dried. Yield, about 2.0 g. with an activity of 400–600 units/mg. or seven to eight times higher than that of the impurities removed at pH 7.0.

The powder was almost water-free, but contained about 35% ash. The secretin activity/mg. organic dry substance is consequently 600–900 units/mg.

SUMMARY

1. Secretin has been extracted with methanol from the sodium chloride precipitate of an in-

testinal extract, as recommended by Friedman & Thomas.

2. Impurities were removed through repeated isoelectric precipitations from methanol at pH 7.

3. The final product had an activity of from 600 to 900 cat units/mg. ash-free substance, the yield being 1000–1600 units/m. of hog intestine.

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The Free Amino-acids of Invertebrate Nerve

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Many types of animal tissue possess an electrolyte composition which differs markedly from that of the body fluids in two important respects: first, the inorganic cations, largely sodium and potassium, outweigh the inorganic anions, chloride, sulphate and phosphate, i.e. there is an inorganic anion deficit; and, secondly, the concentration of potassium is greater (and, conversely, the concentrations of sodium and chloride are less) than in the extracellular fluid, i.e. there is an internal accumulation of potassium. This type of electrolyte distribution is highly developed in excitable tissues. Thus, the extruded axoplasm of giant squid nerves contains over 300 m-moles/l. of potassium and only about 50 and 70 m-moles/l. of sodium and chloride respectively, but squid blood contains only about 20 m-moles/l. of potassium and considerably more than 400 m-moles/l. of both sodium and chloride. In both vertebrate and invertebrate muscle tissue various phosphorus compounds account for most of the inorganic anion deficit; and this may be true also for vertebrate nerve which contains a very high concentration of phosphorus, much of which, however, may not contribute to the anion content of the axoplasm since it is combined with nitrogenous bases to form the phospholipins of the myelin sheath. But in the nerves of marine invertebrates such as the Crustacea and the cephalopod molluscs there is much less phosphorus, and here much of the deficit must be made up by purely organic anions.

The function of these internal anions is largely unknown. But they are almost certain to be important in connexion with the maintenance of a high internal potassium concentration, possibly by some mechanism of the type described by Boyle & Conway (1941). It is therefore important to know what the internal anions are, and this paper deals with the identification of the principal ones present in the nerves of certain marine invertebrates.

The first important attempt to identify the anions in these invertebrate nerves was made by Schmitt and his collaborators working first with extruded squid axoplasm (Bear & Schmitt, 1939) and later with aqueous extracts of lobster nerves (Schmitt, Bear & Silber, 1939; Silber, 1941). This work culminated in the isolation by Silber (1941) of large quantities of alanine and aspartic acid, the separate amounts of which he estimated from analyses of the lobster nerve extracts for total amino nitrogen and total amino-acid carboxyl groups assuming that no other amino-acids were present. The values he obtained—133 and 90 m-moles/kg. of alanine and aspartic acid respectively—left a large fraction of the anion deficit unaccounted for. But the presence of other amino-acids would invalidate these values; so a re-investigation of the amino-acid content of these nerves was essential before beginning a search for other anions.

A preliminary, qualitative survey showed that the nerves from all the seven marine invertebrate species tested contained appreciable amounts of

aspartic acid and alanine, and glutamic acid. All contained considerable amounts of a fourth amino-acid; in some this was glycine and in others the aminosulphonic acid taurine. Accurate analyses were therefore made of the free amino-acid content of nerves from three invertebrate species: *Carcinus maenas*, the shore crab, *Sepia officinalis*, the cuttlefish, and *Homarus vulgaris*, the lobster. For this purpose a micromethod was developed for the estimation of the amino-acids which were first separated by partition chromatography on filter paper. The concentrations of the major inorganic constituents in *Carcinus* nerve were also determined, and a cation-anion balance-sheet drawn up, from which it was clear that, in this tissue at least, very little of the anion deficit remained to be explained. An osmotic balance-sheet was also drawn up and showed that the unexplained osmotic deficit was also small when account was taken of the neutral amino-acids.

METHODS

General techniques

Preparation of material. All the analyses were carried out on freshly dissected tissues. The *Carcinus* leg nerves were dissected out by the procedure described by Keynes & Lewis (1951*a*). The nerve trunks so obtained were 4–5 cm. long; about 5 mm. was cut off each end, and the central portion was briefly, but carefully, blotted on filter paper and weighed on a microbalance. Single samples usually weighed 4–10 mg. Leg nerves from the other Crustacea were prepared in the same way but were normally longer and heavier than those from *Carcinus*. Single *Sepia* axons and samples of squid axoplasm were prepared in the way described by Keynes & Lewis (1951*b*) and averaged 1–2 mg. in weight. The *Carcinus* nerves were dissected out in the Ringer's solution described by Keynes & Lewis (1951*a*); nerves from the other species were dissected out in sea water.

