

STUDIES ON THE CHEMICAL COMPOSITION OF MUSCLE TISSUE

III. THE MANTLE MUSCLE OF CEPHALOPOD MOLLUSCS

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I. INTRODUCTION

This is the third of a series of papers on the composition of muscles of some lower vertebrates and invertebrates, particularly from the standpoints of the osmotic concentration of various constituents and the accumulation and reduction of ions in muscle relative to those of the blood plasma. Mantle muscle of three species of cephalopods has been analysed for all the inorganic and organic compounds believed to be of osmotic significance, and the relative roles of inorganic ions, phosphates and nitrogenous constituents compared in muscle and plasma. Estimates have been made of the extracellular fluid space in the muscle, so that by appropriate correction the intracellular concentrations of ions and compounds could be calculated. Some direct determinations of the osmotic concentration of muscle, plasma and sea water have been made in one species.

The cephalopods studied were the cuttlefish *Sepia officinalis* (L.), the squid *Loligo forbesi* Steenstrup and the lesser octopus *Eledone cirrhosa* (Lamarck).

Blood was obtained by pipette from the anterior vena cava (*Sepia*, *Eledone*) or posterior mantle veins (*Loligo*), and after centrifugation the plasma was analysed as described previously (Robertson, 1949, 1960*a*). Pieces of mantle muscle, taken from the same specimens, were blotted and the outer epithelium and connective tissue were carefully removed on each side. Weighed samples of the prepared muscle were then taken for the determination of inorganic cations, chloride, sulphate and water content by methods used previously (Robertson, 1960*b*). The specimens of *Sepia* and *Eledone* had been collected 1 or 2 days previously and had been allowed to equilibrate for 24 hr. in sea water of the same salinity as that of their previous habitat. *Loligo* could not be kept alive, and was analysed directly after capture.

Muscle and plasma from other specimens were taken for estimations of lactate, nitrogenous compounds and phosphates, using the same techniques as in the study of *Nephrops* (Robertson, 1961). Glycerol in muscle was determined in zinc hydroxide filtrates using Lambert & Neish's (1950) method.

For measurements of extracellular fluid in muscle, both blood and interstitial fluid, 1-2 ml. of an 8% solution of inulin in sea water were injected into the anterior vena cava in *Eledone* and *Sepia*. After periods varying from 4 to 8 hr. in different specimens samples of blood and muscle were removed and zinc hydroxide filtrates were prepared, in which fructose was determined photometrically after hydrolysis of

inulin (Roe, Epstein & Goldstein, 1949). Isosmotic sucrose and sodium thiosulphate were also used as possible indicators of extracellular space, 2–5 ml. of the former being injected according to the size of the specimens (e.g. 5 ml. into a *Sepia* of 900 g.), and 0.5 ml. of the latter. Thiosulphate was measured in tungstic acid filtrates according to Gilman, Philips & Koelle (1946) and the fructose content of the sucrose usually as above. Some determinations of sucrose were also made by measuring the increase of reducing sugar in filtrates after hydrolysis of the sucrose with an invertase preparation. Most of these had to be discarded after it was realized that sufficient time was not always given to overcome the strong inhibition of the enzyme by muscle extracts, an inhibition absent in the plasma extracts.

Chloride was estimated by Volhard titration in the same zinc hydroxide or tungstic acid filtrates, giving chloride 'spaces' for comparison with the presumed extracellular spaces.

An estimate of the volume of blood in muscle is given by comparing the copper contents of blood and muscle, since haemocyanin is the chief copper-containing compound in the muscle. For these estimates, copper was determined in trichloroacetic acid filtrates by the method of Eden & Green (1940).

II. INORGANIC IONS

(1) *Composition of whole muscle and plasma*

As seen in Table 1, three comparisons of muscle, plasma and sea water with respect to the major inorganic ions and total phosphate show close agreement within a species and relatively minor differences between the species.

In all, the sum of the investigated ions in muscle comes to slightly less than half of the total concentration in plasma and sea water, leaving half the total osmotic concentration to be made up by organic compounds other than those of phosphorus (see § III below). The concentrations of ions in the plasma are within 2% of those in the sea water in which the animals had been kept (*Sepia*, *Eledone*) or from which they had been trawled (*Loligo*). Most of these plasma analyses have been discussed previously in another form (Robertson, 1949, 1953). Compared with sea water, the plasma has higher concentrations of potassium, calcium and chloride, an almost equivalent magnesium concentration, and lower values of sodium and sulphate.

In relation to the plasma, the pattern of ions in the muscle is consistent in all specimens of the three species: high values of potassium and lower values of sodium, chloride, calcium, magnesium and sulphate, with very high levels of acid-soluble phosphorus. *Sepia* has the highest level of potassium ions (170–180 mg. ions/kg. water) and the lowest level of sulphate (1.7–3.4 mg. ions) of the three species, *Eledone* the highest sodium (88–95 mg. ions) and chloride values (106–147 mg. ions), and *Loligo* the highest phosphorus values (133–169 mg. atoms).

(2) *Apparent extracellular spaces in muscle*

The chloride space in muscle is obtained by expressing the chloride concentration in muscle as a percentage of that in plasma (both on a water-content basis). If chloride were completely absent from muscle cells, the chloride space would represent the volume of muscle water which is extracellular, with the same chloride concentration

Table 1. Ionic composition of cephalopod whole muscle and blood plasma

		mg. ions/kg. water						Total P	Total mg. ions	Water g./kg. or g./l.
Species		Na	K	Ca	Mg	Cl	SO ₄			
<i>Sepia officinalis</i>										
♂ (1)	Muscle	74.3	180.0	2.91	21.7	95.5	1.9	138.0	514	742
	Plasma	460	23.8	10.8	56.9	589	4.9	—	1145	894
♀ (2)	Muscle	69.1	170.1	2.76	23.3	90.0	3.4	120.3	479	749
	Plasma	465	21.4	12.5	58.7	589	8.1	—	1155	895
♀ (3)	Muscle	59.8	174.1	2.46	21.9	88.6	1.7	130.2	479	741
	Plasma	470	20.6	11.5	57.6	594	5.8	—	1160	888
Means										
	Muscle	67.7	174.7	2.71	22.3	91.4	2.4	129.5	491	744
	Plasma	465	21.9	11.6	57.7	591	6.3	—	1154	892
	Sea water	492	10.5	10.8	56.1	575	29.6	—	1174	989
<i>Loligo forbesi</i>										
♂ (1)	Muscle	71.4	150.2	2.74	13.4	81.5	2.9	133.0	450	767
	Plasma	414	17.3	11.0	50.3	514	8.6	—	1015	868
♂ (2)	Muscle	68.0	156.3	3.39	13.1	79.8	3.1	137.2	459	763
	Plasma	417	22.8	11.3	51.3	525	6.0	—	1033	871
— (3)	Muscle	94.6	149.6	2.96	19.0	112.6	1.8	168.8	549	765
	Plasma	426	21.6	11.6	53.2	527	7.4	—	1047	860
Means										
	Muscle	78.0	152.0	3.03	15.2	91.3	2.6	146.3	488	765
	Plasma	419	20.6	11.3	51.6	522	7.3	—	1032	866
	Sea water	433	9.2	9.5	49.4	506	26.1	—	1033	989
<i>Eledone cirrhosa</i>										
♀ (1)	Muscle	93.5	142.7	4.19	20.3	106.3	7.0	116.2	490	756
	Plasma	438	13.0	11.0	54.6	513	20.7	—	1050	891
	Sea water	436	9.3	9.6	49.8	510	26.3	—	1041	989
— (2)	Muscle	87.8	140.7	3.32	25.4	114.5	7.5	117.8	497	769
	Plasma	412	13.1	11.0	51.0	498	17.9	—	1003	895
	Sea water	425	9.0	9.3	48.4	496	25.6	—	1013	989
♀ (3)	Muscle	94.5	150.2	5.30	22.6	147.0	6.3	120.7	547	762
	Plasma	445	17.0	11.7	57.1	536	23.3	—	1090	902
	Sea water	454	9.7	10.0	51.7	530	27.3	—	1083	989
Means										
	Muscle	91.9	144.5	4.27	22.8	122.6	6.9	118.2	511	762
	Plasma	432	14.4	11.2	54.2	516	20.6	—	1048	896
	Sea water	438	9.3	9.6	50.0	512	26.4	—	1046	989

The figures for P (trichloroacetic acid-soluble P) are really mg. atoms. The mg. ions are slightly lower, since the adenosine triphosphate molecule has 3 atoms P. The sex of *Loligo* (3) and *Eledone* (2) was not recorded.

as the plasma. In *Sepia* and *Eledone* these chloride spaces vary round a mean of 16% in the former and 22% in the latter (Table 2).

