again caused a considerable increase in sodium and decrease in potassium content. The average changes caused by 30 min. of stimulation at 200 imp./sec. were +103 m.mol. Na/kg. axoplasm and −88 m.mol. K/kg.

Taking the density of axoplasm as 1·04 g./cm.$^3$, these figures correspond to a sodium entry of $3{\cdot}5 \times 10^{-12}$ mol./cm.$^2$/imp. and a potassium exit of $3{\cdot}0 \times 10^{-12}$ mol./cm.$^2$/imp. It is possible that a rather higher result might have been obtained if the axons had been stimulated at a lower frequency. On the other hand the resting exchange was ignored in calculating the results, and this would have made them slightly too high. The standard deviation for each analysis was $\pm 7\%$ of the mean (from the resting figures), whence the S.D. of each figure for the Na entry was about $\pm 0{\cdot}35 \times 10^{-12}$ mol./cm.$^2$/imp. The variation

TABLE 5. Sodium and potassium contents of squid axoplasm

| Axon no. | Axon diam. ($\mu$.) | Time from decap. (hr.) | Na content (m.mol./kg.) | K content (m.mol./kg.) | Na entry ($\mu\mu$mol./cm.$^2$/imp.) | K exit ($\mu\mu$mol./cm.$^2$/imp.) |
|---|---|---|---|---|---|---|
| 209S | 480 | 4·0 | 168 | 224 | 4·2 | 3·4 |
| 211R | 449 | 0·8 | 44 | 318 | — | — |
| 212S | ,, | 1·4 | 118 | 242 | 2·3 | 2·6 |
| 213R | 449 | 1·9 | 44 | 294 | — | — |
| 214S | ,, | 2·7 | 173 | 224 | 4·1 | 3·2 |
| 215R | 494 | 0·8 | 44 | 340 | — | — |
| 216S | ,, | 1·4 | 154 | 230 | 3·9 | 3·3 |
| 217R | 507 | 2·0 | 51 | 339 | — | — |
| 218S | ,, | 2·6 | 130 | 253 | 3·1 | 2·6 |
| Average resting | | | 46 | 323 | — | — |
| Average stimulated | | | 149 | 235 | 3·5 | 3·0 |

The samples bracketed together were taken from the same axon, either resting (R) or after stimulation (S) for 30 min. at 200 imp./sec. Samples 211–14 came from one animal, and 215–18 from another. The weight of the samples varied between 1·37 and 3·20 mg. Net losses and gains were calculated with reference to the overall average resting contents, and no account was taken of the time from decapitation. Axoplasm density was taken as 1·04 g./cm.$^3$.

between the individual axons was therefore genuine ($P < 0{\cdot}001$). For the K exit the S.D. was larger, $\pm 0{\cdot}55 \times 10^{-12}$ mol./cm.$^2$/imp., since the change in potassium content was smaller in proportion to the initial content, and there was no significant variation between the axons ($P = 0{\cdot}75$). Although in four cases out of five the sodium entry was appreciably larger than the potassium exit, the difference between the means was not statistically significant ($P = 0{\cdot}2$), and the fact that it was exactly equal to the chloride entry (see below) was purely fortuitous.

Rothenberg (1950) found that squid axons gained $4{\cdot}5 \times 10^{-12}$ mol./cm.$^2$/imp. of $Na^{24}$ on stimulation for 30 min. at 100 imp./sec. Grundfest & Nachmansohn (1950, and personal communication from Dr Grundfest) found a total gain of $Na^{24}$ amounting to 50 m.mol./kg. after 15 min. of stimulation at 100 imp./sec., and 64 m.mol./kg. after stimulation for twice as long. Taking the axon diameter as 500 $\mu$. these figures correspond to $Na^{24}$ entries of 6·9 and $4{\cdot}4 \times 10^{-12}$ mol./cm.$^2$/imp. respectively. Both these results are quite consistent with the net sodium entry of $3{\cdot}5 \times 10^{-12}$ mol./cm.$^2$/imp. found here, if in squid axons, as in *Sepia* (Keynes, 1951*b*), there is a large turn-over of sodium during activity in

addition to a net gain, since the total gain of $Na^{24}$ would then be larger than the true net entry of sodium, and would approach it more closely when the total number of impulses conducted was increased.