Determination of the inorganic constituents. The values for the Na^+ , K^+ and total P contents of *Carcinus* nerve and the ways they were obtained have already been given by Keynes & Lewis (1951*a, b*). Cl^- was determined by the colorimetric method of Conway (1935) for amounts between 7 and 35 $\mu\text{g.}$; pairs of nerves were soaked for at least 4 hr. in 2.5 ml. of water, and two 1 ml. portions then taken for analysis. For the determination of Mg^{++} , the method devised by Robertson & Webb (1939) for the analysis of samples of marine invertebrate blood was adapted to much smaller amounts. This method involves two precipitations with oxine (the second in the presence of only a slight excess of reagent), bromination of the oxine in the precipitate, and titration of the excess BrO_3^- with $\text{Na}_2\text{S}_2\text{O}_4$ after the addition of KI. The procedure used by them was scaled down by a factor of 50 so that analyses on about 30 mg. of nerve were possible. A solution of 0.02N- $\text{Na}_2\text{S}_2\text{O}_4$ delivered from a horizontal Conway burette was used for the titration, and the filtrations were carried out with a small sintered-glass filter-stick and asbestos pad similar to that used by Cunningham, Kirk & Brooks (1941). An accuracy of 5–10% was obtained; this was sufficient for the present purpose since Mg^{++} is only a minor constituent of these invertebrate nerves.

Estimation of the extracellular space in *Carcinus* nerve. The reference substance used was a levan polysaccharide isolated from Italian rye-grass. A sample of this levan was very kindly supplied by Dr J. Beattie; some of its properties have been discussed by Palmer (1951) and by Bell & Palmer (1949). This polysaccharide was preferred to inulin since, though of a similar mean molecular weight, it is much more soluble in sea water at room temperature and so made possible the analysis of very small samples of tissue. Freshly dissected nerves were soaked in *Carcinus* Ringer containing 1% levan for periods of 2.5–5 hr., and each was then transferred to 1 ml. of distilled water for a similar period. After removal of the nerve residue, the amount of polysaccharide was determined by the method described by Harrison (1942); 2 ml. of the diphenylamine reagent were used for each analysis, and the intensity of the colour produced was determined photoelectrically with the apparatus, described below, which was used for the amino-acid analyses.

Micro-analysis of the amino-acids

The development of a suitable technique. For the determination of the principal free amino-acids in freshly dissected *Carcinus* nerve it would have been possible to use one of the established analytical methods, such as the chromatographic technique of Moore & Stein (1948) or, where applicable, one of the many enzymic methods—sufficient material could have been obtained by pooling many nerves. Some much more sensitive method was needed, however, for the determination of the composition of a single *Sepia* axon and for any investigation of the effect of various factors on the composition of individual *Carcinus* nerves. What was needed was a rapid method of estimating amino-acids in quantities of the order of 0.2–0.5 $\mu\text{g.}$ amino N with an accuracy of about 5%. None of the existing methods satisfied all three requirements of speed, sensitivity and accuracy, but it appeared that colorimetric estimation of the blue compound formed with ninhydrin offered the most promising basis for the development of a suitable technique, the individual amino-acids being first separated by partition chromatography on filter paper.

Development of the colour in aqueous solution after extraction of the amino-acid from the paper did not give sufficiently accurate results, probably because full colour development does not occur in dilute solution. The use of a reducing agent such as SnCl_2 is said to improve the reproducibility of colour development (see Landua & Awapara, 1949), but as this also leads to high and variable blank values it is unsatisfactory for the analysis of small samples. Development actually on the paper followed by extraction of the colour was found to give much better results, and the following method of extraction was found to be the most suitable: the circle of paper containing the coloured spot was rolled inside a vertical piece of glass tubing and a jet of steam directed into the open end from above; the colour was rapidly extracted down the paper, and the extract was collected from the lower end of the tube into which was fitted a thin disk of sintered glass to retain any fibres of filter paper detached by the steam. The extraction tube used in the present work was 3 cm. long and tapered from an internal diameter of 8 mm. at the top to one of about 3 mm. at the bottom. In this tube a piece of filter paper 4–5 sq.cm. in area can be extracted completely in 5 min. into a volume of less than 1 ml.