The fraction of total muscle water calculated as containing copper in the same concentration as in the plasma is within 0.8–2.8% in both species. This would seem to set a limit to the volume of extracellular fluid in the blood capillaries of the muscle, and is taken as equivalent to the maximum volume or weight of plasma water in the total water of the muscle.

Table 2. *Apparent extracellular spaces or 'permeations' in cephalopod muscle (concentrations in muscle as percentages of those in plasma, on a water-content basis)*

Extracellular spaces as % total muscle water						Muscle (mg./g. water)		Time allowed for distribution (hr.)
Chloride	Inulin	Sucrose	Sodium thiosulphate	Copper	Copper $\times 10^{-3}$	Chloride		
<i>Sepia</i>								
—	15.51	7.29	—	—	—	3.17	4.5	
—	15.62	8.40	—	—	0.88	3.19	6.3	
—	14.44	7.55	—	—	1.16	2.98	6	
♂	11.40	7.99	—	—	0.92	2.28	5	
♂	12.74	7.90	—	—	1.15	2.56	6	
♀	12.43	11.77	—	—	0.82	2.51	7	
♂	20.11	—	14.58	—	—	4.15	2	
♂	—	—	15.80	—	—	—	2	
♀	26.07	—	14.21	—	—	6.05	4	
♂	—	—	13.64	—	—	—	6.5	
—	—	—	—	32.43	—	—	2	
—	—	—	—	46.22	—	—	4	
—	—	—	—	42.68	—	—	6	
Mean	16.04	8.48	14.56	40.44	0.99	3.36	—	
S.E.	± 1.72	± 0.68	± 0.46	± 4.13	± 0.07	± 0.43	—	
<i>Eledone</i>								
—	22.10	16.66	—	—	1.74	4.16	5	
—	19.57	13.90	—	—	2.79	3.63	6	
—	21.60	13.52	—	—	—	4.03	8	
♀	25.38	—	21.12	—	—	4.85	1	
—	21.54	—	22.56	—	—	4.11	3	
Mean	22.04	14.69	21.84	—	2.27	4.16	—	
S.E.	± 0.94	± 0.99	± 0.72	—	± 0.52	± 0.20	—	

Average copper content of plasma in *Sepia* was 0.22 (0.205–0.243) mg. Cu/g. water, in *Eledone* 0.26 mg. (0.255–0.270).

The calculated spaces in the muscle into which injected inulin, sucrose and sodium thiosulphate penetrate differ, the thiosulphate space in *Sepia* being higher than any of the chloride spaces, the sucrose space usually smaller, and the inulin space in both species being about half to two-thirds of the chloride space. The inulin spaces, averaging 8.5% in *Sepia* and 14.7% in *Eledone*, are the smallest, and will be taken as approximating the extracellular space; ample time (4.5–8 hr.) was given for the inulin to distribute itself uniformly in the muscle, and the large size of its molecule should make it difficult for this carbohydrate to enter cells. Accepting this, a small amount of chloride must be present inside muscle cells (see below, Table 3), and sucrose seems to have begun to penetrate the cells during the 1–6.5 hr. of the experiments

with this disaccharide. *Sepia* muscle cells appear to be fairly permeable to the thiosulphate ion, as after 2–6 hr. 40% of total muscle water had this ion in the same concentration as in the plasma.

From the differences between estimated blood volume (from copper concentrations) and total extracellular fluid (from inulin concentrations), it may be inferred that about 7.5% of the muscle water in *Sepia* and 12.4% in *Eledone* belongs to the interstitial fluid.

(3) Intracellular ionic concentrations of muscle

Accepting extracellular spaces of 8.5% in *Sepia* and 14.7% in *Eledone* muscle, and assuming the whole of the extracellular fluid in muscle has the same composition as the respective plasmas, one can calculate intracellular concentrations. Thus for *Sepia* the values for 85 g. extracellular fluid (based on plasma) are subtracted from the mean concentrations of ions in a kilogram muscle water (Table 1), and the remaining intracellular concentrations in 915 g. expressed per kg. water; similarly for *Eledone*.

Table 3. Intracellular composition of cephalopod muscle compared with blood plasma

	mg. ions/kg. water							Total
	Na	K	Ca	Mg	Cl	SO ₄	P*	
<i>Sepia</i>								
Muscle cells	30.8	189	1.88	19.0	45.0	2.04	141.4	429
Plasma	465	21.9	11.6	57.7	591	6.3	1.23	1155
Ratio: $\frac{\text{muscle cell}}{\text{plasma}}$	0.066	8.6	0.16	0.33	0.076	0.32	115	0.37
<i>Eledone</i>								
Muscle cells	33.3	167	3.08	17.4	54.8	4.5	138.5	419
Plasma	432	14.4	11.2	54.2	516	20.6	0.43	1049
Ratio: $\frac{\text{muscle cell}}{\text{plasma}}$	0.077	11.6	0.28	0.32	0.106	0.22	322	0.40

Water content of cells: *Sepia* 735 g./kg.; *Eledone* 746 g./kg.

* mg. atoms.

Differences between whole muscle (Table 1) and intracellular concentrations (Table 3) include reductions in sodium and chloride of the latter, owing to the subtraction of the sodium- and chloride-rich extracellular fluid. The higher values of potassium and phosphate inside the muscle cells compared with whole muscle are not enough to balance the decrease of sodium and chloride, and the ionic concentration inside the cells decreases from 491 to 429 mg. ions in *Sepia*, and from 511 to 419 mg. ions in *Eledone*. Of the ions studied only potassium and phosphate are accumulated in the cells. Calcium, magnesium and sulphate of the cell are below a third of their respective plasma concentrations, while sodium and chloride are usually less than a tenth.

If it is assumed that the potassium of the cell is held electrostatically by non-diffusible organic ions, particularly phosphates, that the cell is permeable to small ions, and that the low value of sodium is maintained by a process of active extrusion

of sodium ions (e.g. Ussing, 1949; Conway, 1957), the internal potassium concentration should be in Donnan equilibrium with the outside potassium, and

$$\frac{[K_i]}{[K_o]} = \frac{[Cl_o]}{[Cl_i]} = r.$$

In *Sepia* these ratios are 8.6 and 13.2, in *Eledone* 11.6 and 9.4. Thus it is difficult to reconcile the potassium and chloride content of the muscle cells with a simple Donnan equilibrium. A tentative explanation would be to assume that in *Eledone* a small fraction of the potassium is present in an unionized complex, and that in *Sepia* some active extrusion of cell chloride takes place. Of these assumptions only the first has some support (§IV (2)).

III. ORGANIC CONSTITUENTS

(1) *The phosphorus compounds*

As in the previous study of *Nephrops* (Robertson, 1961), the phosphate compounds in a trichloroacetic extract of muscle were separated into four fractions by appropriate treatment of the barium-soluble and barium-insoluble compounds, and determination of total phosphorus (Table 4).

