THE INORGANIC CONSTITUENTS OF THE SEA-URCHIN EGG

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INTRODUCTION

Previous work. The most detailed investigations into the inorganic constituents of the sea-urchin egg are those of Page (1927) and Białaszewicz (1929). Their results, together with those of certain other workers in this field, are given in Table 1 and, in accordance with modern practice, the concentrations of the various substances have been converted into millimoles (mM) per kilogram of water in the eggs. The dry weight of sea-urchin eggs is about 24% of the wet weight (Wetzel, 1907; Ephrussi, 1933; Ballentine, 1940; Hutchens, Keltch, Krahl & Clowes, 1942).

Table 1. *Inorganic constituents of sea-urchin eggs, in mM/kg. water in eggs*

	Sea water, chlorinity 19·00‰	*Arbacia punctulata* (Page, 1927)	*A. punctulata* (Harvey, 1932)	*A. lixula* (Białaszewicz, 1929)	*A. lixula* (Monroy-Oddo, 1946)	*Paracentrotus lividus* (Białaszewicz, 1929)	*Strongylocentrotus droebachiensis* (Malm & Wachtmeister, 1950)	*Psammechinus miliaris* (Malm & Wachtmeister, 1950)
Sodium	475	321	—	280	—	25	316	343
Potassium	10	354	96	162	—	245	200	266
Calcium (+Sr)	10	269	38	16	17	14	—	—
Magnesium	54	1044	17	41	21	28	—	—
Chloride	554	30	—	384	—	371	298	332
Sulphate	29	(0·0301)	—	—	—	—	—	—
Total phosphorus	—	181	—	132	—	142	110	148

The molalities of the commoner ions in sea water are also given in Table 1. The great discrepancies between the values obtained by different workers for the inorganic constituents of sea-urchin eggs are a sufficient reason for re-examining this subject. It seems, for example, unlikely that the magnesium content of two different batches of unfertilized eggs of *Arbacia* should vary by some 6100%; that the chloride content of different species should differ by about 1300%; or even, perhaps, that the sodium content of different species should differ by 1400%.

Apart from the results given in Table 1, a certain number of observations have been made on the concentration and exchange rates of particular substances in sea-urchin eggs. Runnström, for example, said in 1925 that potassium was at least

eight times as concentrated in the eggs of *A. lixula* as in sea water. Many of these observations have, however, been published as brief notes and, in the absence of subsequent papers of normal length, it is difficult to know how much weight should be attached to these preliminary communications. This applies to three notes on the penetration of ^{32}P (Abelson, 1948; Brooks & Chambers, 1948; Chambers, E. L., Whiteley, Chambers, R. & Brooks, 1948) and to two notes on the uptake of ^{42}K (Chambers, 1949; Chambers, White, Jeung & Brooks, 1948). Shapiro & Davson (1941) state, also in a brief note, that the eggs of *A. punctulata* contain about twenty times as much potassium as there is in sea water, but the methods by which they arrived at this conclusion are not given.

Interstitial water. All the results quoted in Table 1 were obtained by centrifuging the eggs and analysing them after removal of the supernatant suspending medium. There was, therefore, an unknown amount of interstitial sea water or suspending medium between the eggs, and it is incorrect to assume that this interstitial liquid can be ignored. In some preliminary experiments using the Trypan Blue method (see later), which are not of sufficient importance to include in the Results Section, it was found that the amount of interstitial sea water remaining after centrifugation of unfertilized eggs of *Echinus esculentus* for 15 min. at $\times$ 12,000 g. varied from 2% to 9%. This source of error may not be of great importance when the substance under examination has about the same concentration in the eggs as in sea water. But if it is much more concentrated in the latter than the former, as in the case of sodium according to Białaszewicz, or chloride according to Page, a knowledge of the amount of fluid between the eggs is essential if the analyses are to have any meaning. If, as Page claimed, the concentration of chloride in eggs is one-eighteenth of what it is in sea water, and if, after centrifugation, there remains 5% of interstitial sea water which is ignored, the apparent concentration of chloride in the eggs will be 185% too high. Some investigators tried to get round this difficulty by washing and suspending the eggs in isotonic glucose solution (Blanchard, quoted by Harvey, 1932), isotonic lithium nitrate (Białaszewicz, 1929), or a molar solution of urea (Monroy-Oddo, 1946). In the absence of experimental evidence that these treatments do not cause an outward diffusion of electrolytes from the eggs, these methods of attempting to circumvent the problem of interstitial sea water are unsatisfactory. Malm & Wachtmeister (1950) may have realized the importance of this problem; they state (p. 448) that 'almost all sodium chloride found in the unfertilized eggs originates from the intercellular water and not from the eggs themselves'. In justification of this contention they say that Białaszewicz showed in 1927 that 'unfertilized sea-urchin eggs contain but little sodium'; but reference to Table 1 shows that in 1929 Białaszewicz came to an entirely different conclusion, so far as the eggs of *Arbacia lixula* were concerned.