Development of a chromatogram by spraying with a

dilute solution of ninhydrin in butanol and heating to about 80° for 10–20 min. in an oven gives a very variable yield of colour, even the shade of colour varying from day to day. The influence of a number of factors on the colour development was therefore studied, and it was found that the best results could be obtained by adding collidine to the spraying fluid and then developing the chromatogram in a moist atmosphere. The use of collidine was suggested by the observation that chromatograms flowed with this solvent always gave very intense blue spots, and by the work of Harding & MacLean (1915) who found that the rate of formation and the yield of the blue compound produced in aqueous solution with excess ninhydrin were increased in the presence of a weak base such as pyridine. Since this technique was perfected, Woiwod (1949) has advocated the use of collidine in CHCl_3 solution. The possibility that the presence of water was necessary for optimum development was suggested by the fact that the formation of the blue compound is essentially an ionic reaction. No further improvement could be obtained by the addition of buffer solutions to the spraying mixture: both adverse pH and high concentrations of salts impair development. Under the conditions used for the quantitative analyses it was found that development was independent of the concentration of ninhydrin in the spraying fluid if this was greater than 0.2% (w/v).

Preparation of nerve extracts. Three methods were used. In the first experiments the nerve was extracted actually on the filter paper with alternate drops of CHCl_3 saturated with water and water saturated with CHCl_3 . This method was used because tracer experiments carried out in this laboratory (Keynes, unpublished work) had shown that saturation of sea water with CHCl_3 causes a rapid escape of the internal K^+ from these nerves. Alternatively, the nerve was suspended from a loop of Pt wire and extracted on to the paper, this procedure obviating the removal of the extracted residue from the paper. Extraction of the residues indicated that quantitative transfer of the amino-acids on to the paper was achieved. For the majority of the analyses, however, the nerve was transferred to a tiny test tube containing a weighed quantity of distilled water, and after about 1 hr. portions of the supernatant were transferred to the paper with a micropipette. Control experiments showed that the amino-acids escaped from the nerve with a half-time of 4–6 min., and that after less than 1 hr. the concentrations of amino-acids in the residue of the nerve and in the supernatant were effectively the same. No significant differences were observed in results obtained by the three methods, but the third was adopted for the routine analyses because there were fewer possible sources of error. The *Sepia* axons were soaked in water for only 30 min. since the amino-acids diffuse out very rapidly from these single fibres.

Chromatographic technique. One-dimensional chromatograms were used for the accurate quantitative analyses; samples of nerve extract and known volumes of a standard solution were placed alternately along the starting line at about 3 cm. intervals; and when the third method of extraction was used the same pipette was employed for both the extract and the standard solution. The method of flowing the chromatograms was essentially that used by Dent (1948); phenol and 'collidine' were the two solvents used, phenol always being the first solvent for two-dimensional chromatograms, but ammonia and diethylamine were not added as suggested, since they might interfere with the quantitative determinations. When aspartic and glutamic acids,

taurine and alanine were the four main amino-acids present, as was the case in most of the nerve extracts analysed, a complete separation could be obtained with a flow of about 20 cm. in phenol, which took about 7 hr. on the Whatman no. 4 filter paper used. Unfortunately, glycine and taurine coincide on a phenol chromatogram; so when both were present a parallel chromatogram was run in collidine, which separates taurine from all the other amino-acids. The amount of taurine present in the extract was determined from this collidine chromatogram, and the amount of glycine determined by difference from the phenol chromatogram. When the flow was complete, the excess solvent was removed at a temperature below 70° since higher temperatures cause significant losses of amino-acids (see Fowden, 1951).

Development and estimation of the colour formed with ninhydrin. When the paper was dry it was uniformly sprayed with a solution of 0.4% (w/v) ninhydrin in 10% (v/v) aqueous isopropanol to which 5% of collidine was added immediately beforehand. In the absence of the collidine this ninhydrin solution is stable indefinitely if protected from sunlight; isopropanol was preferred to the more normally used *n*-butanol which has an objectionable smell. The chromatogram was then suspended in a lead-lined tank in the bottom of which was fitted an electrically-heated water bath and a battery of electric light bulbs. Here, in a moist atmosphere at a temperature of 80–90°, development was allowed to proceed for 20–25 min.

Each spot was cut out in turn and extracted, by the method already described, into a tiny test tube containing a few crystals of sodium acetate. The extract was transferred quantitatively to a weighed colorimeter cell and the depth of colour and the weight of solution measured. The colorimetric measurements were made on a Hilger model H 760 Spekker absorptiometer with Ilford 606 filters; special cell-carriages were made so that small 1 cm. cells requiring only about 2.3 ml. of solution could be used. If protected from sunlight the chromatograms did not deteriorate for several hours at least; and the extracts were quite stable when the sodium acetate was included, the colour fading by less than 2% overnight.

Accuracy. In these analyses the composition of the standard solution was so adjusted that the amount of each amino-acid in the standard spot on the chromatogram was within $\pm 25\%$ of that in the sample spot. The amounts of the individual amino-acids in each spot ranged from about 0.025 μmole for glutamic acid and alanine to about 0.10 μmole for aspartic acid. In most of the experiments the standard error of a single estimation was $\pm 4\%$, but occasional chromatograms gave a larger standard error for no predictable reason. Duplicate estimations were normally made, two spots of the extract being included on the same chromatogram.