Table 4. *Acid-soluble phosphate fractions in cephalopod muscle*

		mg. ions/kg. water				
		Inorganic phosphate	Arginine phosphate	Adenosine triphosphate	Hexose phosphate, etc.	Total phosphate
♂	<i>Sepia</i>	84.3	3.9	7.5	11.2	106.9 (121.9)
♀	<i>Loligo</i>	111.7	0	5.0	16.3	133.0 (143.0)
♂	<i>Eledone</i>	45.2	7.9	6.9	6.7	66.7 (80.5)
♀	<i>Eledone</i>	75.6	7.0	5.0	19.8	107.4 (117.4)

Figures in brackets are mg. atoms P.

Very little arginine phosphate (phosphorylarginine) was found. How far these low concentrations represent average values in the living animal is difficult to judge. Some breakdown of the compound into free arginine and inorganic phosphate may have taken place in the extraction period. The average values for arginine from Table 5a (obtained after presumed complete breakdown of any arginine phosphate) are 62 mM. for *Sepia* 55 mM. for *Loligo* and 46 mM. for *Eledone*. Thus some 60–70% of the inorganic phosphate could be bound as arginine phosphate in *Sepia* and the *Eledone* female, 50% or so in *Loligo*.

Previous workers have had difficulty in finding arginine phosphate in *Sepia*. For example, Needham, Needham, Baldwin & Yudkin (1932) found 0, 6 and 7 mg. ions/kg. fresh weight in the mantle muscle of three specimens. The mean sum of inorganic and arginine phosphate in their specimens was 64.4 mg. ions/kg. fresh weight, equal to about 86 mg. ions/kg. water (cf. 88 mM. in Table 4). Baldwin (1933) found higher arginine phosphate values in *Eledone moschata*, 13–29 mg. ions/kg. fresh weight, by

a method which could have included some phosphate from breakdown of adenosine triphosphate.

In the three genera inorganic+arginine phosphate forms 77–84% of the total phosphate, adenosine triphosphate 4–10% (compared with 8–13% in *Nephrops* muscle), the remaining fraction 10–18%. The total phosphate in mg. atoms P compares well with that of the specimens in Table 1, except for the male *Eledone*, a small animal, which had a much lower value.

Phosphate compounds if free in the muscle cells would form 9–10% of the total osmotic concentration of the muscle.

(2) Nitrogenous constituents

Cephalopod muscle is known to contain free amino acids and the nitrogenous bases betaine and trimethylamine oxide in considerable quantities (e.g. Endo, Hujita & Simidu, 1962). Data for the three species being studied are given in Table 5. Chloride analyses are also given, since it has sometimes been apparent that low amino acid and high chloride figures go together (*Sepia* specimen 4, *Eledone* specimen 10). While poor condition of decapod crustaceans and cephalopods is usually associated with a raised sodium and chloride content of their muscles, all the animals from which the data of Table 5 were obtained appeared healthy.

In *Sepia* and *Loligo* the mean values for amino acids, 442 and 388 mM., are about double the sum of the other two constituents, which themselves are each of the order of 80–120 mM. From the osmotic standpoint free amino acids constitute about 40% of the osmotic concentration of the muscles, the nitrogenous bases about 15–20% (see §IV). Amino acids are not so abundant in *Eledone* muscle, the mean value being 279 mM., equal to about 26% of the osmotic concentration. Betaine values in *Eledone* are approximately the same as in *Sepia* and *Loligo*, but trimethylamine oxide is much lower, with a mean of 34 millimoles.

Volatile base (ammonia) is low, about 1–2 mg. ions in muscle extracts kept at 0° C. and analysed within a few hours, but may rise to about 6 mg. ions in a week or so. The rise is due to increase in ammonia, not to free trimethylamine which was never found, even in old extracts.

Of the nineteen free amino acids found in mantle muscle of *Sepia esculenta* by Endo *et al.* (1962), the chief are arginine, proline, taurine, glycine and alanine. These are also important in *Loligo pealeii* muscle (Koechlin, 1955).

Some analyses of proline and arginine are given in Table 5a. Fairly wide variations in proline were found, but less variable amounts of arginine, which forms part of the arginine phosphate molecule in cephalopod muscle (Lohmann, 1936). The arginine values given are those after splitting of the compound and include both free and bound arginine.

(3) Lactate, glycerol and reducing sugar

These compounds were found to be present in concentrations practically negligible from an osmotic viewpoint (Table 6). The low values of lactate found by Boyland (1928) in cephalopod muscle, 2–3 mg. ions if calculated on a water-content basis, were confirmed in *Eledone*. *Loligo forbesi* muscle taken from dead specimens had two to three times these values.

Estimates of glycerol were also small, 1–4 mM./kg. water. These may be compared with a value of 5 mM. found in the axoplasm from giant nerve fibres of a Chilean squid, *Dosidicus gigas* (Deffner, 1961). (Much higher values given by Deffner & Hafter (1960) for the axoplasm of *Dosidicus* (70 mM./kg. water) and *Loligo pealeii* (98 mM.) were later shown by Deffner (1961) to be due to contamination.)

Table 5. *Nitrogenous constituents of cephalopod muscle*

Species and specimen no.	mM./kg. water			
	Amino acids (α -amino N)	Trimethylamine oxide	Betaine	Chloride
<i>Sepia</i>				
— 1	463	119.2	72.7	87.1
— 2	566	68.3	74.0	73.7
— 3	471	65.3	111.2	97.2
— 4	342	57.8	147.9	106.5
♀ 5	392	89.1	—	82.4
♂ 6	439	77.2	—	95.8
♂ 7	421	74.6	89.4	82.6
Mean	442	78.8	99.0	89.3
S.E.	± 26.6	± 7.7	± 14.0	± 4.2
<i>Loligo</i>				
♂ 1	394	109.9	61.1	—
♂ 2	423	122.0	102.4	—
— 3	346	118.9	49.9	—
Mean	388	116.9	71.1	—
S.E.	± 22.4	± 3.6	± 16.0	—
<i>Eledone</i>				
♂ 1	—	51.5	—	—
♂ 2	280	12.1	106.1	151.6
♀ 3	—	42.2	—	—
♀ 4	255	49.1	110.4	111.0
♀ 5	285	53.3	90.2	94.9
♀ 6	297	38.6	100.3	123.1
♀ 7	248	24.5	74.6	147.0
♀ 8	323	28.0	93.8	91.2
♀ 9	322	28.4	111.9	125.2
♀ 10	202	11.0	110.4	212.4
♀ 11	271	—	—	135.2
♀ 12	267	—	—	124.9
♀ 13	289	—	—	92.5
♀ 14	303	—	—	129.7
Mean	279	33.9	99.7	128.2
S.E.	± 9.7	± 4.9	± 4.6	± 9.6

Volatile base (ammonium ions) of *Sepia* specimens 6 and 7 was 1.95 and 2.03 mg. ions/kg. water, of *Eledone* 1 and 3, 1.24 and 0.92. Amide nitrogen of the same *Sepia* specimens was 7.03 and 7.51 mM. respectively, and of *Eledone* 3, 5.70 mM.

Reducing sugar as measured by a copper reduction method, and, more specifically, glucose measured with glucose oxidase, are small in terms of osmotic concentration, some 3–6 mM. in *Sepia* and *Eledone* muscle, and 1 mM. in plasma. Boyland (1928) had previously found 1.4 mM. in a sample of *Sepia elegans* muscle, and 7.5 mM. in fatigued muscle from the head of *Eledone cirrhosa*. He also found myo-inositol in the

Sepia mantle muscle to the extent of 0.07%, about 5 mm./kg. water. Slightly higher values are given for squid axoplasm by Deffner (1961), 8.7 mm. in *Loligo pealeii* and 11.0 mm. in *Dosidicus*.