### *Phosphorus determinations*

Although the phosphorus content of the nerves was not of primary interest in the present connexion, it was worth examining the results to see whether they were self-consistent and whether they agreed with the analyses made by other workers using conventional methods.

The $P^{32}$ in the samples originated mostly from $P^{31}$, the normal stable isotope, by an $n,\gamma$ reaction, but there were also small amounts formed in the reactions $S^{31}(n,p)P^{32}$ and $Cl^{35}(n,\alpha)P^{32}$. The relative yields from the three reactions, in millicuries per mole of parent element, are 162:4:0·5. Taking the concentrations of sulphur and chloride in *Sepia* axons as 70 and 130 m.mol./kg. respectively (see below), the last two nuclear reactions would have given an amount of $P^{32}$ equivalent to that formed from about 2 m.mol./kg. of phosphorus. After allowing for this the phosphorus content of resting *Sepia* axons was $27{\cdot}9 \pm 0{\cdot}7$ m.mol./kg. (13 samples), while in axons which had been stimulated it was $26{\cdot}2 \pm 2{\cdot}5$ m.mol./kg. (5 samples). There was thus no significant difference between the resting and stimulated axons, and the average phosphorus content corrected for the two subsidiary sources of $P^{32}$ was about 27 m.mol./kg.

The corrected phosphorus content was $25{\cdot}3 \pm 0{\cdot}6$ m.mol./kg. in four samples of resting squid axoplasm, while in the stimulated samples it was $28{\cdot}8 \pm 1{\cdot}0$ m.mol./kg. (see Table 6). Taking each stimulated sample with its resting control there was apparently a significant increase ($P = 0{\cdot}03$) in the stimulated samples, amounting to $3{\cdot}5 \pm 1{\cdot}0$ m.mol./kg., or 14% of the resting value. The corrected average phosphorus content was again 27 m.mol./kg., which is not far from the figure of 30·5 m.mol./kg. for squid axoplasm reported by Bear & Schmitt (1939). It is impossible to tell without doing further experiments what importance should be attached to the apparent increase during activity. It might conceivably have been caused by a shrinkage of the nerve during stimulation, though it seems more likely from the work of Hill (1950) that there would have been a very small swelling. The total amount of phosphorus in the sea water in which the nerves were stimulated was less than $2 \times 10^{-8}$ moles, so that they could not possibly have derived more than 0·1% of the observed increase from this source. Alternatively there may perhaps be some variation in phosphorus content along the length of the axon: the resting controls were always taken from its distal end.

In six samples of *Carcinus* nerve the $P^{32}$ was equivalent to $51{\cdot}7 \pm 0{\cdot}9$ m.mol./kg. of phosphorus. The corrected phosphorus content was therefore about 50 m.mol./kg. Schmitt, Bear & Silber (1939) found 42 m.mol./kg. in lobster nerves.

### *Chloride determinations*

Since the only long-lived component present in any large quantity in the irradiated samples was $S^{35}$, and since this was formed mostly from an $n,p$ reaction with $Cl^{35}$, determination of the $S^{35}$ in the samples gave a measure of their chloride content. The results were less reliable than the sodium and potassium analyses, partly because of counting errors due to the softness of the $S^{35}$ $\beta$-radiation, and partly because of the difficulty of making accurate allowance for the small amount of long-lived $\beta$-radiation arising from parent elements other than chlorine, although this only introduced second-order errors when the difference in [Cl] between two groups of axons was considered. Estimation of chloride by activation analysis is therefore not to be preferred to existing techniques (Glick, 1949), but it was of considerable interest to deter-

mine the change in chloride content of the axoplasm during stimulation, in the samples for which sodium and potassium figures were already available. The method described here was the simplest and most convenient way of making these chloride analyses.