RESULTS

A preliminary survey

Preliminary, semi-quantitative analyses were made of the principal free amino-acids in nerves from seven species of marine invertebrates—five Crustacea and two cephalopod molluscs—and the results are shown in Table 1. Both one-dimensional and two-dimensional chromatograms of the nerve extracts were run, and the intensities of the spots

compared visually with those of standards flowed in parallel; the spots were not extracted for colorimetric estimation since the main purpose was to make a rapid survey of the occurrence of the individual amino-acids as a preliminary to a more detailed study of one or two individual species. A comparative survey was not contemplated, though such a survey should prove of interest since the variations, particularly of taurine and glycine, observed between different species were far greater than those between individual members of the same species.

Positive identification of the amino-acids

The identities of the five principal amino-acids were confirmed in a number of ways. Identification based solely on the position and colour of spots on chromatograms is inadequate and can be misleading. Thus, when a two-dimensional chromatogram of lobster nerve was run for the first time an intense brown spot with approximate R_f values of 0.41 and 0.22 was observed, and was assumed to be due to asparagine and not to glycine, which flows to almost the same position but is supposed to give

Table 1. *The approximate distribution of the principal free amino-acids in the nerves of some marine invertebrates and in Carcinus muscle*

(The results of a preliminary, semi-quantitative survey.)

Species	Tissue	Aspartic acid	Glutamic acid	Alanine	Taurine	Glycine
<i>Carcinus maenas</i>	Nerve	+++	++	++	++	-
<i>Cancer pagurus</i>	Nerve	+++	++	++	++	±
<i>Maia squinado</i>	Nerve	+++	+	+	+	++
<i>Homarus vulgaris</i>	Nerve	+++	+	++	±	++
<i>Palinurus vulgaris</i>	Nerve	+++	+	++	±	+
<i>Sepia officinalis</i>	Nerve	+++	++	+	+++	-
<i>Loligo forbesi</i>	Nerve	+++	++	++	+++	±
<i>Carcinus</i>	Muscle	-	±?	++	+++	+++

Probable concentrations in m-moles/kg. wet weight of tissue are: +++ = > 75; ++ = 30-90; + = 15-35; ± = 5-15; - = < 5. These ranges are approximate.

Ninhydrin-reacting substances other than the five given in Table 1 were often observed, though in traces only. Thus most of the chromatograms showed a yellow spot a short distance behind the phenol front, which was almost certainly due to proline, and a reddish spot a little further behind, which may have been due to carnosine. Frequently there were one or two faint spots which had moved only a short distance in collidine; these did not correspond to known amino-acids, but they were a normal blue in colour and were probably due to small molecular weight peptides or to simple derivatives of known amino-acids. Up to the present time, however, investigations have been confined to the five amino-acids listed in Table 1, which were responsible for the intense spots.

Since nerve and muscle have many features in common a few samples of invertebrate muscle were also analysed and the results are included for comparison in Table 1. Unlike nerve, *Carcinus* muscle contains no aspartic acid and little if any glutamic; traces of a ninhydrin-reacting substance which could be glutamic acid were found but were insufficient for identification. Alanine, taurine and glycine, however, are all present in large amounts together with a substance, at present unidentified, which flows slightly faster than alanine in phenol but only very slowly in collidine.

the normal blue or purple colour (Dent, 1948). Only later was this spot shown to be due entirely to glycine, which under the development conditions used here sometimes gives a spot that is brown at first and may not turn blue for several hours. The course and speed of colour development with ninhydrin are often of diagnostic value. Aspartic acid goes through a complex series of colour changes; a vivid emerald green can be obtained at an intermediate stage by developing the chromatogram under very dry conditions, and the development of the 'aspartic-acid spot' on nerve chromatograms matches at every stage that of a standard spot flowed in parallel. The colour changes of glycine mentioned above are also typical; and during the development of chromatograms of nerve extracts the spot due to glutamic acid is the first to appear and that due to taurine is the last.