Table 5a. Some amino acids in cephalopod muscle

Species and specimen no.	mm./kg. water			
	Total α -amino N	Proline	Arginine	Glycine, taurine, etc. (by difference)
<i>Sepia</i>				
1	463	54	64	345
2	566	90	71	405
3	471	83	66	322
4	342	14	48	280
Mean	461	60	62	338
S.E.	± 45.9	± 17.3	± 4.9	± 26.1
<i>Loligo</i>				
1	394	155	50	189
2	423	136	64	223
3	346	120	50	176
Mean	388	137	55	196
S.E.	± 22.4	± 10.1	± 4.7	± 14.0
<i>Eledone</i>				
2	280	14	38	228
4	255	3	47	205
5	285	13	44	228
6	297	2	47	248
7	248	3	41	204
8	323	22	61	240
9	322	13	46	263
10	202	4	29	169
11	271	6	45	220
12	267	3	52	212
13	289	20	66	203
14	303	7	35	261
Mean	279	9	46	223
S.E.	± 9.7	± 2.0	± 9.4	± 7.9

IV. OSMOLALITY AND CATION-ANION BALANCE

(1) Direct measurements of osmolality

Four measurements of the osmotic concentration of plasma and of the juice expressed from muscle were made on specimens of *Eledone*, each specimen being equilibrated in sea water of very similar salinity (Table 7). Plasma was isosmotic with sea water within 1.1%, with a mean concentration of 100.6% that of the medium.

Juice expressed from muscle showed a slightly higher concentration. Compared with plasma, it was hyperosmotic by 2.5%, with a mean concentration of 103.2%, a significant difference by the *t*-test ($P < 0.02$). The higher values of specimens 2 and 3 are those in which 2-4 hr. had elapsed before the measurements had been completed. Slight breakdown of labile compounds such as arginine phosphate is probably responsible for this difference. In life the muscle cells must be virtually isosmotic with the plasma and interstitial fluid.

Table 6. *Lactate, glycerol and reducing sugar of muscle and plasma*

Species	Lactate (mg. ions/kg. water)		Glycerol (mm./kg. water)		Reducing sugar (mm./kg. water)	
	Plasma	Muscle	Plasma	Muscle	Plasma	Muscle
<i>Eledone</i>						
♂	0.60 (3) (0.30-0.76)	2.71 (3) (1.83-3.68)	—	2.24 (1)	—	—
♀	0.30 (2) (0.28-0.31)	1.42 (1)	—	1.11 (5) (0.77-1.49)	1.05* (1)	6.19* (4) (2.67-9.76)
♂+♀	0.48 (5)	2.39 (4)	—	1.30 (6)	1.44 (4)	6.19* (4)
S.E.	± 0.11	± 0.50		± 0.22	± 0.39	± 1.51
<i>Sepia</i>						
♂	—	—	—	—	0.97 (3) (0.27-1.37)	4.14 (4) (2.42-5.73)
♀	—	—	—	—	1.18 (2) (0.98-1.37)	2.59 (2) (2.02-3.16)
♂+♀	—	—	—	3.82 (2) (3.71-3.93)	1.05 (5)	2.74 (10)
S.E.				± 0.11	± 0.21	± 0.52
<i>Loligo</i>						
♂	—	6.52 (2) (4.79-8.24)	—	0.71 (3) (0.67-0.76)	—	—
S.E.		± 1.72		± 0.03		

Figures in parentheses are numbers of animals and range of values.

A few specimens in which sex was not determined are included in total.

* Glucose, as determined by a glucose oxidase method.

Table 7. *Osmotic concentrations of plasma and muscle-juice of Eledone (Krogh-Baldes method)*

Specimen	Sea water	Plasma	Muscle-juice*	Plasma as % sea water	Muscle-juice as % plasma
1	3.065	3.100	3.156 (0.8 hr.)	101.1	101.8
2	3.098	3.130	3.279 (2 hr.)	101.0	104.8
3	3.053	3.038	3.152 (4 hr.)	99.5	103.6
4	3.000	3.018	3.091 (1 hr.)	100.6	102.4
Mean	3.054	3.072	3.170	100.6	103.2
S.E.	± 0.0204	± 0.0262	± 0.0394	± 0.37	± 0.67

* Figures in parentheses are times between removal of muscle and completion of estimation.

Concentrations are expressed in relation to % NaCl solution (g./100 g. water). S.D. of method 0.75% ($N = 9$).

Very similar findings were obtained in the previous study of crustacean muscle (Robertson, 1961).

(2) Osmolality by summation of chemical constituents

In Table 8 a summation of the concentrations of ions and neutral organic constituents has been made for the three cephalopods. Concentrations inside the muscle cells have been calculated on the basis of an extracellular fluid volume in whole muscle of 8.5% in *Sepia* and 14.7% in *Eledone* (§II (3)), assuming that the interstitial fluid round cells has the same composition as the plasma.

The total osmotic concentration of analysed constituents in muscle cells of *Sepia* is within 1% of that in whole muscle, and is 5-6% below that of the plasma, which

itself is within 1.5 % of the sea-water concentration. An excess of 3 % osmotic constituents is apparent in *Loligo* muscle compared with plasma, whereas in *Eledone* a deficit of some 13–15 % is found in the muscle and muscle cells.

Table 8. Osmotic concentration of plasma, whole muscle and muscle cells

Constituent	mg. ions or mM./kg. water							
	<i>Sepia</i>			<i>Eledone</i>			<i>Loligo</i>	
	Plasma	Whole muscle	Muscle cells	Plasma	Whole muscle	Muscle cells	Plasma	Whole muscle
Sodium	465	67.7	30.8	432	91.9	33.3	419	78.0
Potassium	21.9	174.7	189	14.4	144.5	167	20.6	152.0
Calcium	11.6	2.71	1.88	11.2	4.27	3.08	11.3	3.03
Magnesium	57.7	22.3	19.0	54.2	22.8	17.4	51.6	15.2
Ammonium	—	2.0	2.2	0.023	1.1	1.3	—	2.0
Chloride	591	91.4	45.0	516	122.6	54.8	522	91.3
Sulphate	6.3	2.4	2.0	20.6	6.9	4.5	7.3	2.6
Bicarbonate	—	—	—	7.5	—	—	—	—
Lactate	—	6.2	6.8	0.48	2.4	2.7	—	6.5
Inorganic phosphate	1.23	84.3	92.0	0.37	75.6	88.6	—	111.7
Arginine phosphate	—	3.9	4.3	—	7.0	8.2	—	0
Adenosine tri-phosphate	—	7.5	8.2	—	5.0	5.9	—	5.0
Remaining acid-soluble phosphate	—	11.2	12.2	0.06	19.8	23.2	—	16.3
Amino acids	3.91	442	483	3.08	279	326	—	388
Trimethylamine oxide	0	78.8	86.1	—	33.9	39.7	—	116.9
Betaine	—	99.0	108.2	—	99.7	116.9	—	71.1
Reducing sugar	1.05	2.7	2.9	1.05*	6.2*	7.1*	—	—
Glycerol	—	3.8	4.2	0	1.30	1.5	—	0.71
Total	1160	1103	1098	1061	924	901	1032	1060

Plymouth sea water (*Sepia*) 1176 mg. ions/kg. water; Millport sea water (*Eledone*) 1048 mg. ions and (*Loligo*) 1035 mg. ions.

* Glucose (glucose oxidase method).

These discrepancies must be considered further. First, some organic constituent(s) of not negligible importance may have been omitted in the analyses. Secondly, there is possible error involved in adding up constituents from different specimens. While one series of animals was used for the comparative inorganic analyses of muscle and plasma, a second series was used for the nitrogenous constituents. As already mentioned, a low content of amino acids in muscle is usually associated with a high chloride content. However, from Table 5 it may be noted that the amino acid value of 279 mM. in *Eledone* was found in conjunction with 128 mg. ions chloride, the latter figure only slightly higher than the 123 mg. ions in Table 8, from the three *Eledone* used for the inorganic data.