One can, of course, use the analyses based on centrifuged eggs to establish maximum or minimum concentrations, according to whether the substance under examination is less or more concentrated in the eggs than in the sea water. This method of establishing concentration limits, which involves the assumption that high-speed centrifugation does not affect the concentration of substances in the

egg, has been used in pilot experiments done before those described in this paper. There are two other methods of estimating the amounts of sea water and eggs in a suspension. The first is to add a known amount of some non-penetrating solute to a known volume of egg suspension, mix, and measure the solute's concentration in the suspending medium. The difference between the concentration of the solute, assuming no eggs in the suspension, and the observed concentration enables the volumes of eggs and sea water in the suspension to be calculated. This method was tried out with two different non-penetrating solutes. The first was a levan polysaccharide of high molecular weight obtained from Italian rye-grass, kindly supplied by Dr J. Beattie. This method was unsatisfactory because of the high reducing activity of the eggs. A small amount of egg cytolysis, which is inevitable in experiments of this type, interfered with the colorimetric determinations to a degree which made reasonable precision unattainable. The second solute was Trypan Blue. Although this substance does not penetrate the eggs, it appeared in some cases to become adsorbed on to the egg surfaces and on debris. In addition, the Trypan Blue method did not give reproducible results, and it was therefore decided to abandon these methods and estimate the number of eggs in the suspension, together with their volumes. The procedure is described in the next section.

Precision of measurements. Little attention has been paid to this question, though it is difficult to know what importance should be attached to measurements unless some indication is given of the errors associated with them. In two cases (Blanchard, quoted by Harvey, 1932, and Monroy-Oddo, 1946) estimates are followed by plus or minus some figure, which may mean the standard error of the mean of some number of estimates. But the practical object of working out standard errors is to define an interval which encloses the true value with some assigned probability. When estimates are based on very few measurements, the standard error cannot be used for an accurate determination of the interval which encloses the true value unless the distribution under consideration is normal, or if some transformation is known to make it normal. For this reason and because of the interstitial-water problem mentioned above, it has not been thought necessary or desirable to examine previous results in great detail, nor to try to explain the discrepancies between the results of different workers.

EXPERIMENTAL PROCEDURE AND CALCULATIONS

Estimation of volume of egg suspension and total egg volume. There are three parts to these estimations: first, determination of egg radii, from which the mean volume $\bar{v}$ of an egg and its variance $V(\bar{v})$ are obtained; secondly, estimation of the number of eggs in the suspension under examination, together with the precision of the estimate; thirdly, estimation of the volume of the egg suspension under examination. The eggs of *Paracentrotus lividus* do not flatten under gravity, so that $\bar{v}$ and $V(\bar{v})$ were determined by measuring two diameters, at right angles to each other, of fifteen to twenty-five eggs. The number of eggs in the suspension was estimated as follows: the parent egg suspension, which consisted of jelly-free unfertilized eggs (the jelly being removed by the usual treatment with acidified sea water) and sea