Duplicate two-dimensional chromatograms of *Carcinus* nerve, to one of which appropriate amounts of the four amino-acids had first been added, showed no obvious differences in the distribution of colour in parallel spots; and preliminary treatment with basic copper acetate, as described by Crumpler & Dent (1949), left only the taurine spot unaffected. The separate components were also isolated by the method of Consden, Gordon & Martin (1947) from a 'multi-spot chromatogram' of a *Carcinus* nerve

extract flowed with phenol. The aspartate, glutamate and taurine fractions were found to be stable to the action of 6N-HCl for 24 hr. at 105°; and the glycine fraction similarly isolated from *Carcinus* muscle was also stable to this treatment. (No trace of aspartic acid was formed from the glycine fraction; so asparagine was absent.) The alanine fraction isolated from the nerve extract, however, contained traces of substances other than α -alanine, though this was certainly the major component. Several extra spots appeared on hydrolysis, including one probably due to glutamic acid; so there was definitely a small amount of a peptide present and perhaps also some glutamine. The amounts were small however; and the identity of the major component, which was stable to hydrolysis, cannot be in doubt, particularly in view of the findings of Silber (1941). In the next section the analytical figures given for alanine have not been corrected for the presence of these impurities, but the error thereby introduced should be small and should not materially affect any of the subsequent arguments.

The accurate determination of the amino-acids

The results of accurate analyses of nerves from three species are given in Table 2. Nerves from *Carcinus* and *Sepia* were analysed because they have been used for recent quantitative work in this laboratory and their electrolyte composition was therefore of especial interest. (Squid axons have also been used, but analyses of their axoplasm have not yet been made since the animals would not survive transport to Cambridge.) A few *Homarus* nerves were also analysed because Schmitt and his

Table 2. *The concentrations of the principal free amino-acids in the nerves of some marine invertebrates*

(All values in m-moles/kg. wet weight of nerve, $\pm$ S.E. All ten *Sepia* axons were obtained from three animals.)

Amino-acid	<i>Carcinus</i> whole leg nerve	<i>Sepia</i> single axon	<i>Homarus</i> whole leg nerve
Aspartic acid	138 $\pm$ 4.5	82	112
Glutamic acid	35 $\pm$ 1.5	39	25
Alanine	33 $\pm$ 3.5	21	33
Taurine	65 $\pm$ 5	103	c12
Glycine	< 5	< 5	35
Anion total	173 $\pm$ 5	122	137
No. of analyses	16	10	8
No. of nerves analysed	9	10	3

collaborators used the American lobster for their work, and also partly because *Homarus* was one of the species found to contain considerably more glycine than taurine. The standard errors of the means for *Carcinus* are included in Table 2; since biological variation is the main cause of uncertainty in these results the mean values given for *Sepia* and

Homarus are probably somewhat less reliable because fewer animals were used for the analyses. Though these results for *Homarus* differ widely from those arrived at by Silber (1941) they would give closely similar values for the total amino N and α -amino CO₂ determinations, upon which Silber's conclusions were based, as can be seen from Table 3 where the two sets of results are compared.

Table 3. *The amino-acids of lobster nerve; a comparison of the amino N and α -amino CO₂ values obtained by Silber (1941) with those deduced from results given in Table 2*

(All values are expressed as m-moles/kg. of nerve. The experimental procedure used by Silber was stated (Silber & Schmitt, 1940) to reveal the second carboxyl group of aspartate to the extent of 90% and that of glutamate to the extent of 3%; and the values for the α -amino CO₂ were calculated on the basis of these figures.)

	Concentration of amino-acid	Calculated values for	
		Amino N	α -Amino CO ₂
From values deduced by Silber:			
Aspartate	90	90	171
Alanine	133	133	133
Totals		223	304
From values given in Table 2:			
Aspartate	112	112	213
Glutamate	25	25	26
Alanine	33	33	33
Taurine	12	12	0
Glycine	35	35	35
Totals		217	307

The cation-anion balance in Carcinus nerve

In order to assess the importance of these dicarboxylic amino-acids as internal anions a cation-anion balance-sheet was drawn up for *Carcinus* nerve and is reproduced in Table 4. The concentrations of the major constituents (sodium, potassium, magnesium, chloride, total phosphorus, aspartate and glutamate) were determined by direct analysis of freshly dissected nerves. There is considerable biological variation; so sufficient analyses were made to reduce the standard error of the mean for each constituent to about 4 m-equiv./kg. or less. The concentrations of sodium, potassium and total phosphorus were of interest in other connexions, and values have already been published by Keynes & Lewis (1951a, b). Values of 257 and 262 m-moles/kg. for the potassium content were obtained by two different methods of analysis and 260 is used here as a reasonable average figure. Values for some minor constituents were arrived at from figures given in the literature for other marine crustacean nerves; variations between species are unlikely to introduce a large absolute error since the estimated total contribution made by the ions concerned (calcium, bicarbonate, lactate and keto acids) is only about 50 m-equiv./kg., i.e. some 6% of the total electrolyte

content. Estimation of the total anion contribution made by the phosphorus compounds was complicated by the fact that no one complete analysis of them appears to have been made. Use was therefore made of two partial analyses of the phosphorus fractions in *Homarus* nerve, those of the acid-soluble fraction by Engel & Gerard (1935) and of the lipid fraction by Brante (1949), from which the total anion contribution in *Carcinus* was estimated on the assumption that the relative distribution of phosphorus compounds is the same in both species. It was also assumed that the pH of the axoplasm was about 7.0. Such an estimate is obviously only approximate but is unlikely to be in error by much more than 5 m-equiv./kg.