Thirdly, some 30–40 mM. of the arginine included in the total amino acids of the three species may have been combined with inorganic phosphate as arginine phosphate in the living animal. This would tend to reduce the total of the estimated compounds and ions by that amount. Fourthly, some proportion of the analysed constituents may be bound to protein molecules, reducing the effective osmotic concentration. And finally, the osmotic coefficients of the various compounds and

salts (or ions) should be taken into consideration. It is probable that such different media as plasma and cell solution are affected differently by these last two factors.

(a) *The binding of ions.* Juice expressed from muscle in a tissue press has approximately the same osmotic pressure as the plasma; in *Eledone* it is at most 3% hyperosmotic (Table 7), due probably to breakdown of labile compounds. When analysed it is seen to show considerable differences from muscle in both *Eledone* and *Sepia* (Table 9). Apart from its much higher water content, not very different from that of the blood, all its ions except chloride are less than those of the corresponding muscle. Calcium and magnesium show the greatest reductions, usually to well below half their values in the muscle, sodium, potassium and total acid-soluble phosphorus to 70–90%. Only the chloride concentration is essentially the same (within 1–2%) in muscle and muscle-juice.

Table 9. *Composition of muscle-juice*

	mg. ions/kg. water						Water (g./kg.)
	Na	K	Ca	Mg	Cl	P*	
<i>Sepia</i>							
Muscle	73.2	168.9	2.92	23.7	91.8	—	747 (±2.9)
Muscle-juice	52.4	124.6	0.99	10.0	93.9	—	922 (±7.9)
Juice as % muscle	72	74	34	42	98	—	—
No. of paired estimations	2	3	1	2	3	—	7
<i>Eledone</i>							
Muscle	90.1	172.6	2.99	23.7	103.3	112.3	754 (±6.4)
Muscle-juice	78.0	153.5	1.28	14.0	102.2	102.2	890 (±6.5)
Juice as % muscle	87	89	43	59	99	91	—
No. of paired estimations	2	2	3	2	2	1	9

* mg. atoms. (±) = S.E.

These features of muscle-juice have already been found in *Nephrops* (Robertson, 1961), and it is difficult to avoid the conclusion drawn previously that most of the reduction is due to the holding back by the proteins of muscle of a proportion of the 'ions', those bound in complexes. It is not simply a case of cations being held back electrostatically to balance negatively charged soluble proteins of the cell. If this were so, one would expect a Donnan equilibrium in such ions as Na^+ , K^+ and Cl^- . Instead of a much higher chloride content in the muscle-juice expected on the basis of Donnan forces, the chloride values in juice and muscle are essentially similar.

Muscle-juice, apart from its small content of extracellular fluid, contains the free ions and nitrogenous compounds of the muscle cells, the osmotically active portion which is in equilibrium (or steady state) with the plasma and interstitial fluid (Table 7). While the proportion of extracellular fluid in muscle-juice has not been measured, it may be assumed to be similar to that in whole muscle, 8.5% of the total water in *Sepia*, 14.7% in *Eledone*. Accepting this and assuming that the composition of plasma (Table 8) is that of extracellular fluid as a whole, a calculation can be made of the average composition of the muscle cell and the cellular portion of the muscle-juice in the specimens of Table 9. This calculated composition is given in Table 10.

All the calcium of *Sepia* and *Eledone* muscle cells, as well as over 60% of the magnesium, seems to be bound. Most or all of the chloride is free. Because of the subtraction of the chloride-rich extracellular fluid in the calculation, the small

analytical differences of 1–2% in chloride content of muscle and muscle-juice, barely significant in themselves, lead to larger calculated differences of 4–5% between the chloride of the cell and the cellular portion of the juice, which may not be significant. In contrast to the predominantly or wholly free nature of the chloride, only 38–54% of the sodium and 73–89% of the potassium is calculated to be free, and 91% of the total acid-soluble phosphorus. If adenosine triphosphate is partly or wholly bound (e.g. Nanninga, 1961), this would concern up to 17–18 mg. atoms phosphorus in *Eledone* cells (Table 8), an amount which slightly exceeds the 9% of phosphorus calculated to be bound.

Table 10. *Approximate amount of ion-binding (based on data of Table 9)*

	mg. ions/kg. water					
	Na	K	Ca	Mg	Cl	P*
<i>Sepia</i>						
Muscle cell	36.8	182.6	2.11	20.5	45.4	—
Cellular portion of muscle-juice†	14.1	134.1	0.004	5.6	47.7	—
Free ions (%)	38	73	0	27	105	—
Bound ions (%)	62	27	100	63	—	—
<i>Eledone</i>						
Muscle cell	31.2	199.9	1.57	18.4	32.2	131.6
Cellular portion of muscle-juice†	17.0	177.5	0.01	7.1	30.9	119.7
Free ions (%)	54	89	1	39	96	91
Bound ions (%)	46	11	99	61	4	9

* mg. atoms.

† That is excluding extracellular component of juice.

(b) *Conversion to milliosmoles.* In order to relate solute concentrations (mg. ions or millimoles) to osmotic pressure, one must consider the appropriate osmotic coefficients of salts and non-electrolytes. It is useful to accept Dick's (1959) suggestion that osmoles and milliosmoles be defined in relation to an ideal non-electrolyte as units of osmotic pressure. Thus a solution of $\Delta 1.858^\circ \text{C.}$ contains 1000 m-osmoles. Sea water of this osmotic pressure contains 1129 mg. ions per kg. solvent water, being isosmotic with 0.553 M-NaCl (Sverdrup, Johnson & Fleming, 1942; Robinson, 1954). The over-all osmotic coefficient for sea water of this total concentration is thus 0.886. Since inorganic ions form over 99% of the total osmotic concentration of the plasma in the three cephalopods studied, and individually depart little from the corresponding ions of sea water, it is probably safe to use the same coefficient for plasma. Thus the 1160 mg. ions of *Sepia* plasma would equal 1028 m-osmoles. *Eledone* and *Loligo* plasmas of slightly less concentration, would equal 941 and 914 m-osmoles, respectively.

For muscle cells it can be assumed that amino acids and nitrogenous compounds would behave approximately as ideal non-electrolytes, and that the inorganic ions and phosphates would behave like plasma ions. As discussed previously (Robertson, 1961), the osmotic coefficients of certain amino acids and nitrogenous compounds for which data are available do depart from unity in some cases. Thus at 1 molal the coefficients of glycine, proline and alanine are 0.928, 1.046 and 1.003, and that of

betaine is 1.115. These differences may possibly cancel each other out when related to the actual concentrations present. Lacking any information on trimethylamine oxide and on the amino acid taurine which is abundant in mollusc muscle (Simpson, Allen & Awapara, 1959; Endo *et al.*, 1962), we may assume for present purposes that the nitrogenous substances show ideal behaviour like alanine. Thus in *Sepia* the 677 mM. nitrogenous bases and amino acids would equal 677 m-osmoles, and the cellular ions equal 366 m-osmoles (osmotic coefficient 0.886). Together with the 7 mM. of low-molecular weight carbohydrates, the total cellular osmolality comes to 1050 m-osmoles, compared with 1028 m-osmoles for plasma.

Table 11. *Osmotic concentration of plasma and muscle cells*

	mg. ions or mM./kg. water		m-osmoles/kg. water			
			Uncorrected		Corrected for ion-binding	
	Plasma	Muscle cells	Plasma	Muscle cells	Plasma	Muscle cells
<i>Sepia</i>						
Inorganic ions and phosphates, etc.	1155	413	1023	366	1022	283
Amino acids	4	483	4	483	4	483
Nitrogenous bases	—	194	—	194	—	194
Carbohydrates	1	7	1	7	1	7
Total	1160	1097	1028	1050	1027	967
<i>Eledone</i>						
Inorganic ions and phosphates, etc.	1057	410	937	363	936	316
Amino acids	3	326	3	326	3	326
Nitrogenous bases	—	157	—	157	—	157
Carbohydrates	1	9	1	9	1	9
Total	1061	902	941	855	940	808

Table 11 gives a revised osmotic balance sheet, taking into account these estimates of osmolality (milliosmoles) and the binding of ions in undissociated complexes, as indicated by the data of Table 10. The correction for ion-binding in the plasma is small, since it concerns only 8% of the calcium in *Eledone* (Robertson, 1949) and 16% of the calcium in *Sepia* (Robertson, 1953). But in muscle cells the analytical totals of 413 and 410 mg. ions in *Sepia* and *Eledone* are reduced to 319 and 357 mg. ions when the fraction of bound ions is subtracted. These values when multiplied by the osmotic coefficient of 0.886 give 283 and 316 m-osmoles, respectively.