water in a boiling tube (diameter 2·3 cm., length 15 cm.), was gently inverted ten times to suspend the eggs uniformly in the sea water. A known volume of this parent suspension was then removed with a pipette and diluted to 50 ml. in a stoppered flask with sea water containing 5% formalin. After agitation to suspend the eggs uniformly, samples of known volume were removed from the flask and the number n of eggs in these samples counted. A similar method was described by Shapiro in 1935. The volume of the counting tube was 0·096 ml. in all experiments. In a typical experiment eight egg counts were made, $\bar{n}$ being 163† and $V(\bar{n})$ 16·786. In this experiment $V(n)$ equalled 134·288, a value which is not inconsistent with a Poisson distribution of n, though this was not always the case. Estimation of the volume of the egg suspension upon which the chemical analyses were done was based on a series of weighings, from which, by calculation of the appropriate densities, the volume of the egg suspension was obtained. The original egg suspension was concentrated by very gentle centrifugation, for about $\frac{1}{2}$ min. at less than $\times 50$ g., after which most of the supernatant sea water was removed by suction. After determination of the volume of this concentrated egg suspension, it was evaporated in a steam oven and the dried material (eggs + sea water) analysed (see pp. 538–40).

Estimation of concentration of substances in eggs. Let V_F = final volume of the suspension, K_F = amount of potassium (for example) in V_F, K_{SW} = concentration of potassium in sea water, and k = the unknown concentration of potassium in the eggs. Let $x = \bar{n}\bar{v}$ and A be the dilution factor used when estimating $\bar{n}$. Then

$$K_F = K_{SW}(V_F - Ax) + kAx. \tag{1}$$

Solving for k,

$$k = \frac{K_F - K_{SW}V_F}{Ax} + K_{SW}. \tag{1·1}$$

Bearing in mind that if $u = f(z)$, $V(u) = \{f'(z)\}^2 V(z)$,

$$V(k) = \frac{(K_F - K_{SW}V_F)^2}{(Ax)^4} A^2 V(x), \tag{2}$$

where $V(x) = \bar{n}^2 V(\bar{v}) + \bar{v}^2 V(\bar{n})$.

Equation (2) gives the precision of k. In practice, however, a number of estimates of the concentration of potassium (and other substances) were made; so that in the case of potassium, for example, we have a series of estimates k_i, each with variance $V(k_i)$, $i = 1, \ldots, N$. As the errors in chemical analysis were small compared with the errors involved in estimating $\bar{n}$ and $\bar{v}$, and as inter-batch variations were large compared with intra-batch errors (see later), the best estimate of k, k^*, is simply $(1/N)(\Sigma k_i)$, with variance $V(k^*) = \Sigma(k_i - k^*)^2/N(N-1)$. Inter-batch variation may be quantitatively estimated by subtracting $\overline{V(k_i)}$, the average value of the individual batch variances, from $V(k) = \Sigma(k_i - k^*)^2/(N-1)$. k^* and $V(k^*)$ may be used to define an interval within which the true mean of the observed values of k

† $\bar{n}$ must be multiplied by the appropriate dilution factor to obtain the number of eggs in the parent suspension.

lies with any desired probability, while the estimate of inter-batch variation may be used to define another interval within which a particular k_i will lie, with any desired probability.

Chemical analyses

The methods used for the estimation of cations were similar to those described by Robertson & Webb (1939), who developed reliable micro-methods for the analysis of 1 ml. of sea water or body fluid, with a maximum error of 2%. Minor modifications and details of the other methods used are described below.

Ashing. The egg suspension, whose volume was 2–7 ml., was ashed after evaporation to dryness with 2 ml. concentrated H_2SO_4, re-ashed if necessary, and taken up in a small quantity of distilled water with the addition of 0·3 ml. concentrated HNO_3. After making up to 10 ml., portions were taken for determination of the various cations.

Sodium (*zinc uranyl acetate method*). Phosphate interferes with the sodium analysis since it precipitates as zinc uranyl phosphate. A small quantity of finely powdered $Ca(OH)_2$ was therefore added to the solution for analysis and, after shaking several times while standing for 30 min., the precipitate of calcium phosphate and excess $Ca(OH)_2$ was removed by centrifugation. A portion of the supernatant was then taken for analysis.

Potassium (*silver cobaltinitrite method*). The precipitate was not left to stand overnight but was kept in the refrigerator for 4 hr. Ice-cold water was used for washing the precipitate instead of a saturated solution of the potassium salt.