In addition to the $S^{35}$ formed from $Cl^{35}$, some originated in the reaction $S^{34}(n,\gamma)S^{35}$. Owing to the low abundance of $S^{34}$—4·2% of natural sulphur—100 m.mol./kg. of sulphur in the samples would have given a quantity of $S^{35}$ corresponding to only 8 m.mol./kg. of chloride. No figures for the total sulphur content of invertebrate nerves could be found in the literature, but it was estimated from the amount of the amino-sulphonic acid taurine in crab and *Sepia* nerves (Lewis, 1951*b*) that it was probably of the order of 70 m.mol./kg. The correction to be subtracted for $S^{34}$ was therefore 5·6 m.mol./kg. Taking the total nitrogen content of squid axons as 8 g./kg. (Bear & Schmitt, 1939), the correction to be made for $C^{14}$ formed in the reaction $N^{14}(n,p)C^{14}$ was calculated as 1·0 m.mol./kg. Taking the calcium content as 7·5 m.mol./kg. as in crustacean nerve (Tipton, 1934), the correction for $Ca^{44}(n,\gamma)Ca^{45}$ was 0·6 m.mol./kg. Using the figures given by Webb (1937) for the amounts of trace elements in marine animals, radiation from $Co^{60}$, $Fe^{59}$, $Cs^{134}$ and other isotopes formed in even smaller quantities would have been unlikely to have caused an error of more than 1 m.mol./kg. in all. The total correction for isotopes other than $S^{35}$ was therefore estimated as 2·6 m.mol./kg. No attempt was made to verify these calculations by estimating the various long-lived isotopes separately, as this would have been a most difficult task. But two determinations of the half-life of the long-lived component—which was $83 \pm 2$ days for the squid axoplasm sample of Fig. 3, and $89{\cdot}9 \pm 1{\cdot}3$ days for a sample of *Carcinus* nerve whose decay was followed for almost a year—gave results close enough to the correct one for $S^{35}$ (87·1 days) to confirm that little else was present.

The final figure to be subtracted from the apparent chloride content of the samples was thus $5{\cdot}6 + 2{\cdot}6$, which was taken as 8 m.mol./kg. Eight unstimulated *Sepia* axons then had a mean corrected chloride of $129 \pm 4$ m.mol./kg. Three stimulated *Sepia* axons, each of which had conducted about 110,000 impulses, contained $130 \pm 6$ m.mol./kg. of chloride. Although these figures show that stimulation caused no significant change in the chloride, it must be remembered that in a whole *Sepia* axon about 100 m.mol./kg. of the chloride is extracellular, so that only rather large changes in the intracellular chloride could have been measured reliably.

As Table 6 shows, four resting samples of squid axoplasm had an average chloride content of $72 \pm 6$ m.mol./kg., after making a correction of 8 m.mol./kg. as before. This was entirely intracellular, and agrees well with the figure of 75 m.mol./kg. obtained by Steinbach (1941) for axons soaked in sea water for an hour or more. Four stimulated samples had an average chloride of $89 \pm 5$ m.mol./kg. Taking each stimulated sample with its unstimulated control the

average gain in chloride was $17 \pm 4{\cdot}6$ m.mol./kg. This increase was statistically significant, a $t$-test giving $P = 0{\cdot}04$. As the stimulated axons had conducted 360,000 impulses, and as the mean axon diameter was $475\,\mu$., the corresponding figure for the net chloride entry was $0{\cdot}55 \times 10^{-12}$ mol./cm.$^2$/imp. The finding of a difference in phosphorus content between stimulated and control samples suggested that there might have been a longitudinal variation in the properties of the axoplasm, which might also have accounted for some of the difference in

TABLE 6. The phosphorus and chloride contents of squid axoplasm

| Resting samples | | | Stimulated samples | | |
|---|---|---|---|---|---|
| Sample no. | P content (m.mol./kg. axoplasm) | Cl content (m.mol./kg. axoplasm) | Sample no. | P content (m.mol./kg. axoplasm) | Cl content (m.mol./kg. axoplasm) |
| 211R | 26·6 | 81 | 212S | 28·9 | 105 |
| 213R | 25·4 | 70 | 214S | 31·5 | 83 |
| 215R | 23·8 | 57 | 216S | 27·9 | 82 |
| 217R | 25·4 | 80 | 218S | 27·0 | 86 |
| Mean | $25{\cdot}3 \pm 0{\cdot}6$ | $72 \pm 6$ | — | $28{\cdot}8 \pm 1{\cdot}0$ | $89 \pm 5$ |