Thus in *Sepia* about 94% and in *Eledone* about 86% of the osmotic concentration of the cell have been accounted for by free ions, amino acids, nitrogenous bases and carbohydrates.

No data have been obtained on ion-binding in *Loligo*, nor have estimations been made of the extracellular space in muscle. The analytical data for whole muscle and plasma if converted to m-osmoles would be 1005 and 914 respectively.

A rough calculation has been made of the total m-osmoles in *Loligo* muscle cells by assuming a 12% extracellular space and applying the average ion-binding found in the other two cephalopods. The value found, 961 m-osmoles, is slightly higher than

the 912 m-osmoles for plasma (914 less 2 mg. ions for bound calcium). This estimate that *Loligo* muscle cells are hyperosmotic to the plasma does not take into account the probability that a considerable part of the inorganic phosphate and of the arginine included in the total amino acids is present in the living animal as the phosphagen, arginine phosphate. This alone might make the calculated total too high by 20-40 m-osmoles.

(3) Cation-anion balance

By taking valency into account, the cation-anion balance can be calculated from the analytical data of Table 8. However, an estimate of cell pH is desirable since the valency of the predominant phosphate anions varies with pH.

Table 12. *Measurements of pH (glass electrode) on Eledone*

Blood	Ground muscle	Muscle mince	Muscle juice
7.40	6.75	—	—
	6.79*	—	—
7.40	6.86	—	—
	6.87*	—	—
7.34	6.87	6.86	—
	6.86*	—	—
7.50	6.79	6.77	6.90
Calculated intracellular pH	6.72-6.82	6.71-6.81	6.85

* Second sample.

The data in Table 12 were obtained on ground muscle, muscle-mince, muscle-juice and blood of *Eledone*. Calculation of intracellular pH was made on the assumption that the 14.7% of extracellular fluid had the same pH as the blood. Caldwell (1958) had previously found close agreement between the pH of *Carcinus* muscle fibres and muscle-mince, using a micro-glass electrode. It is uncertain whether these results on *Eledone* muscle approach the true internal pH, since this would require a balancing of two potential errors arising from stimulation by grinding and pressing, the alkalinizing effect of the breakdown of arginine phosphate and the acidifying effect of the production of lactic acid and carbon dioxide. Buffering by the phosphates and proteins of the muscle would tend to neutralize any pH changes, and the data may perhaps be taken at their face value as indicating an internal pH of 6.7-6.8. This is similar to results obtained on *Nephrops* muscle (Robertson, 1961). The pH of *Eledone* blood is practically identical with that found in *Loligo forbesi* by Caldwell (1958), 7.4-7.5.

Cation-anion balance for both *Sepia* and *Eledone* plasma and muscle cells is given in Table 13, the milli-equivalents of the phosphate compounds being calculated for both pH 6 and 7.

It has been assumed that at cell pH, 6.5-7.0, the positive and negative charges of the dipolar ions betaine and trimethylamine oxide cancel each other out, as with most of the amino acids. Arginine is an important cation on account of its net positive charge. Aspartic and glutamic acids, with their two carboxyl groups as against one amino group, would act as anions, but their concentration in muscle as distinct from nerve is small (Endo *et al.* 1962; Deffner, 1961). In *Sepia esculenta* mantle muscle

Table 13. *Cation-anion balance*

	m-equiv./kg. water		
	Plasma	Muscle cell constituents	
		Free + bound	Free
<i>Sepia</i>			
Sodium	465	30.8	11.7
Potassium	21.9	189	138
Calcium	23.2	1.9	0
Magnesium	115.4	38.0	10.3
Ammonium	—	2.2	2.2
Arginine (net positive charge)	—	64	64
Total cations	625.5	325.9	226.2
Chloride	591	45.0	45.0
Sulphate	12.6	4.0	4.0
Lactate	—	6.8	6.8
Inorganic phosphate	2.2	148.1*—103.5	148.1*—103.5
Arginine phosphate	—	4.5—4.3	4.5—4.3
Adenosine triphosphate	—	31.1—26.7	0
Hexose monophosphate, etc.	—	23.7—17.6	23.7—17.6
Total anions	605.8	263.2—207.9	232.1—181.2
<i>Eledone</i>			
Sodium	432	33.3	18.0
Potassium	14.4	167	149
Calcium	22.4	3.1	0
Magnesium	108.4	34.8	13.6
Ammonium	0.02	1.3	1.3
Arginine	—	46	46
Total cations	577.2	285.5	227.9
Chloride	516	54.8	54.8
Sulphate	41.2	9.0	9.0
Bicarbonate	7.5	—	—
Lactate	0.48	2.7	2.7
Inorganic phosphate	0.67	142.6*—99.7	142.6*—99.7
Arginine phosphate	—	8.5—8.2	8.5—8.2
Adenosine triphosphate	—	22.4—19.2	0
Hexose monophosphate, etc.	—	45.0—33.4	45.0—33.4
Total anions	565.9	285.0—227.0	262.6—207.8

* At intracellular pH of 7.0; 2nd figure for pH 6.0.

Calculations for equivalents of phosphates from data of Sørensen (1912): inorganic phosphate; Meyerhof & Lohmann (1928): arginine phosphate; Alberty, Smith & Bock (1951): adenosine triphosphate; Meyerhof & Lohmann (1927): hexose monophosphate.

they together have a concentration of about 2 mM./kg., and about 3 mM. in the squid *Ommastrephes* (Endo *et al.*).

One of the difficulties in interpreting cation-anion balance in cephalopods is that changes may have occurred during the preparation of the appropriate extracts for analysis. As previously considered for osmotic balance, some of the free arginine may have been combined in the living animal as arginine phosphate, on the other side of the balance sheet. A minor point is that the lactate concentration of *Sepia* (and *Loligo* muscle) would probably have been less, more like that of *Eledone*, if more rigid precautions had been taken prior to its estimation (immediate grinding in ice-cold trichloroacetic acid). Bicarbonate was not estimated in muscle, but is likely to be below the value found in the plasma of *Eledone* (cf. *Nephrops*, Robertson, 1961).

The cation-anion balance of the plasma is satisfactory (Table 13). About 3.7 m-equiv. in *Sepia* and 1.8 m-equiv. in *Eledone* have to be subtracted from the cations as being the amount of calcium which is bound in a complex with protein. In *Eledone* the deficit in anions is now 9.5 m-equiv. and probably represents protein anions. If the bicarbonate and lactate values of *Sepia* are assumed to be similar to those of *Eledone*, *Sepia* cations are 622, anions 614, the deficit being 8 m-equiv., probably proteinate.

In muscle cells if binding of a proportion of cations and anions is neglected, cations apparently exceed anions in *Sepia* at any pH between 6 and 7, and roughly balance anions in *Eledone* at pH 7. If, however, binding is taken into account, cations would balance anions at a pH between 6 and 7 in both species.