Table 4. *The cation-anion balance in Carcinus nerve*

(All values are expressed as m-equiv./kg. wet weight.)

Electrolyte	Ionic concentrations		Anion deficit remaining
	Mean $\pm$ S.E.	Group totals	
Sodium	152 $\pm$ 5	—	—
Potassium	260 $\pm$ 4	—	—
Magnesium	23 $\pm$ 3	—	—
Calcium*	13	448	448
Chloride	145 $\pm$ 2	—	—
Sulphate†	9	—	—
Bicarbonate‡	9	163	285
Phosphorus compounds§	45	—	—
Keto acids‡	14	—	—
Lactate	13	72	213
Aspartate	138	173	40
Glutamate	35 $\pm$ 5		

* Value obtained by Tipton (1934) for *Libinia* and *Homarus*.

† Calculated value for sulphate in the extracellular space where it is assumed to be at the same concentration as in Ringer.

‡ Values calculated from figures of Silber's (1941) for *Homarus*.

§ The method used to estimate the contribution of the phosphorus compounds is given in the text.

|| Value obtained by Holmes (1929) for *Cancer*.

No account has been taken of the base-binding power of the proteins of the axoplasm or of any peptides that may be present. The dicarboxylic amino-acids are assumed to bind one equivalent of cation and the mono-carboxylic amino-acids none; the small contribution which taurine may make by virtue of its strongly acidic sulphonc acid group is neglected. Values are given in the table for lactate and keto acids; but there are a number of other acidic intermediates of carbohydrate metabolism, such as acetic and succinic acids, which may be present in significant amounts. The presence of organic cations would naturally increase the anion deficit, but they rarely occur in high concentrations in biological tissues. All these omissions from the table do not, however, radically affect the general picture or the conclusion that the anion deficit which remains un-

explained is quite small. That the deficit due to unidentified small molecular weight anions may be even less than indicated is suggested by a few analyses of the nerve residues obtained after soaking in distilled water to release the amino-acids. When calculated in terms of m-moles/kg. of water, the concentrations of taurine and alanine in the nerve residue approximated to those in the supernatant and the concentrations of aspartic and glutamic acids were a little less, though not significantly so, whereas some 8 % of the total sodium and potassium of the nerve and 45 % of the total phosphorus were retained in excess of that necessary for equivalence with the supernatant. These results suggest that the nerve residue contains fixed, or very slowly diffusing, anions which set up a Donnan equilibrium between the interior of the residue and the supernatant; and this would account for the fact that Silber (1941) failed to extract the last 10 % of the cations from lobster nerve. It is obvious that the unextracted phosphorus must account for much of the cation-binding power of the residue; but if the value of 45 m-equiv./kg. given in Table 4 for the total anion contribution of phosphorus compounds is correct, about 15 m-equiv./kg. of sodium and potassium must be retained by other anions. These considerations reduce the overall deficit to the order of 25 m-equiv./kg., a value so small that there is unlikely to be any organic anion in *Carcinus* nerve present in amounts comparable with those of aspartate and glutamate. This conclusion is not justified for *Sepia*, however, where the balance does not appear to be so complete since the sum of aspartate and glutamate is about 50 m-equiv./kg. less than in *Carcinus*, although the sum of sodium and potassium is almost the same. Owing to the very small weight of a single *Sepia* axon no attempt has yet been made to look for other anions which might account for this difference. *Homarus* appears to be intermediate, and although aspartate is certainly the principal organic anion there may be unidentified anions which are as important as glutamate. Nevertheless, it is true to say that in all the seven invertebrate species studied aspartate and glutamate must make up a large fraction of the total internal anions and therefore balance much of the potassium present.

The osmotic balance in Carcinus nerve

Just as the aspartate and glutamate help to make up the total anionic concentration necessary to balance the internal potassium, so, it may be argued, the other amino-acids help to make up the total solute concentration necessary to maintain osmotic equilibrium with the blood. This appears to be a justifiable argument since Hill (1950) has concluded that single *Sepia* axons behave as almost perfect osmometers so that the maintenance of the

internal osmotic pressure is probably important for optimum functioning of the nerves. An approximate osmotic balance sheet was therefore drawn up for *Carcinus* nerve and is reproduced in Table 5. Until more is known about the physical conditions existing in the axoplasm an exact treatment of this problem is impossible; so in the table all the solute species are assumed to have osmotic activities proportional to their molal concentrations. Only