Magnesium (*8-hydroxyquinoline complex method*). As an adaption to smaller quantities (0·2–0·3 mg.) the whole magnesium analysis was done in a centrifuge tube. 1 ml. of oxine was added to the solution (about 8 ml.), followed by one drop of NH_4OH (50%, v/v), and the complex, after precipitation at 70–80° C. with stirring, allowed to stand for 10 min. at about 90° C. After centrifugation and removal of the supernatant, the precipitate was washed with 2% NH_4OH, re-dissolved in three drops of 2N-HCl and re-precipitated by the addition of 0·3 ml. reagent followed by two drops of 50% NH_4OH, the conditions being maintained as in the first precipitation. The titration was carried out in the usual way.

Calcium. This was estimated as calcium oxalate, the latter being titrated with standard ceric sulphate. The first precipitate of calcium oxalate was re-dissolved and then re-precipitated to remove magnesium in the original precipitate.

Chloride. A more direct method was used than that of Robertson & Webb. After cytolysing the eggs by vigorous shaking with distilled water the solution was deproteinized with Somogyi's zinc sulphate-barium hydroxide reagent (1945). The precipitate was separated by centrifugation and filtration, the solution made up to 50 ml. and portions titrated by Volhard's method. After adding HNO_3 and excess standard $AgNO_3$ solution (about 0·02N) to the aliquot and centrifuging off the precipitated halides, it was back-titrated with NH_4CNS (about 0·01N, previously standardized against the $AgNO_3$ solution), using 1 ml. of saturated ferric alum solution as indicator. The total volume was less than 20 ml.

Sulphate. Robertson & Webb's method was not used. After cytolysis with

distilled water followed by de-proteinization with 5% $HgCl_2$, the sulphate was determined gravimetrically as $BaSO_4$, taking the standard precautions for this method and weighing the precipitate on a micro-balance.

Phosphorus. For determination of phosphate fractions the egg suspension was cytolysed with distilled water and made up to a known volume. The total phosphorus was determined on an aliquot which was transferred to a boiling tube, evaporated and ashed with H_2SO_4. After clearing with 100 vol. M.A.R. H_2O_2 and neutralization with NaOH, the sample was made up to a known volume, and the phosphate was determined by the Fiske-Subbarow method (1925) using the Spekker absorptiometer and red filters (Ilford 608). A second aliquot was treated with ice-cold trichloroacetic acid (final concentration 10%) and, after centrifugation, the inorganic phosphate was determined directly on one aliquot of the supernatant. The acid-soluble phosphorus was determined on a second aliquot after ashing, clearing and neutralization.

Accuracy of the analytical results. Robertson & Webb claimed that the errors associated with their methods did not exceed 2%, and inspection of their tables shows that the errors were often much less than this. During this investigation 1 ml. samples of sea water, whose chlorinity had been determined by the standard Knudsen technique, were taken through the ashing procedure and analysed. The accuracy of the results can be assessed by a comparison of the ionic ratios obtained with those given by Lyman & Fleming (1940). The results are given in Table 2.

Table 2. *Ratio of ions in sea water to chlorinity.*

Cl, Volhard (micro), 18·46‰. Standard Knudsen Method, 18·44‰.		
Ion	Found	Best value
Potassium	0·02043	0·0200
Sodium	0·5548	0·5556
Calcium (+Sr)	0·2113	0·2152
Magnesium	0·06701	0·06695
SO_4	0·1384	0·1394

Regardless of the absolute concentrations of the salts, the ratios between the common ions in sea water are virtually constant. This constancy of composition is not only the basis of the chlorinity-salinity density relations; it also provides a means of estimating the concentration of all the so-called major constituents when one is known. The major 'constituent' most easily determined with accuracy is the silver precipitating halides and their concentration is expressed as chlorinity. Chlorinity is defined by Knudsen as the weight in grams (*in vacuo*) of the chlorides contained in 1 kg. of sea water (*in vacuo*) when all the bromides and iodides have been replaced by chlorides, the values being calculated using Knudsen's Tables and Copenhagen Normal Water as standard. It is desirable to maintain an unvarying standard for chlorinity independent of atomic weights so that the apparent chlorinity of the oceans will not vary with changes in atomic weights. Jacobsen & Knudsen (1940) have established a permanent standard (in terms of weight of silver)

identical with the previous one but independent of the store of Normal Water at Copenhagen. The ionic ratios are conveniently expressed in relation to the chlorinity. Strictly speaking, the ratios only hold good at chlorinities in the neighbourhood of 19‰, although the divergences are small for comparatively wide changes of salinity.