Phosphorus was estimated from total $P^{32}$, and a correction of 2 m.mol./kg. has been subtracted (see text). Chloride was estimated from total $S^{35}$, and a correction of 8 m.mol./kg. has been subtracted. For grouping of samples see Table 5; all the resting samples were taken from the distal end of the axon. The S.E.'s of the means are given. The nerves were stimulated for 30 min. at 200 imp./sec.

chloride content. However, Steinbach (1941) found no consistent difference in the chloride content of squid axoplasm extruded from the proximal and distal parts of the axon. Moreover, it can be seen from Table 6 that there was no good evidence of any parallel variation in [P] and [Cl], though too few figures were available for any rigorous test of the correlation to be made. The increase in chloride content, +24% of the resting value, was nearly double that in phosphorus, and it seems probable that at least part of it was a real effect of activity.

## DISCUSSION

Enough has been said about the method of analysis to make clear its advantages and disadvantages. Its two chief merits are its extremely high sensitivity and the simplicity of the operations which have to be performed in applying it—though it may be questioned whether the method as a whole, necessitating as it does a neutron pile for irradiating the samples, can justifiably be termed simple. The smallest quantities of sodium on which it has been used were of the order of $0{\cdot}3\,\mu$g., but this is not the limit of sensitivity. By increasing the counting times, and by using a scintillation counter to obtain higher $\gamma$-counting rates, the same accuracy could be achieved for quantities 10 times smaller without too much trouble. It cannot, however, be emphasized too strongly that in using the method on tissues other than those considered here great care should be taken to see that there are no interfering trace elements present in sufficient

amounts to cause large errors. If serious interference were encountered, or if the ratio of sodium to potassium were much greater than it is in nerve and muscle, it might be necessary to add inactive carriers to the irradiated samples and then to separate either the $Na^{24}$ or $K^{42}$ by chemical means before attempting to count them.

The results show that in resting *Sepia* axons the net rates of potassium loss and of sodium gain did not differ significantly, and that their average value was about 10 m.mol./kg. axoplasm/hr. This agrees with the finding of Steinbach & Spiegelman (1943) that in squid axons the sum of the sodium and potassium contents was roughly constant, the net leakage rate being rather higher than in *Sepia*—about 20 m.mol./kg./hr.—probably because, as has already been mentioned, their axons were dissected at room temperature instead of being cooled to 10° C. The tracer experiments described in the preceding paper (Keynes, 1951*b*) also showed a sodium-potassium exchange in resting *Sepia* axons, and it was pointed out there that the running down of dissected axons was partly a consequence of the depolarization of the membrane caused by the flow of injury currents from the cut ends. Hodgkin & Katz (1949) have demonstrated that Steinbach & Spiegelman's figures for the magnitudes of the ionic leakage currents in squid axons, whose actual membrane potential is again considerably lower than the theoretical potassium diffusion potential, are consistent with their electrical data. Another factor which would cause a potassium leakage from resting axons is that the potassium concentration in sea water—about 10 mM.—is somewhat lower than that in *Sepia* and squid blood plasma. Manery (1939) and Robertson (1949) found potassium concentrations in squid and *Sepia* blood ranging from 15 to 22 mM. It therefore seems that experiments on cephalopod axons should ideally be done in a Ringer's solution containing more potassium than sea water.

It has been assumed throughout this work that the net ionic leakage currents can be regarded as constant, and that there is no tendency for the axons to come into a state of equilibrium with their surroundings—in other words that it is justifiable to fit a straight line to the points plotted in Figs. 7 and 8 rather than an exponential. This assumption is in contradiction with the suggestion of Steinbach & Spiegelman that the sodium-potassium exchange in squid axons reaches completion after about 4 hr. On the data presented here it would not be possible to reach any definite conclusion one way or the other, since too few axons were analysed and the results had rather a large scatter, but this applies equally to Steinbach & Spiegelman's analyses, none of their axons having been soaked in sea water for more than 4 hr. In experiments with $Na^{24}$ on *Sepia* axons (Keynes, 1951*b*), however, the resting net inward sodium current was found to increase rather than decrease with time, and according to the constant field equations derived by Hodgkin & Katz (1949) a steady state would not be reached until the ionic contents of the axoplasm were virtually