Table 5. *The osmotic balance in Carcinus nerve*

Water content ... Osmotic activities ...	Nerve 865 g./kg. (mg. ions/kg. nerve)		Ringer 992 g./l. (mg. ions/l. Ringer)
	Individual activities	Running total	
Sodium	152	152	495
Potassium	260	412	11
Chloride	145	557	532
Calcium, magnesium and sulphate	12	569	49
		Total	1087
Acid-soluble phosphorus compounds	16	585	
Bicarbonate, lactate and keto acids	36	621	—
Aspartate and glutamate	173	794	—
Taurine and alanine	98	892	—
Unexplained deficit	56	948	—
Totals (in mg. ions/kg. water)		1096	1096

that part of the calcium and magnesium which is in the extracellular space is included since most of the intracellular fraction is probably bound to proteins. The osmotic contribution of the proteins themselves is also neglected but is certainly quite small. The unexplained osmotic deficit is small (56 mg. ions/kg. of nerve) and is little larger than the unexplained anion deficit; so it is unlikely that appreciable amounts of undiscovered solutes exist within the axoplasm. The osmotic deficit would be considerable but for the taurine and alanine which together form more than 10% of the total solutes in these nerves.

The extracellular space of Carcinus nerve and the probable intracellular ionic concentrations

It is often desirable to know the actual electrolyte concentrations in the axoplasm, and since these can be calculated from whole-nerve analyses only

if the fraction of extracellular space is known, an attempt was made to measure this fraction in *Carcinus* nerve. The mean of seven determinations was 0.242 ± 0.011 l./kg. This value is probably slightly low since it was obtained with a polysaccharide sample which, though it had a mean molecular weight of 5000–6000, almost certainly contained traces of high molecular weight material which might penetrate some parts of the true extracellular space only very slowly. A maximum value of 0.272 l./kg. can be deduced, however, from the figures for the chloride concentrations, 532 m-moles/l. in the *Carcinus* Ringer and 145 m-moles/kg. in the fresh nerve, on the assumption that the nerve axoplasm contains no chloride, but this value is probably too high since the large fibres in both squid and *Sepia* have been shown to contain appreciable internal chloride (Steinbach, 1941; Keynes & Lewis, 1951b). Thus the true mean value for the extracellular space can reasonably be assumed to lie between 0.24 and 0.27, and these two extremes were used to calculate the pairs of values for the intracellular concentrations given in Table 6.

DISCUSSION

This investigation began as a search for the organic anions which balance the internal potassium of invertebrate nerves. It has shown that the amino-acids are a conspicuous group of electrolytes in these nerves; in *Carcinus* nerve, for example, the aspartate and glutamate are together sufficient to balance about two-thirds of the internal potassium, and the alanine and taurine, whilst they have no significant cation-binding power, contribute over 10% of the total osmotic pressure of the axoplasm.

These amino-acids undoubtedly exist in the free state in the axoplasm. When a nerve trunk is placed in distilled water they come out with a half period of about 5 min. compared with a half period of about 3 min. for the loss of radioactive sodium ions from the extracellular spaces of a nerve placed in inactive Ringer (Keynes & Lewis, 1951a). Thus the half period for the escape of the amino-acids is so short that their release from the axoplasm must be very rapid, so rapid that their formation by enzymic action would be impossible since the 'free'

Table 6. *The probable intracellular concentrations of the principal electrolytes in Carcinus nerve*

Electrolyte	Concentration		Quantity in 1 kg. of nerve		Probable intracellular concentration (m-moles/kg. water)
	In nerve (m-moles/kg.)	In Ringer (m-moles/l.)	Extracellular (m-moles)	Intracellular (m-moles)	
Sodium	152	495	119–134	33–18	53–30
Potassium	260	11	3	257	412–432
Chloride	145	532	128–144	17–1	<27
Aspartate and glutamate	173	—	—	173	276–291
Alanine and taurine	98	—	—	98	157–165

amino-acids form 25 % of the total dry weight of the nerve!

Substances present in such large amounts are likely to have important functions to perform. It seems reasonable to suppose that two very important functions of these amino-acids are to provide, on the one hand, the anions needed to balance the essential internal cations, and, on the other hand, the extra solutes necessary for osmotic equilibrium; and support for this supposition is provided by the two balance sheets drawn up for *Carcinus* nerve since both the cation-anion and the osmotic balances are very close when account is taken of the contributions made by the amino-acids.