Webb (1938) has pointed out that strontium is chemically indistinguishable from calcium in sea-water analyses by the usual techniques, and that this leads to high values for the apparent calcium content. The ratio (Ca + Sr)/Cl is 0·02152.

In the experiments described in this paper (apart from the check experiments already mentioned), the sea-water concentrations were calculated from Lyman & Fleming's ratios after accurate salinity determinations using a standard Knudsen burette and Knudsen's Tables. When concentrations were required in terms of grams per litre, the density was also calculated by the use of these tables. The chlorinity of Roscoff sea water was found not to vary significantly from 19·37‰ during these experiments.

To investigate the accuracy of the phosphate fraction estimations, duplicate analyses were done on different portions of an egg suspension derived from one sea-urchin; the results are given in Table 3.

Table 3. *Accuracy of phosphate estimations, mg./ml.* (Echinus esculentus). *Duplicate analyses,* i *and* ii, *were done on two samples of eggs from one sea-urchin*

	Total P		Acid-soluble P		Inorganic P	
Analysis ...	i	ii	i	ii	i	ii
Sample 1	2·97	3·02	1·64	1·64	0·91	0·93
Sample 2	2·94	2·98	1·72	1·72	1·05	1·04

RESULTS

Table 4 gives the results of the analyses, in mg./ml. eggs and in millimoles per kg. of water in eggs, of unfertilized, jelly-free eggs of *P. lividus*. The molalities of the same ions and radicals in Roscoff sea water are included for comparison. There are, perhaps, four features of these analyses which require special mention: first, sodium is nine times as concentrated in sea water as in the eggs; secondly, potassium is twenty-one times as concentrated in the eggs as in sea water; thirdly, there is nearly seven times as much chloride in sea water as in the eggs; and fourthly, there appears to be a large anion deficit in the eggs, which contain 292 m.equiv. of cations per kg. of water in the eggs and 194 m.equiv. of anions. In arriving at the anion figure, 1·5 charges have been allotted to each phosphorus atom, which implies that the phosphorus content of the egg consists of equal amounts of HPO_4 and H_2PO_4 at a pH of about 7 (see, for example, Needham & Needham, 1926). These assumptions certainly exaggerate the contribution of phosphate to electroneutrality because the acid-soluble phosphorus only accounts for somewhat more than half the total phosphorus present in the eggs. The proportions of acid-soluble

and inorganic phosphorus are shown in Table 5. The pooled estimate of total phosphorus, together with the precision of this estimate, given in Table 4, is derived from the data in Table 5.

Table 4. *Inorganic constituents of unfertilized eggs of* Paracentrotus lividus

	c	S.E. of *c*	*n*	*V*	mM	Sea water (mM)
Sodium	0·9996	0·2939	10	0·8626	52	485
Potassium	6·7917	0·3970	9	1·1653	210	10
Calcium	0·1338	0·0192	7	0·0499	4	11
Magnesium	0·2124	0·0860	6	0·2066	11	55
Chloride	2·3354	0·9568	3	1·5261	80	566
Sulphate	0·4441	0·1541	4	0·2967	6	29
Total phosphorus	2·0974	0·0497	3	—	—	—

c, mg./ml. eggs. This is the best estimate, e.g. k^*, see p. 537.
S.E. of *c*, standard error of *c*, i.e. $\sqrt{[V(k^*)]}$.
n, number of independent determinations.
V, inter-batch variance. In the case of phosphorus the inter-batch and intra-batch variations were of the same order, so that *c* was calculated by the formulae

$$k^* = \Sigma\{I(k_i)\, k_i\}/\Sigma I(k_i) \quad \text{and} \quad I(k^*) = \Sigma I(k_i),$$

where $I(p) = 1/V(p)$.

mM, *c* in terms of millimoles per kilogram of water in the eggs (dry weight of eggs, 24%; density, 1·09).
Sea water (mM), millimoles per kilogram of water, chlorinity 19·37‰.