V. DISCUSSION

Previous studies on the inorganic composition of cephalopod muscle have shown that the major cations sodium, potassium, calcium and magnesium, together with chloride, account for less than half of its osmotic concentration (Henze, 1905; Bialaszewicz & Kupfer, 1936; Hayes & Pelluet, 1947). These former analyses show only fair agreement with the present ones, if all are expressed in the same terms, mg. ions/kg. fresh tissue, the ranges to include all the analyses being Na 50–156, K 98–198, Ca 1–9, Mg 3–26, Cl 62–179. Bialaszewicz & Kupfer's high potassium values of 190–198 mg. ions for *Loligo vulgaris* and *Sepia officinalis* are out of line with the remaining analyses, 98–130, and with a value of 114 mg. ions for *Loligo pealeii* (Manery, 1939). Their values for calcium (6.6–8.4) are also higher than those of Hayes & Pelluet and myself (1.3–3.6 mg. ions); on the other hand, their values for magnesium, 19.8–23.1 are closer to mine, 11.6–17.4, and Hayes & Pelluet's low values of 3.5 for magnesium in *Sepia* and 6.0 for *L. pealeii* may be underestimates.

Henze's (1905) analyses of *Octopus* muscle, using classical gravimetric methods, compare well with the more recent analyses, except for his much higher sodium (156 mg. ions) and chloride (179) values. Perhaps the adherent sea water and connective tissue had not been completely removed, since his sodium value exceeds the potassium value of 120 mg. ions/kg. fresh weight, a finding contrary to any later analysis.

Even when phosphate values of some 110–130 mg. ions are added, the total ions in the mantle muscle of the three cephalopods studied here still do not come to half the osmotic concentration of the plasma (or sea water). But the addition of various nitrogenous constituents goes far to make up the balance. Of these, free amino acids are quantitatively the most important, forming 30–40% of the osmotic concentration of whole muscle, betaine and trimethylamine oxide forming about 12–18%.

The presence of free amino acids and other nitrogenous compounds in mollusc muscle has long been known. Taurine and glycine were found in *Pecten opercularis* adductor muscle by Kelly (1904) in amounts which may be calculated to be about 129 and 21 mM. respectively per kg. solvent water. In *Octopus* muscle Henze (1905) found about 52 mM. taurine/kg. water, and also later identified the nitrogenous bases betaine (Henze, 1911) and trimethylamine oxide (Henze, 1914).

Quantitative data for these nitrogenous constituents in cephalopod muscle are, however, very slight. The most comprehensive are those of Endo *et al.* (1962). Their figures for *Sepia esculenta*, in mm./kg. solvent water, may be compared with those of *S. officinalis* (in brackets, this paper): amino acids 193–390, mean 262 (442), trimethylamine oxide 49–135, mean 105 (79), and betaine 58–94, mean 81 (99). The separation of individual amino acids and nitrogenous bases resulted in an average recovery of 56% of the extractive nitrogen in the muscle, so that the true values for total amino acids may have been higher; it is improbable that nearly half the non-protein nitrogen is in unknown forms. Indeed, more recently Endo, Hujita & Simidu (1963) found that in the squid *Sepioteuthis lessoniana* amino acids, octopine (which arises from arginine *post mortem*), trimethylamine oxide and betaine constituted 94% of the total extractive nitrogen, nucleotides and related compounds about 5%, leaving 1% unknown.

Average values for *Ommastrephes sloani pacificans* are amino acids 149 mm/kg. water, trimethylamine oxide 123 mm., and betaine 61 mm (Endo *et al.* 1962). Some further published values for trimethylamine oxide show reasonable agreement with the above data, 107–111 mm./kg. fresh weight for *Loligo opalescens* (Norris & Benoit, 1945), 79 and 87 mm. for two specimens of *L. pealeii* (Dyer, 1952), but values for the mantle muscle of three species of Japanese octopus are only 7–29 mm./kg. (Asano & Sato, 1954).

The fast portion of the adductor muscle of the bivalve *Mytilus* has a total amino acid concentration of 238 mm./kg. muscle water (Potts, 1958), a value comparable to the 279 mm. of *Eledone*, but considerably lower than the 442 mm. of *Sepia*.

Investigation of the extracellular space in the mantle muscle of *Eledone* and *Sepia* suggests that inulin injected into a blood-vessel distributes itself throughout the blood and interstitial fluid of the muscle without entering cells to any extent, whereas some sucrose appears to enter the muscle cells in both species, and sodium thio-sulphate enters *Sepia* cells markedly. A maximum value for the blood in the muscle capillaries can be estimated from copper analyses of blood and muscle. *Sepia* had the smaller blood space (expressed as a percentage of total muscle water), 1.0% compared to 2.3% in *Eledone*, and the smaller total extracellular space, 8.5% compared to 14.7%. Similar values for the abdominal muscles of the decapod crustacean *Nephrops* (Robertson, 1961) are blood space 4.1% and total extracellular space 12.1%, as measured by inulin. The higher blood space of *Nephrops* muscle is perhaps to be expected in an animal in which blood is not confined in capillaries but bathes the muscles.

Calculated intracellular ionic concentrations of muscle, obtained by appropriate correction of whole muscle analyses for extracellular fluid, show fairly close agreement in *Sepia* and *Eledone*. Potassium ion concentrations of 189 and 167 mg. ions/kg. cellular water are 5–6 times those of sodium; chloride at 45–55 mg. ions is less than half of the phosphate anions, while magnesium at 17–19 mg. ions is 5–8 times the calcium concentration, 2–3 mg. ions. In the past it has often been assumed that all the chloride of muscle is extracellular. Thus Hayes & Pelluet (1947) calculated on this basis that the cells of lamellibranch and cephalopod muscle contained only potassium, and no sodium, calcium or magnesium. It is evident from the present work

on *Sepia* and *Eledone* and that on *Mytilus* by Potts (1958) (see below) that the muscle cells of these animals contain some proportion of all the major inorganic ions.

Intracellular nitrogenous compounds are abundant in *Sepia* muscle, forming over half of the osmotically active constituents of the muscle cells. In *Eledone* they form less than half, the major fraction of free amino acids being 326 mM. as against 483 mM.

The only comparable analyses in molluscs are those of Potts (1958), who studied muscles of the bivalves *Mytilus edulis* and *Pecten maximus*. He obtained intracellular concentrations by allowing for the extracellular space as measured by inulin. The muscles studied, in order of their increasing speed, were the anterior byssus retractor (*Mytilus*) and the slow and fast portions of the adductor muscles (*Mytilus* and *Pecten*). The *Mytilus* muscles showed inulin spaces of 29.5% (retractor), 24.5 and 19.3% (slow and fast adductor), and calculated intracellular concentrations of decreasing sodium (95–79 mg. ions/kg. fibre water) and chloride (152–94), with little difference in potassium (153, 148 and 152 mg. ions). Thus these *Mytilus* muscles have greater extracellular spaces, higher intracellular concentrations of sodium and chloride, and slightly lower concentrations of potassium than has cephalopod mantle muscle. The fast adductor of *Pecten*, with an extracellular space of 6.0%, low sodium (43) and chloride (34) and high potassium (160 mg. ions), is more comparable.

With 295 mM. amino acids (including 113 mM. taurine) and only 39 mg. ions total phosphate, *Mytilus* fast adductor differs considerably from cephalopod mantle muscle. The sum of analysed constituents in this *Mytilus* muscle can be calculated to be 709 mg. ions + mM., compared to 1183 mg. ions in the blood plasma, leaving some 40% of the osmotic concentration still to be accounted for. Trimethylamine oxide and betaine presumably would make up much of this.

In these lamellibranch muscles the distribution of potassium and chloride inside and outside the muscle fibres does not conform to a Donnan equilibrium; for example $[K_i]/[K_o]$ and $[Cl_o]/[Cl_i]$ are 12.2 and 6.1 in *Mytilus* fast adductor, 12.8 and 16.8 respectively in *Pecten* fast adductor (Potts, 1958). A similar discrepancy in the two ratios is found here in the mantle muscle of *Sepia* and *Eledone* (§II (3)), and was found previously in *Nephrops* abdominal muscle (Robertson, 1961).