the same as those in the surrounding medium. The evidence therefore seems to favour the view that equilibrium is not reached, although the point cannot be considered as settled. Steinbach & Spiegelman may perhaps have been unduly influenced by the results obtained earlier by Steinbach (1941) on the chloride content of squid axoplasm, which did show a rapid initial increase immediately after dissection and thereafter only a rather slow increase. But, as Hodgkin & Katz have pointed out, this observation is quite consistent with the constant field equations if the internal chloride concentration is related closely to the membrane potential, and if the membrane potential is lower *in vitro* than in the living animal.

The importance of the measurements of the changes in sodium and potassium content caused by stimulation is that they provide for the first time direct proof that there is a net entry of sodium during nervous activity which roughly balances the loss of potassium. The experiments with $Na^{24}$ and $K^{42}$ on *Sepia* axons (Keynes, 1951*b*) could not prove this conclusively, even after figures for the resting internal ionic concentrations had been obtained by activation analysis, since there was always some uncertainty about the exact values of the outward sodium and potassium fluxes. Hodgkin (1949) has said that a crucial test of the sodium hypothesis of nervous conduction is to see whether the net entry of sodium in one impulse is large enough to charge the membrane capacity to the full extent of the action potential, as it must be if, as he supposes, the entry of sodium is the direct cause of the change in membrane potential. In squid axons the membrane capacity is $1{\cdot}5\,\mu$F./cm.$^2$, and the average action potential is of the order of 90 mV. (Hodgkin & Katz, 1949), so that on the sodium hypothesis the minimum sodium entry is

$$1{\cdot}5 \times 10^{-6} \times 90 \times 10^{-3}/96{,}500 \text{ mol./cm.}^2\text{/imp.},$$

that is $1{\cdot}4 \times 10^{-12}$ mol./cm.$^2$/imp. This is less than half the observed sodium entry, which is therefore quite large enough to perform the function assigned to it. In *Sepia* axons the membrane capacity is $1{\cdot}2\,\mu$F./cm.$^2$ and the height of the action potential is 125 mV. (Weidmann, 1951), giving a minimum entry of $1{\cdot}55 \times 10^{-12}$ mol./cm.$^2$/imp., which is again under half the observed net entry. The minimum outward potassium movement required to bring the membrane potential back to its resting value is the same as the minimum sodium entry, so that both for squid and *Sepia* there is once more, as for *Carcinus* (Keynes, 1951*a*), a considerable margin between the theoretical minimum and the actual potassium leakage during activity. These determinations of the net ionic movements during nervous activity hence provide further direct support for the sodium hypothesis.

Although the values for the chloride content of squid axoplasm were subject to some uncertainty, they show clearly that the net movement of chloride during activity was much smaller than that of sodium or potassium. The gain

of chloride was in fact no greater than might be expected if the internal chloride concentration were governed chiefly by the resting membrane potential. There was no direct evidence to show how the membrane potential was altered by a long period of stimulation, but the potassium concentration ratio between axoplasm and sea water fell to 0·73 of its resting value, corresponding to a drop of 7·8 mV. in the theoretical potassium diffusion potential, while the gain of 24% in chloride corresponds to a potential change of 5·5 mV. The chloride entry was therefore of the right order to have followed passively from the change in internal potassium concentration. It would be necessary to do experiments with radioactive chlorine to find whether there was a change in the chloride permeability of the membrane during activity, but the small size of the chloride entry supports the idea that $Cl^-$ ions play no active part in the transmission of nervous impulses. The figures obtained for squid axoplasm also show a close over-all balance between the movements of cations and anions. The exactness of the agreement must be largely accidental, in view of the appreciable S.E. of the figures, but it provides some evidence that no ions other than sodium, potassium, and chloride are involved in nervous activity.