Table 5. *Phosphorus fractions, in mg./ml., unfertilized eggs of* Paracentrotus lividus

Sea-urchin no.	Acid-soluble P	Inorganic P	Total P
i	1·19	0·69	2·12
ii	1·07	0·61	2·02
iii	1·19	0·79	2·19

DISCUSSION

The asymmetrical distribution of ions between the inside of a cell and the external medium has been the subject of a great number of inquiries and of an almost equal amount of discussion and speculation. The sea-urchin egg is similar in several respects to other tissues, such as nerve, muscle and erythrocytes, which have received much more attention and about which much more precise information is available. The mechanisms responsible for the distribution of ions between these cells and their environments have been discussed in great detail, for example, by Boyle & Conway (1941), Ussing (1949) and Solomon (1952). We therefore do not propose to recapitulate the various arguments but to examine certain fundamental questions so far as the incomplete information permits. The fundamental questions are:

(1) *How is electroneutrality maintained in the egg?* Amino-acids such as glutamine probably make a significant contribution to the anion deficit (Kavanau, 1953).

Bicarbonate is unlikely to contribute to the missing anion in view of the reported pH of the egg interior (Needham & Needham, 1926).

(2) *Are the observed concentrations maintained by active processes?* If the egg is in a steady state and no active work is being done to maintain the observed differences between the concentrations of potassium and chloride inside and outside the egg, but an active process is responsible for the asymmetrical distribution of sodium, the Donnan equilibrium requires that $[K]_i/[K]_o = [Cl]_o/[Cl]_i$, where the square brackets refer to concentrations and the subscripts *i* and *o* refer to the inside and outside of the egg. If an active process is responsible for the asymmetrical distribution of potassium and not for the sodium or chloride distribution, $[Na]_i/[Na]_o = [Cl]_o/[Cl]_i$, while if chloride alone is actively transferred, $[K]_i/[K]_o = [Na]_i/[Na]_o$.

Table 6

Active transport of	Donnan equilibrium	Ratio
Sodium	$[K]_i\,[Cl]_i = 16{,}800$ $[K]_o\,[Cl]_o = 5{,}660$	3·0:1
Potassium	$[Na]_i\,[Cl]_i = 4{,}160$ $[Na]_o\,[Cl]_o = 274{,}510$	1:66·0
Chloride	$[K]_i\,[Na]_o = 101{,}850$ $[K]_o\,[Na]_i = 520$	195·9:1

Table 6 gives the results of substituting the observed concentrations of sodium, potassium, and chloride in these relationships. It shows that if, on the basis of the existing evidence, a decision had to be taken in support of one ion being the subject of active transport, the decision would be in favour of the egg actively pumping out sodium. But the available evidence is far too scanty to warrant such a conclusion at present.

Lack of information about the permeability of the egg surface to potassium, sodium or chloride makes it impossible to say anything about the work which might be necessary to maintain the observed concentration differences. In addition, Chambers, White, Jeung & Brooks (1948) make the 'tentative assumption' (p. 252) that only 20% of the intracellular potassium is readily exchangeable with sea water in the case of the unfertilized eggs of *Arbacia punctulata* and *Strongylocentrotus purpuratus*. In the absence of more detailed information, particularly about the possibility of contamination of the ^{42}K used with ^{24}Na, and about the difficulties in interpreting experiments to demonstrate the existence of 'bound' ions (see Keynes, 1951), it may be premature to accept the findings in this preliminary note of Chambers and his co-workers.

SUMMARY

1. The principal inorganic constituents of the unfertilized egg of *Paracentrotus lividus* have been analysed by chemical methods. The results of the analyses, in millimoles per kg. of water in the eggs (dry weight of eggs, 24%; density, 1·09), were:

Sodium	52 (485)	Magnesium	11 (55)
Potassium	210 (10)	Chloride	80 (566)
Calcium	4 (11)	Sulphate	6 (29)

The figures in brackets are the concentrations of the same substances in Roscoff sea water, chlorinity 19·37‰, in the same units.

2. The total phosphorus content of the eggs was about 2 mg./ml. eggs, somewhat over half of this being acid-soluble phosphorus.

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