Sepia and *Eledone* muscles resemble those of other active invertebrates such as *Nephrops* (Robertson, 1961) and *Carcinus* (Shaw, 1958) both in ionic composition and in the relative importance of free amino acids, trimethylamine oxide and betaine in the osmotic balance of their cells. It is significant that muscle cells of active animals from two different phyla are so similar and yet different types of cell from the same animal or allied species can be so different in certain aspects of their composition.

If the present data for *Sepia* and *Eledone* muscle cells are compared with the data for the dialysable constituents of the axoplasm of the giant nerve fibres of the American squids *Loligo pealeii* and *Dosidicus gigas* (Deffner, 1961), a number of interesting facts emerge. In axoplasm the potassium concentration is over twice that in the muscle cells (344–409 mg. ions/kg. axoplasm, or about 398–473 mg. ions/kg. water as against 167–189 mg. ions), forming 31–34% of the total osmotic concentration of the axoplasm, compared with 17 and 19%. Phosphates form a large fraction of the osmotic concentration (12–15%) and total anions (about 71–79%) of muscle cells, but a very small fraction in the axoplasm (about 1–2% of the concentration and 5–6% of the anions). Values of 6–17 mg. atoms P/kg. axoplasm in *Loligo forbesi* (Caldwell,

1956) compare with 17.8 mg. atoms in *L. pealeii* (Deffner, 1961), and contrast with 98–104 mg. atoms in *Sepia* muscle cells and about 120 mg. atoms in *Loligo forbesi* (on the same fresh-weight basis).

Isethionic acid (a hydroxy analogue of taurine) and aspartic and glutamic acids in the axoplasm form a major fraction, 60–62%, of the anions and 24–25% of the total of osmotic constituents; in contrast the concentration of dicarboxylic amino acids is negligible in cephalopod muscle (0.4–2.3 mM./kg. in *Sepia esculenta* (Endo *et al.* 1962) compared to the 79 mM. aspartic acid and 21 mM. glutamic acid of *Loligo pealeii* axoplasm (Deffner, 1961)), while isethionic acid has not yet been identified in muscle, although it is present in axoplasm to the extent of 165 mg. ions in the American squids and 0.5 mg. ion/l. in *Loligo* blood.

Other features are the insignificance of arginine in the axoplasm, 3–4 mM./kg. as against about 40–50 mM./kg. in the muscle cells of the three cephalopods studied here, the lower total amino acid content of the axoplasm (about 60% of that in muscle cells), and the absence of trimethylamine oxide in nerve fibres.

The differences in composition of nerve and muscle cells must be a reflection of the cells' differing metabolism and physiological roles, impulse propagation and contraction. But while the involvement of certain phosphate compounds in the contractility and energy cycle of the muscle cell has obvious relationship to the high phosphate content, it is difficult to see the purpose of the isethionate ion in the functioning of the nerve cell, apart from its role in cation-anion balance and in osmotic balance.

It has often been tacitly assumed that the osmotic pressure inside muscle and other cells is the same as that of the surrounding interstitial fluid or blood plasma. In molluscan tissues there is positive evidence for this in Potts's (1952) finding that the freezing point of isolated muscle fibres of *Mytilus* is within 1.5% of that of the blood. This confirmed previous vapour-pressure determinations by Krogh (1939) on the juice expressed from *Mytilus* muscle. Direct measurements on juice expressed from *Eledone* muscle (§ IV (1)) have shown that its osmolality is a little higher than that of the blood (mean difference + 3.2%), but this is probably due to a slight breakdown of labile compounds such as arginine phosphate, slightly increasing the osmotic concentration of the juice; in the living octopus the muscle is probably isosmotic with the internal medium, just as the blood is isosmotic with the external medium.

Analyses of this muscle juice in both *Sepia* and *Eledone* has led to the conclusion that only a certain proportion of the cations are free in the cell solution, playing a part in cation-anion balance and in the osmotic relations of the cell with the internal medium, the remaining fraction being bound to cellular organelles such as the sarcolemma and structural proteins. It is estimated that all of the calcium is bound but none of the chloride. A large fraction of the magnesium (61–63%) and sodium (46–62%) is bound, and a smaller proportion of potassium and phosphate, estimated at 11–27% and 9% respectively. These values are quite comparable with those obtained on *Nephrops* muscle (Robertson, 1961), but sodium binding in cephalopod muscle is rather less.

Since the few relevant supporting data for the binding of a proportion of the alkali-metal ions were discussed (Robertson, 1961), some direct measurements have been made. Using cation-selective glass microelectrodes to measure the activities of sodium and potassium, Hinke (1961) estimates that 24% of the sodium in the extruded

axoplasm from the giant nerve fibres of *Loligo forbesi* is bound, if the whole of the potassium is considered to be free. A 10% binding of the potassium would alter the calculated sodium binding to 31.5% of the total sodium. Hinke's 1959 preliminary work on muscle cells of *Homarus vulgaris*, in which marked differences were found between sodium and potassium in the ratios of activity to total concentration (the sodium ratio half that of the potassium), suggests that only a fraction of the sodium in the cytoplasmic solution exists as free ions.

Lev (1964) calculates from similar experiments that the active concentration of sodium ions in frog muscle cytoplasm is about 30% of the total intracellular sodium, the remaining 70% being excluded in some way from the cytoplasmic solution surrounding his microelectrodes, presumably being present in sodium-rich cellular components, including nucleus and mitochondria. He found little difference between the activity coefficients of potassium ions in frog muscle and of potassium ions in a chloride solution of the same concentration, and so assumes that the intracellular potassium concentration represents free potassium ions.

Chemical support is also available for binding of ions in muscle. Experimentally, it has been shown that viscous solutions and suspensions of the rabbit muscle proteins myosin A and B can bind sodium and potassium, but human or bovine serum albumin cannot (Lewis & Saroff, 1957). The specific affinities of ions for proteins and certain phosphate compounds have been considered by Nanninga (1961). Owing to the binding of a proportion of magnesium, calcium and potassium to ATP, creatine phosphate and myosin, the percentage of free ions in frog skeletal muscle is calculated by him to be K 87%, Ca 36% and Mg 30%. The percentage of sodium is considered to be similar to that of potassium, or it may be less if the finding that myosin binds sodium even more than potassium is accepted (Lewis & Saroff, 1957). Nanninga regards these values for free ions as a maximum, since the presence of actin and myogen might reduce them.

There is evidence that most of the calcium in the squid nerve fibre axoplasm is bound. Free ionized calcium in concentrations as low as 0.7 mM. calcium chloride causes the dispersion of the normally gelatinized axoplasm (Hodgkin & Katz, 1949).

In drawing up a final osmotic balance sheet for *Sepia* and *Eledone* muscle cells, account has been taken of the binding of ions as deduced from analyses of muscle-juice, and of probable or possible osmotic coefficients of the inorganic ions and phosphates, and of the amino acids and nitrogenous bases. The calculated total in milliosmoles of *Sepia* cells is then about 94% of the plasma value, and the figure for *Eledone* cells, 86%.

VI. SUMMARY

1. Mantle muscle from *Sepia officinalis*, *Loligo forbesi* and *Eledone cirrhosa* has been analysed for all the major constituents contributing to its osmotic concentration, including inorganic ions, organic phosphates, amino acids and nitrogenous bases.
2. Intracellular concentrations have been calculated for *Sepia* and *Eledone* muscle after appropriate corrections for extracellular fluid as measured by inulin space.
3. From analyses of the juice pressed from muscle it has been concluded that the whole of the cell calcium, much of the magnesium and sodium, and a lesser proportion of potassium is bound to cell constituents; the chloride ion is completely free.

4. Direct measurements of the osmotic concentration of muscle-juice and plasma of *Eledone* show that the juice is slightly hyperosmotic (+3.2% on plasma value), probably owing to some breakdown of labile compounds.

5. The ions and compounds studied make up 94% of the osmotic concentration of the muscle cells in *Sepia*, and 86% in *Eledone*. Over half of the osmotic concentration of the cells is made up by free amino acids, trimethylamine oxide and betaine.

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