The distribution of free amino-acids in other excitable tissues differs markedly from that found in these invertebrate nerves. Thus *Carcinus* muscle (see Table 1) contains little or no aspartate or glutamate but large quantities of alanine, taurine and glycine, and Camien, Sarlet, Duchateau & Florkin (1951) have recently reported the presence of large amounts of glycine and proline in *Homarus* muscle. Taurine has long been known to occur in marine invertebrates. Kelly (1904) isolated it from *Mytilus* muscle, which from her results would appear to contain about 120 m-moles/kg., and similar amounts have been reported to occur in other marine creatures. (Kelly (1904) appears to have been misquoted by several authors. Her value of 5 % for the taurine content of *Mytilus* muscle undoubtedly refers to tissue which had been dried at 110°, but Webb & Young (1940) and Krogh (1939) assume that it refers to fresh tissue: since the dry weight is about one-third of the wet weight, fresh *Mytilus* muscle must, in fact, contain only about 1.6 % taurine.) Baldwin (1947) has suggested that it may play an important part in regulating the intracellular osmotic pressure, and this suggestion is probably correct not only for taurine, but also for other non-toxic, nitrogenous substances, such as glycine, glycine betaine and trimethylamine oxide, which have been reported to occur in significant amounts in the muscle tissues of various marine invertebrates. The comparative absence of aspartate and glutamate in invertebrate muscle may be correlated with the fact that a large fraction of the internal potassium can be balanced by organic phosphorus compounds which are presumably associated with the contractile mechanism, for Engel & Gerard (1935) found a total of 66 m-moles/kg. acid-soluble phosphorus in *Limulus* muscle compared with only 13 m-moles/kg. in *Homarus* nerve.

Vertebrate excitable tissues, on the other hand, contain only small amounts of free amino-acids. Awapara, Landua & Fuerst (1950) have made a fairly comprehensive survey of the amino-acid content of the tissues of the rat; their values for the

excitable tissues have been recalculated and are given in Table 7 in m-moles/kg. of fresh tissue. The relative distribution of the individual amino-acids is closely similar to that found in the invertebrates:

Table 7. *Distribution of free amino-acids in the excitable tissues of the rat*

(Data of Awapara *et al.* (1950) recalculated: all values in m-moles/kg. wet weight of tissue.)

Amino-acid	Muscle	Brain
Aspartate	<1	3.6
Glutamate	1.7	9.2
Alanine	4.4	<1
Taurine	11.6	2.4
Glycine	5.7	<1

taurine and alanine predominate in muscle and the dicarboxylic amino-acids predominate in nerve (i.e. brain), though the proportion of aspartate in the latter is small. Similar values have been reported in other mammalian excitable tissues, and it has been confirmed that concentrations of the same order exist in several types of vertebrate brain and peripheral nerve.

Thus, of the tissues discussed above, only invertebrate nerve contains more than a few m-moles/kg. of aspartate, and its presence there in such large amounts is most probably due to the need for maintaining a very high internal potassium concentration. Similarly, the fact that invertebrate excitable tissues contain far higher concentrations of the three neutral amino-acids than do the vertebrate ones is probably a direct consequence of the much higher internal osmotic pressure which the marine invertebrates have to maintain. Glutamate, though its concentration is less in vertebrate than in invertebrate nerve, balances about the same fraction of the potassium in both; so it may, perhaps, have a fundamental part to play in nervous conduction. These amino-acids may, of course, have important biochemical roles to play since alanine, aspartate and glutamate are all closely connected with aerobic glycolysis; and it is perhaps significant that glutamate appears to be one of the key substances in the biochemistry of brain (see, for example, Weil-Malherbe, 1950; Turner, Eggleston & Krebs, 1950). But in these invertebrate nerves it seems likely that aspartate and glutamate are important principally as internal anions, possibly as indiffusible anions which thereby set up some form of a Donnan equilibrium similar to that suggested by Boyle & Conway (1941) for frog muscle.

SUMMARY

1. A study has been made of the amino-acids present in aqueous extracts of nerves from certain marine Crustacea and cephalopod molluscs.

2. Nerves from all seven species tested contained large amounts of aspartic acid, glutamic acid and alanine, plus taurine in some species and glycine in others. These amino-acids account for over 20 % of the dry weight of the nerves and are almost certainly in free solution in the axoplasm.

3. The concentrations of these amino-acids present in three species were accurately determined by a micromethod developed for the purpose: paper chromatograms of the nerve extracts were developed with ninhydrin and the amount of colour extracted with steam was estimated photoelectrically. As little as 0.05 μ mole of an individual amino-acid could be estimated with a standard error of about $\pm 5\%$.

4. The major inorganic electrolytes in *Carcinus maenas* nerve were estimated, and balance-sheets were drawn up for the cation-anion and osmotic

balances, in both of which the unexplained deficit was small when account was taken of the free amino-acids present.

5. The possible functional significance of these amino-acids is briefly discussed: it is suggested that the dicarboxylic amino-acids function as internal anions to balance the high internal potassium concentration necessary for nervous conduction and that the other, 'neutral', amino-acids function as solute species to maintain the high internal osmotic pressure necessary for equilibrium with sea water.

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