Hill (1950) has suggested from a study of the swelling of *Sepia* axons during stimulation that about half the sodium which enters the axon is accompanied by chloride. An entry of chloride as large as this would almost certainly have been detected in the chloride analyses of stimulated *Sepia* axons, since it would have increased the total chloride in a whole axon by about one-third. It is conceivable that there might be a chloride entry of this magnitude if there were also a mechanism for extruding $Cl^-$ ions very rapidly after activity, since the chloride entry in the 2-min. periods of stimulation used by Hill might then be appreciably greater than in the longer periods used in these experiments. It would be necessary to use tracers to test this possibility. It is difficult, however, to envisage any mechanism which would be capable of extruding chloride at the necessary rate, and it seems rather unlikely that there is any large turn-over of chloride during activity. The chloride entry into stimulated squid axons was only about one-third of that required to account for the degree of swelling observed by Hill, and it therefore appears that in his experiments some other process tending to move water into the axoplasm may have been operative in addition to the entry of osmotically active ions.

## SUMMARY

1. The application of the technique of activation analysis to make simultaneous determinations of the sodium and potassium contents of single cephalopod axons is described. Nerves were irradiated for a week in a neutron pile and the amounts of $Na^{24}$ and $K^{42}$ which had been formed were found by taking $\beta$- and $\gamma$-counts of the irradiated samples with appropriate filters. Some of the earlier $K^{42}$ estimates were done by adding inactive $K_2CO_3$ as a carrier

and separating the potassium by a chemical method; this gave slightly low results, and was less reliable than the radiation discrimination method. Quantities down to 0·3 $\mu$g. of Na and 3 $\mu$g. of K were determined without difficulty, the S.E. of the analytical procedure being about $\pm 2\%$. The presence of $Br^{82}$ in the irradiated samples may have increased the apparent Na content by 1 %, but there was no appreciable interference from any other trace elements.

2. Resting *Sepia* axons, kept at about 10° C., gained sodium at a rate of 8·8 m.mol./kg. axon/hr., and lost potassium at roughly the same rate. The sodium content 2 hr. after decapitation of the animal was 32 m.mol./kg. axon, and the potassium content was 268 m.mol./kg. The axons were washed in an isotonic choline solution before analysis in order to remove extracellular sodium, and it was estimated that the results in m.mols./kg. whole axon should be multiplied by 1·25 to express them in m.mol./l. axoplasm.

3. Comparison of the ionic contents of resting and stimulated *Sepia* axons showed that during activity there was a net entry of $3{\cdot}8 \times 10^{-12}$ mol. Na/cm.$^2$/imp., and a net loss of $3{\cdot}6 \times 10^{-12}$ mol. K/cm.$^2$/imp.

4. Analyses of samples of extruded squid axoplasm gave average resting contents of 46 m.mol. Na/kg. axoplasm and 323 m.mol. K/kg. During stimulation at 200 imp./sec. there was a net entry of $3{\cdot}5 \times 10^{-12}$ mol. Na/cm.$^2$/imp. and a net loss of $3{\cdot}0 \times 10^{-12}$ mol. K/cm.$^2$/imp.

5. From the amounts of $P^{32}$ and $S^{35}$ in the irradiated samples rough estimates could be made of their total phosphorus and chloride contents. In *Sepia* axons stimulation had no significant effect on either P or Cl, but in the stimulated samples of squid axoplasm both were slightly increased, the net chloride entry amounting to about $0{\cdot}6 \times 10^{-12}$ mol./cm.$^2$/imp.

We are indebted to Dr A. G. Maddock for much helpful advice. We also wish to express our gratitude to the director and staff of the Laboratory of the Marine Biological Association for their hospitality at Plymouth, and to the Atomic Energy Research Establishment, Harwell, for irradiating our samples. The expenses of the work were met by a grant from the Rockefeller Foundation.

*Note added in proof.* The density of isolated *Sepia* axons has recently been determined by observing whether they sink or float in mixtures of artificial sea water (density 1·025 g./cm.$^3$) and a solution in which the sodium chloride was replaced by dextrose (density 1·056 g./cm.$^3$). Eight axons which were tested all had densities well within the range 1·050–1·056 g./cm.$^3$, and their average density was estimated as 1·053 g./cm.$^3$. The figure of 1·04 g./cm.$^3$ assumed in this paper is thus not far enough wrong to introduce serious errors in the results.

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